frontiers | Research Topics

Nutritional physiology of aquacultured species

Edited by
Mohamed Salah Azaza, Helena Peres, Hien Van Doan and Serhat Turkmen

Published in
Frontiers in Physiology
Frontiers in Marine Science

ISSN 1664-8714
ISBN 978-2-83251-475-7
DOI 10.3389/978-2-83251-475-7

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Nutritional physiology of aquacultured species

Topic editors

Mohamed Salah Azaza — Aquaculture Laboratory, National Institute of Marine Science and Technology, Tunisia

Helena Peres — University of Porto, Portugal

Hien Van Doan — Chiang Mai University, Thailand

Serhat Turkmen — University of Alabama at Birmingham, United States

Citation

Azaza, M. S., Peres, H., Van Doan, H., Turkmen, S., eds. (2023). *Nutritional physiology of aquacultured species*. Lausanne: Frontiers Media SA.

doi: 10.3389/978-2-83251-475-7

Table of contents

frontiers | Frontiers in Physiology

TYPE Editorial
PUBLISHED 12 January 2023
DOI 10.3389/fphys.2022.1130143

OPEN ACCESS

EDITED AND REVIEWED BY
Pung Pung Hwang,
Academia Sinica, Taiwan

*CORRESPONDENCE
Mohamed Salah Azaza,
med.azaza@instm.rnrt.tn

SPECIALTY SECTION
This article was submitted to Aquatic Physiology,
a section of the journal
Frontiers in Physiology

RECEIVED 22 December 2022
ACCEPTED 29 December 2022
PUBLISHED 12 January 2023

CITATION
Azaza MS, Peres H and Turkmen S (2023), Editorial: Nutritional physiology of *Aquacultured species*.
Front. Physiol. 13:1130143.
doi: 10.3389/fphys.2022.1130143

Editorial: Nutritional physiology of *Aquacultured species*

Mohamed Salah Azaza[1]*, Helena Peres[2,3] and Serhat Turkmen[4]

[1]Aquaculture Laboratory, National Institute of Marine Sciences and Technology, University of Carthage, Salammbô, Tunisia, [2]Department of Biology, Faculty of Sciences of University of Porto, Porto, Portugal, [3]Interdisciplinary Center for Marine and Environmental Research, Matosinhos, Portugal, [4]University of Alabama at Birmingham, Birmingham, AL, United States

KEYWORDS

nutrient efficiency, digestibility, oxidative stress, digestive enzymes activities, feed management, metabolic response, feed additives

Editorial on the Research Topic
Nutritional physiology of *Aquacultured species*

Whatever the aqua-cultured species, it is crucial that aqua feed be adequate and sustainable, as feeds represent the main contribution to production costs. In the face of this challenge, in the last few decades, substantial efforts have been oriented to identify alternatives to high-cost ingredients (e.g., fishmeal and fish oil) from unconventional protein sources and carbohydrates, particularly of vegetal origin, with variable success. These attempts may not be enough if the diet profile is not improved so as to boost and maintain the good health, welfare, and immune capacity of rearing species, abreast of growth performance, and feed conversion efficiency (Azaza et al., 2020). Therefore, the Research Topic "Nutritional Physiology of *Aquacultured species*" has been conceived to set out such knowledge and improve understanding of the relationships between nutrition and the related physiological aspects. This Research Topic comprises eleven original research articles.

The limited supply of fishmeal and fish oil associated with the continuous increase of their price has severely impacted farming costs. The search for feasible alternatives to fishmeal and fish oil is a prerequisite for sustainable aquaculture development. Significant progress has been made, and modern aquaculture diets are shifting towards agriculture-based ingredients. In this context, Biasato et al. investigated the growth performance, whole-body proximate composition, and intestinal microbiome of rainbow trout strains when selected and non-selected for weight gain on full-plant-based diets. Results demonstrate that the selected strain showed higher survival, final body weight, weight gain, and specific growth rate when compared to the non-selected strain. Furthermore, decreased whole-body lipid content was identified in the plant protein-fed selected rainbow trout compared to the non-selected strain fed the same diet.

Another study by Liu et al. was conducted on largemouth bass to investigate the effects of low, medium, and high viscous guar gums on growth performance, apparent nutrient digestibility, intestinal development, and morphology. Results indicated that guar gum diets adversely affected intestinal morphology, decreased intestinal digestive and absorptive enzyme activities, and caused poor nutrient digestibility and growth performance in juvenile largemouth bass. In fact, the adverse effects of guar gum are closely related to its viscous level, and high viscous guar gum adversely affects the rearing performance of juvenile largemouth bass.

In addition to the above-mentioned fish species, two investigations were conducted on Pacific white leg shrimp (*Litopenaeus vannamei*), considered the most important farmed

crustacean species. Peng et al. evaluated the effects of increased dietary inclusion of soybean meal on growth performance, apparent digestibility, intestinal digestive enzyme activity, and muscle growth-related gene expression. Results indicated that the final body weight, weight gain, specific growth rate, feed intake, intestinesomatic index, and dressed weight percentage linearly and significantly decreased as dietary soybean meal increased from 20% to 50%. The same trend was also observed in the apparent digestibility coefficients of dry matter, crude protein, crude lipid, and ash. For the same species (i.e., *Litopenaeus vannamei*), Hi et al. investigated the effects of replacing commercial feeds with fresh black soldier fly larvae (BSFL) on the intestinal microbiota, immune enzyme activities, and rearing water quality. Authors reported that proper replacement of commercial feed with fresh BSFL had positively modulated intestinal health and immune-related enzyme activities, as well as the water quality.

Increased diversity of non-nutritive or functional aquafeed additives has been used as a valuable approach to stabilize feed quality, enhance growth, digestibility, feed efficiency, and immune status, and as an alternative strategy for disease-fighting. Functional feed additives include phytogenic compounds, mycotoxin binders, organic acids, immunostimulants, yeast products, probiotics, prebiotics, and enzymes. In this context, four research articles featured in the current Research Topic dealt with using feed additives. Peng et al. investigated the effects of dietary condensed tannins, regarded as a potent antioxidant, anti-inflammatory, and antibacterial activities, on serum metabolites, antioxidant and immune response, liver histomorphology, and glycometabolism enzyme activities of *Chinese seabass (Lateolabtax maculatus)*. Results revealed that condensed tannins dietary supplementation, up to 2 g/kg of diet, reduced serum lipid and glucose levels, enhanced liver antioxidant and immune response, and improved glucose utilization of *L. maculatus*.

The study conducted by Mansour et al. aimed to investigate the effects of increasing dietary supplementation levels with an ethanolic extract from *A. platensis* naturally rich in astaxanthins (circa 98%) on growth performance, feed utilization, immune-related genes expression, and water and intestinal microbiota of Pacific white leg shrimp. Supplementing a commercial diet with 4 g kg^{-1} crude *A. platensis* extract did not affect the survival rate and significantly improved shrimp growth performance and feed conversion ratio compared to the control diet. The superoxide dismutase and immune-related gene expression (*prophenoloxidase*, *lysozyme*, *beta-glucan binding protein*, *transglutaminase*, and *crustin*) were significantly upregulated in groups fed increasing levels of this extract. Besides, results demonstrate that increasing *A. platensis* extract supplementation levels significantly reduced the prevalence of heterotrophic bacteria and *Vibrio* spp.

Wang et al. investigated the effects of glutathione (GSH), as a feed additive in practical diets, on growth, intestinal antioxidant capacity, intestine histology, gene expression, and gut microbiota in juvenile triploid rainbow trout (*Oncorhynchus mykiss*). Based on the broken-line regression analysis, results demonstrate that the optimum dietary GSH level to maximize growth performance was circa 447 mg kg^{-1} of diet. Likewise, feeding juvenile triploid *O. mykiss* 200–800 mg kg^{-1} GSH increased intestinal catalase and superoxide dismutase activities and improved general intestinal health.

Another study on dietary supplementation with a commercial feed additive, betaine, a by-product of sugar beet processing widely used as an attractant, was conducted by Li et al. This study aimed to investigate the mechanism by which betaine modulates reactive oxygen species (ROS) production *via* Wnt10b/β-catenin signaling in zebrafish liver, based on the fact that, under oxidative stress, chronically elevated ROS levels play a crucial role in innate fish immunity. Results showed that betaine enrichment of diet at levels of .1, .2, and .4 g/kg induced Wnt10b and β-catenin gene expression but suppressed GSK-3β expression in zebrafish liver. In addition, irrespective of the betaine supplementation level, betaine led to a reduction of superoxide anion (O_2^{-}), hydrogen peroxide (H_2O_2), and hydroxyl radical (·OH) content. However, dietary betaine enrichment, at .1, .2, or .4 g/kg diet, upregulated hepatic gene expression of antioxidant enzymes and increased activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) and catalase (CAT) in zebrafish, clearly demonstrating that betaine can efficiently inhibit ROS production.

Understanding the functionality of the digestive tract is a prerequisite to optimizing diet formulation for new species. Knowledge of fish digestive biochemistry and its health is essential in determining animal performance, feed utilization efficiency, and aquaculture profitability. To strengthen understanding on lumpfish (*Cyclopterus lumpus*) nutrition, a rapidly expanded cultured species in the last decade, Zhou et al. evaluated the effects of various dietary macronutrient compositions on gut function. The results demonstrated that increased lipid and decreased protein levels in the diet negatively impacted digestive function, including the reduced activity of brush border membrane digestive enzymes and gene expression related to nutrient digestion and transport, ion exchange, immune regulation, and cell remodeling. The effect of dietary lipid to carbohydrate ratio (7.5/18.3, 13.8/14.6, and 18.1/9.5) of isoproteic diets (55% crude protein) on macronutrient digestibility was also studied. Results showed that decreased as the starch level increased, whereas protein digestibility was not affected by the lipid/carbohydrate ratio. This led to conclude that protein sparing effect of lipids negatively affected digestion, absorption, and immune responses in the lumpfish intestine.

Greater amberjack (*Seriola dumerili*) is a pelagic teleost highly interested in marine aquaculture diversification, high growth rates, and exquisite flesh quality. To further acquire scientific knowledge on the digestive physiology of this species, Navarro et al. studied the activity and functional characteristics of key digestive enzymes (i.e., pepsin, trypsin, chymotrypsin, etc.) and the modulatory effect of water temperature. Results demonstrate that chymotrypsin was the most active enzyme in the digestive tract of the greater amberjack, while lipase was the enzyme with lower activity. The activity of trypsin, chymotrypsin, and lipase was responsive to water temperature, even though the highest activity of trypsin was reached at 26°C and of chymotrypsin at 18°C.

The last paper concerns the pearl oysters, *Pinctada fucata martensii* and *P. maxima*, which are the two main farmed species used for producing nucleated round pearls. Ye et al. evaluated the growth performance, physiological energetics, and activity of digestive enzymes and carbonic anhydrase of both species fed with different microalga diets. Results showed that the relative growth rate (RGR) of *P. f. martensii* was higher than that of *P. maxima*. Irrespective of the microalga species, RGR was higher when fed with a microalgae bend than with a single microalga. Amylase, cellulase, lipase, and pepsin activity was higher in *P. f. Martensii* than with *P. maxima* fed with the same diets.

The papers presented in this Research Topic and conducted on diverse species highlight the usefulness of the physiologic approaches to deepen knowledge in feeding farmed aquatic organisms. This helps fish nutritionists to tailor and improve the nutritional profile of the diet and hence to provide more adequate and healthy diets for fish. We sincerely thank all authors and reviewers for their valuable contribution that made the publication of this Research Topic possible.

Author contributions

MSA was responsible for the idea of this special volume and wrote the draft. HP and ST revised the text. All authors contributed to the article and approved the submitted version.

Acknowledgments

Guest editors are thankful to the Frontiers in Marine Science journal for allowing us to edit the present Research Topic and especially to international authors who, independently of the revision result, showed interest and/or participated as external reviewers in this exciting 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

Reference

Azaza, M. S., Saidi, S. A., Dhraief, M. N., and El-Feki, A. (2020). Growth performance, nutrient digestibility, hematological parameters and hepatic oxidative stress response in juvenile nile Tilapia, *Oreochromis niloticus* fed carbohydrate of different complexities. *Animals* 10, 1913–1929. doi:10.3390/ani10101913

frontiers in Physiology

ORIGINAL RESEARCH
published: 29 November 2021
doi: 10.3389/fphys.2021.784852

Effects of Glutathione on Growth, Intestinal Antioxidant Capacity, Histology, Gene Expression, and Microbiota of Juvenile Triploid *Oncorhynchus mykiss*

Chang'an Wang[1,2], Baohui Su[2], Shaoxia Lu[1], Shicheng Han[1], Haibo Jiang[3], Zhuang Li[4], Yang Liu[1], Hongbai Liu[1]* and Yuhong Yang[2]**

[1]Key Open Laboratory of Cold Water Fish Germplasm Resources and Breeding of Heilongjiang Province, Heilongjiang River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Harbin, China, [2]College of Animal Science, Northeast Agricultural University, Harbin, China, [3]College of Animal Science, Guizhou University, Guiyang, China, [4]Fishery Technical Extension Station of Jilin Province, Changchun, China

This study aimed to demonstrate the effects of dietary glutathione (GSH) on growth, intestinal antioxidant capacity, histology, gene expression, and microbiota in juvenile triploid rainbow trout (*Oncorhynchus mykiss*). Different diets (G0-control, G100, G200, G400, and G800) containing graded levels of GSH (0, 100, 200, 400, and 800 mg kg^{-1}) were fed to triplicate groups of 30 fish (initial mean weight 4.12 ± 0.04 g) for 56 days. G400 had significantly improved weight gain and feed conversion rate. Based on the broken-line regression analysis, the optimum dietary GSH level was 447.06 mg kg^{-1}. Catalase and superoxide dismutase activities were significantly higher in G200–G800. G200 had significantly lower malondialdehyde content. The height of the intestinal muscular layer in G400 was significantly higher than that of the control group. Intestinal PepT1 and SLC1A5 gene expression was significantly increased, and the highest was observed in G400. TNF-α, IL-1β, IL-2, and IL-8 expression were significantly decreased than that of G0. Next-generation sequencing of the 16S rDNA showed a significant difference in alpha diversity whereas no differences in beta diversity. On the genus level, LefSe analysis of indicator OTUs showed *Ilumatobacter*, *Peptoniphilus*, *Limnobacter*, *Mizugakiibacter*, *Chelatococcus*, *Stella*, *Filimonas*, and *Streptosporangium* were associated with the treatment diet, whereas *Arcobacter*, *Ferrovibrio*, *Buchnera*, *Chitinophaga*, *Stenotrophobacter*, *Solimonadaceae*, *Polycyclovorans*, *Rhodococcus*, *Ramlibacter*, and *Azohydromonas* were associated with the control diet. In summary, feeding juvenile triploid *O. mykiss* 200–800 mg kg^{-1} GSH improved growth and intestinal health.

Keywords: triploid *O. mykiss*, glutathione, intestinal health, growth, microbiota

OPEN ACCESS

Edited by:
Serhat Turkmen, University of Alabama at Birmingham, United States

Reviewed by:
Beth Cleveland, Cool and Cold Water Aquaculture Research (USDA-ARS), United States
David Huyben, University of Guelph, Canada

****Correspondence:***
Chang'an Wang
gordoncase@126.com
Hongbai Liu
liuhongbai@hrfri.ac.cn
Yuhong Yang
yuhongyang68@163.com

Specialty section:
This article was submitted to Aquatic Physiology, a section of the journal Frontiers in Physiology

Received: *28 September 2021*
Accepted: *25 October 2021*
Published: *29 November 2021*

Citation:
Wang C, Su B, Lu S, Han S, Jiang H, Li Z, Liu Y, Liu H and Yang Y (2021) Effects of Glutathione on Growth, Intestinal Antioxidant Capacity, Histology, Gene Expression, and Microbiota of Juvenile Triploid Oncorhynchus mykiss. Front. Physiol. 12:784852. doi: 10.3389/fphys.2021.784852

INTRODUCTION

Fish in intensive aquaculture are frequently subjected to a range of negative environmental stresses, including high temperatures, overcrowding, deteriorating water quality, pathogen invasion, and disinfectant and antibiotic (Ming et al., 2015). This causes bacterial resistance and residues in aquatic products, transfer of antimicrobial resistance genes into the environment and food, immunosuppression in fish and increased susceptibility to different diseases (Liu et al., 2010), which can lead to significant economic losses. As a consequence, one of the methods is to improve fish immune and stress resistance through dietary approaches.

Glutathione (GSH), a tripeptide consisting of glutamate, cysteine, and glycine residues (Kosower and Kosower, 1978) can eliminate unnecessary free oxygen radicals from cells. It also has many physiological roles, such as increasing antioxidant activity (Doyotte et al., 1997), protecting liver cells (Ponsoda et al., 1999), maintaining DNA synthesis, enhancing immunity (Will, 1999), and alleviating neuron intoxication (Raghunathan et al., 2007). Several GSH studies in freshwater fish and shrimp found growth, antioxidant ability, and immunity could be considerably improved in European bass (*Dicentrarchus labrax*; Zambonino-Infante et al., 1997), Nile tilapia (*Oreochromis niloticus*; Zhou et al., 2013), grass carp (*Ctenopharyngodon idella*; Ming et al., 2015), Atlantic salmon (*Salmo salar*; Ma et al., 2019), and whiteleg shrimp (*Litopenaeus vannamei*; Xia and Wu, 2018).

Rainbow trout (*Oncorhynchus mykiss*) is a typical coldwater fish. As one of China's major farmed species, triploid *O. mykiss* is grown mainly in cold-water regions. Induced triploidy through chromosome set manipulation leads to an additional chromosome package in each somatic cell that makes fish sterile (Thorgaard, 1983). The negative effects of gonad growth with female triploid salmonids can often benefit recirculating aquaculture producers (Good and Davidson, 2016). Due to physiological changes, there may be variations in nutritional needs between ploidies (Fjelldal et al., 2016). This may be either because of increased growth potential or the odd number of chromosomes inherent in genetic variations (Ren et al., 2017). Currently, there is little information on the nutritional needs of triploid *O. mykiss*.

The intestine is a delicate tissue that plays an important role in fish health and nutrition. Maintaining intestinal homeostasis is important to enhance the growth performance and fish health status (Rombout et al. 2011). Intestinal health is determined by host (immunity, mucosal barrier), nutritional, microbial, and environmental factors (Kelly and Salinas, 2017). Thus, intestinal health could be improved through dietary approaches. GSH may benefit intestinal health in aquatic animals. In shrimp, dietary glutathione (150–250 mg kg^{-1}) improved the jejunum wall thickness and villus height of intestine (Wang et al., 2018). To date, there is limited published research on the intestinal health of dietary GSH in fish. Furthermore, the published data were mainly based on the purified diets. Further studies based on the practical diet are needed for the application of GSH or related ingredients in fish feeds, especially for the commercial fish feeds. Therefore, the aim of this research was to study the effects of GSH supplementation in practical diets on growth, intestinal antioxidant capacity, histology, gene expression, and microbiota in triploid *O. mykiss* diets.

MATERIALS AND METHODS

Diets

Experimental diets (G0, G100, G200, G400, and G800) with five GSH levels (0, 100, 200, 400, and 800 mg kg^{-1}) were prepared (**Table 1**). The spectrophotometric approach (Ming et al., 2015) revealed that the dietary GSH levels were 8.52, 106.36, 210.32, 409.51, and 796.55 mg kg^{-1}, respectively. Fish meal, soybean protein concentrate, chicken meal, and extruded soybean were the main protein sources in the experimental diets, with fish oil and soybean oil as lipid sources and wheat middling as a carbohydrate source. Ingredients were finely ground before mixing (<250 μm) and then blended with minerals and vitamins. After adding the lipid source, all the ingredients were thoroughly mixed for 15 min before being mixed again for 10 min. The dough was shaped into feed pellets with a diameter of 1.2 mm using a small-scale extruder (G250; Machine Factory of Muyang, China). After pelleting, feed pellets were dried for approximately 12 h in a ventilated oven at 45°C, then sieved. The pellets were then frozen at −20°C until use.

Feeding Management

Before feeding the basal diet, the fish were acclimatized in the laboratory (Chinese Academy of Fishery Sciences Coldwater Fish Experimental Station, Mudanjiang, China) for 14 days. At the start of the experiment, the fish were starved for 24 h before being pooled. In 15 tanks, 450 fish (initial weight 4.12 ± 0.04 g) were distributed (size: 300 L). Each group had 3 tanks, and each tank was used as a replicate. The fish in each tank were weighed in batches. During the 56-day feeding trial, the fish were hand-fed four times a day (08:00, 11:00, 14:00, and 17:00) until they were satiated. Fish were raised in a water flow-through system (flow rate: 0.2 L s^{-1}). Water quality was measured (YSI 6600 V2-2, Ohio State, United States) daily during the experimental period, water temperature (11.3–15.8°C), pH (7.2–7.5), dissolved oxygen (7.8–9.2 mg L^{-1}), and ammonia nitrogen (<0.2 mg L^{-1}). After a 24-h starvation period, the fish in each tank were weighed again as a batch at the end of the feeding trial.

Sample Collection

At the end of the experiment, all fish had been starved for 24 h. Ten fish were randomly selected from each tank and anesthetized (tricaine methane sulfonate MS-222, 75 mg L^{-1}) before weighing. The weight (precision 0.01 g) and fork length (precision 0.01 cm) of the fish were then determined to calculate the condition factor (*CF*). Further, the total weight of the fish in each tank was measured to calculate the weight gain rate (WGR), specific growth rate (SGR), feed conversion rate (FCR), daily feed intake (DFI). For body composition analysis, four fish were sampled from each tank and stored at −80°C. Three fish were collected from each tank and aseptically killed in an

TABLE 1 | Formulation and chemical proximate composition of the experimental diets.

Ingredients (g kg^{-1})	G0	G100	G200	G400	G800
Soybean protein concentrate[1]	300	300	300	300	300
Fish meal[2]	200	200	200	200	200
Wheat middling[3]	200	200	200	200	200
Extruded soybean[4]	145	145	145	145	145
Chicken meal[5]	50	50	50	50	50
Fish oil[6]	20	20	20	20	20
Soybean oil	30	30	30	30	30
Soybean phospholipid	30	30	30	30	30
Calcium dihydrogen phosphate	10	10	10	10	10
Vitamin premix[7]	3	3	3	3	3
Mineral premix[8]	6	6	6	6	6
GSH (mg kg^{-1})	0	100	200	400	800
Glycine	6	5.9	5.8	5.6	5.2
Proximate analysis of experimental diet					
Moisture	9.23	9.18	9.19	9.20	9.21
Crude protein	437.1	436.8	436.6	435.8	436.9
Crude lipid	109.3	108.6	109.1	109.2	109.5
Ash	6.68	6.71	6.53	6.61	6.56
Gross energy (MJ kg^{-1})	18.65	18.59	18.61	18.56	18.62

[1]*Dalong Feed Company, Harbin, China.*
[2]*Dalong Feed Company, Harbin, China.*
[3]*Huada Feed Company, Harbin, China.*
[4]*Dalong Feed Company, Harbin, China.*
[5]*Dalong Feed Company, Harbin, China.*
[6]*Huludao Chia Tai Feed Corporation, Huludao, China.*
[7]*Vitamin premix (mg kg^{-1}): ascorbic acid 200, alpha-tocopherol 100, menadione sodium bisulfate 5, retinol acetate 5.2, cholecalciferol 0.07, thiamin 25, riboflavin 40, pyridoxine 25, cyanocobalamin 0.8, nicotinic acid 275, folic acid 8, biotin 5, pantothenic acid 100.*
[8]*Mineral premix (mg kg^{-1}): $MgSO_4{\cdot}7H_2O$ 2000, KCl 1,500, $FeSO_4{\cdot}7H_2O$ 1,000, $CuSO_4{\cdot}5H_2O$ 20, $MnSO_4{\cdot}4H_2O$ 100, ZnSO4·$7H_2O$ 150, KI 3, NaCl 500, $CoCl_2$ 5, Na_2SeO_3 3.*

ice bath. The body surfaces of the sampled fish were washed with 70% ethanol, and the fish were dissected using sterile surgical scissors. Then their mid-intestines (located right after the pyloric ceca, 1 inch from the stomach) were collected until being tested for antioxidant enzymes, and faecal samples from the mid-intestine were collected in sterile tubes from each dietary replicated tank and stored at −80°C for microbial analysis. The research protocol was handled following the Chinese Animal Health Protection Law and the Scientific Laboratory Animal Permit Approval (Ethical Approval No. SCXK(YU)2005-0001).

Chemical Analysis

Normal procedures were used to determine the crude protein, crude lipid, ash, moisture, and gross energy of feed and whole body (AOAC, 2012). After acid digestion using a Kjeltec system, crude protein (N × 6.25) was measured using the Kjeldahl method (KDN-102C Autoanalyzer, Xianjian, China). The ether-extraction process was used to measure crude lipid with the Soxtec System (SXT-06-analyzer, Hongji, China). Moisture was measured by oven drying for 6 h at 105°C. Ash was placed in a muffle furnace at 550°C for 12 h. The energy content of the diet was determined using bomb calorimetry (XRY-1A, Jingmi, China).

Intestinal Antioxidant Capacity

Intestinal samples were weighed and mixed with an ice-cold buffer in a 1:9 ratio (0.86% normal saline). The extract was then centrifuged for 15 min at 7,700 *g* and 4°C, and the supernatant was used to determine superoxide dismutase (SOD), and catalase (CAT), glutathione reductase (GR) activities, GSH, and malondialdehyde (MDA) content. The standard was bovine serum albumin, and the spectrophotometer absorbance was determined at 750 nm. Spectrophotometric kits were bought from the Chinese Nanjing Jiancheng Institute of Bioengineering and were used to analyze the MDA content (cat. no. A003-1), GSH content (cat. no. A006-1-1), and the activity of GR (cat. no. A062-1-1), SOD (cat. no. A001-1), and CAT (cat. no. A007-1). The concentrations of MDA, SOD, CAT, GR and GSH were then calculated according to the instructions provided with the respective kits as described by Deng et al. (2015).

Intestinal Gene Expression

Total RNA was extracted from mid-intestines according to the manufacturer's instructions using RNAiso Plus Reagent (TaKaRa, Dalian, China). A spectrophotometer was used to examine the absorbance at 260 nm to determine the RNA concentration. Using agarose gel electrophoresis, the RNA integrity was determined, and the absorbance ratio at A260 nm/A280 nm ranged from 1.8 to 2.0. Tumor necrosis factor (TNF-α), interleukin 1 (IL-1β), interleukin 2 (IL-2), interleukin 8 (IL-8), solute carrier family 1 member 5 (SLC1A5), peptide transporter 1 (Pep T1), and β-actin expression levels were determined using quantitative real-time PCR (ABI 7500, USA) with a reaction length of 20 µl, including 10 µl of 2 SYBR® Premix Ex Taq (TaKaRa, Dalian, China), 0.8 µl for quantitative real-time PCR, specific primers were constructed based on sequences cloned and published in the *O. mykiss* gene bank (**Table 2**). The cycling conditions were 95°C for 30 s followed by 35 cycles of 95°C for 5 s, 59°C for 10 s, and 72°C for 30 s. The housekeeping gene (β-actin) was chosen as a reference gene to normalize the results. $2^{-\Delta\Delta Ct}$ was used to measure the expression values.

Intestine Histology

The intestinal samples were fixed in Bouin's solution for 24 h, rinsed multiple times with water to extract the fixative, dehydrated in ethanol, placed in xylene, embedded in wax for 2 h at 60°C, sectioned to 6 µm thick, with a microtome (Leica-RM2235), stained with hematoxylin–eosin, and sealed with neutral resin. Intestinal morphology was measured using Motic Images Plus 2.0 software after being photographed (Nikon, DS-Ri2). The muscular layer and villus height were determined by randomly selecting 10 villi per slide.

Intestine Bacterial DNA Extraction and Sequencing

Complete DNA was extracted from 0.2 g of intestinal faeces using a DNA Extraction Kit (Beijing Tiangen Biochemical Technology Co. Ltd., China) according to the guidelines for total DNA extraction from intestinal microorganisms. DNA was isolated

TABLE 2 | Primers sequence and annealing temperature in RT-PCR.

Target genes	Forward primer (5′-3′)	Reverse primer (5′-3′)	Accession number
β-Actin	F: GGACTTTGAGCAGGAGATGG	R:ATGATGGAGTTGTAGGTGGTCT	XM_042314795.1
SLC1A5	F:CCTGTCAATCAACGCTGGT	R:CACTGCCCATAATGAACACG	KY775396.1
PepT1	F: CTGGGAGAGGAGGGAGAGAT	R: TCCACGATCTTCCCTGCTAC	XM_014213484.1
IL-1β	F:ACATTGCCAACCTCATCATC	R:GTTCTTCCACAGCACTCTCC	LR584424.1
IL-2	F:TGATGTAGAGGATAGTTGCATTGTTGC	R:GAAGTGTCCGTTGTGCTGTTCTC	NM_001164065.2
IL-8	F:CACAGACAGAGAAGGAAGGAAAG	R:TGCTCATCTTGGGGTTACAGA	AY160981.1
TNF-α	F:GTTGGCTATGGAGGCTGTGT	R:ACCCTCTAAATGGATGGCTG	NM_001124357.1

from the collected intestinal microorganisms using specific primers of the forward sequence 341F (5′-CCTAYGGGRBGCASCAG-3′) and reverse sequence 806R (5′-GGACTACNNGGGTATCTAAT-3′; Sun et al., 2020). The optimized conditions for amplification were as follows: one pre-denaturation cycle at 95°C for 5 min, 27 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 45 s, and a final extension at 72°C for 10 min. The resulting amplicons were purified from 0.7% agarose gel, measured concentration using Qubit dsDNA broad-range assay kit (Life Technologies, United States), and equal concentration (20 ng μl^{-1}) of amplicons were pooled together, and sequenced on the Illumina MiSeq platform (300 bp paired-end reads; Beijing Baimaike Technology Co. Ltd., China).

Intestinal Microbiota Bioinformatics Analysis

Using QIIME (Version 1.8.0, http://qiime.org/), low-quality reads with quality scores <20e were filtered out and raw reads were sorted according to their Barcode sequences (Caporaso et al., 2010). The reads were first cut in poor quality and then separated based on Barcode from the subsequent reads. Through binding the raw reads to the Barcode, the raw reads were then separated from the subsequent reads. To detect chimera, the Barcode and primer sequences were removed from the quality control, and the read sequences were removed using VSearch software (https://github.com/torognes/vsearch/; Li et al., 2015). The chimeric sequences were compared to the species annotation database, and the remaining chimeric sequences were deleted. The chimeric sequences were deleted from the database, leaving only the clean reads. The final correct reads were collected using the Uparse program (Version 7.0.1001, http://www.drive5.com/uparse/) to group all clean reads from all samples (Haas et al., 2011). The sequences were grouped into operative taxonomic units (OTUs) with 97% sequence similarity. The most frequent sequences were chosen as OTUs based on the algorithm (Edgar, 2010). According to the algorithm, the most commonly occurring sequences were chosen as symbolic OTUs. The Mothur system was used to species-annotate the OTUs sequences. Taxon resembling chloroplasts, mitochondria, unknowns, archaea, and eukaryotes were removed. The RDP Classifier Bayesian algorithm (http://sourceforge.net/projects/rdpclassifier/; Wang et al., 2007) was used to cluster the samples into OTUs, as was the GreenGene database[1] for species annotation analysis, and OTUs for abundance. Raw reads were saved to the National Center for Biotechnology Information's (NCBI) Sequence Read Archive (SRA) database (Accession Number: PRJNA714809). At each taxonomic rank, the species abundance, diversity index (Chao1 index, Shannon index and ACE index), and population structure were studied (Lozupone and Knight, 2005).

[1]http://greengenes.lbl.gov/cgibin/nphindex.cgi

Calculations and Statistical Analysis

Weight gain rate (WGR, %) = 100 × (weight gain, g)/(initial weight, g); Survival rate (%) = 100 × (final number of fish)/(initial number of fish); Feed conversion ratio (FCR) = (dry dietary intake, g)/(weight gain, g). Specific growth rate (SGR, % per day) = 100 × [Ln (final weight, g) − Ln (initial weight, g)]/duration (days); Condition factor (CF) = 100 × (body weight, g)/(body length, cm)^3; survival rate (SR, %) = 100 × (final fish number/initial fish number); Daily feed intake (DFI) = (feed consumed, g)/[(initial weight + final weight)/2, g] × (days, d).

All data in tables and figures are expressed as mean ± standard deviation (SD). After normality and homogeneity checking, one-way variance analysis (ANOVA) and Duncan's multiple range tests were used to examine the data. p values <0.05 were considered significantly different. The SPSS statistical package 23.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The sigma plot software version 14.0 is used to draw column graphs and curves.

RESULTS

Growth Performance

The effects of dietary GSH level on weight gain, feed conversion rate, and survival rate are shown in **Table 3**. With increasing dietary GSH level, weight gain rate and specific growth rate gradually increased, reaching the highest level in G400, showing a significant difference with G0 and G100 ($p < 0.05$). The daily feed intake of G200 and G400 was significantly higher than that of the control group ($p < 0.05$). The feed conversion rate decreased with increasing GSH, and G400 was the lowest, which was significantly different from G0 and G100 ($p < 0.05$). The survival rate of G400 was the highest, and significantly higher than that of G0, G100, and G200 ($p < 0.05$). There were no significant differences in condition factor among the groups ($p > 0.05$). According to the relationship between weight gain and dietary GSH levels, the broken line model was selected as a good

TABLE 3 | Growth performances of *O. mykiss* fed the experimental diets (mean ± SD, $n = 3$).

Indices	G0	G100	G200	G400	G800	*p*-values
IBW[1] (g)	4.10 ± 0.12	4.13 ± 0.16	4.15 ± 0.13	4.11 ± 0.13	4.12 ± 0.17	0.991
FBW[2] (g)	19.89 ± 0.35^{a}	20.04 ± 0.40^{a}	20.71 ± 0.16^{ab}	22.05 ± 1.00^{c}	21.74 ± 0.64^{bc}	0.003
WGR[3] (%)	385.45 ± 18.42^{a}	385.62 ± 27.76^{a}	398.93 ± 12.64^{ab}	436.83 ± 17.97^{b}	428.59 ± 33.24^{ab}	0.049
SGR[4] (% d^{-1})	2.82 ± 0.07^{a}	2.82 ± 0.10^{a}	2.87 ± 0.05^{ab}	3.00 ± 0.06^{b}	2.97 ± 0.12^{ab}	0.052
FCR[5]	1.02 ± 0.06^{b}	1.01 ± 0.04^{b}	0.97 ± 0.02^{ab}	0.92 ± 0.01^{a}	0.98 ± 0.04^{ab}	0.083
DFI[6] (g d^{-1})	1.44 ± 0.02^{a}	1.46 ± 0.03^{ab}	1.51 ± 0.01^{b}	1.51 ± 0.06^{b}	1.50 ± 0.03^{ab}	0.035
CF[7]	1.27 ± 0.01	1.28 ± 0.01	1.30 ± 0.03	1.30 ± 0.03	1.29 ± 0.03	0.659
Survival (%)	90.67 ± 3.06^{a}	92.67 ± 3.06^{ab}	92.00 ± 2.00^{ab}	98.00 ± 2.00^{c}	95.33 ± 1.15^{bc}	0.023

Means in the same row with different superscripts are significantly different ($p < 0.05$).
[1]IBW, initial body weight.
[2]FBW, final body weight.
[3]WGR, weight gain rate.
[4]SGR, specific growth rate.
[5]FCR, feed conversion rate.
[6]DFI, daily feed intake.
[7]CF, condition factor.

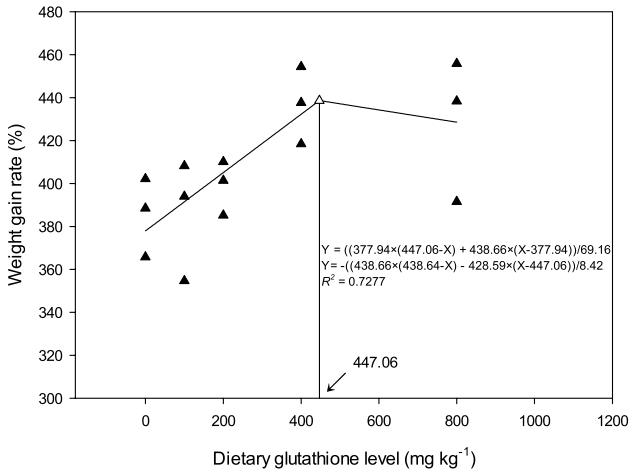


FIGURE 1 | Relationship between weight gain and dietary GSH for *O. mykiss* as described by a broken line regression ($n = 3$). The breakpoint in the broken line is 447.06 mg kg^{-1}.

TABLE 4 | Effects of dietary GSH on body composition of *O. mykiss* (mean ± SD, $n = 12$).

Groups	Moisture (%)	Crude protein (%)	Crude lipid (%)	Ash (%)
G0	74.76 ± 1.05	18.48 ± 0.72	7.25 ± 0.64	2.21 ± 0.13
G100	74.47 ± 0.70	17.82 ± 0.17	6.61 ± 0.95	2.06 ± 0.08
G200	74.25 ± 0.77	18.13 ± 0.47	7.35 ± 0.41	2.28 ± 0.16
G400	73.84 ± 0.66	17.68 ± 0.71	6.59 ± 0.52	2.05 ± 0.08
G800	73.96 ± 0.11	17.93 ± 0.40	7.00 ± 0.72	2.17 ± 0.15
P-values	0.555	0.448	0.536	0.206

fit for the model, and the optimal dietary GSH level was 447.06 mg kg^{-1} (**Figure 1**).

Body Composition

Crude protein, crude lipid, ash, and moisture levels did not change significantly among the groups ($p > 0.05$; **Table 4**).

Antioxidant Capacity

GSH content and GR activity did not vary considerably with increased dietary GSH supplementation (**Table 5**). Compared to the control group, SOD and CAT activities were significantly higher in G200, G400, and G800 ($p < 0.05$). MDA content was significantly lower in G200 than in the other groups ($p < 0.05$).

INTESTINE HISTOLOGY

Table 6 and **Figure 2** show the mid-intestine morphology. No pathological differences in the intestines were found between the different groups. When the dietary GSH level was 0–200 mg kg^{-1}, the height of intestinal muscular layer was not significantly different ($p > 0.05$); however, it was significantly higher in G400 ($p < 0.05$). The intestinal villus height and width were also not significantly different between groups ($p > 0.05$).

Gene Expression

Figure 3 shows the mid-intestine gene expression. Compared to that of the control group, PepT1 and SLC1A5 gene expression in each GSH feeding group were significantly increased ($p < 0.05$); G400 had the highest expression, and there was no significant difference between G100, G200, and G800 ($p > 0.05$). TNF-α, IL-1β, IL-2, and IL-8 gene expressions were significantly reduced than that of the control group, respectively.

Microbial Diversity

The 16S rDNA sequencing produced 1.18 million reads with a mean of 68,310 clean tags per sample. Chloroplast, mitochondria and eukaryotes were removed and reduced the amount of reads by 66.7% to a total of 1.0 million reads. **Table 7** shows the Chao1, ACE, and Shannon indexes. Of all experimental treatments, fish fed a G400 diet had the highest values of Chao1, ACE, and Shannon index. On the phylum level, dietary GSH resulted in reduced Proteobacteria abundance and increased Bacteroidetes,

TABLE 5 | Effects of dietary GSH on the antioxidant capacity of mid-intestine in *O. mykiss* (mean ± SD, $n = 9$).

Groups	SOD/(U mg^{-1} protein)	CAT/(U mg^{-1} protein)	GR/(U mg^{-1} protein)	GSH/(μmol g^{-1} protein)	MDA/(nmol g^{-1} protein)
G0	303.60 ± 16.80^{a}	306.33 ± 12.50^{a}	46.94 ± 2.64	37.88 ± 5.28	0.95 ± 0.03^{c}
G100	320.40 ± 4.33^{a}	328.33 ± 30.35^{ab}	46.04 ± 1.54	36.08 ± 3.09	0.90 ± 0.01^{bc}
G200	384.40 ± 33.13^{b}	391.67 ± 14.22^{d}	46.20 ± 1.74	36.40 ± 3.48	0.77 ± 0.11^{a}
G400	382.80 ± 5.23^{b}	368.33 ± 3.51^{cd}	47.44 ± 0.21	38.88 ± 0.42	0.83 ± 0.03^{ab}
G800	361.20 ± 14.55^{b}	349.00 ± 26.00^{bc}	46.02 ± 0.98	36.04 ± 1.96	0.87 ± 0.01^{bc}
P-values	0.001	0.003	0.771	0.770	0.018

The superscript small letters in the same column mean the significant difference at $p < 0.05$.

TABLE 6 | Effects of dietary GSH on micro-morphology of the intestine of *O. mykiss* (mean ± SD, $n = 9$).

Groups	muscular layer (μm)	villus height (μm)	villus width (μm)
G0	120.94 ± 8.95^{a}	626.04 ± 36.51	208.06 ± 16.52
G100	128.70 ± 4.67^{ab}	634.04 ± 33.48	214.78 ± 21.11
G200	131.04 ± 11.87^{ab}	636.96 ± 87.82	219.98 ± 36.43
G400	142.16 ± 9.44^{b}	685.73 ± 12.11	234.90 ± 53.65
G800	137.58 ± 10.91^{ab}	655.93 ± 50.01	232.47 ± 45.83
P-values	0.020	0.628	0.884

The superscript small letters in the same column mean the significant difference at $p < 0.05$.

Firmicutes, Acideobacteria, and Actinobacteria abundance (**Figure 4**). On the genus level, faeces were mainly composed of *Cetobacterium*, *Nicotiana_otophora*, *Sphingomonas*, *Bacteroides*, *Pseudomonas*, *Bradyrhizobium*, and *Candidatus_ Branchiomona* (**Figure 5**). **Figure 6** depicts a heatmap study of species abundance clustering at the phylum stage. Clustering results showed that the intestinal microbiota composition was classified into three classes (G0, G100; G400, G800; G200).

Principle coordinate analysis (PCoA) illustrated beta diversity. Samples in PCoA using weighted and unweighted UniFrac distances were evenly distributed among the groups (**Figure 7**). The findings revealed that the intestinal microbiota composition of G0 and G100 are similar. However, there was no significant difference in beta diversity using ANOSIM ($R = 0.049$, $p = 0.328$).

Analysis with LefSe noted several indicator bacteria species associated with each diet (**Table 8**). In the faeces, dietary GSH 200–800 mg kg^{-1} increased the abundance of *Ilumatobacter*, *Peptoniphilus*, *Limnobacter*, *Mizugakiibacter*, *Chelatococcus*, *Stella*, *Filimonas*, and *Streptosporangium*, while the control diet had increased abundance of *Ferrovibrio*, *Buchnera*, *Chitinophaga*, *Stenotrophobacter*, *Solimonadaceae*, *Polycyclovorans*, *Rhodococcus*, *Ramlibacter*, *Azohydromonas*, and *Arcobacter*.

Microbial Function

The microbial functions of the intestine were predicted with PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; **Figure 8**). The intestine microbiota was enriched with functions related to carbohydrate metabolism, amino acid metabolism, energy metabolism, the metabolism of cofactors and vitamins, nucleotide metabolism, membrane transport, translation, lipid metabolism, and xenobiotics biodegradation and metabolism. However, the abundant among the groups had little change, and it is speculated that the microbial functions of the intestine were similar.

DISCUSSION

Growth Performance

The results showed that dietary GSH can significantly improve the weight gain rate and survival rate of triploid *O. mykiss*, and reduce the feed conversion rate, which is similar to results obtained for grass carp (Ming et al., 2015) and Atlantic salmon (Ma et al., 2019). GSH can enhance the growth performance of European bass larvae by improving amylase and protease activity (Zambonino-Infante et al., 1997). In a study of Japanese flounder (*Paralichthys olivaceus*), the deamination product of cysteine and hydrolysate of GSH was found to be a component of coenzyme A (Wang et al., 2011). This can also enhance grass carp growth by destroying the growth inhibitory molecules, and promoting the secretion of growth hormone (Xiao and Lin, 2003). It was found that GSH can promote the secretion of pituitary growth hormone and liver insulin-like growth factor-1 level, to promote the synthesis of protein and improve the nutrient utilization rate (Ming et al., 2015). Furthermore, GSH can protect intestinal mucosa from the damage of toxins and peroxides, protect the digestive system, and help the intestinal absorption of nutrients in animals (Aw et al., 1992), which is in line with reports of improving growth and feed conversion rate.

In the present study, the optimal addition of GSH in the feed of triploid *O. mykiss* was 447.06 mg kg^{-1}, which is close to that of grass carp (381 mg kg^{-1}; Ming et al., 2015), but much higher than that of Japanese flounder (368.92 mg·kg^{-1}; Wang et al., 2011) and Nile tilapia (355.13 mg kg^{-1}; Zhou et al., 2013). The differences in these results may be related to the culture environment, species and size, feed formula, feeding strategy, and raw material processing technology. Adding an appropriate amount of GSH to the feed can improve the growth performance, but excessive GSH will result in negative effects. The weight and protein efficiency of yellow catfish (*Pelteobagrus fulvidraco*) first increased and then decreased, and reached a maximum value at 300 mg kg^{-1}, and the high GSH levels possibly had a toxic effect on the fish (Zhou et al., 2017). A low GSH concentration can inhibit lipid peroxidation, whereas a high GSH concentration can promote mitochondrial lipid peroxidation, which is related to the mutual transformation of reduced glutathione and oxidized glutathione in the body (Gao et al., 1998). Moreover, GSH can be synthesized with many compounds,

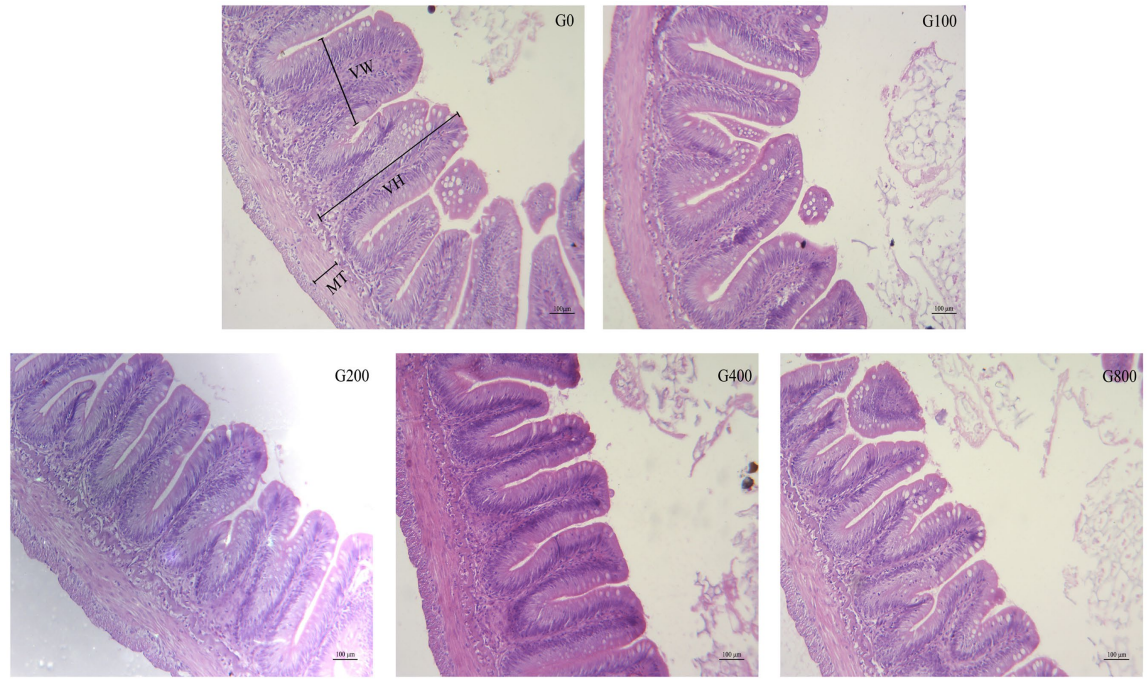


FIGURE 2 | The light micrograph in the triploid *O. mykiss* fed GSH diets. VH, VW and MT represent villus height, villus width and muscular thickness.

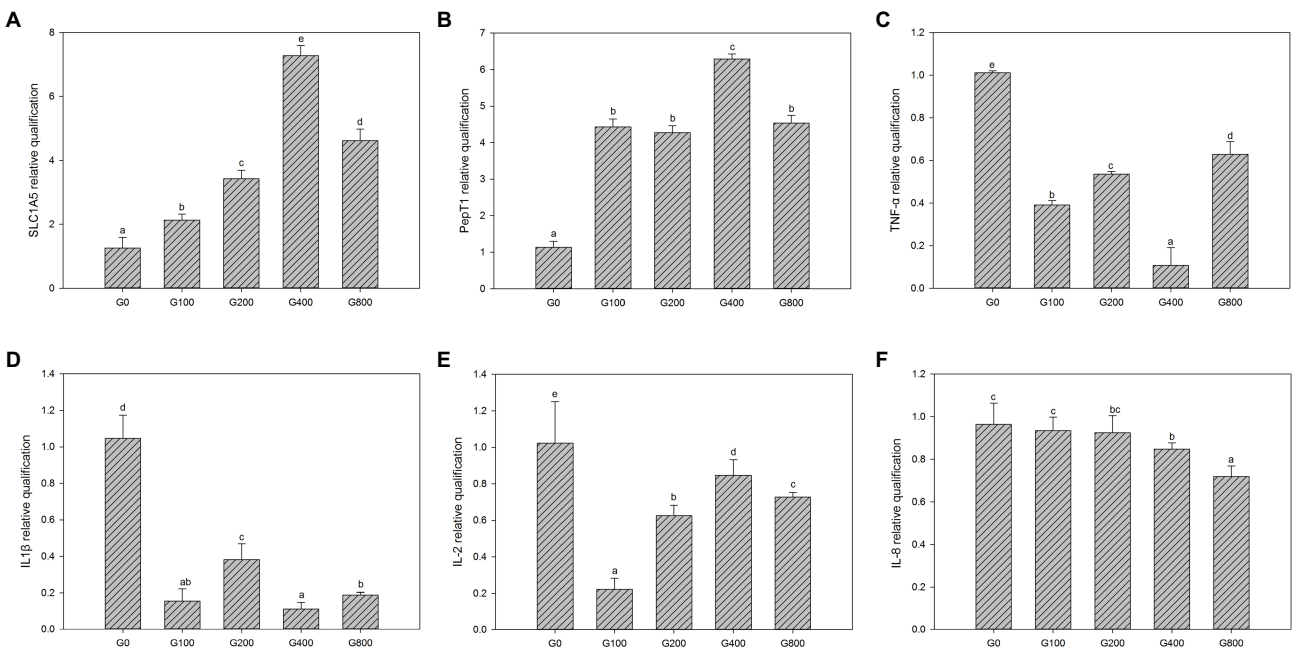


FIGURE 3 | The gene expression of the intestine in each group (mean ± SD, $n = 3$). **(A)** SLC1A5, **(B)** pepT1, **(C)** TNF-α, **(D)** IL-1β, **(E)** IL-2, and **(F)** IL-8 [β-actin was chosen as a reference gene to normalize the results, different letters indicate significant differences between the different groups ($p < 0.05$)].

such as aromatic epoxides, halogenated hydrocarbons, to produce toxic metabolites, which cause DNA damage and have adverse effects on the body (Kleider et al., 2016).

Body Composition

The effects of GSH on fish body composition varied with different species. There was no significantly different effect of GSH on the

TABLE 7 | Effects of dietary GSH on alpha diversity index of intestinal microbiota in *O. mykiss* (mean ± SD, $n = 3$).

Groups	OTU	ACE index	Chao1 index	Shannon index	Coverage(%)
G0	1282.67 ± 658.73	1222.18 ± 91.65^{a}	1052.81 ± 77.73^{a}	6.05 ± 0.04^{a}	99.88
G100	1069.67 ± 189.83	1213.19 ± 48.52^{a}	1246.05 ± 49.31^{a}	6.12 ± 0.13^{ab}	99.85
G200	2572.00 ± 1587.76	3978.54 ± 208.12^{bc}	4007.93 ± 216.42^{cb}	6.76 ± 0.35^{c}	98.84
G400	2881.00 ± 1317.55	4211.87 ± 233.62^{c}	4304.58 ± 205.83^{c}	6.62 ± 0.22^{c}	98.44
G800	2431.33 ± 1022.59	3060.39 ± 137.16^{b}	3139.11 ± 196.50^{b}	6.47 ± 0.03^{bc}	99.41
p-values	0.485	0.032	0.021	0.043	—

The superscript small letters in the same column mean the significant difference at $p < 0.05$.

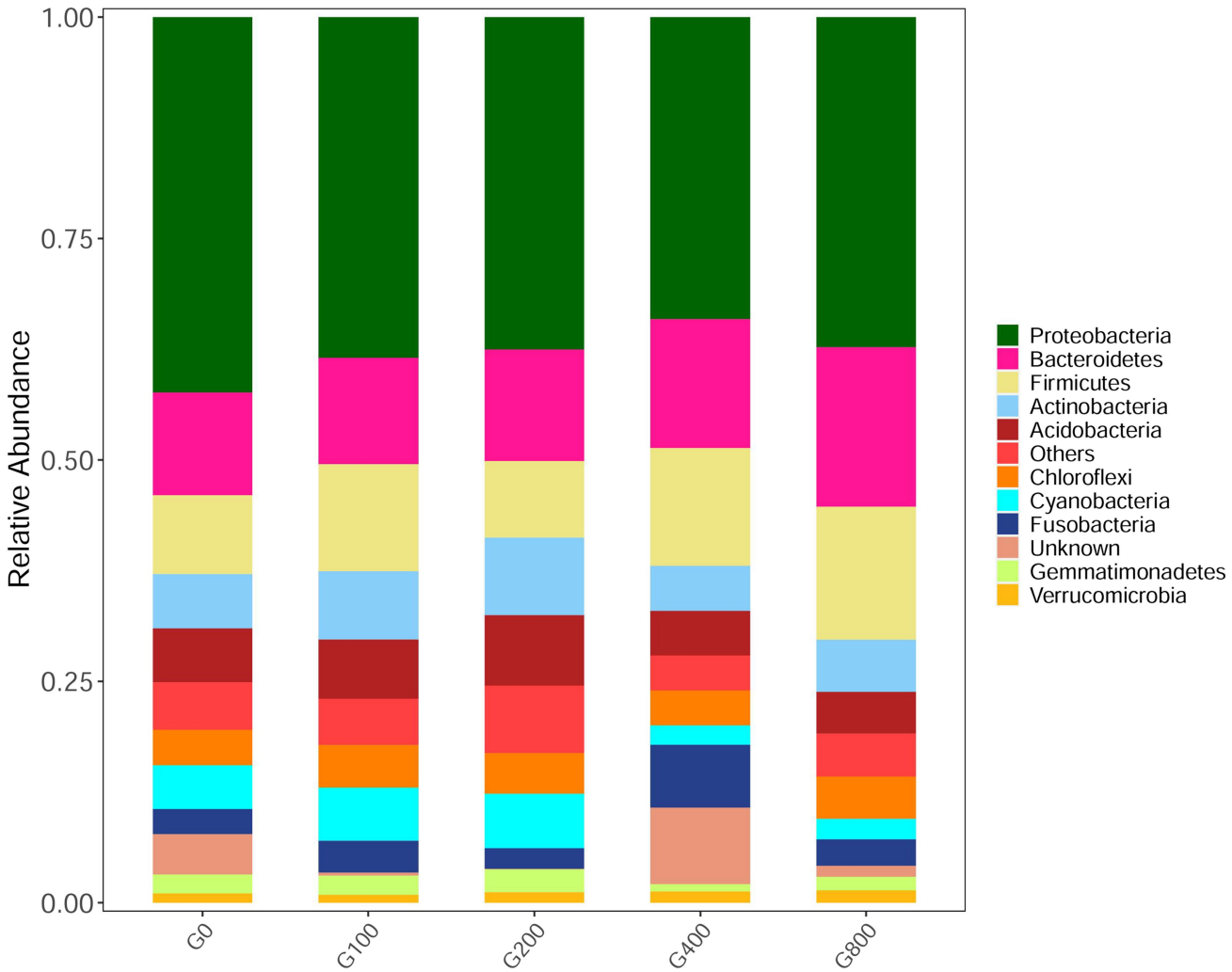


FIGURE 4 | Composition and relative abundance of bacterial community-based 16S rDNA sequences in phylum level ($n = 3$).

dry matter, crude lipid, and ash content of grass carp (Ming et al., 2015). Similarly, there were no significant differences among the groups for body composition in this study. However, crude lipid and protein in yellow catfish were higher than that of the control group (Zhou et al., 2017). This may be related to GSH participating in the transport of amino acids and small peptides, which promotes amino acid absorption (Meister and Anderson, 1983).

Histology

Increased intestinal villus height can enhance digestion and absorption by improving the interaction between the intestine and nutrients (Long et al., 2018). The results in this study were similar to previous reports of Atlantic salmon (Refstie et al., 2000) and meager (*Argyrosomus regius*; Ribeiro et al., 2015). The small peptide can improve the intestinal development of fish, and increase

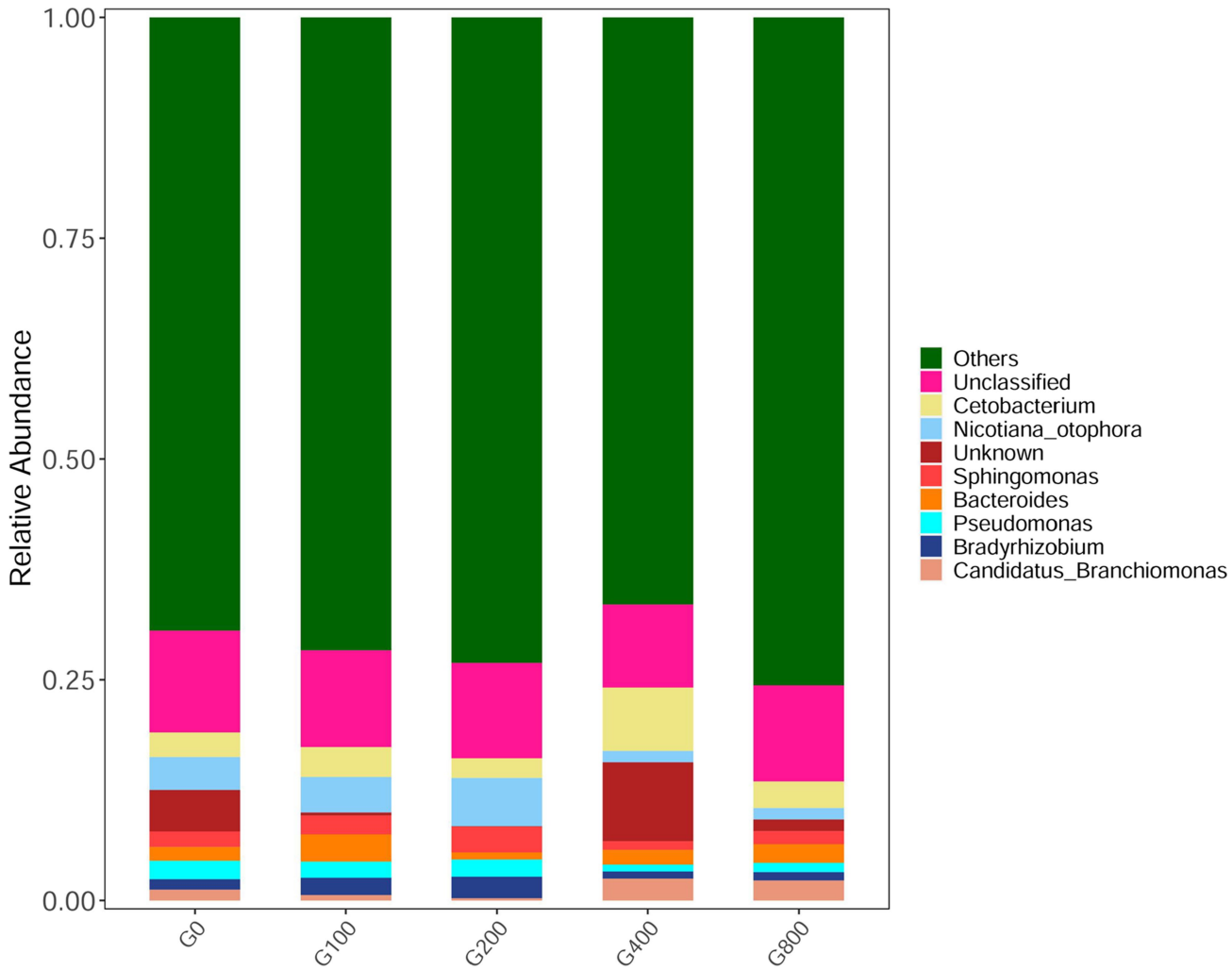


FIGURE 5 | Composition and relative abundance of bacterial community-based 16S rDNA sequences in genus level ($n = 3$).

the height of small intestinal villi (Murashita et al., 2015). It has been reported that adding GSH to the feed of Pacific white shrimp improved intestinal development, promoted intestinal villi growth, and thickened the muscle layer (Wang et al., 2018). It was found that when fish were fed dietary GSH at 400 mg kg^{-1}, the myometrium thickness and intestinal villus height increased significantly. The results showed that GSH could enhance intestinal tract development by affecting the tissue structure of the small intestine, improve the utilization rate of feed nutrition, and enhance the growth and development of triploid *O. mykiss*.

Antioxidant Capacity

In the present study, the SOD, CAT, GSH, and glutathione reductase (GR) activities in the mid-intestine when fish fed GSH were higher than those of the control group. Similarly, the SOD activity also increased as dietary GSH levels increased (Zhou et al., 2012). It has been speculated that exogenous GSH can reduce the oxygen-free radical damage to the body and antioxidant stress of *O. mykiss* (Pena-Llopis et al., 2001). However, the SOD activity of Japanese flounder was not affected by the amount of GSH added to the feed (Wang et al., 2011). It is possible that the effect of GSH on SOD activity is different for different species. With the increase of GSH, CAT activity in tilapia increased first and then decreased; therefore, CAT could possibly eliminate hydrogen peroxide in the body and protect cells from its toxicity (Zhou et al., 2012). The change in CAT activity was due to the activation of the antioxidant enzyme system in the body. In this study, the mid-intestine CAT activity in triploid *O. mykiss* was higher than that of the control group. Therefore, adding appropriate GSH could regulate body antioxidant status, decrease the concentration of hydrogen peroxide in the cells, and reduce the degree of cell damage. In this experiment, the mid-intestine GSH and GR activities in triploid *O. mykiss* are higher than those in the control group.

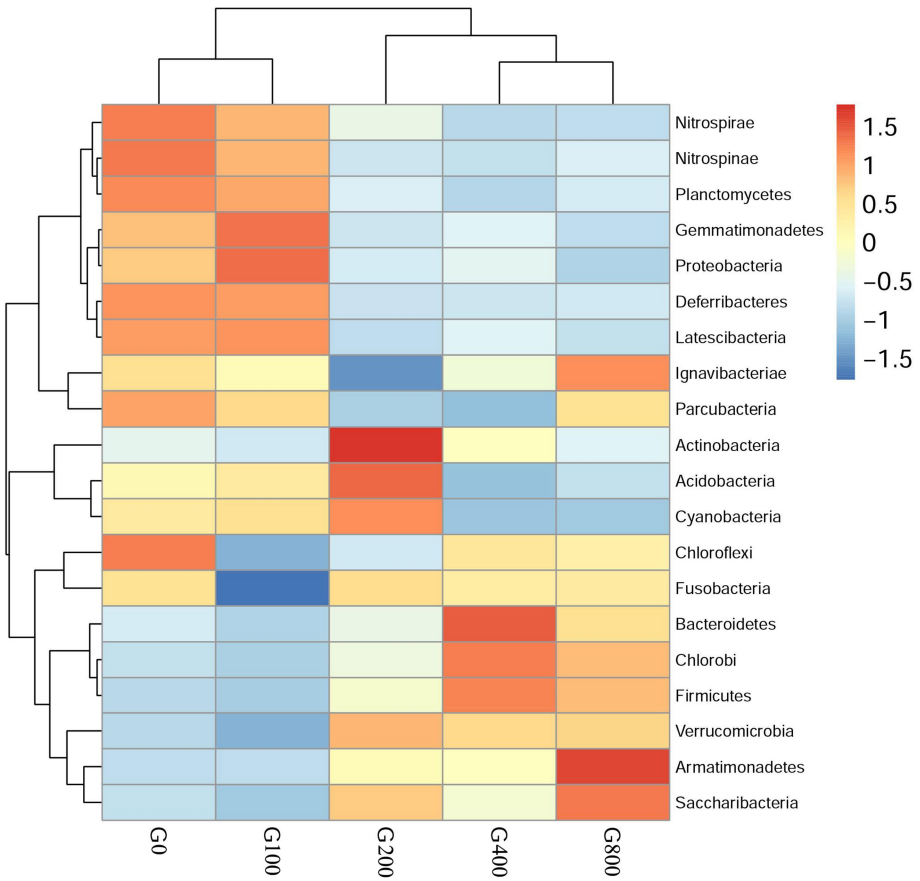


FIGURE 6 | Heatmap analysis of the species abundance clustering in the top 20 on the phylum level (mean ± SD, $n = 3$).

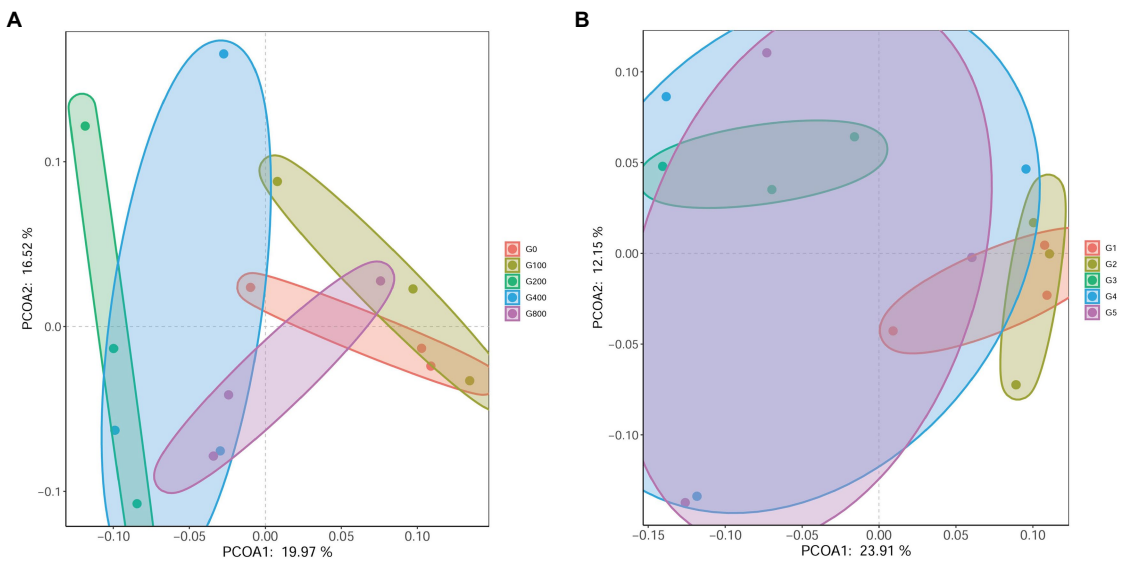


FIGURE 7 | Principle coordinate analysis (PCoA) based on weighted-unifrac **(A)** and unweighted-unifrac **(B)** analysis of bacterial profiles from intestines of *O. mykiss* ($n = 3$).

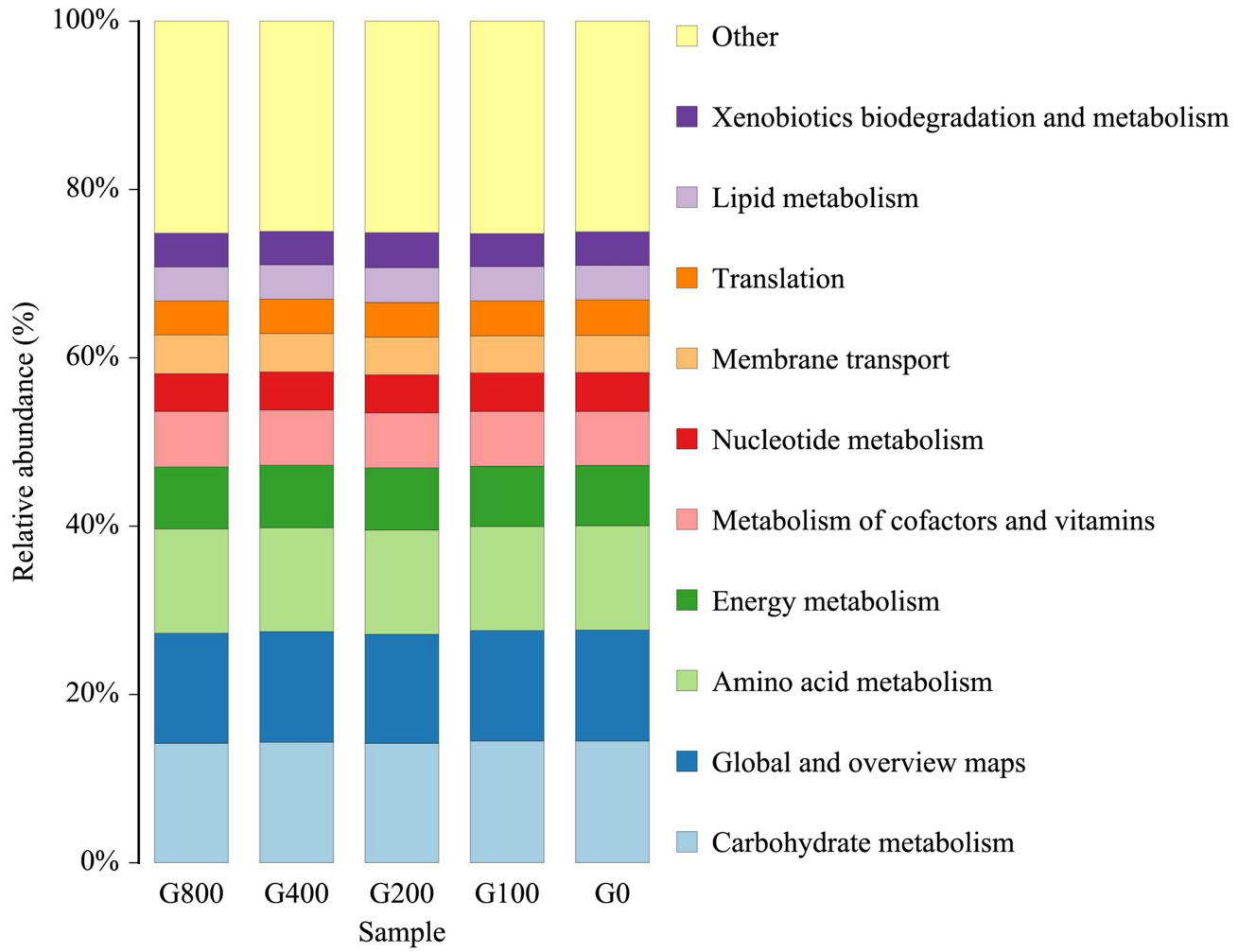


FIGURE 8 | Microbial functions in the top 10 of level-2 for intestine samples (mean ± SD, $n = 3$).

It is speculated that exogenous GSH could activate a reaction and metabolism related to GSH and increase the demand for GSH. Therefore, the GR activity that can convert oxidized GSH into GSH (Ming et al., 2015) also increased accordingly. Furthermore, intestinal MDA content when fish were fed GSH was lower than that of the control group, which is associated with previous study findings (Wang et al., 2011). It is possible that an appropriate amount of GSH could reduce the toxic effect of lipid peroxidation in cells, reducing the degree of cell damage (Birnie-Gauvin et al., 2017).

Gene Expression

When protein ingestion increases, the PepT1 expression in the brush border of the small intestine increases, transport efficiency of small peptides increases, and the use of protein, tripeptides, and free amino acids will be improved through enhanced amino acid uptake efficiency (Ostaszewska et al., 2010). SLC1A5, an amino acid transporter, transports amino acids using the concentration gradient of Na^+ inside and outside the cell membrane and is the most important transporter for cells to absorb exogenous glutamine. Compared to that of the control group, the PepT1 and SLC1A5 gene expression in the intestinal oligopeptide transporter by dietary GSH supplementation increased significantly, suggesting that dietary glutamine utilization was improved, which enhances intestinal development.

Proinflammatory factors are necessary to signal cytokines to initiate and regulate this reaction, which mainly includes tumor necrosis factor-α, interleukin-1β, interleukin-2, and interleukin-8. Interleukin-1β can promote the proliferation and activation of immune cells, such as thymocytes and T cells, and also promote the synthesis and secretion of immune proteins by B cells, and mediate the inflammatory response (Sims and Smith, 2010). Interleukin-2 is primarily generated by activated T cells, which can promote the growth, proliferation, and differentiation of lymphocytes, enhance NK cell function and play an important role in immune response and antiviral infection (Koreth et al., 2011). Interleukin-8 regulates immunity, promoting cell mitosis, and stimulating capillary formation (Hoffmann et al., 2002). Tumor necrosis factor-α is a member of the cytokine

TABLE 8 | Linear discriminant analysis effect size (Lefse) of indicator bacteria species that were significantly ($p < 0.05$) associated with each group.

Groups	Phylum	Family/Genus	LDA	*p*-values
G0	Proteobacteria	*Buchnera*	2.712	0.028
G0	Bacteroidetes	*Chitinophaga*	2.696	0.040
G0	Acidobacteria	*Stenotrophobacter*	2.318	0.019
G0	Proteobacteria	Solimonadaceae	2.312	0.035
G0	Proteobacteria	*Polycyclovorans*	2.259	0.032
G0	Actinobacteria	*Rhodococcus*	2.230	0.035
G0	Proteobacteria	*Ramlibacter*	2.193	0.039
G0	Proteobacteria	*Ferrovibrio*	2.100	0.014
G0	Proteobacteria	*Azohydromonas*	2.056	0.026
G0	Proteobacteria	*Arcobacter*	2.008	0.045
G200	Actinobacteria	*Streptosporangium*	2.021	0.029
G200	Bacteroidetes	*Filimonas*	2.023	0.039
G400	Actinobacteria	*Ilumatobacter*	2.763	0.029
G400	Proteobacteria	*Mizugakiibacter*	2.265	0.034
G400	Proteobacteria	*Chelatococcus*	2.235	0.042
G400	Proteobacteria	*Stella*	2.161	0.039
G800	Proteobacteria	Rhodobacteraceae	3.514	0.042
G800	Firmicutes	*Peptoniphilus*	2.511	0.041
G800	Proteobacteria	*Limnobacter*	2.365	0.043

family, which can induce apoptosis of tumor cells and coordinate non-specific immune response (Paul, 1985), which is produced in the early stage of inflammatory response. When the level of intestinal pro-inflammatory factors increases, it promotes the production of the inflammatory response, causing damage to intestinal mucosa tissue cells, destroying the barrier function of the intestinal epithelium, increasing intestinal permeability, making pathogens and endotoxins enter the blood circulation, causing functional damage to more organs, resulting in a systemic inflammatory response of the body (Costantini et al., 2010). It has been found that GSH can reduce intestinal injury, the expression of intestinal inflammatory factors, incidence of pathogen and endotoxin translocation in the intestinal tract of rats with acute necrotizing pancreatitis, and play a role in protecting the intestinal mucosa (Aw et al., 1992). In addition to interleukin-8, the expression of proinflammatory factors in the intestinal tract in the treatment group was significantly lower than that of the other groups. The mechanism of action may be related to the NF-κB/MLCK pathway inhibition by GSH's nitroso derivatives, to protect the structure and function of closely connected intestinal epithelial cells (Koeberle et al., 2020).

Intestinal Microbiota

Feed composition is a key aspect influencing fish intestinal microbiota (Ringø et al., 2006). In this study, when fish were fed GSH, the Chao1 index, ACE index, and Shannon index increased, which indicated that the abundance and evenness of the intestinal microbiota of triploid *O. mykiss* increased. The abundance of the intestinal microbiota reached a significant level when fish were fed 200–800 mg kg^{-1} GSH. Results have shown that several species with low microbiotal abundance in the intestines could benefit from dietary GSH supplementation. The change in alpha diversity might be related to the antibacterial properties of GSH, and the mechanism needs further study. In the present study, the predominant intestinal microbiota were Proteobacteria, Firmicutes, and Bacteroidetes, which were similar to those of *O. mykiss* (Huyben et al., 2017; Lyons et al., 2017; Huyben et al., 2021). Some studies have shown that Firmicutes and Bacteroides in the intestine are related to fat deposition, and can ferment more short-chain fatty acids, and promote fat deposition when the proportion of Bacteroides increases (Turnbaugh et al., 2006). This study showed that the abundance of Firmicutes/Bacteroides in each group increased, and the proportion of Actinobacteria with the addition of GSH increased compared with that of the control group. Proteobacteria includes many pathogenic bacteria, such as *Escherichia coli*, *Vibrio cholerae*, and *Salmonella enterica* (Holben et al., 2002), which are usually present in the intestines. In this study, although there were no significant differences in beta diversity among the groups, dietary GSH 200–800 mg kg^{-1} increased the abundance of *Ilumatobacter*, *Peptoniphilus*, *Limnobacter*, etc. Moreover, the control diet had an increased abundance of *Arcobacter*, which is responsible for causing diseases in fish. The results in the present study indicated that 200–800 mg kg^{-1} dietary GSH may decrease the probability of triploid *O. mykiss* being infected by pathogenic bacteria. This may be related to the role of GSH in scavenging intracellular peroxides and free radicals, protecting cells from oxidative damage. This would maintain the intestinal mucosal barrier, promoting immune cell proliferation, and inhibiting the expression of pro-inflammatory factors, resulting in increased abundance and homogeneity of microbiota in the intestines of triploid *O. mykiss*. However, the microbial functions of the intestine were similar through the functional analysis. Therefore, the dynamics of the microbiota structure and mechanism of action between the altered microbiota and intestine function are still not well understood and need more research.

CONCLUSION

In conclusion, dietary GSH is beneficial for triploid *O. mykiss* and may be related to the ability of GSH to scavenge peroxides and free radicals in cells, protect cells from oxidative damage, maintain the intestinal mucosal barrier, promote immune cell proliferation, inhibit the expression of the pro-inflammatory factors, and increase the abundance and evenness of intestinal microbiota. The mechanism of interaction is still unclear, and further research is needed. In addition, more studies are required to elucidate the exact regulatory mechanisms involved by which GSH causes these coordinated responses and explore products (e.g., yeast, wheat germ, and animal liver which contains approximately 1–100 mg g^{-1} GSH) or use feed ingredients with high glutathione content appropriately to generate substantial economic returns for the aquaculture industry.

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 at: https://www.ncbi.nlm.nih.gov/genbank/, PRJNA714809.

ETHICS STATEMENT

The animal study was reviewed and approved by Chinese Animal Health Protection Law and the Scientific Laboratory Animal Permit Approval [Ethical Approval No. SCXK(YU)2005-0001]. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

CW, HL and YY designed the study. BS, YL, and SH carried out the rearing work. CW, BS, HJ, and ZL tested the samples and analyzed the results. CW wrote the manuscript with contributions from the other authors. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the Natural Science Funds of Heilongjiang (YQ2019C036), China Agriculture Research System of MOF and MARA (CARS-46), China Scholarship Council (Grant No.202003260012), the Science and Technology Project of Guizhou Province (20162502 and 20162511), the Guizhou Science and Technology Plan Project (QKHZC20172532), the Guizhou Technology Innovation Team Project (QKHRCTD20154016), and the Beijing Sturgeon & Trout Innovation Team (BAIC08-2018).

ACKNOWLEDGMENTS

The authors thank the participants who gave their time to the trial.

REFERENCES

AOAC, 2012. *Official Methods for Analysis*, 19th edn. Association of Official Analytical Chemists, Washington, DC.

Aw, T. Y., Williams, M. W., and Gray, L. (1992). Absorption and lymphatic transport of peroxidized lipids by rat small intestine in vivo: role of mucosal GSH. *Am. J. Physiol. Gastrointest. Liver Physiol.* 262, G99–G106. doi: 10.1152/ajpgi.1992.262.1.G99

Birnie-Gauvin, K., Costantini, D., Cooke, S. J., and Willmore, W. G. (2017). A comparative and evolutionary approach to oxidative stress in fish: a review. *Fish Fish.* 18, 928–942. doi: 10.1111/faf.12215

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Costantini, T. W., Bansal, V., Krzyzaniak, M., Putnam, J. G., Peterson, C. Y., Loomis, W. H., et al. (2010). Vagal nerve stimulation protects against burn-induced intestinal injury through activation of enteric glia cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* 299, G1308–G1318. doi: 10.1152/ajpgi.00156.2010

Deng, J., Mai, K., Chen, L., Mi, H., and Zhang, L. (2015). Effects of replacing soybean meal with rubber seed meal on growth, antioxidant capacity, non-specific immune response, and resistance to Aeromonas hydrophila in tilapia (*Oreochromis niloticus* × *O. aureus*). *Fish Shellfish Immunol.* 44, 436–444. doi: 10.1016/j.fsi.2015.03.018

Doyotte, A., Cossu, C., Jacquin, M., Babut, M., and Vasseur, P. (1997). Antioxidant enzymes, glutathione and lipid peroxidation as relevant biomarkers of experimental or field exposure in the gills and the digestive gland of the freshwater bivalve *Unio tumidus*. *Aquat. Toxicol.* 39, 93–110. doi: 10.1016/S0166-445X(97)00024-6

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Fjelldal, P. A., Hansen, T. J., Lock, E. J., Wargelius, A., Fraser, T., Sambraus, F., et al. (2016). Increased dietary phosphorous prevents vertebral deformities in triploid Atlantic salmon (*Salmo salar* L.). *Aquac. Nutr.* 22, 72–90. doi: 10.1111/anu.12238

Gao, S., Liu, D., and Luo, G. (1998). ESR study on the mechanism of anti-lipid peroxidation of antioxidants. *Chin. J. Magn. Reson.* 15, 139–143.

Good, C., and Davidson, J. (2016). A review of factors influencing maturation of Atlantic salmon, *Salmo salar*, with focus on water recirculation aquaculture system environments. *J. World Aquacult. Soc.* 47, 605–632. doi: 10.1111/jwas.12342

Haas, B. J., Gevers, D., Earl, A. M., Feldgarden, M., Ward, D. V., Giannoukos, G., et al. (2011). Chimeric 16S rRNA sequence formation and detection in sanger and 454-pyrosequenced PCR amplicons. *Genome Res.* 21, 494–504. doi: 10.1101/gr.112730.110

Hoffmann, E., Dittrich Breiholz, O., Holtmann, H., and Kracht, M. (2002). Multiple control of interleukin-8 gene expression. *J. Leukoc. Biol.* 47, 605–632. doi: 10.1111/jwas.12342

Holben, W. E., Williams, P., Saarinen, M., Särkilahti, L. K., and Apajalahti, J. H. (2002). Phylogenetic analysis of intestinal microflora indicates a novel mycoplasma phylotype in farmed and wild salmon. *Microb. Ecol.* 44, 175–185. doi: 10.1007/s00248-002-1011-6

Huyben, D., Chiasson, M., Lumsden, J. S., Pham, P. H., and Chowdhury, M. A. K. (2021). Dietary microencapsulated blend of organic acids and plant essential oils affects intestinal morphology and microbiome of rainbow trout (*Oncorhynchus mykiss*). *Microorganisms* 9:2063. doi: 10.3390/microorganisms9102063

Huyben, D., Nyman, A., Vidaković, A., Passoth, V., Moccia, R., Kiessling, A., et al. (2017). Effects of dietary inclusion of the yeasts *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* on gut microbiota of rainbow trout. *Aquaculture* 473, 528–537. doi: 10.1016/j.aquaculture.2017.03.024

Kelly, C., and Salinas, I. (2017). Under pressure: interactions between commensal microbiota and the teleost immune system. *Front. Immunol.* 8:559. doi: 10.3389/fimmu.2017.00559

Kleider, C., Esch, H. L., and Lehmann, L. (2016). Reactivity against DNA bases despite previous conjugation to glutathione–A new mechanism of toxicity for α, β-unsaturated carbonyls? *Toxicol. Lett.* 258:S254. doi: 10.1016/j.toxlet.2016.06.1899

Koeberle, S. C., Gollowitzer, A., Laoukili, J., Kranenburg, O., Werz, O., Koeberle, A., et al. (2020). Distinct and overlapping functions of glutathione peroxidases 1 and 2 in limiting NF-κB-driven inflammation through redox-active mechanisms. *Redox Biol.* 28:101388. doi: 10.1016/j.redox.2019.101388

Koreth, J., Matsuoka, K., Kim, H. T., McDonough, S. M., Bindra, B., Alyea, E. P. III, et al. (2011). Interleukin-2 and regulatory T cells in graft-versus-host disease. *New Engl. J. Med.* 365, 2055–2066. doi: 10.1056/NEJMoa1108188

Kosower, N. S., and Kosower, E. M. (1978). The glutathione status of cells. *Int. Rev. Cytol.* 54, 109–160. doi: 10.1016/S0074-7696(08)60166-7

Li, S., Zhang, C., Gu, Y., Chen, L., Ou, S., Wang, Y., et al. (2015). Lean rats gained more body weight than obese ones from a high-fibre diet. *Brit. J. Nutr.* 114, 1188–1194. doi: 10.1017/S0007114515002858

Liu, B., Ge, X., He, Y., Xie, J., Xu, P., He, Y., et al. (2010). Effects of anthraquinones extracted from Rheum officinale Bail on the growth, non-specific immune response of Macrobrachium rosenbergii. *Aquaculture* 310, 13–19. doi: 10.1016/j.aquaculture.2010.09.020

Long, M., Yang, S., Li, P., Song, X., Pan, J., He, J., et al. (2018). Combined use of *C. butyricum* Sx-01 and *L. salivarius* C-1-3 improves intestinal health and reduces the amount of lipids in serum via modulation of gut microbiota in mice. *Nutrients* 10:810. doi: 10.3390/nu10070810

Lozupone, C., and Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71, 8228–8235. doi: 10.1128/AEM.71.12.8228-8235.2005

Lyons, P. P., Turnbull, J. F., Dawson, K. A., and Crumlish, M. (2017). Exploring the microbial diversity of the distal intestinal lumen and mucosa of farmed

rainbow trout *Oncorhynchus mykiss* (Walbaum) using next generation sequencing (NGS). *Aquac. Res.* 48, 77–91. doi: 10.1111/are.12863

Ma, J., Zhang, J., Sun, G., Lou, Y., and Li, Y. (2019). Effects of dietary reduced glutathione on the growth and antioxidant capacity of juvenile Atlantic salmon (*Salmo salar*). *Aquac. Nutr.* 25, 1028–1035. doi: 10.1111/anu.12920

Meister, A., and Anderson, M. E. (1983). Glutathione. *Annu. Rev. Biochem.* 52, 711–760. doi: 10.1146/annurev.bi.52.070183.003431

Ming, J., Ye, J., Zhang, Y., Xu, P., and Xie, J. (2015). Effects of dietary reduced glutathione on growth performance, non-specific immunity, antioxidant capacity and expression levels of IGF-I and HSP70 mRNA of grass carp (*Ctenopharyngodon idella*). *Aquaculture* 438, 39–46. doi: 10.1016/j.aquaculture.2014.12.038

Murashita, K., Fukada, H., Takahashi, N., Hosomi, N., Matsunari, H., Furuita, H., et al. (2015). Effect of feed ingredients on digestive enzyme secretion in fish. *Bull. Fish. Res. Agency* 40, 69–74.

Ostaszewska, T., Dabrowski, K., Kamaszewski, M., Grochowski, P., Verri, T., Rzepkowska, M., et al. (2010). The effect of plant protein-based diet supplemented with dipeptide or free amino acids on digestive tract morphology and PepT1 and PepT2 expressions in common carp (*Cyprinus carpio* L.). *Comp. Biochem. Physiol. A* 157, 158–169. doi: 10.1016/j.cbpa.2010.06.162

Paul, W. E. (1985). Fundamental immunology. *Fundam. Immun.* 14, 549–550. doi: 10.3109/08820138509022683

Pena-Llopis, S., Pena, J. B., Sancho, E., Fernandez-Vega, C., and Ferrando, M. D. (2001). Glutathione-dependent resistance of the European eel *Anguilla anguilla* to the herbicide molinate. *Chemosphere* 45, 671–681. doi: 10.1016/S0045-6535(00)00500-2

Ponsoda, X., Bort, R., Jover, R., Gómez-Lechón, M. J., and Castell, J. V. (1999). Increased toxicity of cocaine on human hepatocytes induced by ethanol: role of GSH. *Biochem. Pharmacol.* 58, 1579–1585. doi: 10.1016/S0006-2952(99)00249-X

Raghunathan, V. K., Ellis, E. M., Tettey, J. N., and Grant, M. H. (2007). Involvement of reduced glutathione and glutathione reductase in the chronic toxicity of hexavalent chromium to monocytes in vitro. *Toxicology* 231, 105–106. doi: 10.1016/j.tox.2006.11.030

Refstie, S., Korsøen, Ø. J., Storebakken, T., Baeverfjord, G., Lein, I., and Roem, A. J. (2000). Differing nutritional responses to dietary soybean meal in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). *Aquaculture* 190, 49–63. doi: 10.1016/S0044-8486(00)00382-3

Ren, J., Wu, P., Trampe, B., Tian, X., Lübberstedt, T., and Chen, S. (2017). Novel technologies in doubled haploid line development. *Plant Biotechnol. J.* 15, 1361–1370. doi: 10.1111/pbi.12805

Ribeiro, L., Moura, J., Santos, M., Colen, R., Rodrigues, V., Bandarra, N., et al. (2015). Effect of vegetable based diets on growth, intestinal morphology, activity of intestinal enzymes and haematological stress indicators in meagre (*Argyrosomus regius*). *Aquaculture* 447, 116–128. doi: 10.1016/j.aquaculture.2014.12.017

Ringø, E., Sperstad, S., Myklebust, R., Refstie, S., and Krogdahl, Å. (2006). Characterisation of the microbiota associated with intestine of Atlantic cod (*Gadus morhua* L.): the effect of fish meal, standard soybean meal and a bioprocessed soybean meal. *Aquaculture* 261, 829–841. doi: 10.1016/j.aquaculture.2006.06.030

Rombout, J. H., Abelli, L., Picchietti, S., Scapigliati, G., and Kiron, V. (2011). Teleost intestinal immunology. *Fish Shellfish Immunol.* 31, 616–626. doi: 10.1016/j.fsi.2010.09.001

Sims, J. E., and Smith, D. E. (2010). The IL-1 family: regulators of immunity. *Nat. Rev. Immunol.* 10, 89–102. doi: 10.1038/nri2691

Sun, X., Li, M., Xia, L., Fang, Z., Yu, S., Gao, J., et al. (2020). Alteration of salivary microbiome in periodontitis with or without type-2 diabetes mellitus and metformin treatment. *Sci. Rep.* 10, 15363–15314. doi: 10.1038/s41598-020-72035-1

Thorgaard, G. H. (1983). 8 chromosome set manipulation and sex control in fish. *Fish Physiol.* 9, 405–434. doi: 10.1016/S1546-5098(08)60308-8

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., and Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444, 1027–1031. doi: 10.1038/nature05414

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Wang, X., Xu, W., Zhou, H., Zhang, Y., Gao, W., Zhang, W., et al. (2018). Reduced glutathione supplementation in practical diet improves the growth, anti-oxidative capacity, disease resistance and gut morphology of shrimp *Litopenaeus vannamei*. *Fish Shellfish Immunol.* 73, 152–157. doi: 10.1016/j.fsi.2017.11.043

Wang, F., Zhang, W., Mai, K., Xu, W., Wang, X., and Liufu, Z. (2011). Effects of dietary reduced glutathione on growth and antioxidative capability of Japanese flounder, *Paralichthys olivaceus*. *Period. Ocean Univ. China* 4, 55–60. doi: 10.3969/j.issn.1672-5174.2011.04.008

Will, Y. (1999). Overview of glutathione function and metabolism. *Curr. Protoc. Toxicol.* 1, 1–6. doi: 10.1002/0471140856.tx0601s00

Xia, Z., and Wu, S. (2018). Effects of glutathione on the survival, growth performance and non-specific immunity of white shrimps (*Litopenaeus vannamei*). *Fish Shellfish Immunol.* 73, 141–144. doi: 10.1016/j.fsi.2017.12.015

Xiao, D., and Lin, H. (2003). Effects of cysteamine—a somatostatin-inhibiting agent—on serum growth hormone levels and growth in juvenile grass carp (*Ctenopharyngodon idellus*). *Comp. Biochem. Physiol. A* 134, 93–99. doi: 10.1016/S1095-6433(02)00191-5

Zambonino-Infante, J. L., Cahu, C. L., and Peres, A. (1997). Partial substitution of di-and tripeptides for native proteins in sea bass diet improves *Dicentrarchus labrax* larval development. *J. Nutr.* 127, 608–614. doi: 10.1093/jn/127.4.608

Zhou, T., Cao, J., Huang, Y., Wang, G., Zhao, H., Sun, Z., et al. (2012). Effects of dietary glutathione on the mRNA expression of growth and antioxidant related genes in liver of GIFT *Oreochromis niloticus*. *Guang. Agric. Sci.* 14, 152–155. doi: 10.3969/j.issn.1004-874X.2012.14.045

Zhou, W., Liang, Q., Xu, Y., Gurr, G. M., Bao, Y., Zhou, X., et al. (2013). Genomic insights into the glutathione S-transferase gene family of two rice planthoppers, *Nilaparvata lugens* (Stål) and *Sogatella furcifera* (Horváth) (Hemiptera: Delphacidae). *PLoS One* 8:e56604. doi: 10.1371/journal.pone.0056604

Zhou, Y., Sun, Y., Cao, J., Huang, Y., Wang, G., Chen, B., et al. (2017). Effects of dietary glutathione on growth performance, body composition, serum biochemical indices and anti-ammonia-nitrogen stress ability of juvenile yellow catfish (*Pelteobagrus fulvidraco*). *Chin. J. Ani. Nutr.* 29, 4189–4197. doi: 10.3969/j.issn.1006-267x.2017.11.043

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: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Physiology

ORIGINAL RESEARCH
published: 28 April 2022
doi: 10.3389/fphys.2022.877178

Betaine Regulates the Production of Reactive Oxygen Species Through Wnt10b Signaling in the Liver of Zebrafish

Ao Li[1], Yaqi Gu[1], Xiuzhen Zhang[1], Hairui Yu[2], Dongwu Liu[1,3] and Qiuxiang Pang[1]**

[1]Anti-Aging & Regenerative Medicine Research Institution, School of Life Sciences and Medicine, Shandong University of Technology, Zibo, China, [2]College of Biological and Agricultural Engineering, Weifang Bioengineering Technology Research Center, Weifang University, Weifang, China, [3]School of Agricultural Engineering and Food Science, Shandong University of Technology, Zibo, China

When fish are under oxidative stress, levels of reactive oxygen species (ROS) are chronically elevated, which play a crucial role in fish innate immunity. In the present study, the mechanism by which betaine regulates ROS production *via* Wnt10b/β-catenin signaling was investigated in zebrafish liver. Our results showed that betaine enrichment of diet at 0.1, 0.2 and 0.4 g/kg induced Wnt10b and β-catenin gene expression, but suppressed GSK-3β expression in zebrafish liver. In addition, the content of superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH) was decreased by all of the experimental betaine treatments. However, betaine enrichment of diet at 0.1, 0.2 and 0.4 g/kg enhanced gene expression and activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) and catalase (CAT) in zebrafish liver. In addition, Wnt10b RNA was further interfered in zebrafish, and the results of Wnt10b RNAi indicated that Wnt10b plays a key role in regulating ROS production and antioxidant enzyme activity. In conclusion, betaine can inhibit ROS production in zebrafish liver through the Wnt10b/β-catenin signaling pathway.

Keywords: betaine, Wnt10b, β-catenin, reactive oxygen species, zebrafish

OPEN ACCESS

Edited by:
Mohamed Salah Azaza, National Institute of Marine Science and Technology, Tunisia

Reviewed by:
Ines Ben Khemis, Institut National des Sciences et Technologies de la Mer, Tunisia
Feriel Ghribi, Tunis El Manar University, Tunisia

****Correspondence:***
Dongwu Liu
liudongwu@sdut.edu.cn
Qiuxiang Pang
pangqiuxiang@sdut.edu.cn

Specialty section:
This article was submitted to Aquatic Physiology, a section of the journal Frontiers in Physiology

Received: *16 February 2022*
Accepted: *17 March 2022*
Published: *28 April 2022*

Citation:
Li A, Gu Y, Zhang X, Yu H, Liu D and Pang Q (2022) Betaine Regulates the Production of Reactive Oxygen Species Through Wnt10b Signaling in the Liver of Zebrafish. Front. Physiol. 13:877178. doi: 10.3389/fphys.2022.877178

1 INTRODUCTION

Due to various stress factors in the aquatic environment, fish are prone to stimulate inflammatory responses (Saurabh and Sahoo, 2008; Troncoso, et al., 2012). In addition, the antioxidant status is closely related to the innate immunity of fish species (Kuang, et al., 2012; Tort, et al., 2003). Generally speaking, the existence of various antioxidant enzymes, the generation and elimination of ROS in fish are in a state of dynamic equilibrium. However, if the homeostasis is disrupted, excess ROS can be produced in fish tissues. Excessive production of ROS is closely related to lipid peroxidation, cellular damage and protein oxidation (Cooke, et al., 2003; Martínez-Álvarez, et al., 2005; Maynard, et al., 2009). Furthermore, high levels of ROS exacerbate oxidative stress and induce the production of pro-inflammatory cytokines (Naik and Dixit, 2011). To protect animals from ROS-mediated damage, various antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) and catalase (CAT), play key roles in regulating the balance of ROS levels (Martínez-Álvarez, et al., 2005).

TABLE 1 | Composition and proximate analysis of the experimental diets.

Items	Betaine level (g/kg diet)			
	0	0.1	0.2	0.4
Ingredients (g/kg diet)				
Casein	420	420	420	420
Gelatin	105	105	105	105
Dextrin	190	190	190	190
Lard oil	83	83	83	83
Linseed oil	17	17	17	17
Cellulose	100	99.9	99.8	99.6
Sodium carboxymethylcellulose	20	20	20	20
Vitamin premix[a]	10	10	10	10
Mineral premix[b]	40	40	40	40
$Ca_2(H_2PO_4)_2$	10	10	10	10
Choline chloride	5	5	5	5
Betaine	0	0.1	0.2	0.4
Total	1,000	1,000	1,000	1,000
Proximate composition				
Moisture (g/kg diet)	97.4	98.2	96.6	98.1
Crude protein (g/kg diet)	480.6	481.2	482.6	482.4
Crude lipid (g/kg diet)	103.4	102.9	104.5	103.5
Crude ash (g/kg diet)	61.2	63.2	63.8	63.2

Notes.

[a]*Vitamin premix contained (mg/g mixer) thiamin hydrochloride, 5 mg; riboflavin, 5 mg; calcium pantothenate, 10 mg; nicotic acid, 6.05 mg; L-ascorbyl-2-monophosphate-Mg, 3.95 mg; alpha-tocopherol acetate, 50 mg; pyridoxine hydrochloride, 4 mg; folic acid, 1.5 mg; inositol, 200 mg; menadione, 4 mg; retinyl acetate, 60 mg; biotin, 0.6 mg. All ingredients were diluted with alpha-cellulose to 1 g.*

[b]*Mineral premix contained (g/kg diet) calcium biphosphate, 13.58 g; calcium lactate, 32.7 g; $FeSO_4{\cdot}6H_2O$, 2.97 g; magnesium sulfate, 13.7 g; potassium phosphate dibasic, 23.98 g; sodium biphosphate, 8.72 g; sodium chloride, 4.35 g; $AlCl_3{\cdot}6H_2O$, 0.015 g; KI, 0.015 g; $CuCl_2$, 0.01 g; $MnSO_4{\cdot}H_2O$, 0.08 g; $CoCl_2{\cdot}6H_2O$, 0.1 g; $ZnSO_4{\cdot}7H_2O$, 0.3 g.*

The chemical structural formula of betaine is $(CH_3)_3N^+–CH_2COO^-$, which is a naturally occurring metabolite in animals (Craig, 2004). In addition, betaine, a by-product of sugar beet processing, is used commercially as a feed additive in aquaculture fish feeds (Eklund, et al., 2005). Today, betaine is widely used as an attractant in aquaculture. Moreover, betaine plays an important role in regulating cellular osmotic pressure (Nakanishi, et al., 1990). Previously, betaine was observed to accumulate in mammalian renal medulla cells and chicken fibroblasts under osmotic stress, thereby functioning to regulate osmotic pressure and monitor water content (Bagnasco, et al., 1986; Eklund, et al., 2005; Kidd, et al., 1997; Petronini, et al., 1992). In addition, betaine reduces the osmotic pressure of Atlantic salmon and improves the ability to maintain osmotic balance (Virtanen, et al., 1989). Addition of betaine to fish feed significantly improves feed utilization, survival, and fish growth (Craig, 2004; Liang, et al., 2001; Luo, et al., 2011; Wu and Davis, 2005).

Cytoplasmic accumulation of β-catenin is a decisive event in cells in the canonical Wnt signaling pathway. In addition, the molecule GSK-3β is involved in the regulation of cytoplasmic β-catenin levels (Logan and Nusse, 2004; Veeman, et al., 2003; Zeng, et al., 1997). After the Wnt molecule binds to the receptor complex on the cell membrane, the activity of GSK-3β decreases and β-catenin accumulates, which further regulates the expression of target genes (Bhanot, et al., 1996; Molenaar, et al., 1996). In a previous study, it was observed that the Wnt/β-catenin signaling pathway is involved in the regulation of oxidative stress in MC3T3-E1 cells (Qi, et al., 2016).

Under oxidative stress, ROS production in animals is chronically increased, disrupting cellular metabolism and fish immunity (Lushchak, 2014). Previously, betaine has been shown to have antioxidant properties in a variety of animals (Doğru-Abbasoğlu, et al., 2018; Heidari, et al., 2018; Wen, et al., 2019). However, the regulatory mechanism of betaine on ROS production *via* Wnt10b signaling remains unknown in fish species. Previous studies have shown that betaine increases stress resistance, and the Wnt/β-catenin signaling pathway plays a role in regulating oxidative stress (Kim, et al., 2014; Li, et al., 2019; Qi, et al., 2016; Zhang, et al., 2018). It is interesting to examine whether betaine regulates ROS production in fish through the Wnt10b signaling pathway. The aim of this study was to investigate the mechanism by which betaine modulates ROS production *via* Wnt10b/β-catenin signaling in zebrafish liver.

2 MATERIALS AND METHODS

2.1 Diet and Animals

Betaine was purchased from Sunwin Biotechnology Co., Ltd. (Weifang, China). Casein and gelatin were used as protein sources. Four isoenergetic and isonitrogenic diets containing 0, 0.1, 0.2, and 0.4 g/kg betaine were prepared according to **Table 1**. The ingredients were ground into powder then passed through a 120 μm sieve, and mixed with lard and linseed oil. Then, the appropriate amount of water (300 ml/kg dry ingredient) was added to the ingredients and blended with a tablet machine to form flakes. Finally, the flakes were dried in a ventilated oven at 40°C for 10 h, pulverized, sieved into small particles (200–300 μm), and stored at −20°C for later use.

Male zebrafish (AB strain, ~3.2 cm) were cultured in 3.0 L flow-through glass jars with circulating dechlorinated water at 28°C on a 14 h light:10 h dark cycle. The fish were then fed ad libitum twice a day at 8:00 and 18:00 with commercial feed obtained from Sanyou Landscaping Feed Technology Co., Ltd. (Beijing, China) for 2 weeks of acclimation. Two different experimental rearings were performed, the first involving betaine levels in the experimental diet and the second involving Wnt10b RNA interference.

2.2 Animals Treatments

First, experimental feeding on the four betaine level diets was performed as follows. 180 male zebrafish were assigned to 12 tanks (15 fish per tank) and each betaine level (0, 0.1, 0.2 and 0.4 g/kg betaine) was distributed into three tanks. The fish were fed ad libitum twice a day at 8:00 and 18:00. 6 weeks later, after zebrafish were anesthetized with 0.1 g/L MS 222, a liver sample was collected from five fish per tank for gene expression analysis. In addition, another liver sample was collected from five fish in each tank for biochemical indicator analysis. Three replicates were used for each betaine level treatment group.

TABLE 2 | PCR primers for Wnt10b RNA interference.

PCR primers	Forward (5′-3′)	Reverse (5′-3′)
ORF	CAATGACATCCTCGGCCTGAAG	TCACTTGCACACATTAACCCACTC
dsRNA	GATCACTAATACGACTCACTATAGGGGAGACCAGCGCTGGAACTGCTC	GATCACTAATACGACTCACTATAGGGCCACTCTGTTATTACGAATCC

TABLE 3 | Real-time quantitative PCR primers for genes of zebrafish.

Target gene	Forward (5′-3′)	Reverse (5′-3′)	Size (bp)	GenBank
Wnt10b	TCCTGAAACAGGCTCGAAGT	GCTGCTCACTTGCACACATT	112	AY182171.1
GSK-3β	TCTGCTCACCGTTTCCTTTC	CTCCGACCCACTTAACTCCA	115	NM_131381.1
β-catenin	GGAGCTCACCAGCTCTCTGT	TAGCTTGGGTCGTCCTGTCT	120	NM_001001889.1
CAT	CAAGGTCTGGTCCCATAAA	TGACTGGTAGTTGGAGGTAA	227	BC051626
SOD	GTCCGCACTTCAACCCTCA	TCCTCATTGCCACCCTTCC	217	BC055516
GSH-PX	AGATGTCATTCCTGCACACG	AAGGAGAAGCTTCCTCAGCC	94	AW232474
β-actin	CCGTGACATCAAGGAGAAGC	TACCGCAAGATTCCATACCC	194	AF057040.1

2.3 Wnt10b RNA Interference

First, the Wnt10b gene (AY182171.1) was cloned according to the primers listed in **Table 2**. Then, double-stranded RNA (dsRNA) was synthesized using the primers listed in **Table 2** according to the previous method (Arockiaraj, et al., 2014). Experimental rearing for Wnt10b RNA interference (Wnt10b RNAi) was performed as follows. After 2 weeks of acclimation, 90 male zebrafish (~3.8 cm) were allocated to six tanks for Wnt10b RNAi. For the RNAi group (3 tanks), fish were intraperitoneally injected with 500 ng of dsRNA diluted in DEPEC-treated water, while fish in the control group (3 tanks) were intraperitoneally injected with 5 μl of DEPEC-treated water. Then, the two groups of zebrafish were fed ad libitum a diet containing 0.4 g/kg betaine at 8:00 and 18:00 every day. After 6 days, the fish were sampled for analysis as the betaine treatment experiments.

2.4 Assay for Liver Level of Reactive Oxygen Species and Antioxidant Enzyme Activity

Liver samples were homogenized and centrifuged at 5,000 ×g for 10 min at 4°C, and supernatants were collected for biochemical analysis. The supernatant ·OH, H_2O_2, $O_2^{\cdot-}$ levels, and CAT, SOD, GSH-PX activity were measured using commercial kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The protein concentration in the supernatant was detected at 595 nm according to the Bradford method (Bradford, 1976). SOD activity was measured at 550 nm by measuring inhibition of the reduction rate of cytochrome c, and CAT activity was measured by analyzing residual H_2O_2 absorbance at 405 nm. Activity of GSH-PX was detected by analyzing the rate of NADPH oxidation at 412 nm. In addition, since H_2O_2 can form a stable complex with ammonium molybdate, the content of H_2O_2 was detected at 405 nm. The production of ·OH was detected according to the production of hydrogen peroxide, and the production of $O_2^{\cdot-}$ was measured at 550 nm by the xanthine oxidase method.

2.5 RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction

The primer sequences for various target genes and reference gene (β-actin) were shown in **Table 3**. Subsequently, real-time PCR was performed in a quantitative thermal cycler (ROCHE, Lightcycler 480, Switzerland) using SYBR® Premix Ex Taq™ II (Takara, Japan). The program for quantitative RT-PCR was as follows: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. Finally, the relative gene expression level was detected by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.6 Statistical Analysis

Results were expressed as mean ± standard error of the mean (sem). SPSS 16.0 was used to analyze statistical differences. Then, after testing for normality and homogeneity of variance between groups, the results were subjected to a one-way analysis of variance (ANOVA) followed by Tukey's test. In addition, RNAi experiments were analyzed by t-test analysis using independent samples. Differences were set at $p < 0.05$.

3 RESULTS

3.1 Effect of Betaine on the Gene Expression Related to Wnt10b/β-Catenin Signaling in Zebrafish

Compared with control, 0.2 and 0.4 g/kg betaine treatments significantly induced Wnt10b gene expression, while 0.1, 0.2 and 0.4 g/kg betaine treatments significantly decreased GSK-3β gene expression (**Figures 1A,B**). However, the gene expression of GSK-3β was not significantly different between 0.1, 0.2 and 0.4 g/kg betaine treatments (**Figure 1B**). Furthermore, betaine enrichment of diet at 0.2 and 0.4 g/kg significantly enhanced β-catenin gene expression (**Figure 1C**).

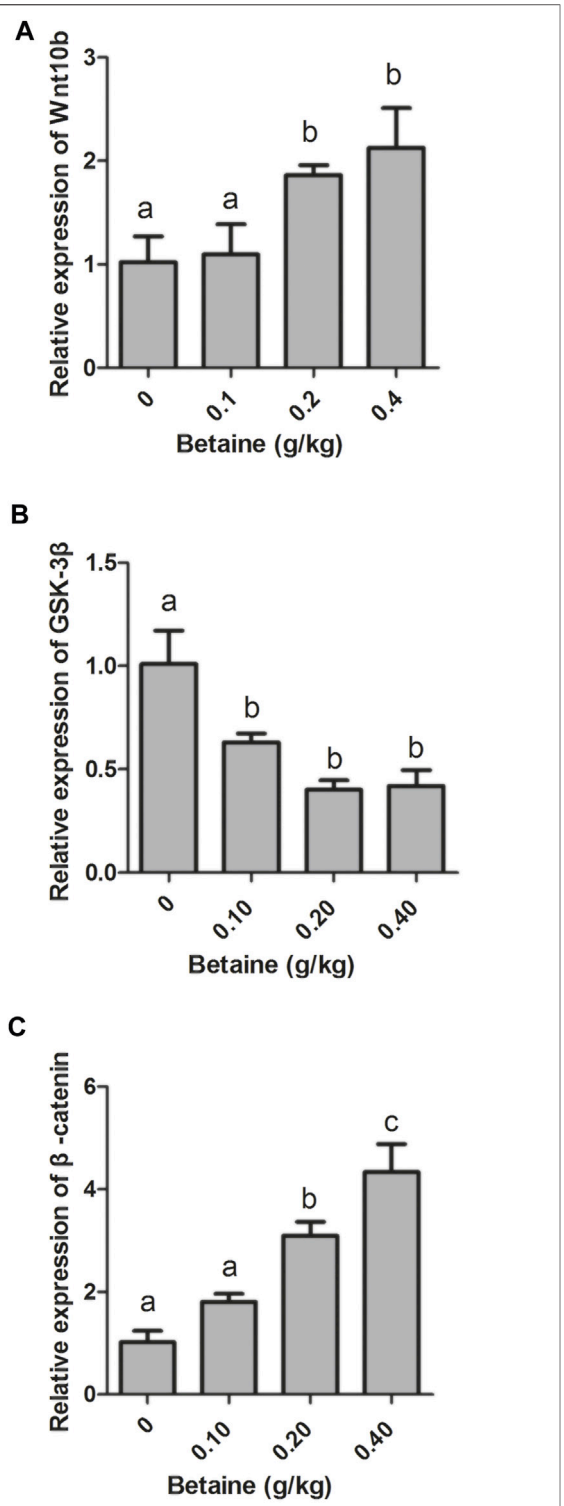


FIGURE 1 | Effect of betaine on the mRNA expression of Wnt10b, GSK-3β, and β-catenin in the liver of zebrafish. **(A)** The mRNA expression of Wnt10b; **(B)** The mRNA expression of GSK-3β; **(C)** The mRNA expression of β-catenin. Values are expressed as means ± sem (n = 3). Statistically significant differences are denoted by different letters (p < 0.05).

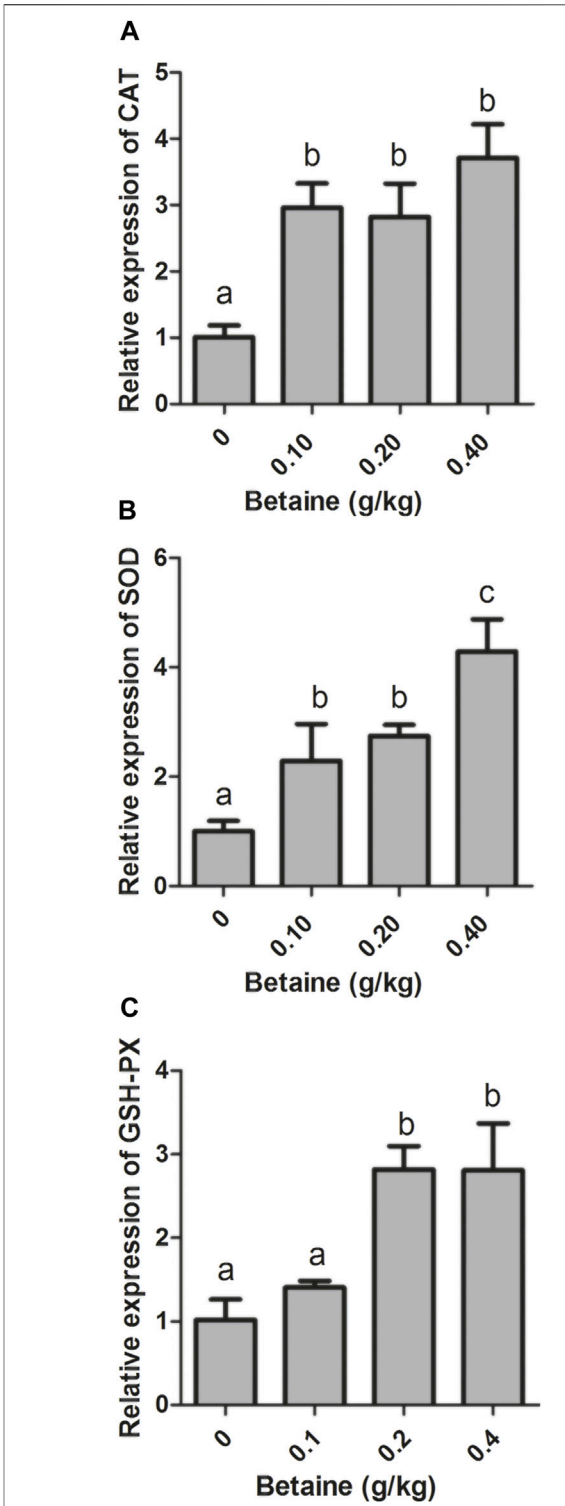


FIGURE 2 | Effect of betaine on the mRNA expression of CAT, SOD, and GSH-PX in the liver of zebrafish. **(A)** The mRNA expression of CAT; **(B)** The mRNA expression of SOD; **(C)** The mRNA expression of GSH-PX. Values are expressed as means ± sem (n = 3). Statistically significant differences are denoted by different letters (p < 0.05).

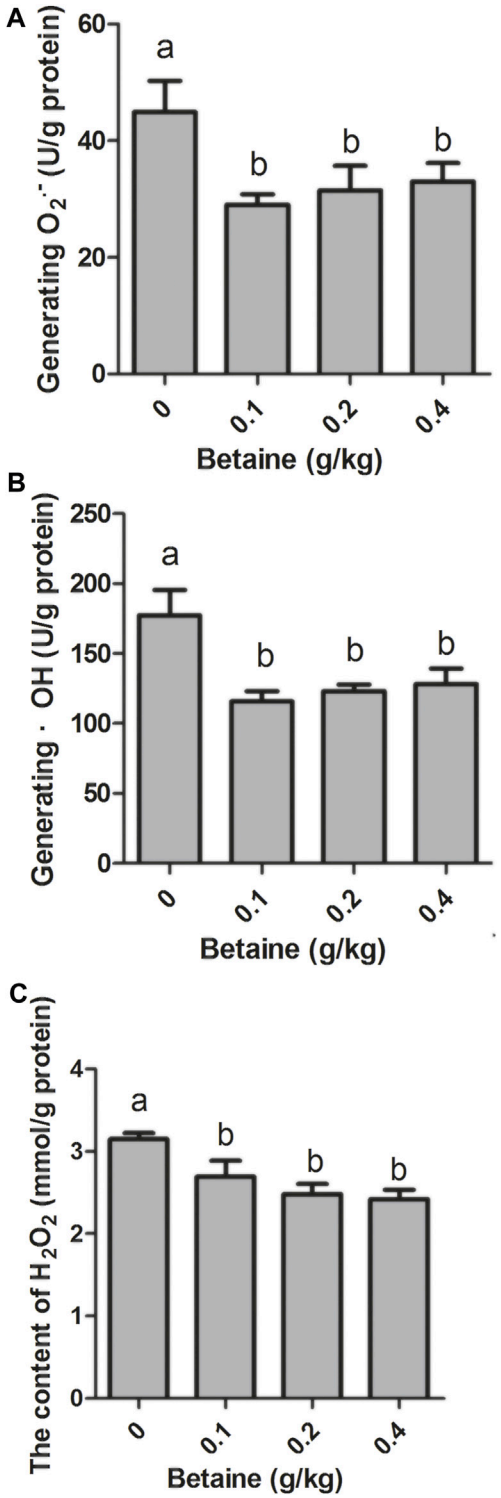


FIGURE 3 | Effect of betaine on the level of ROS in the liver of zebrafish. **(A)** The level of $O_2^{\cdot-}$; **(B)** The level of ·OH; **(C)** The level of H_2O_2. Values are expressed as means ± sem (n = 3). Statistically significant differences are denoted by different letters (p < 0.05).

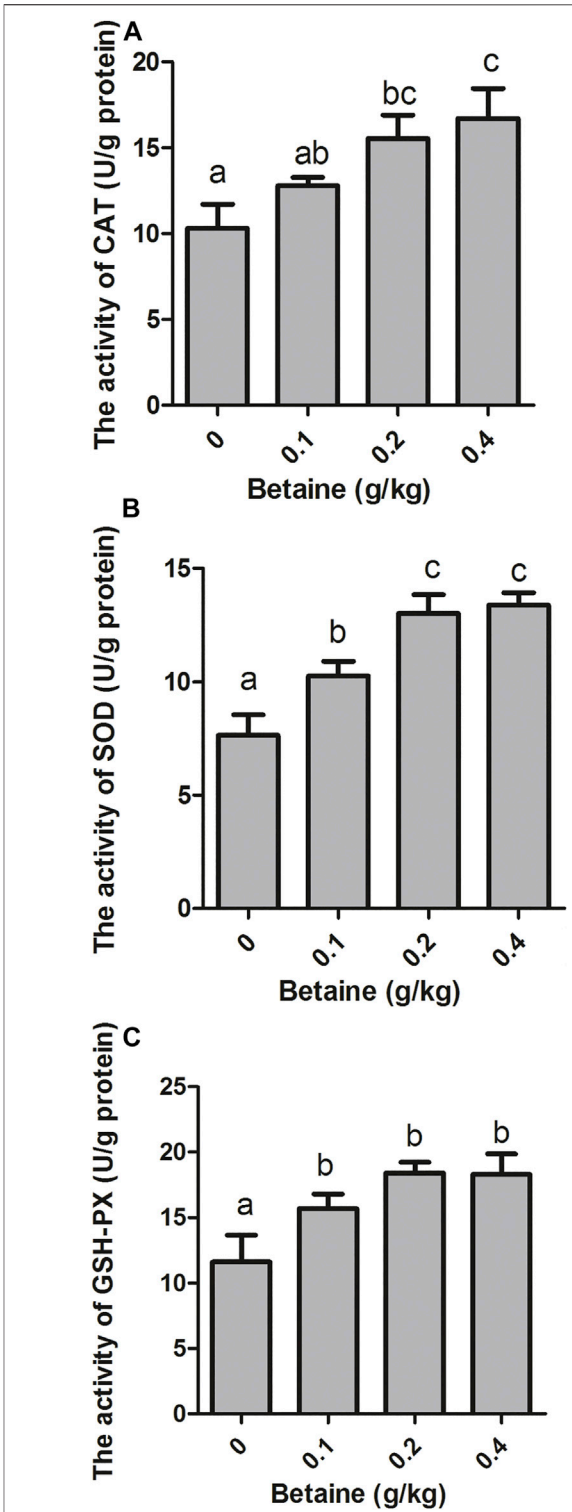


FIGURE 4 | Effect of betaine on the activities of antioxidant enzymes in the liver of zebrafish. **(A)** The activity of CAT; **(B)** The activity of SOD; **(C)** The activity of GSH-PX. Values are expressed as means ± sem (n = 3). Statistically significant differences are denoted by different letters (p < 0.05).

TABLE 4 | Effect of Wnt10b RNA interference on the gene expression level in the liver of zebrafish.

Genes	Control group (0.4 g/kg betaine)	RNAi group (Wnt10b RNAi and 0.4 g/kg betaine)	*p* Value
Wnt10b	1.02 ± 0.23	0.42 ± 0.09	<0.05
GSK-3β	1.01 ± 0.19	3.32 ± 0.82	<0.05
β-catenin	1.02 ± 0.25	0.57 ± 0.06	<0.05
CAT	1.03 ± 0.27	0.47 ± 0.05	<0.05
SOD	1.00 ± 0.03	0.51 ± 0.11	<0.05
GSH-PX	1.01 ± 0.13	0.48 ± 0.03	<0.05

*Values are expressed as means ± sem (*n *= 3). Statistically significant differences are denoted by* p *values.*

3.2 Effect of Betaine on the Gene Expression of Antioxidant Enzymes in Zebrafish

0.1, 0.2 and 0.4 g/kg betaine treatments significantly induced CAT and SOD gene expression compared with control (**Figures 2A,B**). However, there was no significant difference in CAT gene expression between three betaine treatments (**Figure 2A**). In addition, 0.2 and 0.4 g/kg betaine treatments significantly induced GSH-PX gene expression (**Figure 2C**).

3.3 Effect of Betaine on the Level of Reactive Oxygen Species in Zebrafish

0.1, 0.2 and 0.4 g/kg betaine treatments significantly reduced $O_2^{\cdot-}$ level, but there was no significant difference between 0.1, 0.2 and 0.4 g/kg betaine treatments (**Figure 3A**). In addition, three betaine treatments significantly reduced the levels of ·OH and H_2O_2 (**Figures 3B,C**).

3.4 Effect of Betaine on the Antioxidant Enzyme Activity in Zebrafish

0.2 and 0.4 g/kg betaine treatments significantly increased CAT activity, while no significant difference was observed between 0.2 and 0.4 g/kg betaine treatments (**Figure 4A**). Furthermore, betaine enrichment of diet at 0.1, 0.2 and 0.4 g/kg significantly increased the activities of SOD and GSH-PX (**Figures 4B,C**).

3.5 Effect of Wnt10b RNAi on the Gene Expression Related to Wnt10b/β-Catenin Signaling in Zebrafish

Interference of Wnt10b RNA significantly reduced Wnt10b gene expression and significantly increased GSK-3β gene expression (**Table 4**). In addition, Wnt10b RNAi significantly reduced the expression of β-catenin gene (**Table 4**).

3.6 Effect of Wnt10b RNAi on the Gene Expression of Antioxidant Enzymes in Zebrafish

Interference with Wnt10b RNA significantly reduced the gene expression of CAT and SOD compared to controls (**Table 4**). In addition, Wnt10b RNAi in zebrafish liver also significantly suppressed GSH-PX gene expression (**Table 4**).

3.7 Effect of Wnt10b RNAi on the Level of Reactive Oxygen Species and Activity of Antioxidant Enzymes in Zebrafish

Wnt10b RNAi treatment significantly increased the level of $O_2^{\cdot-}$ (**Table 5**). Interference of Wnt10b RNA also significantly increased the levels of ·OH and H_2O_2 (**Table 5**). Furthermore, Wnt10b RNAi treatment significantly reduced SOD, CAT and GSH-PX activities in zebrafish liver (**Table 5**).

4 DISCUSSION

Wnt molecules play a key role in the Wnt/β-catenin signaling pathway (Cossu and Borello, 1999; Ridgeway, et al., 2000; Taelman, et al., 2010; Valenta, et al., 2012). GSK-3β activity is hindered as Wnt molecules bind to receptors on the cell membrane (Taelman, et al., 2010). In addition, β-catenin levels are closely related to the activity of GSK-3β (Huelsken and Behrens, 2002). In the present study, gene expression of both Wnt10b and β-catenin was induced, but GSK-3β gene expression was decreased by betaine treatment in zebrafish liver. Our findings suggest that betaine can stimulate the Wnt/β-catenin signaling pathway by inducing Wnt10b and inhibiting GSK-3β expression (**Figure 5**).

Under conditions of oxidative stress, ROS production in fish will be enhanced over time. However, overproduction of ROS can damage cellular components and interfere with cellular metabolism (Lushchak, 2014). Previously, betaine has been shown to have antioxidant properties in various animals. Betaine attenuates oxidative stress in the liver of rat fed with the high-fructose and thioacetamide diet (Doğru-Abbasoğlu, et al., 2018; Heidari, et al., 2018). In addition, betaine reduces the negative effects of heat stress-stimulated oxidative status in broilers (Wen, et al., 2019), and betaine alleviates heat stress in dairy cows (Hall, et al., 2016; Zhang, et al., 2014). In this study, different concentrations of betaine in diet reduced the levels of $O_2^{\cdot-}$, ·OH and H_2O_2 within fish liver. Furthermore, the increasing betaine content in diet significantly induced the gene expression and activity of CAT, SOD and GSH-PX in zebrafish liver. In the current study, we also found that the higher content of betaine can better reduce the level of ROS and increase the activity of antioxidant-related enzymes, which is consistent with the results of previous studies on betaine.

TABLE 5 | Effect of Wnt10b RNA interference on the level of ROS and activities of antioxidant enzymes in the liver of zebrafish.

ROS level and antioxidant enzyme activity	Control group (0.4 g/kg betaine)	RNAi group (Wnt10b RNAi and 0.4 g/kg betaine)	*p* Value
$O_2^{\cdot-}$ level (U/g protein)	54.30 ± 3.73	64.63 ± 4.01	<0.05
·OH level (U/g protein)	132.50 ± 6.21	150.54 ± 4.94	<0.05
H_2O_2 level (mmol/g protein)	3.48 ± 0.38	4.50 ± 0.15	<0.05
CAT activity (U/g protein)	10.90 ± 0.40	9.03 ± 0.33	<0.05
SOD activity (U/g protein)	6.85 ± 0.68	5.38 ± 0.39	<0.05
GSH-PX activity (U/g protein)	12.30 ± 1.01	10.27 ± 0.57	<0.05

*Values are expressed as means ± sem (*n *= 3). Statistically significant differences are denoted by* p *values.*

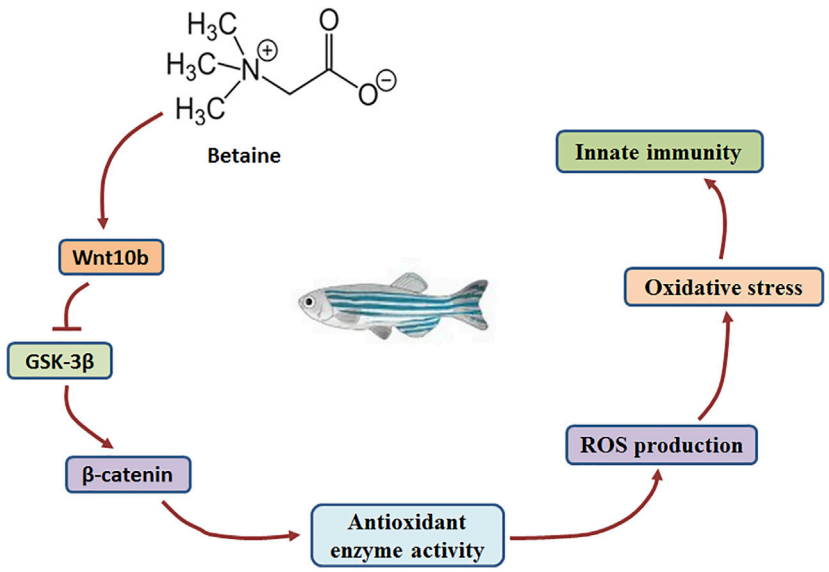


FIGURE 5 | The mechanism by which betaine regulates the production of ROS through Wnt10b/β-catenin signaling pathway in the zebrafish liver.

To confirm that Wnt10b plays a key role in regulating ROS levels, fish were intraperitoneally injected with Wnt10b dsRNA and ROS production was further detected in zebrafish liver. The results showed that β-catenin gene expression was inhibited, and the Wnt/β-catenin signaling pathway was inhibited by Wnt10b RNAi. In addition, Wnt10b RNAi increased the levels of $O_2^{\cdot-}$, ·OH and H_2O_2 in zebrafish liver, while decreasing the activities of SOD, CAT and GSH-PX. Wnt/β-catenin signaling pathway was previously found to play a role in regulating oxidative stress in MC3T3-E1 cells (Qi, et al., 2016). Our findings also suggest that the Wnt/β-catenin signaling pathway plays a key role in regulating antioxidant-related enzyme activities and ROS levels. Therefore, for betaine-induced Wnt/β-catenin signaling, it clearly demonstrated that betaine can inhibit ROS production in zebrafish liver through the Wnt/β-catenin signaling pathway (**Figure 5**).

In addition, betaine has three active methyl groups and plays a unique role in animal nutrition metabolism. Methyl groups are necessary for animal metabolism and cannot be synthesized by animals themselves. Therefore, betaine is one of the most efficient active methyl donors due to its unique structure (Kidd, et al., 1997). It has been previously found that if animals have enough betaine in their bodies, they can store more methionine for other metabolic activities (Lipiński, et al., 2012; Pereira, et al., 2018). Betaine is involved in regulating various physiological activities of animals (Zhang, et al., 2016). In addition, betaine has the ability to modulate osmotic changes and water imbalance during heat stress (Mahmoudnia and Madani, 2012; Zhou, et al., 2012). In the poultry industry, betaine can maintain the moisture of poultry cells and improve the ability of poultry to resist heat stress (Saeed, et al., 2017). In the current study, we observed that betaine reduced ROS levels and increased the activities of antioxidant-related enzymes. The ability of betaine to reduce ROS levels may also be related to osmoprotectant and methyl-donating properties. However, the specific mechanism needs to be further studied in future research.

5 CONCLUSION

In conclusion, a mechanism by which betaine modulates oxidative stress via Wnt10b/β-catenin signaling was discovered in zebrafish liver. Our findings suggest that betaine can induce Wnt10b/β-catenin signaling in zebrafish liver. ROS levels are reduced in zebrafish liver,

but betaine enhances the activity of antioxidant-related enzymes. Furthermore, the results of Wnt10b RNAi indicated that the Wnt10b/β-catenin signaling pathway plays a key role in regulating ROS production and antioxidant-related enzymatic activities. Taken together, betaine can inhibit ROS production in zebrafish liver through Wnt10b/β-catenin signaling pathway.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care Committee of Shandong University of Technology under the Guidelines for the Correct Conduct of Animal Experimentation (Science Council of China).

AUTHOR CONTRIBUTIONS

DL contributed to the conception and design of the work. AL, HY, YG, XZ and QP contributed to data acquisition, analysis, and interpretation. AL, DL, XZ and QP drafted and critically revised the work.

FUNDING

This study was supported by the Natural Science Foundation of China (31701845), Key Technology Research and Development Program of Shandong (2019JZZY020710), and Natural Science Foundation of Shandong Province of China (ZR2017MC066 and ZR2017BC005).

REFERENCES

Arockiaraj, J., Palanisamy, R., Bhatt, P., Kumaresan, V., Gnanam, A. J., Pasupuleti, M., et al. (2014). A Novel Murrel Channa Striatus Mitochondrial Manganese Superoxide Dismutase: Gene Silencing, SOD Activity, Superoxide Anion Production and Expression. *Fish. Physiol. Biochem.* 40, 1937–1955. doi:10.1007/s10695-014-9981-0

Bagnasco, S., Balaban, R., Fales, H. M., Yang, Y. M., and Burg, M. (1986). Predominant Osmotically Active Organic Solutes in Rat and Rabbit Renal Medullas. *J. Biol. Chem.* 261, 5872–5877. doi:10.1016/s0021-9258(17)38464-8

Bhanot, P., Brink, M., Samos, C. H., Hsieh, J.-C., Wang, Y., Macke, J. P., et al. (1996). A New Member of the Frizzled Family from Drosophila Functions as a Wingless Receptor. *Nature* 382, 225–230. doi:10.1038/382225a0

Bradford, M. M. (1976). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* 72, 248–254. doi:10.1016/0003-2697(76)90527-3

Cooke, M. S., Evans, M. D., Dizdaroglu, M., and Lunec, J. (2003). Oxidative DNA Damage: Mechanisms, Mutation, and Disease. *FASEB J.* 17, 1195–1214. doi:10.1096/fj.02-0752rev

Cossu, G., and Borello, U. (1999). Wnt Signaling and the Activation of Myogenesis in Mammals. *EMBO J.* 18, 6867–6872. doi:10.1093/emboj/18.24.6867

Craig, S. A. (2004). Betaine in Human Nutrition. *Am. J. Clin. Nutr.* 80, 539–549. doi:10.1093/ajcn/80.3.539

Doğru-Abbasoğlu, S., Soluk-Tekkeşin, M., Olgaç, V., and Uysal, M. (2018). Effect of Betaine Treatment on the Regression of Existing Hepatic Triglyceride Accumulation and Oxidative Stress in Rats Fed on High Fructose Diet. *Gen. Physiol. Biophys.* 37, 563–570.

Eklund, M., Bauer, E., Wamatu, J., and Mosenthin, R. (2005). Potential Nutritional and Physiological Functions of Betaine in Livestock. *Nutr. Res. Rev.* 18, 31–48. doi:10.1079/nrr200493

Hall, L. W., Dunshea, F. R., Allen, J. D., Rungruang, S., Collier, J. L., Long, N. M., et al. (2016). Evaluation of Dietary Betaine in Lactating Holstein Cows Subjected to Heat Stress. *J. Dairy Sci.* 99, 9745–9753. doi:10.3168/jds.2015-10514

Heidari, R., Niknahad, H., Sadeghi, A., Mohammadi, H., Ghanbarinejad, V., Ommati, M. M., et al. (2018). Betaine Treatment Protects Liver through Regulating Mitochondrial Function and Counteracting Oxidative Stress in Acute and Chronic Animal Models of Hepatic Injury. *Biomed. Pharmacother.* 103, 75–86. doi:10.1016/j.biopha.2018.04.010

Huelsken, J., and Behrens, J. (2002). The Wnt Signalling Pathway. *J. Cell Sci* 115, 3977–3978. doi:10.1242/jcs.00089

Kidd, M. T., Ferket, P. R., and Garlich, J. D. (1997). Nutritional and Osmoregulatory Functions of Betaine. *World's Poult. Sci. J.* 53, 125–139. doi:10.1079/WPS19970013

Kim, D. H., Sung, B., Kang, Y. J., Jang, J. Y., Hwang, S. Y., Lee, Y., et al. (2014). Anti-inflammatory Effects of Betaine on AOM/DSS-induced colon Tumorigenesis in ICR Male Mice. *Int. J. Oncol.* 45, 1250–1256. doi:10.3892/ijo.2014.2515

Kuang, S.-Y., Xiao, W.-W., Feng, L., Liu, Y., Jiang, J., Jiang, W.-D., et al. (2012). Effects of Graded Levels of Dietary Methionine Hydroxy Analogue on Immune Response and Antioxidant Status of Immune Organs in Juvenile Jian Carp (*Cyprinus carpio* Var. Jian). *Fish Shellfish Immunol.* 32, 629–636. doi:10.1016/j.fsi.2011.12.012

Li, C., Wang, Y., Li, L., Han, Z., Mao, S., and Wang, G. (2019). Betaine Protects against Heat Exposure-Induced Oxidative Stress and Apoptosis in Bovine Mammary Epithelial Cells via Regulation of ROS Production. *Cell Stress and Chaperones* 24, 453–460. doi:10.1007/s12192-019-00982-4

Liang, M.-q., Yu, H., Chang, Q., and Chen, C. (2001). Effects of Feeding Stimularnt on Intake and Growth of Red Seabream *Pagrosomus Major*. *Zhongguo shui chan ke Xue = J. Fish. Sci. China* 8, 58–61.

Lipiński, K., Szramko, E., Jeroch, H., and Matusevičius, P. (2012). Effects of Betaine on Energy Utilization in Growing Pigs-A Review. *Ann. Anim. Sci.* 12, 291–300.

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods* 25, 402–408. doi:10.1006/meth.2001.1262

Logan, C. Y., and Nusse, R. (2004). The Wnt Signaling Pathway in Development and Disease. *Annu. Rev. Cell Dev. Biol.* 20, 781–810. doi:10.1146/annurev.cellbio.20.010403.113126

Luo, Z., Tan, X.-Y., Liu, X.-J., and Wen, H. (2011). Effect of Dietary Betaine Levels on Growth Performance and Hepatic Intermediary Metabolism of GIFT Strain of Nile tilapia *Oreochromis niloticus* Reared in Freshwater. *Aquac. Nutr.* 17, 361–367. doi:10.1111/j.1365-2095.2010.00805.x

Lushchak, V. I. (2014). Free Radicals, Reactive Oxygen Species, Oxidative Stress and its Classification. *Chemico-Biological Interactions* 224, 164–175. doi:10.1016/j.cbi.2014.10.016

Mahmoudnia, N., and Madani, Y. (2012). Effect of Betaine on Performance and Carcass Composition of Broiler Chicken in Warm Weather-A Review. *Int. J. Agrisci* 2, 675–683.

Martínez-Álvarez, R. M., Morales, A. E., and Sanz, A. (2005). Antioxidant Defenses in Fish: Biotic and Abiotic Factors. *Rev. Fish. Biol. Fish.* 15, 75–88. doi:10.1007/s11160-005-7846-4

Maynard, S., Schurman, S. H., Harboe, C., de Souza-Pinto, N. C., and Bohr, V. A. (2009). Base Excision Repair of Oxidative DNA Damage and Association with Cancer and Aging. *Carcinogenesis* 30, 2–10. doi:10.1093/carcin/bgn250

Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., et al. (1996). XTcf-3 Transcription Factor

Mediates β-Catenin-Induced Axis Formation in Xenopus Embryos. *Cell* 86, 391–399. doi:10.1016/s0092-8674(00)80112-9

Naik, E., and Dixit, V. M. (2011). Mitochondrial Reactive Oxygen Species Drive Proinflammatory Cytokine Production. *J. Exp. Med.* 208, 417–420. doi:10.1084/jem.20110367

Nakanishi, T., Turner, R. J., and Burg, M. B. (1990). Osmoregulation of Betaine Transport in Mammalian Renal Medullary Cells. *Am. J. Physiology-Renal Physiol.* 258, F1061–F1067. doi:10.1152/ajprenal.1990.258.4.F1061

Pereira, R., Menten, J., Longo, F., Lima, M., Freitas, L., and Zavarize, K. (2018). Sources of Betaine as Methyl Group Donors in Broiler Diets. *Iran J. Appl. Anim. Sci.* 8, 325–332.

Petronini, P. G., De Angelis, E. M., Borghetti, P., Borghetti, A. F., and Wheeler, K. P. (1992). Modulation by Betaine of Cellular Responses to Osmotic Stress. *Biochem. J.* 282 (Pt 1), 69–73. doi:10.1042/bj2820069

Qi, H.-H., Bao, J., Zhang, Q., Ma, B., Gu, G.-Y., Zhang, P.-L., et al. (2016). Wnt/β-catenin Signaling Plays an Important Role in the Protective Effects of FDP-Sr against Oxidative Stress Induced Apoptosis in MC3T3-E1 Cell. *Bioorg. Med. Chem. Lett.* 26, 4720–4723. doi:10.1016/j.bmcl.2016.08.043

Ridgeway, A. G., Petropoulos, H., Wilton, S., and Skerjanc, I. S. (2000). Wnt Signaling Regulates the Function of MyoD and Myogenin. *J. Biol. Chem.* 275, 32398–32405. doi:10.1074/jbc.M004349200

Saeed, M., Babazadeh, D., Naveed, M., Arain, M. A., Hassan, F. U., and Chao, S. (2017). Reconsidering Betaine as a Natural Anti-heat Stress Agent in Poultry Industry: a Review. *Trop. Anim. Health Prod.* 49, 1329–1338. doi:10.1007/s11250-017-1355-z

Saurabh, S., and Sahoo, P. K. (2008). Lysozyme: an Important Defence Molecule of Fish Innate Immune System. *Aquaculture Res* 39, 223–239. doi:10.1111/j.1365-2109.2007.01883.x

Taelman, V. F., Dobrowolski, R., Plouhinec, J.-L., Fuentealba, L. C., Vorwald, P. P., Gumper, I., et al. (2010). Wnt Signaling Requires Sequestration of Glycogen Synthase Kinase 3 inside Multivesicular Endosomes. *Cell* 143, 1136–1148. doi:10.1016/j.cell.2010.11.034

Tort, L., Balasch, J., and Mackenzie, S. (2003). Fish Immune System. A Crossroads between Innate and Adaptive Responses. *Inmunología* 22, 277–286.

Troncoso, I. C., Cazenave, J., Bacchetta, C., and Bistoni, M. d. l. Á. (2012). Histopathological Changes in the Gills and Liver of *Prochilodus lineatus* from the Salado River basin (Santa Fe, Argentina). *Fish. Physiol. Biochem.* 38, 693–702. doi:10.1007/s10695-011-9551-7

Valenta, T., Hausmann, G., and Basler, K. (2012). The many Faces and Functions of β-catenin. *EMBO J.* 31, 2714–2736. doi:10.1038/emboj.2012.150

Veeman, M. T., Axelrod, J. D., and Moon, R. T. (2003). A Second Canon. *Dev. Cell* 5, 367–377. doi:10.1016/s1534-5807(03)00266-1

Virtanen, E., Junnila, M., and Soivio, A. (1989). Effects of Food Containing Betaine/amino Acid Additive on the Osmotic Adaptation of Young Atlantic salmon, *Salmo salar* L. *Aquaculture* 83, 109–122. doi:10.1016/0044-8486(89)90065-3

Wen, C., Chen, Y., Leng, Z., Ding, L., Wang, T., and Zhou, Y. (2019). Dietary Betaine Improves Meat Quality and Oxidative Status of Broilers under Heat Stress. *J. Sci. Food Agric.* 99, 620–623. doi:10.1002/jsfa.9223

Wu, G., and Davis, D. A. (2005). Interrelationship Among Methionine, Choline, and Betaine in Channel Catfish *Ictalurus punctatus*. *J. World Aquac. Soc.* 36, 337–345.

Zeng, L., Fagotto, F., Zhang, T., Hsu, W., Vasicek, T. J., Perry, W. L., 3rd, et al. (1997). The Mouse Locus Encodes Axin, an Inhibitor of the Wnt Signaling Pathway that Regulates Embryonic Axis Formation. *Cell* 90, 181–192. doi:10.1016/s0092-8674(00)80324-4

Zhang, D., Jing, H., Dou, C., Zhang, L., Wu, X., Wu, Q., et al. (2018). Supplement of Betaine into Embryo Culture Medium Can Rescue Injury Effect of Ethanol on Mouse Embryo Development. *Sci. Rep.* 8, 1761. doi:10.1038/s41598-018-20175-w

Zhang, L., Ying, S. J., An, W. J., Lian, H., Zhou, G. B., and Han, Z. Y. (2014). Effects of Dietary Betaine Supplementation Subjected to Heat Stress on Milk Performances and Physiology Indices in Dairy Cow. *Genet. Mol. Res.* 13, 7577–7586. doi:10.4238/2014.September.12.25

Zhang, M., Wu, X., Lai, F., Zhang, X., Wu, H., and Min, T. (2016). Betaine Inhibits Hepatitis B Virus with an Advantage of Decreasing Resistance to Lamivudine and Interferon α. *J. Agric. Food Chem.* 64, 4068–4077. doi:10.1021/acs.jafc.6b01180

Zhou, Y., Holmseth, S., Hua, R., Lehre, A. C., Olofsson, A. M., Poblete-Naredo, I., et al. (2012). The Betaine-GABA Transporter (BGT1, Slc6a12) Is Predominantly Expressed in the Liver and at Lower Levels in the Kidneys and at the Brain Surface. *Am. J. Physiology-Renal Physiol.* 302, F316–F328. doi:10.1152/ajprenal.00464.2011

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: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Physiology

ORIGINAL RESEARCH
published: 20 May 2022
doi: 10.3389/fphys.2022.892550

Efficacy of Utilization of All-Plant-Based and Commercial Low-Fishmeal Feeds in Two Divergently Selected Strains of Rainbow Trout (*Oncorhynchus mykiss*): Focus on Growth Performance, Whole-Body Proximate Composition, and Intestinal Microbiome

Ilaria Biasato[1], *Simona Rimoldi*[2], *Christian Caimi*[1], *Sara Bellezza Oddon*[1], *Giulia Chemello*[3], *Marino Prearo*[4], *Marco Saroglia*[2], *Ronald Hardy*[5], *Laura Gasco*[1]* *and Genciana Terova*[2]

[1]*Department of Agricultural, Forest and Food Sciences, University of Turin, Grugliasco (TO), Italy,* [2]*Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy,* [3]*Department of Life and Environmental Sciences, Marche Polytechnic University, Ancona, Italy,* [4]*The Veterinary Medical Research Institute for Piedmont, Liguria and Aosta Valley, Torino, Italy,* [5]*Hagerman Fish Culture Experiment Station, University of Idaho, Hagerman, United States*

The present study aimed to investigate the growth performance, whole-body proximate composition, and intestinal microbiome of rainbow trout strains when selected and non-selected for weight gain on all-plant protein diets. A 2x2 factorial design was applied, where a selected (United States) and a non-selected (ITA) rainbow trout strain were fed using either an all-plant protein (PP) or a commercial low-FM diet (C). Diets were fed to five replicates of 20 (PP) or 25 (C) fish for 105 days. At the end of the trial, growth parameters were assessed, and whole fish (15 pools of three fish/diet) and gut samples (six fish/diet) were collected for whole-body proximate composition and gut microbiome analyses, respectively. Independent of the administered diet, the United States strain showed higher survival, final body weight, weight gain, and specific growth rate when compared to the ITA fish ($p < 0.001$). Furthermore, decreased whole-body ether extract content was identified in the PP-fed United States rainbow trout when compared to the ITA strain fed the same diet ($p < 0.001$). Gut microbiome analysis revealed the *Cetobacterium* probiotic-like genus as clearly associated with the United States rainbow trout, along with the up-regulation of the pathway involved in starch and sucrose metabolism. In summary, the overall improvement in growth performance and, to a lesser extent, whole-body proximate composition observed in the selected rainbow trout strain was accompanied by specific, positive modulation of the intestinal microbiome.

Keywords: rainbow trout, selective breeding, performance, vegetable proteins, gut microbiome

OPEN ACCESS

Edited by:
Mohamed Salah Azaza, National Institute of Marine Science and Technology, Tunisia

Reviewed by:
Ines Ben Khemis, Institut National des Sciences et Technologies de la Mer (Tunisia), Tunisia
Sebahattin Ergun, Onsekiz Mart University, Turkey

***Correspondence:**
Laura Gasco
laura.gasco@unito.it

Specialty section:
This article was submitted to Aquatic Physiology, a section of the journal Frontiers in Physiology

Received: *09 March 2022*
Accepted: *11 April 2022*
Published: *20 May 2022*

Citation:
Biasato I, Rimoldi S, Caimi C, Bellezza Oddon S, Chemello G, Prearo M, Saroglia M, Hardy R, Gasco L and Terova G (2022) Efficacy of Utilization of All-Plant-Based and Commercial Low-Fishmeal Feeds in Two Divergently Selected Strains of Rainbow Trout (Oncorhynchus mykiss): Focus on Growth Performance, Whole-Body Proximate Composition, and Intestinal Microbiome. Front. Physiol. 13:892550. doi: 10.3389/fphys.2022.892550

INTRODUCTION

Genetic selection has progressively gained a foothold in the aquaculture sector, as it allows obtaining individuals with desired phenotypic traits. Traditional selective breeding—where only fish that exhibit desirable characteristics for one or multiple traits are chosen and bred—represents the most commonly adopted genetic enhancement technique. It is mainly directed towards the maximization of growth potential, improvement in fillet quality attributes, or development of resistance to stress and pathogens (Xu et al., 2015). In particular, rainbow trout (*Oncorhynchus mykiss*) has frequently been the object of selective breeding, with current programs being predominantly based on mass selection for growth rates (Cleveland et al., 2020) and resistance to viral or bacterial diseases (Vallejo et al., 2020; Zuo et al., 2020). Among all the selective breeding programs carried out to improve the growth potential of rainbow trout, one of the most impactful is related to the efficiency of digesting all-plant protein feeds (Overturf et al., 2013; Lazzarotto et al., 2015; Callet et al., 2017). It is, indeed, well known that the aquaculture sector has been trying to overcome sustainability challenges for the past decade, as worldwide production from aquaculture has grown rapidly and overtaken capture fisheries, and per capita consumption of fish has doubled (FAO, 2020). Therefore, the unsustainability of a "fish-to-fish" feeding has progressively made it necessary to replace fishmeal (FM) by alternative protein sources of plant origin in diets for farming fish. However, all-plant protein feeds do not represent the optimal choice for salmonids, with reductions in feed intake, weight gain, feed efficiency, alteration of gut microflora and immune response, and intestinal dysfunction and inflammation (mainly affecting the distal gut) being commonly observed (Krogdahl et al., 2010). In the light of such negative outcomes, genetic selection has successfully proved to be an effective counteracting measure to create rainbow trout strains that outperform the commercial, parental lines in utilizing all-plant protein diets without showing worsened growth performance or developing distal enteritis (Burr et al., 2012; Abernathy et al., 2017; Callet et al., 2017).

So far, the efficacy of selective breeding for utilization of all-plant protein diets in rainbow trout has been investigated by comparing selected and non-selected strains when fed either an FM or an all-plant protein diet in terms of growth response, nutrient retention, plasma amino acid (AA) patterns, and gut, liver, and muscle transcriptomic profiles (Abernathy et al., 2017; Callet et al., 2017; Brezas and Hardy, 2020; Brezas et al., 2021; Callet et al., 2021). In particular, candidate genes for protein (i.e., intestinal AA transporters and ribosomal proteins), lipid (i.e., long-chain polyunsaturated fatty acids biosynthesis), and purine–thiamine metabolisms, as well as energy production and inflammation (i.e., interleukin receptors), have previously been linked to synchronization of AA absorption and protein digestion rates and, in turn, improved growth and protein retention in the selected strains (Abernathy et al., 2017; Brezas et al., 2021; Callet et al., 2021). However, as gut microbiome alterations can influence fish performance (Vargas-Albores et al., 2021), and the intestinal microbiome itself may be, in turn, modulated by dietary changes (Rimoldi et al., 2018 and, 2021; Gaudioso et al., 2021; Terova et al., 2021), the characterization of the gut microbiome in selected rainbow trout could provide novel insights into the adaptation mechanisms of the fish to all-plant protein feeds. Nowadays, it is widely accepted that there is a correlation between fish genotype and gut microbial communities, but to date, there is very little information on how genetic selection drives differences in intestinal microbiota composition and how this could affect diet plasticity, health, and disease resistance in both marine and freshwater fish species (Blaufuss et al., 2020; Piazzon et al., 2020).

Therefore, the present study was designed to investigate the growth performance, whole-body proximate composition, and intestinal microbiome of rainbow trout strains when selected and non-selected for weight gain on all-plant protein feeds in response to all-plant protein and commercial low-FM diets.

MATERIALS AND METHODS

Experimental Fish and Diets

A feeding trial was conducted at the Experimental Facility of the Department of Agricultural, Forest and Food Sciences (DISAFA) of the University of Turin (Italy) using two different rainbow trout strains. The selected strain (United States) was developed by the United States Department of Agricultural–Agricultural Research Service (USDA-ARS) and the University of Idaho by introgression of nine domesticated commercial and conservation stocks (Overturf et al., 2013). The selection was based on growth performance when trout were fed an all-plant protein feed. Besides dietary utilization, the selected strain also displays an enhanced non-specific pathogen resistance and a concomitant resistance to the development of distal intestine enteritis (Venold et al., 2012; Abernathy et al., 2017). The non-selected strain (ITA) was selected for different performance traits when fed a commercial low-FM diet by the Italian hatchery Fratelli Leonardi (Trento, Italy). To understand the effects of both diet and genotype, a 2x2 factorial design was applied. In particular, each rainbow trout strain was fed using two different experimental diets: a diet containing only plant proteins (PP, provided by the University of Idaho), and a commercial low-FM diet (C) containing both the plant meals and FM (provided by Naturalleva, Cologna Veneta, Verona, Italy). No information was provided by the feed company about the C diet formulation as it was considered confidential. Both the diets were provided as extruded feeds.

In detail, the United States embryonated eggs were received from the University of Idaho and, after hatching in hatchery tanks, were fed commercial diets (crumbled extruded feeds of different sizes, with 55–62% crude protein [CP] and 11–18% ether extract [EE]; Skretting Italia Spa, Mozzecane, Verona, Italy) until the beginning of the experimental trial. In parallel, ITA fingerlings (mean weight of 2.0 ± 0.01 g) were provided by Fratelli Leonardi and fed the same commercial diets until the beginning of the feeding trial as well. After reaching the juvenile stage, 250 juvenile rainbow trout (125 from each strain) underwent light

TABLE 1 | Feed ingredients (g/kg, as fed) and proximate composition of the experimental diets.

	PP	C
Ingredients (g/kg, as fed)		
Soy protein concentrate	265.8	-
Soybean meal, dehulled	250.0	-
Corn gluten meal (CP 60%)	170.0	-
Wheat gluten meal	29.2	-
Fish oil	194.2	-
L-lisin HCl	18.4	-
DL-methionine	4.4	-
Threonine	2.0	-
Taurine	5.0	-
Vitamin premix[a]	10.0	-
Stay C 35%	2.0	-
Dicalcium phosphate	15	-
Potassium chloride	5.6	-
Sodium chloride	2.8	-
Monocalcium phosphate	24.6	-
Trace mineral premix[b]	1.0	-
Proximate composition[c]		
DM, g/kg	91.32	90.71
CP, g/kg DM	48.97	49.71
EE, g/kg DM	18.11	26.56
Ash, g/kg DM	6.04	7.19
GE, MJ/Kg[d]	22.80	24.07

DM, dry matter; CP, crude protein; EE, ether extract; GE, gross energy; [a]Vitamin premix supplied the following per kg diet: vitamin A, 2.4 mg; vitamin D, 0.15 mg; vitamin E, 267 mg; vitamin K as menadione sodium bisulfite, 20 µg; thiamin as thiamin mononitrate, 32 mg; riboflavin, 64 mg; pyridoxine as pyridoxine-HCl, 64 mg; pantothenic acid as Ca-d-pantothenate, 192 mg; niacin as nicotinic acid, 240 mg; biotin, 0.56 mg; folic acid, 12 mg; vitamin B_{12}, 50 µg; and inositol as meso-inositol, 400 mg [d]Trace mineral premix supplied the following (mg/kg diet): Zn (as $ZnSO_4.7H_2O$), 75; Mn (as $MnSO_4$), 20; Cu (as $CuSO_4.5H_2O$), 1.54; I (as KIO_3), 10. [c]Values are reported as mean of duplicate analyses; [d]Determined by calorimetric bomb.

anesthesia (MS-222; PHARMAQ Ltd., United Kingdom; 60 mg/L) and were individually weighed (10.63 ± 0.01 g) by using electronic scales (KERN PLE-N v. 2.2; KERN and, Sohn GmbH, Balingen-Frommern, Germany; d: 0.1). Then, fish from each strain were randomly distributed into ten 100-L, rectangular-shaped tanks (five replicate tanks per strain, 25 fish per tank) connected to a flow-through open system supplied with artesian well water (constant temperature of 13 ± 1°C, 8 L min-1, dissolved oxygen ranged between 7.6 and 8.7 mg L), and fed the C diet (USA-C and ITA-C treatments). In parallel, at the same rearing conditions, 200 juvenile rainbow trout (100 from each strain; initial body weight of 13.47 ± 0.01 g) were randomly allotted to ten 100-L, rectangular-shaped tanks (five replicate tanks per strain, 20 fish per tank), and fed the PP diet (USA-PP and ITA-PP treatments). In order to update the daily amount of feed, the biomass in each tank was weighed in bulk every 14 days. Fish were fed by hand twice a day (08:00 and 15:00) and 6 days per week, up to a maximum of 2.5% of the tank biomass the first 6 weeks of the feeding trial. Then, according to the fish growth, the daily amount of feed per tank was progressively reduced to 1.7%. Feed intake was checked at each administration, as feed distribution was immediately suspended when fish stopped eating. Undistributed feed was weighed and this data was used for the correct calculation of the growth parameters. Mortality was daily recorded. The feeding trial lasted 105 days.

Chemical Analyses of Feed

Feed samples were ground using a cutting mill (MLI 204; Bühler AG, Uzwil, Switzerland) and analyzed for dry matter (DM, AOAC #934.01), CP (AOAC #984.13), and ash (AOAC #942.05) contents according to AOAC International (2000). Feed samples were also analyzed for EE (AOAC #2003.05) content according to AOAC International (2003). The gross energy (GE) content was determined using an adiabatic calorimetric bomb (C7000; IKA, Staufen, Germany). All the chemical analyses of the feeds were performed in duplicate. The proximate composition of the experimental diets is shown in **Table 1**.

Growth Performance

At the end of the feeding trial, the feed was withheld from the fish for 1 day, and then they were individually weighted after light anesthesia. The following performance indexes were calculated per tank:

Survival (%) = 100—[(number of dead fish/number of fish at start) × 100].

Individual weight gain (iWG, g) = individual mean final body weight (iFBW, g)—individual mean initial body weight (iIBW, g).

Feed conversion ratio (FCR) = total feed supplied (g, DM)/WG (g).

Protein efficiency ratio (PER) = WG (g)/total protein fed (g, DM).

Specific growth rate (SGR, % day-1) = [(lnFBW–lnIBW)/number of days] × 100.

Whole-Body Proximate Composition

At the end of the feeding trial, 3 fish per tank (15 fish per dietary treatment) were sacrificed by an overdose of anesthetic (MS-222, PHARMAQ Ltd., United Kingdom; 500 mg/L) and then frozen at -20°C. Frozen fish were finely ground with a knife mill (Grindomix 174 GM200; Retsch GmbH, Haan, Germany) to obtain one pool of three fish per tank (5 pools per dietary treatment). All the fish pools were freeze-dried and used for the determination of the final whole-body proximate composition. The proximate composition of fish whole body was determined following the same procedures used for feed analyses (AOAC International, 2000 and 2003).

Sampling and Processing

At the end of the feeding trial, six fish per dietary treatment were sacrificed by an overdose of anesthetic, and the whole intestine was aseptically dissected out. The gut autochthonous microbiota was obtained by scraping the mucosa of the entire intestine (excluding pyloric caeca) with a sterile swab as reported in detail by Rimoldi et al. (2019). Briefly, each swab head was cut and immediately transferred to a sterile 1.5 ml microtube containing 200 µl of Xpedition Lysis/Stabilization Solution. The tube was then vortexed for shaking out the bacteria from

the swab tip and stored at room temperature for up to 24 h until bacterial DNA extraction.

Bacterial DNA Extraction

The DNA was extracted from 200 µl of bacterial suspension and from three aliquots (200 mg) of each experimental feed using the DNeasy PowerSoil® Kit (Qiagen, Milan, Italy), according to the manufacturer's instructions. The samples were mechanically lysed in PowerBead Tubes by means of a TissueLyser II (Qiagen) for 2 min at 25 Hz. The extracted DNA was spectrophotometrically quantified using the NanoDropTM 2000 spectrophotometer (Thermo Scientific, Milan, Italy) and stored at -20°C until the PCR reaction was performed.

16S rRNA Gene Amplicon Library Preparation and Sequencing

Preparation of the 16S metagenomic library and sequencing were performed by GalSeq srl (Italy), targeting the variable V4 region and applying Illumina protocol "16S Metagenomic Sequencing Library Preparation for Illumina MiSeq System" (#15044223 rev. B). Details of the methodology used have previously been described (Rimoldi et al., 2019; Terova et al., 2019). Briefly, primers are tailed with sequences to incorporate indexing barcodes, and samples are pooled into a single library for sequencing on the Illumina MiSeq device using the pair-ended sequencing (2 × 250) strategy. All FastQ sequence files were submitted to the European Nucleotide Archive (EBI ENA).

Analysis of Metabarcoding Data

The raw sequence files were imported in QIIME™ 2 (v. 2018.4) (Bolyen et al., 2019) and processed at the default setting. Artefacts and primers were trimmed, the remaining sequences were filtered for base quality (Q > 30), and forward and reverse reads were merged. Filtered reads were then dereplicated and chimeras removed using the denoise-paired command of the DADA2 plug-in. The output was an amplicon sequence variant (ASV) table, analog to the OTU table, which records the number of times each exact ASV was observed in each sample. The representative sequences were classified using the Silva reference database (https://www.arb-silva.de/) down to the genus level. All sequences assigned to eukaryotes (chloroplasts and mitochondria) in gut samples were discarded. Alpha and beta diversity analyses were performed using QIIME scripts "alpha_rarefection.py" and "jackknifed_beta_diversity_.py", respectively. Principal Coordinates Analysis (PCoA) based on both unweighted UniFrac and weighted UniFrac distance matrices (Lozupone and Knight, 2005; Lozupone et al., 2007) was conducted to visualize similarities or dissimilarities between bacterial communities.

Predictive Functional Analysis of Bacterial Communities

The PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) software package was used to predict the functional profiling of microbial communities on the basis of 16S rRNA gene sequences (Langille et al., 2013) as previously described (Rimoldi et al., 2021). The inferred metagenomic functions were predicted using the Kyoto Encyclopedia of Genes and Genomes (KEGG database). The maximum allowed Nearest Sequenced Taxon Index (NSTI) value was set to two. Analysis of the output generated by PICRUSt was made using the Statistical Analysis of Metagenomic Profiles (STAMP) software package (Parks et al., 2014) applying a two-sided Welch t-test ($p < 0.05$) to identify differences in microbial metabolic pathways between two groups.

Bioinformatics and Statistical Analysis

The experimental unit for statistical analyses was the tank for growth performance and whole-body proximate composition, and the fish for microbiome analysis.

The statistical analysis of growth performance and whole-body proximate composition was performed using IBM SPSS Statistics v. 28.0 (IBM, Armonk, NY, United States). The Shapiro–Wilk test assessed the normality distribution of the residuals, while the assumption of equal variances was assessed by Levene's homogeneity of variance test. The growth performance and whole-body proximate composition data were analyzed by fitting a general linear model that allowed them to depend on two fixed factors (strain and diet) and their interaction. The interactions between the levels of the fixed factors were evaluated by pairwise contrasts. The results obtained were expressed as the least square mean and pooled standard error of the mean (SEM). p values ≤ 0.05 were considered statistically significant.

The statistical analysis of gut microbiome data was performed using Past3 software (Hammer et al., 2001). All the data were checked for normality and homogeneity of variance using Shapiro–Wilk and Levene's test, respectively. Concerning gut microbiota composition, only those taxa with an overall abundance of more than 0.5% (up to order level) and 0.01% at family and genus level were considered for statistical analysis. Before being statistically analyzed, the percentage values were angular transformed (arcsine of the square root). To test the null hypothesis ($p < 0.05$), a two-way ANOVA was performed considering the influence of two main factors (strain and diet) and their interaction. Multivariate analysis of beta diversity was tested using a two-way non-parametric permutational multivariate analysis of variance (two-way PERMANOVA) with 999 permutations.

RESULTS

Growth Performance

The growth performance of the rainbow trout in the present study is summarized in **Table 2**. Survival was high for all the dietary treatments (range: 95.00–100), with a significant influence of both the strain ($p < 0.05$) and the interaction between strain and diet ($p < 0.001$) being identified. In particular, the United States rainbow trout showed higher survival than the ITA group ($p < 0.05$), but greater survival was observed in the United States strain fed the PP diet when compared to the PP-fed

TABLE 2 | Growth performance of the rainbow trout depending on strain, diet, and their interaction (n = 5).

	Strain (S)		Diet (D)		SEM		*p* value		
	ITA	United States	C	PP	S	D	S	D	S × D
Survival, %	97.10	98.40	98.00	97.50	0.36	0.36	0.010	0.323	<0.001
iFBW, g	69.82	76.18	61.18	84.82	0.80	0.80	<0.001	<0.001	0.628
iWG, g	54.96	59.40	51.27	63.09	0.88	0.88	<0.001	<0.001	0.010
FCR, n	0.96	0.94	0.96	0.94	0.02	0.02	0.176	0.345	0.171
PER, n	2.04	2.10	2.10	2.04	0.03	0.03	0.173	0.240	0.162
SGR, % day-1	1.61	1.70	1.66	1.65	0.01	0.01	<0.001	0.634	0.371

ITA, Italian strain; United States, American strain; C, commercial diet; PP, all-plant protein diet; SEM, standard error of the mean; P, probability; iIBW, individual initial body weight; iFBW, individual final body weight; iWG, individual weight gain; SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio.

TABLE 3 | Final whole-body proximate composition (as is) of the rainbow trout depending on strain, diet, and their interaction (n = 5).

	Strain (S)		Diet (D)		SEM		*p* value		
	ITA	United States	C	PP	S	D	S	D	S × D
Moisture, %	71.71	72.82	72.90	71.63	0.17	0.17	<0.001	<0.001	0.042
Ash, %	2.42	2.39	2.46	2.35	0.05	0.05	0.622	0.139	0.031
CP, %	16.25	16.16	15.75	16.66	0.16	0.16	0.652	<0.001	0.652
EE, %	9.27	8.38	8.60	9.06	0.07	0.07	<0.001	<0.001	<0.001

ITA, Italian strain; United States , American strain; C, commercial diet; PP1, all-plant protein diet; SEM, standard error of the mean; P, probability; DM, dry matter; CP, crude protein; EE, ether extract.

ITA fish only (100.00 ± 0.51 vs. 95.00 ± 0.51; $p < 0.001$). In parallel, the ITA rainbow trout fed the C diet displayed higher survival than the C-fed United States group (99.20 ± 0.51 vs. 96.80 ± 0.51; $p = 0.001$). Higher survival was also observed in both the United States and the ITA strains when fed the PP and C diets, respectively ($p < 0.001$). Differently, the iFBW was influenced by both strain and diet ($p < 0.001$). The United States fish showed greater iFBW than the ITA group ($p < 0.001$), with higher values being also observed when the fish were fed the PP diet in comparison with the C one ($p < 0.001$). The iWG depended on all the considered variables (strain and diet: $p < 0.001$; strain × diet: $p = 0.010$). In particular, the United States rainbow trout showed higher iWG than the ITA group ($p < 0.001$), with greater iWG being also observed when fish were fed the PP diet in comparison with the C one ($p < 0.001$). However, the United States rainbow trout displayed higher iWG than the ITA strain when they were fed the C diet only (55.12 ± 1.25 vs. 47.43 ± 1.25; $p < 0.001$). The FCR and the PER were not affected by any of the considered variables ($p > 0.05$). Finally, the SGR depended on strain only ($p < 0.001$), with higher values being identified in the United States rainbow trout in comparison with the ITA fish ($p < 0.001$).

Whole-Body Proximate Composition

Table 3 summarizes the whole-body proximate composition of the rainbow trout in the current research. The moisture depended on all the considered variables (strain and diet: $p < 0.001$; strain × diet: $p = 0.042$). In particular, the ITA rainbow trout showed lower moisture content than the United States group ($p < 0.001$), with a reduction in the whole-body moisture being also observed when fish were fed the PP diet in comparison with the C one ($p < 0.001$). However, the ITA strain displayed lower moisture content than the United States rainbow trout when they were fed the PP diet only (70.83 ± 0.25 vs. 72.43 ± 0.25; $p < 0.001$). The ash content was influenced by the strain × diet interaction only ($p < 0.05$), with the C-fed United States fish displaying increased whole-body ash in comparison with the USA-PP group (2.52 ± 0.07 vs. 2.26 ± 0.07; $p < 0.05$). Differently, the CP content uniquely depended on diet ($p < 0.001$). In detail, the PP diet led to higher whole-body CP than the C feed ($p < 0.001$). On the contrary, the EE content was affected by all the considered variables ($p < 0.001$). In particular, the ITA fish displayed higher whole-body EE than the United States strain ($p < 0.001$), with an increase in the EE content being also observed when fish were fed the PP diet in comparison with the C one ($p < 0.001$). However, greater whole-body EE was observed in the ITA rainbow trout fed the PP diet when compared to the PP-fed United States group only (9.84 ± 0.10 vs. 8.28 ± 0.10; $p < 0.001$). Furthermore, the ITA strain showed increased EE content when fed the PP diet (9.84 ± 0.10 vs. 8.71 ± 0.10 [C]; $p < 0.001$).

Microbial DNA Sequencing

Illumina sequencing of the 30 samples (24 mucosae + six feeds) yielded 1,015,591 high-quality reads, which were successfully taxonomically assigned according to the Silva database. The average number of reads (mean + SD) per sample was 24,838 ± 5,381 and 37,356 ± 4,810 for feed and gut mucosa samples, respectively (Supplementary data file S1). Rarefaction analysis of the chao1 index showed most of the curves approximating the saturation, with one intestinal sample from group United States_C failing only (**Supplementary Figure S1**). To ensure the adequateness of the remaining samples, the good

TABLE 4 | Alpha diversity indices (mean ± SD, rarefied at 17,778 reads) of microbial communities of feed (*n* = 3) and gut mucosa (*n* = 6) samples. diet, D; strain, S.

	Chao 1	Faith_PD	Observed OTUs	Shannon	Simpson
ITA_C	81 ± 46	1.41 ± 0.58	74 ± 43	3.69 ± 0.26	0.85 ± 0.02
ITA_PP	73 ± 36	1.27 ± 0.24	66 ± 33	3.61 ± 0.14	0.84 ± 0.01
USA_C	97 ± 45	1.27 ± 0.23	86 ± 39	3.83 ± 0.43	0.85 ± 0.03
USA_PP	118 ± 34	1.40 ± 0.38	104 ± 35	3.88 ± 0.52	0.85 ± 0.03
Sig	D: 0.661	D: 0.965	D: 0.737	D: 0.961	SxD: 0.676
	S: 0.081	S: 0.985	S: 0.123	S: 0.184	S: 0.431
	SxD: 0.401	SxD: 0.411	SxD: 0.423	SxD: 0.676	SxD: 0.739
Feed_C	269 ± 7	3.47 ± 0.11	260 ± 7	4.92 ± 0.02	0.90 ± 0.00
Feed_PP	288 ± 1	4.05 ± 0.22	261 ± 7	4.66 ± 0.12	0.89 ± 0.01
t-test	0.010	0.014	0.827	0.024	0.010

ITA, Italian strain; United States , American strain; C, commercial diet; PP, all-plant protein diet.

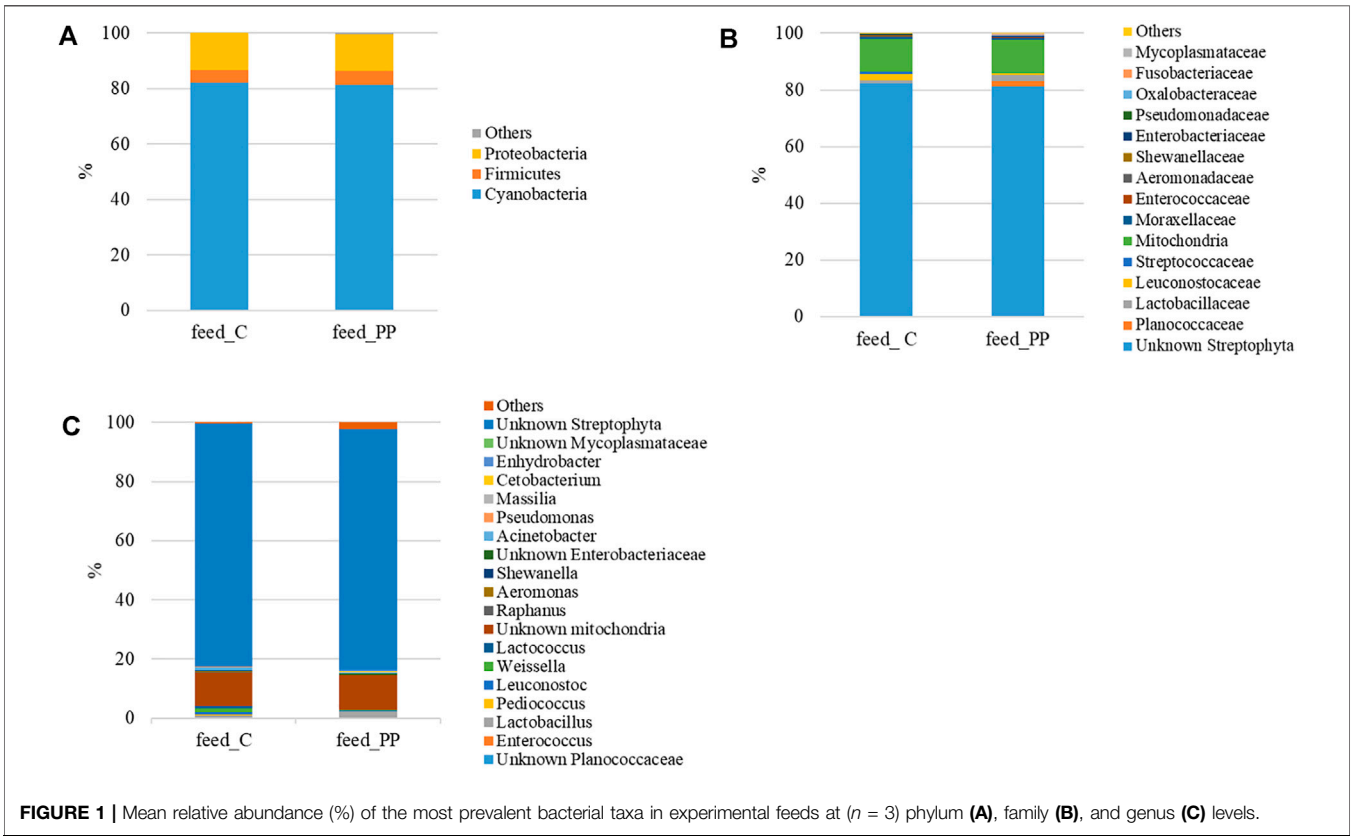


FIGURE 1 | Mean relative abundance (%) of the most prevalent bacterial taxa in experimental feeds at (*n* = 3) phylum **(A)**, family **(B)**, and genus **(C)** levels.

coverage was also calculated, resulting in a good range (ranged 0.99–1) in all the samples.

When comparing the bacterial alpha diversity of gut mucosa samples, no significant differences were found in species richness and biodiversity indices (**Table 4**). Differently, the plant protein feed-related microbiota was characterized by higher species richness (Chao1 and Faith_PD) and lower diversity (Shannon and Simpson indices) than the C diet (**Table 4**).

Feed-Related Microbial Community Profiles

The microbial community profiles of the feeds were outlined up to the genus level. Most of the sequences (81–82%) were of plant origin (p_Cyanobacteria and c_Chloroplast), deriving from vegetable ingredients of the diets. Considering only the most representative taxa, the overall feed-related microbial community comprised three phyla, four classes, five orders, 14 families, and 14 genera. The profiles of the feed-related microbial communities are shown at the phylum, family, and genus level in **Figure 1**. The list of the most abundant bacterial taxa, their relative abundance values, and statistical analysis are reported in **Supplementary Table S1**. Proteobacteria and Firmicutes were the dominant bacterial phyla in feeds, with a relative abundance of 13% and 4%, respectively (**Figure 1A**).

Accordingly, Alphaproteobacteria and Bacilli were the predominant classes of bacteria in the feed samples. At the order level, Lactobacillales only differed between the two feeds,

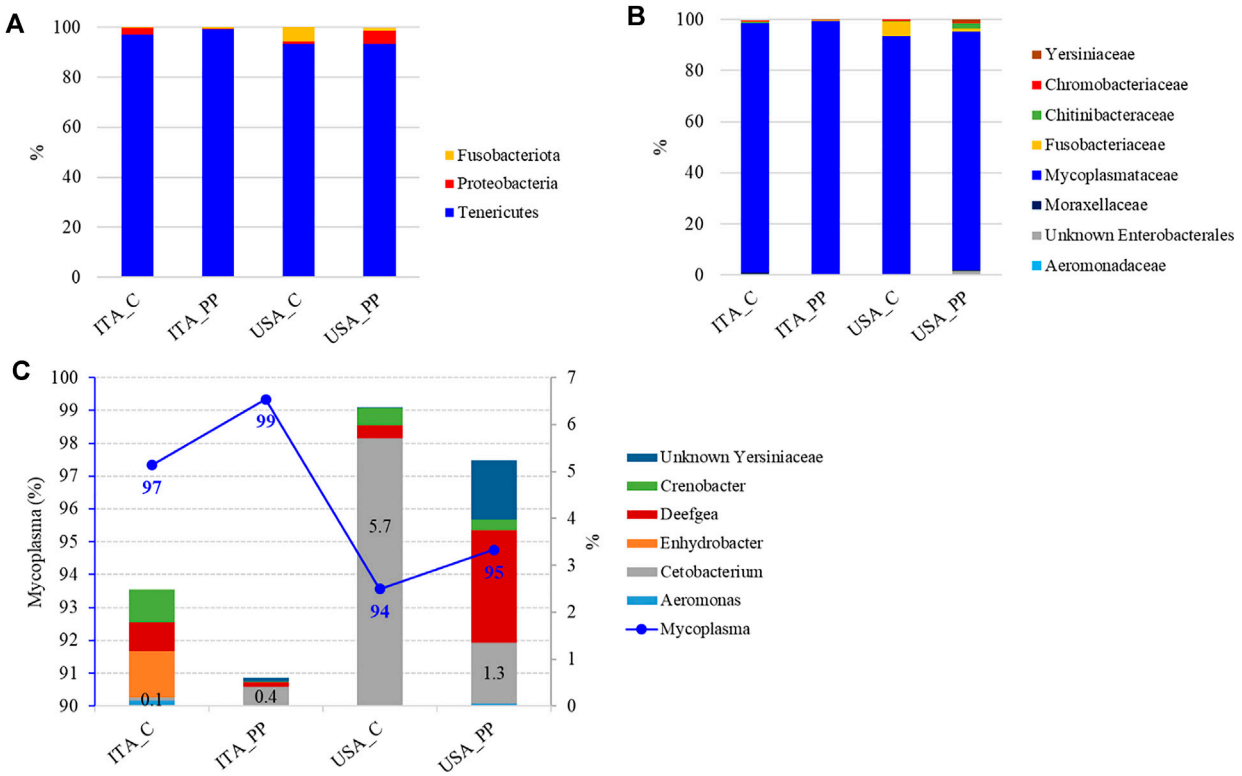


FIGURE 2 | Mean relative abundance (%) of the most prevalent bacterial taxa in gut mucosa of two rainbow trout strains (the United States and ITA) fed experimental diets (C and PP) at phylum **(A)**, and family **(B)**, and genus **(C)** level. ITA_C (n = 6), ITA_PP (n = 6), United States_C (n = 5), and United States_PP (n = 6). Percentages of the Cetobacterium genus are indicated on histograms. Blueline and the corresponding values indicate the amount of Mycoplasma in each experimental feeding group.

resulting in two times more abundant in the C diet than in the PP feed. Bacteria assigned to the Leuconostocaceae, Streptococcaceae, Aeromonadaceae, and Shewanellaceae families were preferentially associated with the C diet, while Fusobacteriaceae and Mycoplamataceae were found in the PP feed only (**Figure 1B**). Similarly, at the genus level, *Pediococcus*, *Leuconostoc*, *Weissella*, *Lactococcus*, *Aeromonas*, *Shewanella*, and *Acinetobacter* were significantly higher in the C diet when compared to the PP feed (**Figure 1C**). The relative abundance of the *Lactobacillus* genus only was increased in the PP diet, whereas *Cetobacterium* and *Anhydrobacter* genera were solely detected (**Figure 1C**).

Effects of Genotype and Diet on the Gut Microbiome Composition

The overall intestinal microbial community, considering only the most representative taxa, was mainly composed of three phyla, three classes, five orders, seven families, and six genera. The profiles of the intestinal microbial communities for each dietary treatment are shown at phylum, family, and genus level in **Figure 2**. The most abundant bacterial taxa with their relative abundance data and statistical analysis are listed in **Supplementary Table S2**.

To determine the impact of strain and diet on intestinal phylotypic diversity, UniFrac analysis was performed. Principal Coordinates Analysis (PCoA) of unweighted UniFrac distances revealed an effect of both the strain and the diet on gut microbial community profiles, with the first principal coordinate PC1 explaining up to 48.9% of the variation among the individuals (**Figure 3**).

Intestinal samples appeared clearly separated from the feed ones, thus indicating that observed differences between intestinal bacterial communities were not simply a consequence of undigested feed-related bacteria. Gut communities from the ITA strain clustered separately according to the diet. Similarly, distinct clusters were observed between the trout strains fed the C diet. Results of unweighted UniFrac PCoA were wholly confirmed by the non-parametric multivariate statistical test PERMANOVA (**Table 5**). The unweighted data showed a significant interaction between strain and diet. Results of pairwise comparisons showed differences between strains fed the same diet, while an overall effect of the diet was evident for the ITA strain only (p = 0.004). The weighted UniFrac analysis was also performed, but no significant differences were found among the groups (data not shown).

The autochthonous gut microbiome of rainbow trout was mostly dominated, regardless of the diet, by the Tenericutes phylum, mainly represented by the *Mycoplasma* genus, with a relative abundance ranging between 93 and 99% in all the samples (**Figure 2**). Proteobacteria and Fusobacteria completed the microbiota profile, while Firmicutes were unexpectedly not detected. The difference in gut microbiota composition

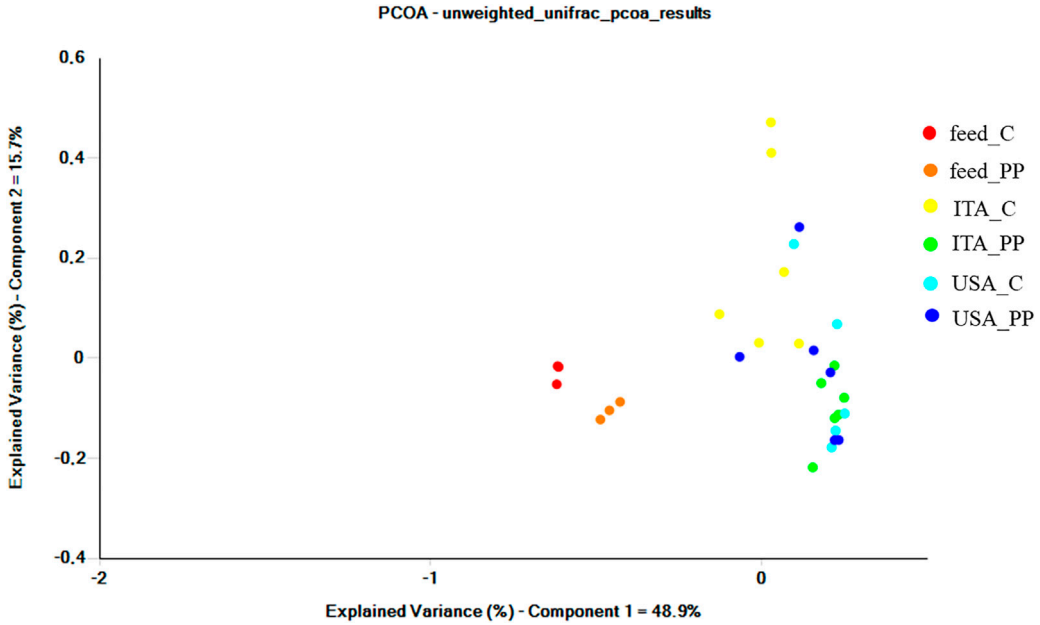


FIGURE 3 | Beta diversity metrics. Principal coordinate analysis (PCoA) of unweighted UniFrac distances of microbial communities associated with gut mucosa and feeds. The figure shows the bi-dimensional plot of individual fish and feeds samples according to their microbiome profile.

TABLE 5 | Results of non-parametric multivariate analysis PERMANOVA on the unweighted UniFrac data of intestinal and feed samples. Significant *p* values ($p \leq 0.05$) are in bold.

Two-way PERMANOVA		
Permutation N		
	999	
Source	Pseudo-F	P
Strain	1.354	0.220
Diet	4.800	**0.021**
Interaction	3.904	**0.012**
Pairwise comparisons		
	Pseudo-F	P
Feed C vs. feed PP	48.5	0.103
ITA_C vs. ITA_PP	5.435	**0.004**
ITA_C vs. USA_C	3.296	**0.022**
ITA_C vs. USA_PP	1.964	0.101
ITA_PP vs. USA_C	1.933	0.095
ITA_PP vs. USA_PP	2.339	**0.014**
USA_C vs. USA_PP	1.245	0.264

ITA, Italian strain; United States , American strain; C, commercial diet; PP, all-plant protein diet; P, probability.

between the two strains was limited to the *Cetobacterium* genus, belonging to the Fusobaceriaceae family (**Figure 2**). Two-way ANOVA indicated that the relative abundance of this genus was influenced by genotype only, with results being higher in the United States strain when compared to the ITA fish (**Figure 2**, **Supplementary Table S2**).

Functional Analysis of the Gut Microbiome

To attain a comprehensive analysis of the functional composition of the metagenome of each gut microbial community based on 16S rRNA sequencing, the PICRUSt method was used at Level 3 KEGG. Comparative analysis of PICRUSt functional inferences revealed an increased abundance of genes responsible for replication and repair pathway in both the ITA and the United States strains fed the all-plant protein diet (**Figure 4**). A total of six pathways were, instead, significantly different between the two strains when fed the C feed. Specifically, the pathway involved in starch and sucrose metabolism was more abundant in the United States trout in comparison with the ITA strain. On the contrary, the ITA trout showed an over-representation of the ubiquinone biosynthesis pathway (**Figure 4**).

DISCUSSION

Growth Performance

Independent of the administered diet, the United States rainbow trout strain in the present study overall showed better growth performance (in terms of increased FBW, iWGm, and SGR) when compared to the ITA one. Furthermore, a clear strain×diet interaction indicating an improvement in the iWG in the United States fish was observed when the C diet only was provided. This represents an interesting finding, as the selected strains usually outperform the non-selected ones on the plant-based selection diet (Overturf et al., 2013). Therefore, the ability of the United States rainbow trout to grow more efficiently when fed either the low-FM or the all-plant protein diets–with even more pronounced effects when using the former–reflects the genetic selection directed towards the utilization of the vegetable proteins, which efficiently integrates the physiological, carnivorous feeding behavior of this fish species.

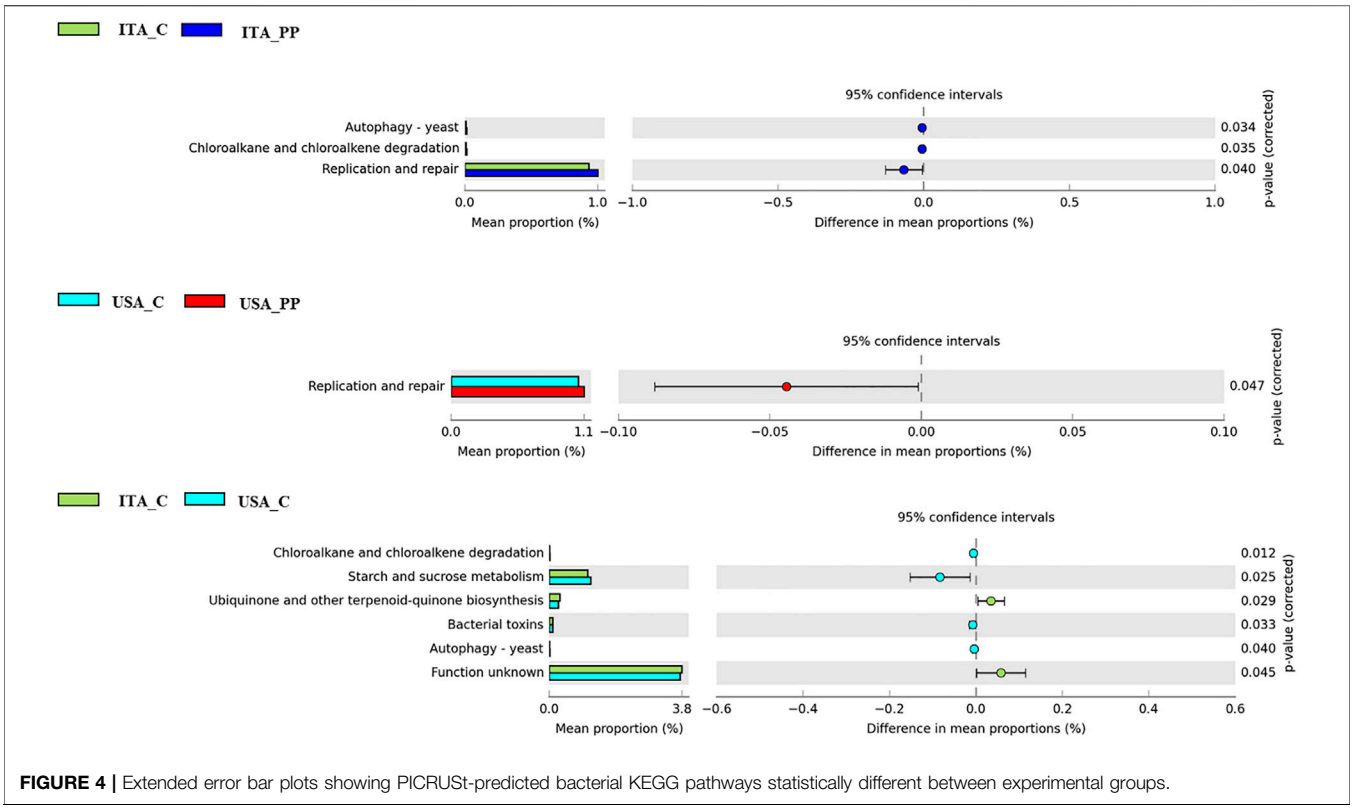


FIGURE 4 | Extended error bar plots showing PICRUSt-predicted bacterial KEGG pathways statistically different between experimental groups.

Analogously to what was reported by Overturf et al. (2013), the selected strain of the current research displayed similar FCR to that of the non-selected one, thus still not excluding a primary role of the feed intake–rather than the feed efficiency–in the improvement of the growth performance (Callet et al., 2017). Differently, the percent survival of the rainbow trout of the present study–while remaining high (≥ 95%)—displayed a clear strain × diet interaction, with the United States strain surviving more when fed the all-plant protein diet, and the ITA fish behaving similarly when fed the low-FM diet. This is in partial agreement with Callet et al. (2017) that observed higher survival in the selected strain fed the plant-based diets in comparison with the non-selected fish, thus reasonably reflecting genetic selection. As a final aspect to consider, independently of the strain, the rainbow trout used in the current research showed greater FBW and iWG when fed the PP diet. This may be related to the high protein digestibility of all-plant protein diets that have previously been highlighted, as such diets are usually formulated with highly digestible protein concentrates (Gaylord et al., 2008; Callet et al., 2017; Brezas and Hardy, 2020). Furthermore, the ability of rainbow trout to grow efficiently on a diet containing soy protein concentrate-based blend as a replacement of high percentages (until 87%) of the FM in the formulation has already been reported (Burr et al., 2012).

Whole-Body Proximate Composition

Independent of the considered strain, the PP diet led to the lower whole-body moisture content in comparison with the C one, as previously reported (Overturf et al., 2013). Furthermore, a clear strain × diet interaction indicating a reduction in the moisture content of the ITA fish was observed when the PP diet only was provided. This may represent the logical consequence of the increased whole-body EE identified in the PP-fed ITA rainbow trout when compared to the United States fish fed the same diet. As far as ash content is concerned, the C diet determined an overall increase in the United States strain only. Overturf et al. (2013) previously reported that whole-body ash was unaffected in either the selected or the non-selected rainbow trout in response to both a FM- or PP-based diet, but no information about selection-related ash digestibility changes is currently available. Therefore, the increased ash content herein highlighted may result from a better ash digestibility, thus potentially strengthening the effect of the higher ash content of the C diet than the PP feed. Differently, the United States strain showed that the whole-body CP content was similar to the non-selected one, as previously reported (Overturf et al., 2013). This also confirms that CP digestibility is not different in the selected rainbow trout as previously observed (Callet et al., 2017; Brezas and Hardy, 2020). However, independently of the considered strain, the PP diet led to an increased CP content in comparison with the C feed, thus reflecting the above-mentioned high digestibility of the vegetable proteins (Gaylord et al., 2008; Callet et al., 2017; Brezas and Hardy, 2020). As a final aspect to consider, the whole-body EE content of the rainbow trout of the present study displayed

a clear strain×diet interaction, with the ITA strain body containing more EE than the United States fish when fed the all-plant protein diet. Genetic selection towards the utilization of vegetable proteins has been reported to not influence the whole-body EE content of rainbow trout (Overturf et al., 2013; Callet et al., 2017; Brezas and Hardy, 2020). However, the higher whole-body EE content of the PP-fed ITA rainbow trout when compared to the United States fish may result from a synergic effect of both the physiological response to all-plant protein diets (Burel et al., 1998; Kaushik et al., 2004; Lazzarotto et al., 2015) and the use of a C diet (characterized by higher EE content) across the generations.

Microbiome Analysis

The high-throughput sequencing on the Illumina MiSeq platform has been used in the present research for intestinal microbiome characterization. In line with the previous studies performed on rainbow trout, results of the metabarcoding analysis showed that the most abundant phylum of autochthonous gut microbiota in rainbow trout, regardless of the diet and genotype, was Tenericutes, mainly represented by the *Mycoplasma* genus (Rimoldi et al., 2019). Accordingly, it has recently been suggested that *Mycoplasma*, due to its dominance in the distal intestine of rainbow trout and other farmed salmonids, could have a mutualistic relationship with its host (Rasmussen et al., 2021). In Atlantic salmon (*Salmo salar*), the abundance of *Mycoplasma* has positively been associated with improved growth, carotenoid utilization, and disease resilience of the host (Bozzi et al., 2021). In addition, this genus harbors genes for long-chain polymer degradation, such as chitin, which is particularly abundant in insects, that are part of the natural diet of juvenile salmonids. This ability of *Mycoplasma* could be beneficial for its host, which can utilize all the nutritional value of a chitin-rich diet. Indeed, an increased presence of *Mycoplasma* was also shown in the gut of rainbow trout fed an insect-based diet (Rimoldi et al., 2019). An unexpected result was the absence of bacteria belonging to the Firmicutes phylum in all the gut samples, even in those fish fed the plant-based diet. Members of this phylum are known to play a key role in the fermentation of dietary carbohydrates (Corrigan et al., 2015), and are usually associated with plant ingredients, while low-FM diet generally favors the presence of Proteobacteria (Desai et al., 2012; Ingerslev et al., 2014a and b; Rimoldi et al., 2018). An increase in Firmicutes to Proteobacteria ratio was, however, observed by Blaufuss et al. (2020) in analogously selected trout for growth on an all-plant protein diet when fed all-plant protein feeds.

In terms of alpha diversity, no differences were detected between ITA and United States strains in microbial richness and biodiversity, regardless of the administered diet. Comparison of the overall microbial composition among all the samples according to weighted and unweighted UniFrac showed an overlap among all the gut samples, with the feed samples clustering separately. The composition of feed-related microbial communities was, indeed, significantly different from gut microbiota, resulting mainly constituted of cyanobacteria, Proteobacteria, and Firmicutes phyla. According to PERMANOVA analysis, unweighted UniFrac indicated that either the diet or the host genotype plays a crucial role in shaping the gut microbiome, showing differences between the two strains fed the same diet and an overall effect of diet for ITA strain only. This result could explain the differences observed in growth performance between the two strains, with the United States trout growing more efficiently than the ITA fish when fed either the low-FM or the all-plant protein diets.

The current results seemed to indicate an existence of a relationship between the selected United States strain and the *Cetobacterium* genus, belonging to the Fusobacteriaceae family, whose relative abundance was an order of magnitude larger than that detected in the ITA strain. Similarly, higher abundances of the phyla Bacteroidetes and Fusobacteria phyla are associated with trout selected for high-muscle yield (Chapagain et al., 2020). Fusobacteria is commonly found in the freshwater fish guts, and *Cetobacterium* is an important commensal bacterium (Navarrete et al., 2012). Metabolites produced by *Cetobacterium* include, indeed, short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate, and vitamin B12, which can enhance fish health (Xie et al., 2021).

The *in silico* prediction of the metabolic capability of the bacterial populations revealed an up-regulation of the pathway related to starch and sucrose metabolism in the United States strain fed the C diet, thus indicating a better ability to utilize carbohydrates. This is not surprising, as the United States strain has been selected for its growth performance when fed all-plant protein feeds (rich in fibers and non-digestible carbohydrates). The fatty acid metabolism pathway has previously been reported to be enriched in high-muscle yield selected trout (Chapagain et al., 2020). However, despite the different pathways being activated, an up-regulation of bacterial pathways involved in energy supply–which is essential for host growth–was herein analogously observed.

CONCLUSION

In conclusion, the selected strain of rainbow trout overall displayed improved growth performance and (to a lesser extent) whole-body proximate composition when compared to the non-selected one. However, the absence of negative changes in the non-selected strain in response to all-plant protein feed is indicative of a proper adaptation of the fish to such protein sources over time, thus suggesting that a complete FM replacement in diets for rainbow trout is technically feasible. Furthermore, differences between microbial communities were mainly driven by host genetic selection, with the *Cetobacterium* probiotic-like genus being associated with the gut of the selected rainbow trout. Such microbiota signature, along with the up-regulation of the pathway involved in starch and sucrose metabolism, may reasonably predispose the selected strain to efficiently utilize all-plant protein feeds. Future research assessing additional parameters related to the gut health of the fish (i.e., gut mucosa morphology, mucin dynamics, inflammatory response, and metabolomics) are strongly recommended to further

investigate the role of genetic selection in the digestion of all-plant protein diets.

DATA AVAILABILITY STATEMENT

All fastq sequencing files were deposited in the EBI ENA public database under the accession project code PRJEB51166.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical Committee of the University of Turin.

AUTHOR CONTRIBUTIONS

LG, GT, MS, and RH designed the study. IB, CC, GC, MP, and SBO carried out the rearing work. CC and GC performed the whole-body proximate composition analysis. SR and GT performed the intestinal microbiome analysis. IB wrote the first draft of the manuscript. All the authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by the AGER project "Fine Feed for Fish (4F)," Rif. nr. 2016-01-01. Dr. Giulia Chemello was supported by a research grant of the University of Turin.

ACKNOWLEDGMENTS

The authors are grateful to Mr. Dario Sola for fish care and technical support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.892550/full#supplementary-material

REFERENCES

Abernathy, J., Brezas, A., Snekvik, K. R., Hardy, R. W., and Overturf, K. (2017). Integrative Functional Analyses Using Rainbow trout Selected for Tolerance to Plant Diets Reveal Nutrigenomic Signatures for Soy Utilization without the Concurrence of Enteritis. *PLoS ONE* 12, e0180972. doi:10.1371/journal.pone.0180972

Blaufuss, P. C., Bledsoe, J. W., Gaylord, T. G., Sealey, W. M., Overturf, K. E., and Powell, M. S. (2020). Selection on a Plant-Based Diet Reveals Changes in Oral Tolerance, Microbiota and Growth in Rainbow trout (*Oncorhynchus mykiss*) when Fed a High Soy Diet. *Aquaculture* 525, 735287. doi:10.1016/j.aquaculture.2020.735287

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Ghalith, G. A., et al. (2019). Reproducible, Interactive, Scalable and Extensible Microbiome Data Science Using QIIME 2. *Nat. Biotechnol* 37, 852–857. doi:10.1038/s41587-019-0209-9

Bozzi, D., Rasmussen, J. A., Carøe, C., Sveier, H., Nordøy, K., Gilbert, M. T. P., et al. (2021). Salmon Gut Microbiota Correlates with Disease Infection Status: Potential for Monitoring Health in Farmed Animals. *Anim. Microbiome* 3, 1–17. doi:10.1186/s42523-021-00096-2

Brezas, A., and Hardy, R. W. (2020). Improved Performance of a Rainbow trout Selected Strain Is Associated with Protein Digestion Rates and Synchronization of Amino Acid Absorption. *Sci. Rep.* 10, 4678. doi:10.1038/s41598-020-61360-0

Brezas, A., Kumar, V., Overturf, K., and Hardy, R. W. (2021). Dietary Amino Acid Supplementation Affects Temporal Expression of Amino Acid Transporters and Metabolic Genes in Selected and Commercial Strains of Rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.* 255, 110589. doi:10.1016/j.cbpb.2021.110589

Burel, C., Boujard, T., Corraze, G., Kaushik, S. J., Boeuf, G., Mol, K. A., et al. (1998). Incorporation of High Levels of Extruded Lupin in Diets for Rainbow trout (*Oncorhynchus mykiss*): Nutritional Value and Effect on Thyroid Status. *Aquaculture* 163, 325–345. doi:10.1016/S0044-8486(98)00241-5

Burr, G. S., Wolters, W. R., Barrows, F. T., and Hardy, R. W. (2012). Replacing Fishmeal with Blends of Alternative Proteins on Growth Performance of Rainbow trout (*Oncorhynchus mykiss*), and Early or Late Stage Juvenile Atlantic salmon (*Salmo salar*). *Aquaculture* 334-337, 110–116. doi:10.1016/j.aquaculture.2011.12.044

Callet, T., Dupont-Nivet, M., Danion, M., Burel, C., Cluzeaud, M., Surget, A., et al. (2021). Why Do Some Rainbow Trout Genotypes Grow Better with a Complete Plant-Based Diet? Transcriptomic and Physiological Analyses on Three Isogenic Lines. *Front. Physiol.* 12, 732321. doi:10.3389/fphys.2021.732321

Callet, T., Médale, F., Larroquet, L., Surget, A., Aguirre, P., Kerneis, T., et al. (2017). Successful Selection of Rainbow trout (*Oncorhynchus mykiss*) on Their Ability to Grow with a Diet Completely Devoid of Fishmeal and Fish Oil, and Correlated Changes in Nutritional Traits. *PLoS One* 12, e0186705. doi:10.1371/journal.pone.0186705

Chapagain, P., Walker, D., Leeds, T., Cleveland, B. M., and Salem, M. (2020). Distinct Microbial Assemblages Associated with Genetic Selection for High- and Low- Muscle Yield in Rainbow trout. *BMC Genomics* 21, 820. doi:10.1186/s12864-020-07204-7

Cleveland, B. M., Gao, G., and Leeds, T. D. (2020). Transcriptomic Response to Selective Breeding for Fast Growth in Rainbow Trout (*Oncorhynchus mykiss*). *Mar. Biotechnol.* 22, 539–550. doi:10.1007/s10126-020-09974-3

Corrigan, A., De Leeuw, M., Penaud-Frézet, S., Dimova, D., and Murphy, R. A. (2015). Phylogenetic and Functional Alterations in Bacterial Community Compositions in Broiler Ceca as a Result of Mannan Oligosaccharide Supplementation. *Appl. Environ. Microbiol.* 81, 3460–3470. doi:10.1128/AEM.04194-14

Desai, A. R., Links, M. G., Collins, S. A., Mansfield, G. S., Drew, M. D., Van Kessel, A. G., et al. (2012). Effects of Plant-Based Diets on the Distal Gut Microbiome of Rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 350-353, 134–142. doi:10.1016/j.aquaculture.2012.04.005

FAO (2020). The State of World Fisheries and Aquaculture 2020." in *Sustainability in Action*. Rome: FAO. doi:10.4060/ca9229en

Gaudioso, G., Marzorati, G., Faccenda, F., Weil, T., Lunelli, F., Cardinaletti, G., et al. (2021). Processed Animal Proteins from Insect and Poultry By-Products in a Fish Meal-Free Diet for Rainbow Trout: Impact on Intestinal Microbiota and Inflammatory Markers. *Int. J. Mol. Sci* 22, 5454. doi:10.3390/ijms22115454

Gaylord, T. G., Barrows, F. T., and Rawles, S. D. (2008). Apparent Digestibility of Gross Nutrients from Feedstuffs in Extruded Feeds for Rainbow Trout,*Oncorhynchus mykiss*. *J. World Aquac. Soc.* 39, 827–834. doi:10.1111/j.1749-7345.2008.00220.x

Hammer, Ø., Harper, D. A. T., and Paul, D. R. (2001). Past: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontol. Electron* 4 (1), 9.

Ingerslev, H.-C., Strube, M. L., Jørgensen, L. v. G., Dalsgaard, I., Boye, M., and Madsen, L. (2014a). Diet Type Dictates the Gut Microbiota and the Immune Response against *Yersinia ruckeri* in Rainbow trout (*Oncorhynchus mykiss*). *Fish Shellfish Immunol.* 40, 624–633. doi:10.1016/j.fsi.2014.08.021

Ingerslev, H.-C., von Gersdorff Jørgensen, L., Lenz Strube, M., Larsen, N., Dalsgaard, I., Boye, M., et al. (2014b). The Development of the Gut Microbiota in Rainbow trout (*Oncorhynchus mykiss*) Is Affected by First Feeding and Diet Type. *Aquaculture* 424-425, 24–34. doi:10.1016/j.aquaculture.2013.12.032

Kaushik, S. J., Covès, D., Dutto, G., and Blanc, D. (2004). Almost Total Replacement of Fish Meal by Plant Protein Sources in the Diet of a marine Teleost, the European Seabass, *Dicentrarchus labrax*. *Aquaculture* 230, 391–404. doi:10.1016/S0044-8486(03)00422-8

Krogdahl, Å., Penn, M., Thorsen, J., Refstie, S., and Bakke, A. M. (2010). Important Antinutrients in Plant Feedstuffs for Aquaculture: an Update on Recent Findings Regarding Responses in Salmonids. *Aquac. Res.* 41, 333–344. doi:10.1111/j.1365-2109.2009.02426.x

Langille, M. G. I., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., et al. (2013). Predictive Functional Profiling of Microbial Communities Using 16S rRNA Marker Gene Sequences. *Nat. Biotechnol.* 31, 814–821. doi:10.1038/nbt.2676

Lazzarotto, V., Corraze, G., Leprevost, A., Quillet, E., Dupont-Nivet, M., and Médale, F. (2015). Three-year Breeding Cycle of Rainbow trout (*Oncorhynchus mykiss*) Fed a Plant-Based Diet, Totally Free of marine Resources: Consequences for Reproduction, Fatty Acid Composition and Progeny Survival. *PLoS One* 10, e0117609. doi:10.1371/journal.pone.0117609

Lozupone, C. A., Hamady, M., Kelley, S. T., and Knight, R. (2007). Quantitative and Qualitative β Diversity Measures Lead to Different Insights into Factors that Structure Microbial Communities. *Appl. Environ. Microbiol.* 73, 1576–1585. doi:10.1128/AEM.01996-06

Lozupone, C., and Knight, R. (2005). UniFrac: a New Phylogenetic Method for Comparing Microbial Communities. *Appl. Environ. Microbiol.* 71, 8228–8235. doi:10.1128/AEM.71.12.8228-8235.2005

Navarrete, P., Magne, F., Araneda, C., Fuentes, P., Barros, L., Opazo, R., et al. (2012). PCR-TTGE Analysis of 16S rRNA from Rainbow trout (*Oncorhynchus mykiss*) Gut Microbiota Reveals Host-specific Communities of Active Bacteria. *PLoS One* 7, e31335. doi:10.1371/journal.pone.0031335

Overturf, K., Barrows, F. T., and Hardy, R. W. (2013). Effect and Interaction of Rainbow trout Strain (*Oncorhynchus mykiss*) and Diet Type on Growth and Nutrient Retention. *Aquac. Res.* 44, 604–611. doi:10.1111/j.1365-2109.2011.03065.x

Parks, D. H., Tyson, G. W., Hugenholtz, P., and Beiko, R. G. (2014). STAMP: Statistical Analysis of Taxonomic and Functional Profiles. *Bioinformatics* 30, 3123–3124. doi:10.1093/bioinformatics/btu494

Piazzon, M. C., Naya-Català, F., Perera, E., Palenzuela, O., Sitjà-Bobadilla, A., and Pérez-Sánchez, J. (2020). Genetic Selection for Growth Drives Differences in Intestinal Microbiota Composition and Parasite Disease Resistance in Gilthead Sea Bream. *Microbiome* 8, 168. doi:10.1186/s40168-020-00922-w

Rasmussen, J. A., Villumsen, K. R., Duchêne, D. A., Puetz, L. C., Delmont, T. O., Sveier, H., et al. (2021). Genome-resolved Metagenomics Suggests a Mutualistic Relationship between Mycoplasma and Salmonid Hosts. *Commun. Biol.* 4, 579. doi:10.1038/s42003-021-02105-1

Rimoldi, S., Antonini, M., Gasco, L., Moroni, F., and Terova, G. (2021). Intestinal Microbial Communities of Rainbow trout (*Oncorhynchus mykiss*) May Be Improved by Feeding a Hermetia Illucens Meal/low-Fishmeal Diet. *Fish. Physiol. Biochem.* 47, 365–380. doi:10.1007/s10695-020-00918-1

Rimoldi, S., Gini, E., Iannini, F., Gasco, L., and Terova, G. (2019). The Effects of Dietary Insect Meal from Hermetia Illucens Prepupae on Autochthonous Gut Microbiota of Rainbow trout (*Oncorhynchus mykiss*). *Animals* 9, 143. doi:10.3390/ani9040143

Rimoldi, S., Terova, G., Ascione, C., Giannico, R., and Brambilla, F. (2018). Next Generation Sequencing for Gut Microbiome Characterization in Rainbow trout (*Oncorhynchus mykiss*) Fed Animal By-Product Meals as an Alternative to Fishmeal Protein Sources. *PLoS One* 13, e0193652–29. doi:10.1371/journal.pone.0193652

Terova, G., Gini, E., Gasco, L., Moroni, F., Antonini, M., and Rimoldi, S. (2021). Effects of Full Replacement of Dietary Fishmeal with Insect Meal from *Tenebrio molitor* on Rainbow trout Gut and Skin Microbiota. *J. Anim. Sci Biotechnol* 12, 30. doi:10.1186/s40104-021-00551-9

Terova, G., Rimoldi, S., Ascione, C., Gini, E., Ceccotti, C., and Gasco, L. (2019). Rainbow trout (*Oncorhynchus mykiss*) Gut Microbiota Is Modulated by Insect Meal from *Hermetia Illucens* Prepupae in the Diet. *Rev. Fish. Biol. Fish.* 29, 465–486. doi:10.1007/s11160-019-09558-y

Vallejo, R. L., Fragomeni, B. O., Cheng, H., Gao, G., Long, R. L., Shewbridge, K. L., et al. (2020). Assessing Accuracy of Genomic Predictions for Resistance to Infectious Hematopoietic Necrosis Virus with Progeny Testing of Selection Candidates in a Commercial Rainbow Trout Breeding Population. *Front. Vet. Sci.* 7, 590048. doi:10.3389/fvets.2020.590048

Vargas-Albores, F., Martínez-Córdova, L. R., Hernández-Mendoza, A., Cicala, F., Lago-Lestón, A., and Martínez-Porchas, M. (2021). Therapeutic Modulation of Fish Gut Microbiota, a Feasible Strategy for Aquaculture? *Aquaculture* 544, 737050. doi:10.1016/j.aquaculture.2021.737050

Venold, F. F., Penn, M. H., Krogdahl, Å., and Overturf, K. (2012). Severity of Soybean Meal Induced Distal Intestinal Inflammation, Enterocyte Proliferation Rate, and Fatty Acid Binding Protein (Fabp2) Level Differ between Strains of Rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 364-365, 281–292. doi:10.1016/j.aquaculture.2012.08.035

Xie, M., Zhou, W., Xie, Y., Li, Y., Zhang, Z., Yang, Y., et al. (2021). Effects of *Cetobacterium Somerae* Fermentation Product on Gut and Liver Health of Common Carp (*Cyprinus carpio*) Fed Diet Supplemented with Ultra-micro Ground Mixed Plant Proteins. *Aquaculture* 543, 736943. doi:10.1016/j.aquaculture.2021.736943

Xu, K., Duan, W., Xiao, J., Tao, M., Zhang, C., Liu, Y., et al. (2015). Development and Application of Biological Technologies in Fish Genetic Breeding. *Sci. China Life Sci.* 58, 187–201. doi:10.1007/s11427-015-4798-3

Zuo, S., Karami, A. M., Ødegård, J., Mathiessen, H., Marana, M. H., Jaafar, R. M., et al. (2020). Immune Gene Expression and Genome-wide Association Analysis in Rainbow trout with Different Resistance to *Yersinia ruckeri* Infection. *Fish Shellfish Immunol.* 106, 441–450. doi:10.1016/j.fsi.2020.07.023

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: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Marine Science

ORIGINAL RESEARCH
published: 24 May 2022
doi: 10.3389/fmars.2022.902633

Condensed Tannins Improve Glycolipid Metabolism but Induce Liver Injury of Chinese Seabass (*Lateolabrax maculatus*)

Kai Peng[1], *Bing Chen*[1], *Hongxia Zhao*[1], *Yuxi Wang*[2] *and Wen Huang*[1]*

[1] *Key Laboratory of Animal Nutrition and Feed Science in South China, Ministry of Agriculture and Rural Affairs, Guangdong Provincial Key Laboratory of Animal Breeding and Nutrition, Institute of Animal Science, Guangdong Academy of Agricultural Sciences, Guangzhou, China,* [2] *Lethbridge Research and Development Centre, Agriculture and Agri-Food Canada, Lethbridge, AB, Canada*

A 63-day feeding trial with 640 juvenile *L. maculatus* was conducted to assess the effects of dietary CT on serum metabolites, antioxidant and immune response, liver histomorphology and glycometabolism enzyme activities of fish. Four diets were formulated to contain 0 (T1), 1 (T2), 2 (T3), and 2 g/kg of CT plus 4 g/kg of polyethylene glycol (PEG) (T4). PEG specifically binds with CT to neutralize CT activity. Fish were randomly distributed into 16 tanks (4 tanks per group and 40 fish per tank) and were fed to apparent satiation twice daily. Results indicated that fish fed T2 and T3 had lower (P<0.05) concentrations of serum albumin, total cholesterol and triacylglycerol, but higher (P<0.05) alanine aminotransferase activity than T1 and T4. T3 had lower (P<0.05) serum glucose but higher (P<0.05) insulin concentrations than other groups. Fish fed T2 and T3 had higher (P<0.05) liver superoxide dismutase, catalase, lysozyme and immune globulin M than T1 and T4. Compared with T1 and T4, hepatocytes in T2 and T3 were shown to have obvious vacuolar degeneration along with different degree of inflammatory cell infiltration. Fish fed T2 and T3 had higher (P<0.05) values of eosinophilic infiltrate and necrosis and greater (P<0.05) pyruvate kinase, but lower (P<0.05) glucokinase activities than those of fish fed T1 and T4. All parameters were similar (P>0.05) between T1 and T4. In summary, dietary CT up to 2 g/kg reduced serum lipid and glucose concentrations, enhanced liver antioxidant and immune response, improved glucose utilization but of *L. maculatus*. CT induced liver injury of *L. maculatus* which provided a caution for its application in aquaculture.

Keywords: condensed tannins, feed additive, glycolipid metabolism, physical health, fish

OPEN ACCESS

Edited by:
Helena Peres,
University of Porto, Portugal

Reviewed by:
Josef Velíšek,
University of South Bohemia, Czechia
Hamdy Soliman,
Sohag University, Egypt

***Correspondence:**
Wen Huang
huangwen549@126.com

Specialty section:
This article was submitted to Aquatic Physiology, a section of the journal Frontiers in Marine Science

Received: *23 March 2022*
Accepted: *25 April 2022*
Published: *24 May 2022*

Citation:
Peng K, Chen B, Zhao H, Wang Y and Huang W (2022) Condensed Tannins Improve Glycolipid Metabolism but Induce Liver Injury of Chinese Seabass (Lateolabrax maculatus). Front. Mar. Sci. 9:902633. doi: 10.3389/fmars.2022.902633

INTRODUCTION

Chinese seabass (*Lateolabtax maculatus*), an important commercial fishery species in China, whose production was nearly 200,000 tons and the annual output value exceed 3 billion yuan in 2020 according to the data from China Fishery Statistics Yearbook (Fishery Administration of Ministry of Agriculture and Rural Affairs, 2021). In recent years, the ever-increasing breeding density of

L.maculatus driven by commercial interests compels fish under stress or in sub-health state for a long time, which threats to fish health and food safety. On the other hand, since antibiotics were completely banned, challenges including metabolic disorders, disease and oxidative stress faced by intensive breeding have increased severely. Therefore, there is an urgent need to find new alternatives to antibiotics to maintain healthy and sustainable development of aquaculture.

Condensed tannins (CT) is a kind of polyphenol compound which widely exists in the plant kingdom (Peng et al., 2016). CT was regarded as a promising alternative to antibiotics owing to its strong antioxidant, anti-inflammatory and antibacterial activities and etc. (Huang et al., 2018). However, limited information is available about the evaluation of effects of CT on physiology and health of fish. It has been reported that the biological activity of CT is closely associated with its dietary concentrations (Waghorn, 2008). Our recent studies showed that dietary CT less than 1 g/kg did not affect growth performance but improved stress resistance of *L. maculatus* (Peng et al., 2020a; Peng et al., 2022a; Peng et al., 2022b). In contrast, other studies documented that dietary CT exceeded 1 g/kg induced intestinal injury of shrimp (*Litopenaeus vannamei*) (Peng et al., 2021a) and impaired immune function of grass carp (*Ctenopharyngodon idella*) (Li et al., 2020). Furthermore, dietary CT up to 1 g/kg was observed to reduce the serum lipid and glucose levels of *L. maculatus* (Peng et al., 2020a), whereas this has not been assessed for high concentration of CT.

Although CT enhanced antioxidant and immune capacity and improved physical health of *L. maculatus*, dietary CT exceeded 1 g/kg may have adverse effects on fish, including growth inhibition and cellular damage according to the observations by previous studies. To verify this hypothesis, this study is conducted to assess the effects of dietary CT on serum metabolites, antioxidant and immune response, liver histomorphology and glycometabolism enzyme activities of *L. maculatus* to provide a rational for the application of CT in fish diets.

MATERIALS AND METHODS

Diet Preparation

Condensed tannins (purity ≥99.5%) was extracted from grape seed and purified according to the method described by Peng et al. (2022a). Polyethylene glycol (PEG, MW 3350, Sigma) specifically binds with CT to neutralize CT activity (Peng et al., 2016). Therefore, comparison between diets with and without PEG supplementation would define the effects of CT. In total of four isoproteic and isolipidic diets (**Table 1**) were formulated to contain 0 (T1), 1 (T2), 2 (T3), and 2 g/kg of CT plus 4 g/kg of PEG (T4). PEG was added to diet by spraying 30 g/mL of PEG solution onto CT to achieve a CT-to-PEG ratio of 1:2 (Makkar et al., 1995). All ingredients were smashed to pass through a 320 μm sieve, mixed thoroughly and then extruded into 2 mm pellets, dried at 55 °C and stored at -20°C until use.

Experimental Design and Feeding Trial

A 63-day feeding trial was carried out using 640 disease-free *L. maculatus* provided by a commercial fish farm in Zhangzhou, Fujian, China. Fish with a mean initial body weight of 2.75 (± 0.03) g were randomly distributed into 16 tanks (four tanks per diet and 40 fish per tank) and were fed to apparent satiation twice daily (08:00 and 20:00). Water temperature was 26.5 ± 1.0°C, dissolved oxygen > 5.0 mg/L, pH was 7.7 ± 0.3, salinity was 1‰, ammonia nitrogen and nitrite < 0.01 mg/L, and the photoperiod regime was 12 h light and 12 h dark. The protocol (no. SC20210402) of this study was approved by the Animal Care and Use Committee of Guangdong Academy of Agricultural Sciences (Guangzhou, China).

Sampling

Before sampling, fish were fasted for 24 h and anesthetized with 40 mg/L of tricaine methanesulfonate (Sigma, USA). Blood were collected from the caudal veins of twelve fish per tank, kept at 25°C for 30 min, and centrifuged at 8000 × *g* for 10 min. The resultant serum was determined for the serum metabolite concentrations. Livers of six fish per tank were randomly collected, of which three livers were analyzed for antioxidant and immune parameters, and another three livers were used for histological examination following the procedures described by Peng et al. (2021a). Livers of three fish per tank were randomly collected for measuring glycometabolism enzyme activities.

Laboratory Analyses

The crude protein, crude lipid and ash contents of the experimental diets were measured using AOAC method (AOAC, 1999). Serum metabolites, including albumin (ALB), globulin (GLOB), total cholesterol (TCHO), triacylglycerol (TG), alanine aminotransferase (ALT), aspartate aminotransferase (AST), high density lipoprotein cholesterol (HDLC), low density lipoprotein cholesterol (LDLC), glucose (GLU), insulin (INS) and glucagon (GLG), and liver antioxidant and immune parameters, including total antioxidant capacity (TAOC), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), malondialdehyde (MDA), lysozyme (LZM), immune globulin M (IgM) and complement 3 (C3), and liver glycometabolism enzyme activities, including phosphofructokinase (PFK), pyruvate kinase (PK) and glucokinase (GK) were determined using commercial kits provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to corresponding manufacture's instructions.

For liver histological analysis, score were attributed to histological changes according to the degree of intensity: 0 (absence of change), 1 (mild change, corresponding to <25% of the organ area), 2 (moderate change, 25% to 50% of the organ area) and 3 (severe alteration, >50% of the organ area) following the method described by Schwaiger et al. (1997) modified by Brum et al. (2018). The following hepatic changes were evaluated: nucleus hypertrophy, hepatocyte hypertrophy, eosinophilic infiltrate, lymphocytic infiltrate, necrosis, karyolysis, karyorrhexis and pyknosis.

Data Calculations and Statistical Analysis

All data were subjected to a one-way analysis of variance using SPSS 17.0 analysis software for Windows followed by the Duncan's multiple-range test with tank as statistical unit and treatment as fixed effect. Differences were regarded as significance when $P<0.05$ and the results are presented as means ± standard error.

TABLE 1 | Ingredients and proximate composition (g/kg DM) of experimental diets.

Items	Diets			
	T1	T2	T3	T4
Ingredients				
Fish meal	180	180	180	180
Casein	180	180	180	180
Soy protein concentrate	160	160	160	160
High gluten	280	280	280	280
Monocalcium phosphate	15	15	15	15
Fish oil	40	40	40	40
Soybean oil	20	20	20	20
Soy lecithin	20	20	20	20
Vitamin premix	2	2	2	2
Mineral premix	5	5	5	5
Choline chloride	5	5	5	5
Vitamin C ester	1.5	1.5	1.5	1.5
Lysine	0.3	0.3	0.3	0.3
Methionine	2.2	2.2	2.2	2.2
Betaine	5	5	5	5
Zeolite powder	30	30	30	30
Cellulose	54.0	53.0	52.0	48.0
Condensed tannins	0	1	2	2
Polyethylene glycol	0	0	0	4
Proximate composition				
Dry matter	937	932	938	932
Crude protein	430	425	426	425
Crude lipid	100	96	98	98
Ash	94	92	94	93

T1, basal diet; T2, basal diet supplemented with 1 g/kg of condensed tannins; T3, basal diet supplemented with 2 g/kg of condensed tannins; T4, basal diet supplemented with 2 g/kg of condensed tannins and 4 g/kg of polyethylene glycol.

One kilogram of diet provided: VA 3,230 IU, VD 1,600 IU, VE 160 mg, VK_3 4 mg, VB_1 4 mg, VB_2 8 mg, VB_6 4.8 mg, VB_{12} 0.016 mg, nicotinic acid 28 mg, pantothenic acid calcium 16 mg, biotin 0.064 mg, folic acid 1.285 mg, inositol 40 mg, Ca 1,150 mg, K 180 mg, Mg 45 mg, Fe 50 mg, Zn 40 mg, Mn 9.5 mg, Cu 7.5 mg, Co 1.25 mg, I 0.16 mg, Se 0.25 mg.

Polyethylene glycol was added to the T4 diet by spraying 30 g/mL of PEG solution onto condensed tannins during mixing so as to achieve 1 CT: 2 PEG ratio in the diet. PEG specifically binds with condensed tannins to neutralize CT activity (Peng et al., 2016).

RESULTS

Serum Metabolites

Fish fed T2 and T3 had lower ($P < 0.05$) ALB, TCHO and TG, but higher ($P < 0.05$) ALT than those of fish fed T1 and T4 (**Table 2**). T3 had lower ($P < 0.05$) ALB, and similar ($P > 0.05$) TCHO and TG, and higher ($P < 0.05$) ALT than T2. T4 had similar ($P > 0.05$) ALB, TCHO, TG and ALT than T1. T3 had lower ($P < 0.05$) GLU but higher ($P < 0.05$) INS than other groups. GLU and INS were similar ($P > 0.05$) among T1, T2 and T4. All fish had similar ($P > 0.05$) GLOB, AST, HDLC, LDLC and GLG among groups.

TABLE 2 | Serum metabolites of *L. maculatus* fed experimental diets.

Items	Diets			
	T1	T2	T3	T4
ALB, g/L	18.2 ± 0.74^a	12.6 ± 0.50^b	9.7 ± 0.44^c	17.0 ± 0.62^a
GLOB, g/L	29.6 ± 2.15	30.4 ± 2.63	30.0 ± 1.13	28.7 ± 1.19
TCHO, mmol/L	4.6 ± 0.24^a	3.0 ± 0.32^b	2.8 ± 0.28^b	4.4 ± 0.20^a
TG, mmol/L	5.6 ± 0.19^a	3.5 ± 0.28^b	3.6 ± 0.32^b	5.3 ± 0.30^a
ALT, U/L	30.5 ± 2.72^c	54.8 ± 3.94^b	74.8 ± 1.38^a	28.2 ± 4.09^c
AST, U/L	144.0 ± 17.50	146.0 ± 19.57	152.8 ± 19.94	157.8 ± 11.15
HDLC, mmol/L	0.9 ± 0.01	0.9 ± 0.01	0.8 ± 0.03	0.9 ± 0.02
LDLC, mmol/L	0.2 ± 0.02	0.2 ± 0.02	0.2 ± 0.02	0.2 ± 0.01
GLU, mmol/L	9.9 ± 0.61^a	9.5 ± 0.52^a	5.3 ± 0.40^b	9.0 ± 0.43^a
INS, pg/mL	320.5 ± 14.34^b	318.0 ± 17.56^b	428.6 ± 15.52^a	344.2 ± 11.41^b
GLG, ng/mL	1.5 ± 0.12	1.4 ± 0.06	1.5 ± 0.14	1.5 ± 0.11

T1, basal diet; T2, basal diet supplemented with 1 g/kg of condensed tannins; T3, basal diet supplemented with 2 g/kg of condensed tannins; T4, basal diet supplemented with 2 g/kg of condensed tannins and 4 g/kg of polyethylene glycol.

ALB, albumin; GLOB, globulin; TCHO, total cholesterol; TG, triacylglycerol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HDLC, high density lipoprotein cholesterol; LDLC, low density lipoprotein cholesterol; GLU, glucose; INS, insulin; GLG, glucagon.

^{a,b,c}Means with different letters differ ($P < 0.05$).

Liver Antioxidant and Immune Parameters

Fish fed T2 and T3 had higher ($P < 0.05$) SOD, CAT, LZM and IgM than those of fish fed T1 and T4 (**Table 3**). T3 had higher ($P < 0.05$) SOD and CAT, but similar ($P > 0.05$) LZM and IgM than T2. T4 had similar ($P > 0.05$) SOD, CAT, LZM and IgM than T1. All fish had similar ($P > 0.05$) TAOC, GPx, MDA and C3 among groups.

Liver Histomorphology

As shown in **Figure 1**, hepatocytes in T1 and T4 were loose, polyhedral in shape with vacuolated cytoplasm, and no obvious inflammatory cell infiltration was detected. However, hepatocytes in T2 and T3 were shown to have obvious vacuolar degeneration along with different degree of inflammatory cell infiltration.

Fish fed T2 and T3 had higher ($P < 0.05$) values of eosinophilic infiltrate and necrosis than those of fish fed T1 and T4 (**Table 4**). T4 had similar ($P > 0.05$) values of eosinophilic infiltrate and necrosis than T1. All fish had similar ($P > 0.05$) values of nucleus hypertrophy, lymphocytic infiltrate, karyolysis, karyorrhexis and pyknosis among treatments.

Liver Glycometabolism Enzyme Activity

All fish had similar ($P < 0.05$) PFK activity among groups (**Figure 2**). The PK activity was ranked as T1<T2<T3 ($P < 0.05$), and was similar ($P > 0.05$) between T1 and T4. Fish fed T2 and T3 had lower ($P < 0.05$) GK activity than T1 and T4. T4 had similar ($P > 0.05$) GK activity than T1.

DISCUSSION

Serum Metabolites

In this study, PEG specifically binds with CT to neutralize CT activity, therefore comparison between diets with and without PEG supplementation would define the effects of CT. This is a typical method used in previous studies (Huang et al., 2015; Peng et al., 2016; Peng et al., 2018; Peng et al., 2021b). Serum metabolites are one of the best non-invasive means for evaluating health condition, as they are stable end-products of biochemical processes in animals. The serum metabolite profiles in this study suggest that dietary CT partly affected physiological metabolism processes of *L. maculatus* as reflected by the corresponding variation parameters. ALB is synthesized mainly in liver which is the predominant protein filtered by the glomerulus and the major serum protein targeted to oxidant stress in fish (Liu et al., 2018). Generally, injured liver cause decreased concentration of serum ALB (Zhang et al., 2018). Conversely, increased serum ALT activity is commonly related to liver damage or hepatocyte necrosis (Wang et al., 2014). Therefore, the decreased serum ALB concentrations along with increased ALT activities in *L. maculatus* fed CT-treated diets indicated CT induced liver injury of fish. This finding suggested that inclusion of CT in fish diet should be cautious, and the liver damage induced by CT should be considered in their application. It is recommended that the supplemental level of CT in *L. maculatus* diets should be less than 1 g/kg. Blood TCHO and TG indexes are critical factors affecting lipid accumulation which has been commonly proved in fish studies (Torstensen et al., 2011; Jiang et al., 2015; Peng et al., 2020a). In this study, the decreased serum concentrations of TCHO and TG in fish fed CT-treated diets indicated that CT decreased blood lipid content. Similarly, the lipid-lowing effect of CT has been previously observed in Nile tilapia (*Oreochromis niloticus*) (Aiura and Carvalho, 2007), Chinese seabass (*L. maculatus*) (Peng et al., 2020a) and grass carp (*Ctenopharyngodon idellus*) (Yao et al., 2022). INS plays a key role in maintaining glucose homeostasis and a negative correlation between INS and GLU was generally observed in fish (Brauge et al., 1994; Liang et al., 2017; Peng et al., 2020b). Declined GLU concentration along with increased INS level in *L. maculatus* fed diet containing 2 g/kg of CT indicated that CT may decrease blood glucose level by stimulating insulin secretion. Peng et al. (2020b) documented that CT improved glucose utilization of *L. maculatus* by the promotion of glycolysis and inhibition of gluconeogenesis. This may account for the hypoglycemic activity of CT as observed in this study. Furthermore, dietary CT was reported to decrease serum GLU concentration of sheep, owing to CT induced pancreatic β-cell regeneration (Peng et al., 2016).

TABLE 3 | Liver antioxidant and immune parameters of *L. maculatus* fed experimental diets.

Items	Diets			
	T1	T2	T3	T4
Antioxidant parameters				
TAOC, U/mg protein	0.6 ± 0.06	0.6 ± 0.06	0.6 ± 0.04	0.6 ± 0.06
SOD, U/mg protein	309.3 ± 11.10^{c}	450.2 ± 30.75^{b}	546.0 ± 20.70^{a}	300.9 ± 9.43^{c}
CAT, U/mg protein	65.5 ± 1.10^{c}	99.4 ± 7.42^{b}	167.1 ± 2.74^{a}	62.1 ± 3.44^{c}
GPx, U/mg protein	47.5 ± 4.64	52.1 ± 4.05	46.9 ± 2.18	47.5 ± 1.96
MDA, nmol/mg protein	0.7 ± 0.15	0.6 ± 0.08	0.5 ± 0.10	0.7 ± 0.14
Immune parameters				
LZM, U/mg protein	20.7 ± 2.42^{b}	34.4 ± 2.19^{a}	36.6 ± 0.90^{a}	20.6 ± 1.78^{b}
IgM, μg/mg protein	44.4 ± 1.58^{b}	57.9 ± 3.63^{a}	58.3 ± 2.91^{a}	46.3 ± 0.66^{b}
C3, μg/mg protein	32.8 ± 1.98	33.5 ± 0.13	32.9 ± 3.10	31.3 ± 1.11

T1, basal diet; T2, basal diet supplemented with 1 g/kg of condensed tannins; T3, basal diet supplemented with 2 g/kg of condensed tannins; T4, basal diet supplemented with 2 g/kg of condensed tannins and 4 g/kg of polyethylene glycol.

TAOC, total antioxidant capacity; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; MDA, malondialdehyde; LZM, lysozyme; IgM, immune globulin M; C3, complement 3.

^{a,b,c}Means with different letters differ ($P < 0.05$).

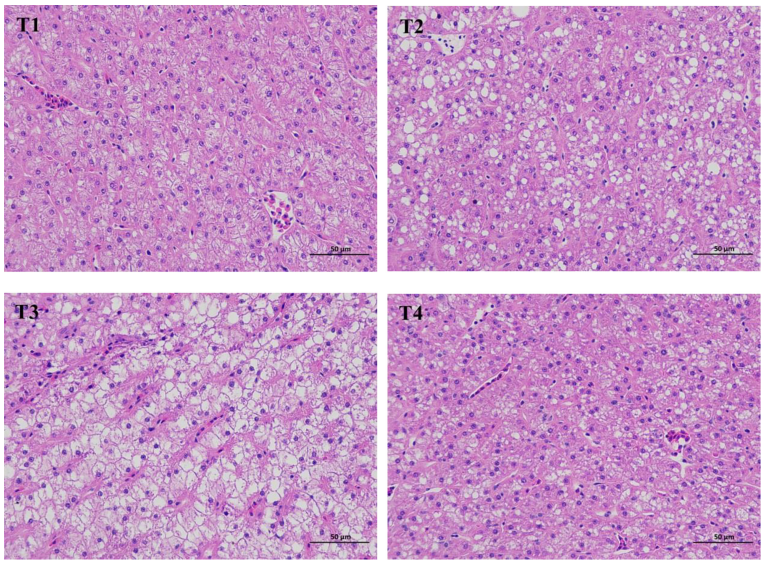


FIGURE 1 | Liver histomorphology (haematoxylin-eosin stain, × 400) of *L. maculatus* fed experimental diets. T1, basal diet; T2, basal diet supplemented with 1 g/kg of condensed tannins; T3, basal diet supplemented with 2 g/kg of condensed tannins; T4, basal diet supplemented with 2 g/kg of condensed tannins and 4 g/kg of polyethylene glycol.

Antioxidant and Immune Response and Histomorphology of Liver

Condensed tannins has been shown to possess strong antioxidant activity both *in vitro* and *in vivo* studies (Beninger and Hosfield, 2003; Peng et al., 2020a; Peng et al., 2021a; Peng et al., 2022a). In this study, dietary CT increased liver SOD and CAT activities. Antioxidant enzymes such as SOD and CAT play a key role in alleviating oxidative stress by scavenging reactive oxygen species (Peng et al., 2022a). SOD catalyzes the dismutation of superoxide anion free radical and plays an important role in defensing oxidant stress (Paulton et al., 2012). CAT protects cells from oxidative damage *via* catalyzing hydrogen peroxide decomposition (Peng et al., 2016). Our previous studies also showed that dietary CT improved antioxidant enzymes activities of *L. maculatus* by activating the Keap1-Nrf2/ARE signaling pathway (Peng et al., 2020a; Peng et al., 2020c). LZM is a key defense protein in innate immune system which plays a critical role in defending pathological change (Brott and Clarke, 2019). IgM is regarded as the first antibody responded to an antigen (Semple et al., 2018). In this study, the increased liver LZM and IgM in fish fed CT-treated diets suggested that CT enhanced immune response of *L. maculatus*. This is similar with our previous observation by Peng et al. (2020a). Generally, CT was regarded as an immunopotentiator (Huang et al., 2018) which enhanced immune function by inhibiting the activation of signaling pathways, such as mitogen activated protein kinases (MAPKs) and nuclear factor kappa B (NF-κB) (Peng et al., 2020d). It has been reported that the biological activity of CT is closely associated with their dietary concentrations (Waghorn, 2008). Previously, Peng et al. (2020a) documented that dietary CT up to 1 g/kg enhanced antioxidant and immune capacity of *L. maculatus*. This study indicated that CT up to 2 g/kg in *L. maculatus* diet still has significantly antioxidant and immune activities.

TABLE 4 | Liver histological indices of L. maculatus fed experimental diets.

Items	Diets			
	T1	T2	T3	T4
Nucleus hypertrophy	0	0	0	0
Eosinophilic infiltrate	0.4 ± 0.12^{c}	1.5 ± 0.22^{b}	2.3 ± 0.58^{a}	0.5 ± 0.11^{c}
Lymphocytic infiltrate	0.2 ± 0.07	0.3 ± 0.03	0.3 ± 0.10	0.3 ± 0.08
Necrosis	0.3 ± 0.04^{b}	1.2 ± 0.10^{a}	1.5 ± 0.22^{a}	0.4 ± 0.13^{b}
Karyolysis	0.1 ± 0.02	0.2 ± 0.04	0.1 ± 0.01	0.1 ± 0.05
Karyorrhexis	0.3 ± 0.10	0.2 ± 0.08	0.3 ± 0.04	0.2 ± 0.07
Pyknosis	0.1 ± 0.05	0.1 ± 0.03	0.1 ± 0.08	0.1 ± 0.05

T1, basal diet; T2, basal diet supplemented with 1 g/kg of condensed tannins; T3, basal diet supplemented with 2 g/kg of condensed tannins; T4, basal diet supplemented with 2 g/kg of condensed tannins and 4 g/kg of polyethylene glycol.

^{a,b,c}*Means with different letters differ ($P < 0.05$).*

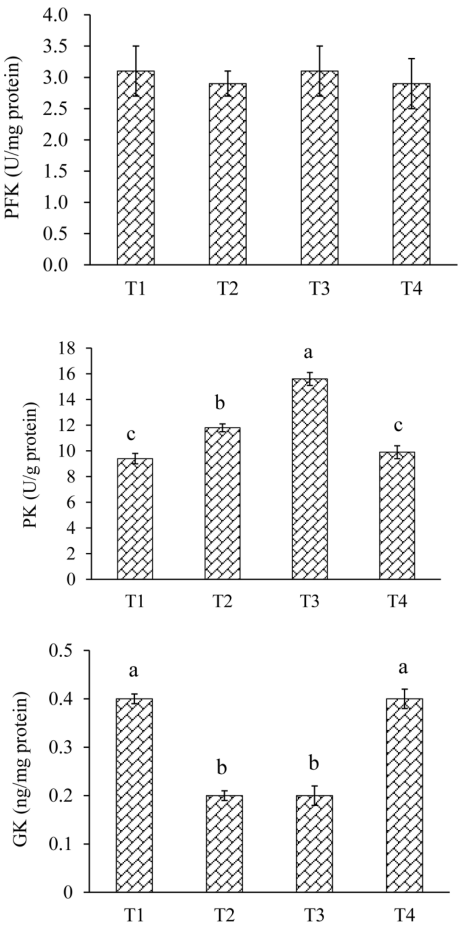


FIGURE 2 | Liver glycometabolism enzyme activities of *L. maculatus* fed experimental diets. PFK, phosphofructokinase; PK, pyruvate kinase; GK, glucokinase. T1, basal diet; T2, basal diet supplemented with 1 g/kg of condensed tannins; T3, basal diet supplemented with 2 g/kg of condensed tannins; T4, basal diet supplemented with 2 g/kg of condensed tannins and 4 g/kg of polyethylene glycol. (a-c) Different lowercase letters above the bars denote significant differences among diets ($P < 0.05$).

As is known to us, histological change is an important aspect in the understanding of pathological alteration related to nutritional intervention in fish (Shi et al., 2017). Although CT was documented to alleviate the stress-induced damage to the liver of fish (Peng et al., 2022b), our recent study reported that no obvious degeneration and inflammatory cell infiltration were observed in the liver of *L. maculatus* fed diets containing CT less than 1 g/kg (Peng et al., 2020a). However, this study indicated that dietary CT exceed 1 g/kg injured the liver of *L. maculatus* accompanied by obvious vacuolar degeneration and inflammatory cell infiltration along with increased values of eosinophilic infiltrate and necrosis, suggesting that effects of CT on liver histomorphology depend on the concentrations of CT in diets. In this study, the histomorphology observation is also consistent with the results of serum metabolites, as reflected by the decreased serum ALB concentrations and the increased ALT activities of fish fed CT-treated diets. This is the first study indicating that dietary CT induced liver damage of fish which provided a caution for the application of CT in aquaculture.

Liver Glycometabolism Enzyme Activity

As the main control center for glycogenesis, glycogenolysis, glycolysis and gluconeogenesis, liver plays a critical role in the regulation of glucose metabolism. Among the glycometabolism enzymes, the PK and GK are critical indicators to diagnose the glucose metabolism in the liver of fish (Peng et al., 2020b). It has been reported that PK is a major enzyme that participates in the last step of glycolysis (Lu et al., 2018), whereas GK promotes glycogen synthesis in the liver (Li et al., 2015). In this study, the increased PK and decreased GK activities in the liver of *L. maculatus* fed CT-treated diets suggested that CT promotes glucose utilization of fish. This is similar with the observation by Peng et al. (2020b) that CT improved glucose utilization of *L. maculatus* due to its positive effects on the activity and gene expression of glycometabolism enzymes. As is well known to us, most carnivorous fish have a low glucose tolerance and poor utilization of carbohydrate (Tan et al., 2009). Our previous study reported that high dietary carbohydrate induced hyperglycemia and abnormal of liver glucose metabolism, and therefore depressed the growth of *L.*

maculatus (Peng et al., 2020b). Results of this study provide a new insight for improving the glucose utilization of fish by application of CT in fish diet. Despite the reason why CT improved glucose utilization of fish is not fully understood, CT and its precursors were reported to accelerate the absorption rate of glucose and promote the synthesis of glycogen by up-regulating gene expression of GLUT-2 (a primary glucose transporter) (Cordero-Herrera et al., 2014) and inhibiting hepatic GK activity (Huang et al., 2013). Howsoever, the findings of this study provided a new explanation from a different perspective to reveal the underlying mechanism regarding CT improve the glucose utilization of fish. Further study is still needed to elucidate the mechanism by which CT improve the glycometabolism of *L. maculatus*.

CONCLUSION

In conclusion, dietary CT up to 2 g/kg reduced serum lipid and glucose concentrations, enhanced liver antioxidant and immune response, and improved glucose utilization of *L. maculatus*. However, dietary CT at 1 and 2 g/kg induced liver injury of *L. maculatus* which provided a caution for their application in aquaculture. It is recommended that the supplemental level of CT in *L. maculatus* diets should be less than 1 g/kg. Further study is still needed to elucidate the mechanism by which CT improve the glycometabolism of fish.

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 Care and Use Committee of Guangdong Academy of Agricultural Sciences.

AUTHOR CONTRIBUTIONS

KP and WH conceived and designed the experiments. KP, BC, HZ, and YW performed the experiments. KP analyzed the data and wrote the manuscript. All authors approved the submitted version.

FUNDING

This study was funded by National Natural Science Foundation of China (31902388), Natural Science Foundation of Guangdong Province of China (2021A1515010850, 2022A1515010545), Science and Technology Planning Project of Guangzhou (202002030378) and Special Fund for Scientific Innovation Strategy-Construction of High-Level Academy of Agriculture Science (R2018QD-075, R2021PY-QY001).

ACKNOWLEDGMENTS

We acknowledge those who donated the experimental animals and those who helped support this study at the Institute of Animal Science, Guangdong Academy of Agricultural Sciences.

REFERENCES

Aiura, F. S., and Carvalho, M. R. B. (2007). Body Lipid Deposition in Nile Tilapia Fed on Rations Containing Tannin. *Pesqui. Agropecu. Bras.* 42, 51–56. doi: 10.1590/S0100-204X2007000100007

AOAC (1999). *Official Methods of Analysis of the Association of Official Agricultural Chemists. 16th ed* (Gaithersburg: AOAC International, 5th rev).

Beninger, C. W., and Hosfield, G. L. (2003). Antioxidant Activity of Extracts, Condensed Tannin Fractions, and Pure Flavonoids From *Phaseolus Vulgaris* L. Seed Coat Color Genotypes. *J. Agr. Food Chem.* 51 (27), 7879–7883. doi: 10.1021/jf0304324

Brauge, C., Medale, F., and Corraze, G. (1994). Effect of Dietary Carbohydrate Levels on Growth, Body Composition and Glycaemia in Rainbow Trout, *Oncorhynchus Mykiss*, Reared in Seawater. *Aquaculture* 123, 109–120. doi: 10.1016/0044-8486(94)90123-6

Brott, A. S., and Clarke, A. J. (2019). Peptidoglycan O-Acetylation as a Virulence Factor: Its Effect on Lysozyme in the Innate Immune System. *Antibiotics* 8, 94. doi: 10.3390/antibiotics8030094

Brum, A., Cardoso, L., Chagas, E. C., Chaves, F. C. M., Mourino, J. L. P., and Martins, M. L. (2018). Histological Changes in Nile Tilapia Fed Essential Oils of Clove Basil and Ginger After Challenge With Streptococcus Agalactiae. *Aquaculture* 490, 98–107. doi: 10.1016/j.aquaculture.2018.02.040

Cordero-Herrera, I., Martin, M., Goya, L., and Ramos, S. (2014). Cocoa Flavonoids Attenuate High Glucose Induced Insulin Signalling Blockade and Modulate Glucose Uptake and Production in Human HepG2 Cells. *Food Chem. Toxicol.* 64, 10–19. doi: 10.1016/j.fct.2013.11.014

Fishery Administration of Ministry of Agriculture and Rural Affairs (2021). *China Fishery Statistical Yearbook* (Beijing, China: China Agriculture Press).

Huang, P. L., Chi, C. W., and Liu, T. Y. (2013). Areca Nut Procyanidins Ameliorate Streptozocin-Induced Hyperglycemia by Regulating Gluconeogenesis. *Food Chem. Toxicol.* 55, 137–143. doi: 10.1016/j.fct.2012.12.057

Huang, Q., Jin, L., Xu, Z., Barbieri, L. R., Acharya, S., Hu, T., et al. (2015). Effects of Purple Prairie Clover (*Dalea Purpurea* Vent.) on Feed Intake, Nutrient Digestibility and Faecal Shedding of *Escherichia Coli* O157:H7 in Lambs. *Anim. Feed Sci. Technol.* 207, 51–61. doi: 10.1016/j.anifeedsci.2015.06.009

Huang, Q., Liu, X., Zhao, G., Hu, T., and Wang, Y. (2018). Potential and Challenges of Tannins as an Alternative to in-Feed Antibiotics for Farm Animal Production. *Anim. Nutr.* 4, 137–150. doi: 10.1016/j.aninu.2017.09.004

Jiang, S., Wu, X., Li, W., Wu, M., Luo, Y., Lu, S., et al. (2015). Effects of Dietary Protein and Lipid Levels on Growth, Feed Utilization, Body and Plasma Biochemical Compositions of Hybrid Grouper (*Epinephelus Lanceolatus* ♂ × *Epinephelus Fuscoguttatus* ♀) Juveniles. *Aquaculture* 446, 148–155. doi: 10.1016/j.aquaculture.2015.04.034

Liang, H., Habte-Tsion, H. M., Ge, X., Ren, M., and Pan, W. (2017). Dietary Arginine Affects the Insulin Signaling Pathway, Glucose Metabolism and Lipogenesis in Juvenile Blunt Snout Bream *Megalobrama Amblycephala*. *Sci. Rep.* 7, 7864. doi: 10.1038/s41598-017-06104-3

Li, M., Feng, L., Jiang, W. D., Wu, P., Liu, Y., Jiang, J., et al. (2020). Condensed Tannins Decreased the Growth Performance and Impaired Intestinal Immune Function in on-Growing Grass Carp (*Ctenopharyngodon Idella*). *Br. J. Nutr.* 123 (7), 737–755. doi: 10.1017/S0007114519003295

Liu, Y., Yang, P., Hu, H., Li, Y., Dai, J., Zhang, Y., et al. (2018). The Tolerance and Safety Assessment of Taurine as Additive in a Marine Carnivorous Fish, *Scophthalmus Maximus* L. *Aquac. Nutr.* 24, 461–471. doi: 10.1111/anu.12579

Li, J. N., Xu, Q. Y., Wang, C. A., Wang, L. S., Zhao, Z. G., and Luo, L. (2015). Effects of Dietary Glucose and Starch Levels on the Growth, Haematological Indices and Hepatic Hexokinase and Glucokinase mRNA Expression of Juvenile Mirror Carp (*Cyprinus Carpio*). *Aquac. Nutr.* 22, 550–558. doi: 10.1111/anu.12278

Lu, S., Wu, X., Gao, Y., Gatlin, D. M., Wu, M., Yao, W., et al. (2018). Effects of Dietary Carbohydrate Sources on Growth, Digestive Enzyme Activity, Gene Expression of Hepatic Gluts and Key Enzymes Involved in Glycolysis-Gluconeogenesis of Giant Grouper *Epinephelus Lanceolatus* Larvae. *Aquaculture* 484, 343–350. doi: 10.1016/j.aquaculture.2017.07.033

Makkar, H. P., Blummel, M., and Becker, K. (1995). Formation of Complexes Between Polyvinyl Pyrrolidones or Polyethylene Glycols and Tannins, and Their Implication in Gas Production and True Digestibility in *In Vitro* Techniques. *Br. J. Nutr.* 73, 897–913. doi: 10.1079/BJN19950095

Paulton, M. P., Thomas, P. C., and Vijayan, K. K. (2012). Identification of Antioxidant Enzyme Genes of the Indian Edible Oyster, *Crassostrea Madrasensis* (Preston) Through Polymerase Chain Reaction. *Indian J. Fish.* 59 (4), 179–181. doi: 10.1016/j.aquaculture.2012.08.041

Peng, K., Chen, B., Zhao, H., Zheng, C., Wang, Y., Luo, C., et al. (2022b). Condensed Tannins Alleviate Aflatoxin B1-Induced Injury in Chinese Sea Bass (*Lateolabrax Maculatus*). *Aquaculture* 552, 738029. doi: 10.1016/j.aquaculture.2022.738029

Peng, K., Huang, W., Zhao, H., Sun, Y., and Chen, B. (2021a). Dietary Condensed Tannins Improved Growth Performance and Antioxidant Function But Impaired Intestinal Morphology of *Litopenaeus Vannamei*. *Aquac. Rep.* 21, 100853. doi: 10.1016/j.aqrep.2021.100853

Peng, K., Jin, L., Niu, Y., Huang, Q., McAllister, T. A., Yang, H. E., et al. (2018). Condensed Tannins Affect Bacterial and Fungal Microbiomes and Mycotoxin Production During Ensiling and Upon Aerobic Exposure. *Appl. Environ. Microbiol.* 84, e02274–e02217. doi: 10.1128/AEM.02274-17

Peng, K., Lv, X., Zhao, H., Chen, B., Chen, X., and Huang, W. (2022a). Antioxidant and Intestinal Recovery Function of Condensed Tannins in *Lateolabrax Maculatus* Responded to *In Vivo* and *In Vitro* Oxidative Stress. *Aquaculture* 547, 737399. doi: 10.1016/j.aquaculture.2021.737399

Peng, K., Shirley, D. C., Xu, Z., Huang, Q., McAllister, T. A., Chaves, A. V., et al. (2016). Effect of Purple Prairie Clover (*Dalea Purpurea* Vent.) Hay and Its Condensed Tannins on Growth Performance, Wool Growth, Nutrient Digestibility, Blood Metabolites and Ruminal Fermentation in Lambs Fed Total Mixed Rations. *Anim. Feed Sci. Technol.* 222, 100–110. doi: 10.1016/j.anifeedsci.2016.10.012

Peng, K., Wang, G., Wang, Y., Chen, B., Sun, Y., Mo, W., et al. (2020c). Condensed Tannins Enhanced Antioxidant Capacity and Hypoxic Stress Survivability But Not Growth Performance and Fatty Acid Profile of Juvenile Japanese Seabass (*Lateolabrax Japonicus*). *Anim. Feed Sci. Technol.* 269, 114671. doi: 10.1016/j.anifeedsci.2020.114671

Peng, K., Wang, Y., Wang, G., and Huang, Y. (2020d). Biological Function of Condensed Tannins and Their Application in Animal Production. *Chin. J. Anim. Nutr.* 32 (8), 3451–3460. doi: 10.3969/j.issn.1006-267x.2020.08.001

Peng, K., Wang, G., Zhao, H., Wang, Y., Mo, W., Wu, H., et al. (2020b). Effect of High Level of Carbohydrate and Supplementation of Condensed Tannins on Growth Performance, Serum Metabolites, Antioxidant and Immune Response, and Hepatic Glycometabolism Gene Expression of *Lateolabrax Japonicus*. *Aquac. Rep.* 18, 100515. doi: 10.1016/j.aqrep.2020.100515

Peng, K., Xu, Z., Nair, J., Jin, L., McAllister, T. A., Acharya, S., et al. (2021b). Conserving Purple Prairie Clover (*Dalea Purpurea* Vent.) as Hay and Silage Had Little Effect on the Efficacy of Condensed Tannins in Modulating Ruminal Fermentation *In Vitro*. *J. Sci. Food Agric.* 101, 1247–1254. doi: 10.1002/jsfa.10913

Peng, K., Zhou, Y., Wang, Y., Wang, G., Huang, Y., and Cao, J. (2020a). Inclusion of Condensed Tannins in *Lateolabrax Japonicus* Diets: Effects on Growth, Nutrient Digestibility, Antioxidant and Immune Capacity and Copper Sulphate Stress Resistance. *Aquac. Res.* 18, 100525. doi: 10.1016/j.aqrep.2020.100525

Schwaiger, J., Wanke, R., Adam, S., Pawert, M., Honnen, W., and Triebskorn, R. (1997). The Use of Histopathological Indicators to Evaluate Contaminant-Related Stress in Fish. *J. Aquat. Ecosyst. Stress Recov.* 6, 75–86. doi: 10.1023/A:1008212000208

Semple, S. L., Kellendonk, C. J., Al-Hussinee, L., MacInnes, J. I., Lumsden, J. S., and Dixon, B. (2018). Serum IgM, MH Class Iiβ Genotype and Respiratory Burst Activity do Not Differ Between Rainbow Trout Families Displaying Resistance or Susceptibility to the Coldwater Pathogen, *Flavobacterium Psychrophilum*. *Aquaculture* 483, 131–140. doi: 10.1016/j.aquaculture.2017.10.020

Shi, X., Luo, Z., Chen, F., Wei, C. C., Wu, K., Zhu, X. M., et al. (2017). Effect of Fish Meal Replacement by Chlorella Meal With Dietary Cellulase Addition on Growth Performance, Digestive Enzymatic Activities, Histology and Myogenic Genes'expression for Crucian Carp *Carassius Auratus*. *Aquac. Res.* 48, 3244–3256. doi: 10.1111/are.13154

Tan, Q., Wang, F., Xie, S., Zhu, X., Lei, W., and Shen, J. (2009). Effect of High Dietary Starch Levels on the Growth Performance, Blood Chemistry and Body Composition of Gibel Carp (*Carassius Auratus* Var. Gibelio). *Aquac. Res.* 40, 1011–1018. doi: 10.1111/j.1365-2109.2009.02184.x

Torstensen, B. E., Espe, M., Stubhaug, I., and Lie, O. (2011). Dietary Plant Proteins and Vegetable Oil Blends Increase Adiposity and Plasma Lipids in Atlantic Salmon (*Salmo Salar* L.). *Brit. J. Nutr.* 106, 633–647. doi: 10.1017/S0007114511000729

Waghorn, G. (2008). Beneficial and Detrimental Effects of Dietary Condensed Tannins for Sustainable Sheep and Goat Production-Progress and Challenges. *Anim. Feed Sci. Technol.* 147, 116–139. doi: 10.1016/j.anifeedsci.2007.09.013

Wang, L. N., Liu, W. B., Lu, K. L., Xu, W. N., Cai, D. S., Zhang, C. N., et al. (2014). Effects of Dietary Carbohydrate/Lipid Ratios on Non-Specific Immune Responses, Oxidative Status and Liver Histology of Juvenile Yellow Catfish *Pelteobagrus Fulvidraco*. *Aquaculture* 426-427 (1), 41–48. doi: 10.1016/j.aquaculture.2014.01.022

Yao, J., Liu, N., Li, N., Li, X., and Hua, X. (2022). Different Metabolomic Responses of Grass Carp (*Ctenopharyngodon Idellus*) to Dietary Tannin and Rapeseed Meal. *Aquac. Fish.* 7, 40–51. doi: 10.1016/j.aaf.2020.06.002

Zhang, Y., Song, L., Hu, B., Mao, S., and Xu, P. (2018). Effect of Curcumin on Growth Performance and Protective Effect of Liver Injury Induced by Carbon Tetrachloride in *Oreochromis Niloticus*. *J. Fish. Sci. China* 25 (6), 1271–1280. doi: 10.3724/SP.J.1118.2018.17437

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: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Physiology

ORIGINAL RESEARCH
published: 23 June 2022
doi: 10.3389/fphys.2022.874172

Growth Performance, Immune-Related and Antioxidant Genes Expression, and Gut Bacterial Abundance of Pacific White Leg Shrimp, *Litopenaeus vannamei*, Dietary Supplemented With Natural Astaxanthin

Abdallah Tageldein Mansour[1,2]*, *Mohamed Ashour*[3]*, *Eman M. Abbas*[3], *Ahmed Saud Alsaqufi*[1], *Mahmoud S. Kelany*[3], *Mohamed A. El-Sawy*[3] *and Zaki Z. Sharawy*[3]

[1]Animal and Fish Production Department, College of Agricultural and Food Sciences, King Faisal University, Al Hofuf, Saudi Arabia, [2]Fish and Animal Production Department, Faculty of Agriculture (Saba Basha), Alexandria University, Alexandria, Egypt, [3]National Institute of Oceanography and Fisheries (NIOF), Cairo, Egypt

The current study examines the effect of dietary supplementation of ethanolic extract of *Arthrospira platensis* NIOF17/003, which is mainly natural astaxanthins (97.50%), on the growth performance, feed utilization, bacterial abundance, and immune-related and antioxidant gene expressions of the Pacific white leg shrimp, *Litopenaeus vannamei*. A total of 360 healthy *L. vannamei* postlarvae (0.19 ± 0.003 g) were divided into four groups (0, 2, 4, and 6 g natural astaxanthins/kg diet) each in three replicates, at an initial density of 30 PLs per tank (40 L capacity). The shrimp were fed the tested diets three times a day at a rate of 10% of their total body weight for 90 days. Diets supplemented with different astaxanthin levels significantly improved shrimp growth performance and feed conversion ratio compared to the control diet. No significant differences were observed in survival rates among all experimental groups. The immune-related genes (*prophenoloxidase*, *lysozyme*, *beta-glucan binding protein*, *transglutaminase*, and *crustin*) mRNA levels were significantly upregulated in groups fed with different concentrations of the natural astaxanthins in a dose-dependent manner. The *prophenoloxidase* gene is the highest immune-upregulated gene (14.71-fold change) in response to astaxanthin supplementation. The superoxide dismutase mRNA level was significantly increased with increasing dietary astaxanthin supplementation. In addition, increasing astaxanthin supplementation levels significantly reduced the count of heterotrophic bacteria and *Vibrio* spp. in the culture water and shrimp intestine. Overall, the current results concluded that diet supplementation with natural astaxanthin, extracted from *Arthrospira platensis*, enhanced the growth performance, immune response, and antioxidant status of *L. vannamei*.

Keywords: astaxanthin, feed additives, immune response, antioxidant, bacterial abundance, marine shrimp

OPEN ACCESS

Edited by:
Serhat Turkmen, University of Alabama at Birmingham, United States

Reviewed by:
Omid Safari, Ferdowsi University of Mashhad, Iran
Po-Tsang Lee, National Taiwan Ocean University, Taiwan

***Correspondence:**
Abdallah Tageldein Mansour
amansour@kfu.edu.sa
orcid.org/0000-0002-5963-5276
Mohamed Ashour
microalgae_egypt@yahoo.com
orcid.org/0000-0002-1595-1197

Specialty section:
This article was submitted to Aquatic Physiology, a section of the journal Frontiers in Physiology

Received: *11 February 2022*
Accepted: *01 June 2022*
Published: *23 June 2022*

Citation:
Mansour AT, Ashour M, Abbas EM, Alsaqufi AS, Kelany MS, El-Sawy MA and Sharawy ZZ (2022) Growth Performance, Immune-Related and Antioxidant Genes Expression, and Gut Bacterial Abundance of Pacific White Leg Shrimp, Litopenaeus vannamei, Dietary Supplemented With Natural Astaxanthin. Front. Physiol. 13:874172. doi: 10.3389/fphys.2022.874172

INTRODUCTION

Shrimp is currently one of the most important aquatic animals worldwide. Due to the increase in global demand, shrimp culture has developed intensively and has priority among the leading aquaculture sectors in many countries (Lukwambe et al., 2019; Abbas et al., 2020). Among all penaeid shrimp species, the Pacific white leg shrimp, *Litopenaeus vannamei,* is the widest species being extensively cultured (Sharawy et al., 2022), accounting for more than 70% of the global shrimp production (Li et al., 2018). However, there are numerous barriers to sustaining aquaculture development globally, including the feed industry, pollution, low survival rates, climatic changes, and poor water quality (Abo-Taleb et al., 2020; Alprol et al., 2021a; Alprol et al., 2021c; Hassan et al., 2021). To cope with the global increase of intensive shrimp farming, the shrimp feed industry has been developed using several strategies. Among these strategies, feed additive supplementation is one of the most important industries that has gained great importance for several shrimp species as growth promoters, immunity enhancers, and an alternative strategy for disease-fighting (Sharawy et al., 2020; Sharawy et al., 2022).

Recently, due to their high concentration of natural bioactive compounds, algal cells (microalgae and seaweeds) have attract great attention for utilization as feed additives, showing improvement in growth performance, feed utilization, and immunity stimulation of cultured animal species, besides improving the water quality (Ashour et al., 2020; Ashour et al., 2021; Mabrouk et al., 2021; Zaki et al., 2021; Mansour et al., 2022). Depending on the algal strain, algal cells contain protein with high essential amino acid content, lipids with high unsaturated fatty acid levels, and carbohydrates (polysaccharides, etc.), which are necessary compounds in shrimp feeding, growth, and metamorphosis (Liñán-Cabello et al., 2003; Wade et al., 2017). Among all the microalgae strains, *Arthrospira* (a filamentous cyanobacterium) is the richest microalgae species in many phytochemicals (Mansour et al., 2021). *Arthrospira platensis* contains high levels of essential amino acids, fatty acids, minerals, and pigments like phycocyanin and astaxanthin, which have important biological functions and serve in several industries (Madkour et al., 2012; El-Shouny et al., 2015; Osman et al., 2016). It could be used for the replacement of fishmeal in the diet of Pacific white shrimp, *Litopenaeus vannamei,* and the obtained results did not show any significant differences with partial or total replacements in the growth performance and feed utilization levels. In addition, the PUFAs was increased significantly with *A. platensis* treatments and the survival of *A. platensis* supplemented groups was significantly increased under hypoxia challenge (Pakravan et al., 2017). The hot-water extract of *A. platensis* improved the growth, genes expression, immune response, and resistance of *L. vannamei* against *Vibrio alginolyticus* (Lin et al., 2010; Tayag et al., 2010). Besides phycocyanin, astaxanthin is the main carotenoid that exists in *A. platensis* (Gouveia et al., 2003; An et al., 2017).

Astaxanthin, a xanthophyll carotenoid, is a fat-soluble red pigment that has more significant biological activities than other carotenoids (Lim et al., 2018). Astaxanthins are extensively used as feed additives in diets of juveniles and adults of several shrimp species. It could be resulting in improved growth performance, survival, feed utilization, immunity responses, digestive enzyme activities, body composition, reproductive performance, spermatophore, egg, and larval qualities, and overcoming the pigment deficiency of Pacific white leg shrimp, *L. vannamei* (Niu et al., 2009; Pei et al., 2009; Chuchird et al., 2015), kuruma shrimp, *Marsupenaeus japonicus* (Wang et al., 2019), *Penaeus monodon* (Chien et al., 2003), red cherry shrimp, *Neocaridina davidi* (Tomas et al., 2020). In addition, astaxanthin dietary supplementation has positive effects on growth, molting cycle, free radical scavenging capacity, and nitrite stress tolerance of *Penaeus japonicus* postlarvae (Petit et al., 1997) and *Pleoticus muelleri* (Díaz et al., 2014). Furthermore, natural astaxanthins derived from the green seaweed, *Enteromorpha intestinalis* were used as feed additives to increase astaxanthin content in shrimp, *Penaeus monodon*, muscles (Mondal et al., 2015). In the present study, astaxanthin was extracted from local strain of *A. platensis* NIOF17/003 as available, cheap, and sustainable source that can be used in a commercial scale as feed additives in shrimp diets. Recently, *A. platensis* was used as an efficient source for astaxanthin that can be increased *via* environment conditions manipulation (Moradi et al., 2021) or inducing mutation (An et al., 2017).

The immune systems of crustaceans depend on innate immunity, that is, mediated by cellular and humoral effectors, which recognize invading microorganisms and trigger various defense mechanisms to eliminate pathogens (Söderhäll and Cerenius, 1992; Mansour et al., 2021; Sharawy et al., 2022). Humoral effectors include the prophenoloxidase system (*ProPO*), hemolymph clotting mechanism, melanization, and antimicrobial immune response (Jiravanichpaisal et al., 2006; Cerenius et al., 2008). The high immune surveillance of invertebrates could be associated with high amounts of hemolymph carotenoids, which could regulate genes expression of several immune-related genes, in particular, the *ProPO* gene (Cornet et al., 2007; Babin et al., 2010), CuZn superoxide dismutase (*SOD*) gene (Han et al., 2016), and other immune genes. Dietary astaxanthin could partially alleviate oxidative stress *via* inducing relatively higher gene expression levels of antioxidant enzymes in *L. vannamei* (Zhang et al., 2013). Meanwhile, the commercially farmed crustaceans did not have the internal mechanism for the *de novo* synthesis of astaxanthin and did not have the access to obtain different carotenoid sources from the environment (Higuera-Ciapara et al., 2006; Seabra and Pedrosa, 2010). Accordingly, dietary supplementation with astaxanthin is a necessity in the formulated diet. Therefore, the current study aimed to investigate the effects of increasing supplementation levels of the acetonic extract of the *A. platensis* NIOF17/003, which mainly consists of natural astaxanthins, as a feed additive on the growth performance, feed utilization,

immune-related genes expression, and bacterial abundance of Pacific white leg shrimp.

MATERIALS AND METHODS

Arthrospira platensis NIOF17/003

Cyanobacterium, *Arthrospira platensis* NIOF17/003 (GenBank accession number: MW396472), isolated from the El-Khadra saline-alkaline Lake, Wadi El-Natrun, Egypt, was molecularly identified, and cultivated, as well as its potential applications in different fields, were determined as described previously (Alprol et al., 2021b; Hassan et al., 2021; Mabrouk et al., 2021; Zaki et al., 2021).

Astaxanthins Extraction, Preparation, and Analysis

Natural astaxanthin, a carotenoid pigment, was extracted from the blue-green alga *A. platensis* NIOF17/003, according to Ju et al. (2009) with some modifications. Briefly, 1 kg of *A. platensis* fine dried powder was soaked in 100% acetone (10% w: v) and extracted three times on a rotary shaker for 72 h at 200 rpm in the dark at room temperature. The extracts were combined and filtered through filter paper (Whatman No. 1). Then the filtrates were concentrated using a rotary evaporator at 40°C under reduced pressure (Elshobary et al., 2020). The crude extract yield was weighed and calculated as a percentage of the initial sample weight. The yield of crude extract was stored at −20°C until further application. To determine the phytochemical profile of *A. platensis* crude extract, GC-Mass Spectrophotometry analysis was performed as previously described by Ashour et al. (2020). The unknown phytochemical compounds were identified based on comparing the obtained mass spectra with those available in the NIST library (National Institute of Standards and Technology, United States).

Experimental Animals

Pacific white leg shrimp, *L. vannamei,* postlarvae (PLs) were brought from a private commercial shrimp hatchery located in Borg El-Arab, Alexandria City, Egypt, to the indoor facilities of the Suez Branch, National Institute of Oceanography and Fisheries (NIOF-Suez). Firstly, the PLs were acclimated for 2 weeks in fiberglass tanks (500 L) under the same controlled conditions of the feeding trial (26–28°C, 31–32 ppt, and continuous aeration). During the acclimated period, PLs were fed a commercial diet (Aller-Aqua, Giza Governorate, Egypt). The compliance with ethical standards in the experimental setup and fish handling was approved by the Research Committee of the NIOF, Egypt.

Experimental Facilities and Design

According to a completely randomized design, the current feeding trial was performed in three replicates for each treatment. After 2 weeks of acclimation, 360 healthy PLs (0.19 ± 0.003 g) at an initial stocking density of 30 PLs per tank were handed out into 12 tanks (40 L capacity). During the experimental period (90 days), all PLs were fed three times a day (6:00, 12:00, and 18:00 h) at a rate of 10% of their total body weight. Every day before the first feeding, all tanks were siphoning to clean and remove the accumulated excreta and unconsumed feed. As a result of the siphoning process, 10% water was replaced daily with filtered, oxygenated seawater (Sharawy et al., 2020).

TABLE 1 | The proximate chemical analysis (% of dry matter) of experimented diets.

Diets[a]	Protein (%)	Fat (%)	Ash (%)	Fiber (%)
D_1	40.59	11.31	9.19	3.51
D_2	39.69	12.60	8.89	4.14
D_3	39.88	12.28	9.21	3.48
D_4	40.51	12.62	9.40	3.41

[a]*Aller-Aqua, Giza Governorate, Egypt. D_1, D_2, D_3, and D_4 are the experimental diets that were supplemented with 0, 2, 4, and 6 g astaxanthins/kg diet, respectively, of the crude acetonic extract of* Arthrospira platensis *NIOF17/003.*

Water Quality Parameters

During the feeding trial, water quality parameters were checked, twice a week, for alkalinity (mg/L), NH_3 (mg/L), PO_4 (mg/L), NO_3 (mg/L), and NO_2 (mg/L) as described by APHA (2005). In addition, to maintain the water quality values as recommended for shrimp, the temperature (°C, using a mercury thermometer suspended at a depth of 30 cm), pH (using a pH meter, Orion, United States), and salinity (ppt, using a refractometer, United States) were investigated daily (1.00 p.m.) (Boyd and Tucker, 2012).

Diets Preparation

In the current feeding trial, four dietary supplementation treatments were performed for 90 days: D_1, a control diet, that the shrimp was fed a commercial diet (Aller-Aqua, Giza Governorate, Egypt, as presented in **Table 1**). The other three diets (D_2, D_3, and D_4) were fed diets supplemented with different levels of the natural astaxanthins (2, 4, and 6 g/kg diet, respectively), as a crude extract of *A. platensis* NIOF17/003. The addition of natural astaxanthins was performed as described by Mehrabi et al. (2012) with some modifications. The control diet was powdered and divided into four equal portions. The specific quantities of the natural astaxanthins (0, 2, 4, and 6 g/kg diet, respectively) were suspended well in 50 ml of corn oil and then sprayed well over the three powdered diets and mixed well. For the control diet (D1), the same volume of corn oil was sprayed without astaxanthins. Then, all four diets were re-pelletized in a pellet mill to obtain the proper diameter, dried at room temperature with forced air, and stored in plastic bags at 4°C until use.

Measured Parameters

Growth Performance and Nutrient Utilization Indices

At the end of the experiment, the PLs weights were recorded to determine the final body weight (FBW, g). Moreover, to determine the growth performance of Pacific white leg shrimp, *L. vannamei*, the weight gain (WG), feed conversion ratio (FCR),

survival (%), and specific growth rate (SGR) were calculated using the following formulas:

$$\text{Weight gain, g} = \text{Final body weight (g)} - \text{Initial body weight (g)} \quad (1)$$

$$\text{Feed conversion ratio} = \frac{\text{Total consumed feed}}{\text{WG}} \quad (2)$$

$$\text{Survival, \%} = \frac{\text{Final number of shrimp}}{\text{Initial number of shrimp}} \times 100 \quad (3)$$

$$\text{Specific growth rate, \%/day} = \frac{\text{Ln FBW} - \text{Ln IBW}}{t} \times 100 \quad (4)$$

where: Ln FBW and Ln IBW are the natural logarithm of final body weight (g) and initial body weight (g); while t is the time in days.

Whole-Body Proximate Chemical Analysis

At the end of the experiment, to determine the whole-body proximate chemical composition, five shrimp from each replicate were selected randomly and homogenized by a blender, dried in an oven, ground, and stored at −20°C for subsequent analysis. Both biochemical analyses of shrimp and diet were applied as described by the standard methods of AOAC (2003). The shrimp, dry matter, crude protein, crude fat, and crude ash were determined, while for diets, crude protein, crude fat, crude ash, and fibre were determined, and the nitrogen-free extract was calculated.

Bacterial Abundance Assessment

The bacterial abundance of water and shrimp intestines was performed according to APHA (2005). At the end of the experiment, three shrimp samples were chosen randomly from each replicate, and the intestines were aseptically extracted to estimate the bacterial count as described by Sharawy et al. (2020). The outwardly surface bacteria were removed by washing each gut three times with sterile distilled water. After that, they were washed in ethanol 96% and homogenized in a mortar separately. At the end of the experiment, samples of culture water (1 ml) and intestines (1 g) were taken from each treatment (three replicates) and supplied with sterile distilled water (9 ml). Later, make dilutions (1:10) and transferred 1–10 ml TSA (Trypticase soy agar) and TCBS (Thiosulphate-Citrate-Bile salts) agar plates and incubated at 37°C for TSA and 28°C TCBS (Sharawy et al., 2020). After 24 h, the colonies in each plate of the TSA and TCBS were counted, and the colonies of *Vibrio* spp. were confirmed using the 0129 test (Thermo Scientific™ Oxoid™ 0129 Discs) (Xie et al., 2020).

Immune-Related Gene Expressions Analysis

Triplicate samples of the shrimp abdominal muscles from each replicate were directly excised with fully sterile dissecting tools under cold conditions. The samples were kept at −80°C until gene expression analysis. Total RNA was extracted from the samples using the TRIzol method (easy-RED, iNtRON Biotechnology) as directed by the manufacturer. The OD ratio at 260/280 nm of RNA purity was determined using a NanoDrop system (BioDrop), and the samples with the highest ratio (A260/A280 1.8) were used for cDNA synthesis (1 ng/μl) for each reaction. Total RNA was treated with DNase I (NEB, United States) as the template for the synthesis of first-strand cDNA using reverse transcriptase (RT-PCR beads, Enzynomics, Korea), and the reaction was carried out using PCR amplification (Applied Biosystems Veriti 96-Well Thermal Cycler, United States) under the manufacturer's conditions. The following cDNA was used in the Real-Time PCR reaction (Bico, Thermo-Fisher): initial denaturation at 95°C for 15 min, 40 cycles with the following parameters (95°C, 10 s; 58–62°C, 20 s; and 72°C, 30 s). Unique and specific products were seen as a melting curve at the end of the last cycle when the temperature increased from (58–62–95°C) in increments of 0.5°C. The studied immune-related genes were prophenoloxidase (*proPO*), lysozyme (*Lys*), beta-glucan binding protein (*Bgp*), superoxide dismutase (*SOD*), transglutaminase (*TGase*), and crustin (*Crus*), and their primers were presented in **Table 2**. The housekeeping gene (β-actin) was used to measure gene expression or fold shift of the target genes (Yang et al., 2013). The values give n-fold difference relative to the calibrator (control) when the $2^{\Delta\Delta}$Ct method is applied in normalizing the critical threshold (Ct) quantities of target genes with quantities β-actin (Livak and Schmittgen, 2001).

Statistical Analysis

The experiment was performed in triplicates and the results of growth performances were presented as the means ± standard deviation (SD). The normality and homogeneity assumptions were confirmed before the statistical analysis of the data. Before analysis, all results in percentages were arc-sin transformed (Zar, 1984). Using the IBM SPSS Statistics software (IBM, v.23), statistical analysis was performed by the One-Way Analysis of Variance (ANOVA), followed by Duncan's post-hoc test, at a significant $p \leq 0.05$.

RESULTS

Astaxanthin of *A. platensis* NIOF17/003

The yield of crude extract of *A. platensis* NIOF17/003 was weighed and calculated as a percentage of the initial weight. The calculated final yield concentration was 27 g/kg (2.7%). The GC-MS analysis of the crude extract of *A. platensis* NIOF17/003 shows three main phytochemical compounds belonging to three retention times (**Table 3**). These different bioactive compounds were astaxanthin ($C_{40}H_{52}O_4$, exact molecular weight: 596.38) with the highest peak area (97.50%) and the highest probability (21.40%). The peak area and probability of the other two bioactive compounds ($C_{35}H_{42}N_6O_2$ and $C_{34}H_{44}ClN_5O_2$) were 0.38%, and 0.65%, respectively, and the probability was 7.07%, and 6.60%, respectively (**Table 3**). The chemical structure of these three phytochemicals were identified using the NIST library as shown in **Figure 1**.

TABLE 2 | Primer sequences for real-time PCR used for gene expression analysis.

Gene	Primer sequence (5′-3′)	Accession no.	Size (bp)
β-actin	F: GCCCATCTACGAGGGATA R: GGTGGTCGTGAAGGTGTAA	AF300705	121
Prophenoloxidase (*proPO*)	F: CGGTGACAAAGTTCCTCTT R: GCAGGTCGCCGTAGTAAG	AY723296	122
Lysozyme (*Lys*)	F: GGACTACGGCATCTTCCAGA R: ATCGGACATCAGATCGGAAC	AY170126	97
Beta-glucan binding protein (*Bgp*)	F: ACGAGAACGGACAAGAAGTG R: TTCAGCATAGAAGCCATCAGG	AY249858	137
Superoxide dismutase (*SOD*)	F: TCATGCTTTGCCACCTCTC R: CCGCTTCAACCAACTTCTTC	AY486424	143
Transglutaminase (*TGase*)	F: TTCACAAGCCTGACATCACC R: GCAGCAGTGGGATAGGGTTA	BE188522	99
Crustin (*Crus*)	F: ACGAGGCAACCATGAAGG R: AACCACCACCAACACCTAC	AF430076	141

TABLE 3 | Phytochemical profile investigated in crude extract of *A. platensis* NIOF17/003.

RT	PA%	Compound's name	P%	CF	EMW
19.610	98.50	Astaxanthin	21.40	$C_{40}H_{52}O_4$	596.38
19.739	0.85	Cyano 5-{5-[5-(5-cyano-3,4-dimethylpyrrol-2-ylidenemethyl)-3,4-dimethyl-1H-pyrrol-2-ylmethylene]-4,4-dimethyl-4,5-dihydro-3H-pyrro-2-ylmethylene}-4,4-dimethylpyrrolidin-2-ylene-acetic acid, tert.-butyl ester	7.07	$C_{35}H_{42}N_6O_2$	578.33
14.937	0.65	[5-(5-Chloro-3,4-dimethyl-1H-pyrrol-2-ylmethylene)-3,4-dimethyl-5H-pyrrol-2-yl]-[5-(5-cyano-4,4,5-trimethyl-4,5-dihydro-3H-pyrrol-2-ylmethylene)-4,4-dimethylpyrrolidin-2-ylene]-acetic acid, tert.-butyl ester	6.60	$C_{34}H_{44}ClN_5O_2$	589.31

RT, retention time; PA, peak area%; P%, Probability%; MF, molecular formula; and EMW, exact molecular weight.

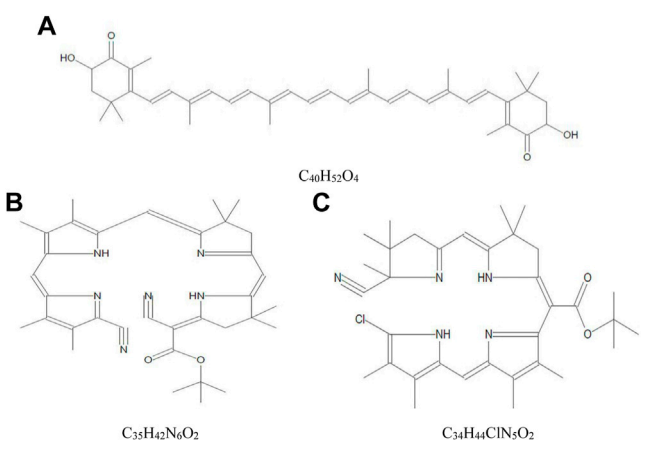


FIGURE 1 | Mass spectra and chemical structure of the three phytochemical compounds identified in crude acetonic extract of *Arthrospira platensis* NIOF17/003. **(A)**: Astaxanthin; **(B)** Cyano 5-{5-[5-(5-cyano-3,4-dimethylpyrrol-2-ylidenemethyl)-3,4-dimethyl-1H-pyrrol-2-ylmethylene]-4,4-dimethyl-4,5-dihydro-3H-pyrro-2-ylmethylene}-4,4-dimethylpyrrolidin-2-ylene-acetic acid, tert.-butyl ester; and **(C)**: [5-(5-Chloro-3,4-dimethyl-1H-pyrrol-2-ylmethylene)-3,4-dimethyl-5H-pyrrol-2-yl]-[5-(5-cyano-4,4,5-trimethyl-4,5-dihydro-3H-pyrrol-2-ylmethylene)-4,4-dimethylpyrrolidin-2-ylene]-acetic acid, tert.-butyl ester.

Water Quality Parameters

Table 4 shows the water quality parameters during the experiments. The results revealed that all recorded water quality conditions (°C, pH, salinity, alkalinity, NH_3, PO_4, NO_3, and NO_2) were in the recommended ranges for shrimp culture. No significant difference was observed among fish fed the control diet and the diets supplemented with different concentrations of astaxanthins.

Growth Performance and Nutrient Utilization Indices

Table 5 shows the effect of dietary supplementation of astaxanthin on the growth performance and feed utilization of *L. vannamei* juveniles. Diets supplemented with different concentrations of astaxanthins (D_2, D_3, and D_4) experienced a significant ($p < 0.05$) improvement of FW, WG, and FCR compared to the control diet. On the other hand, no significant differences ($p < 0.05$) were obtained in survival or SGR among the diets supplemented with astaxanthins (D_2, D_3, and D_4) and the control. While, the response of shrimp, in terms of WG and FCR to increasing inclusion levels of dietary astaxanthin supplementation showed a linear regression pattern with a strong correlation for WG ($r^2 = 0.9112$) and a moderate correlation for FCR ($r^2 = 0.6867$), as presented in **Figure 2**.

Body Proximate Analysis

As presented in **Table 6**, there are significant differences ($p > 0.05$) that were reported in the whole-body chemical composition (dry matter, protein, fat, and ash content) of shrimp *L. vannamei*. The control group had the highest significant ($p < 0.05$) values of dry matter and crude protein

TABLE 4 | Mean values of water quality parameters during the feeding trial.

Water quality parameters	Experimental diets[a]			
	D_1	D_2	D_3	D_4
NH_3 (mg L^{-1})	0.119 ± 0.001	0.116 ± 0.010	0.115 ± 0.015	0.103 ± 0.014
NO_2 (mg L^{-1})	0.119 ± 0.016^{a}	0.109 ± 0.001^{ab}	0.108 ± 0.003^{ab}	0.095 ± 0.015^{b}
NO_3 (mg L^{-1})	0.222 ± 0.028	0.219 ± 0.003	0.225 ± 0.007	0.214 ± 0.023
PO_4 (mg L^{-1})	0.485 ± 0.009	0.495 ± 0.039	0.517 ± 0.022	0.494 ± 0.004
Alkalinity (mg L^{-1})	7.700 ± 0.625	7.725 ± 0.225	7.987 ± 0.137	8.337 ± 0.212
Temperature (°C)	26.84 ± 0.20^{a}	26.55 ± 0.01^{b}	26.57 ± 0.07^{b}	26.65 ± 0.11^{ab}
Salinity (ppt)	32.25 ± 0.09^{b}	32.52 ± 0.02^{a}	32.41 ± 0.07^{ab}	32.52 ± 0.18^{a}
pH	7.79 ± 0.02	7.77 ± 0.01	7.76 ± 0.01	7.76 ± 0.02

[a]D_1, D_2, D_3, and D_4 are the experimental diets that are supplemented with 0, 2, 4, and 6 g astaxanthins/kg diet, respectively, of the crude acetonic extract of Arthrospira platensis *NIOF17/003.*

Data are means ± SD and the n = 3. *Different letters in the same row are significantly different* ($p < 0.05$).

TABLE 5 | Growth performance and feed utilization of Pacific white leg shrimp, *Litopenaeus vannamei*, fed experimental diets for 90 days.

Growth indicators	Experimental diets[a]			
	D_1	D_2	D_3	D_4
IBW (g)	0.19 ± 0.003	0.19 ± 0.003	0.19 ± 0.003	0.19 ± 0.003
FBW (g)	21.87 ± 1.15^{c}	22.03 ± 1.33^{b}	24.61 ± 1.78^{a}	25.75 ± 1.42^{a}
WG (g)	21.68 ± 0.57^{c}	21.84 ± 0.93^{b}	24.42 ± 1.80^{a}	25.56 ± 0.92^{a}
Survival (%)	82.22 ± 3.85	76.67 ± 3.33	77.78 ± 1.92	74.44 ± 5.09
SGR (%/day)	5.27 ± 0.01	5.28 ± 0.038	5.40 ± 0.028	5.45 ± 0.06
FCR	1.53 ± 0.12^{a}	1.43 ± 0.06^{a}	1.25 ± 0.09^{c}	1.33 ± 0.11^{b}

[a]D_1, D_2, D_3, and D_4 are the experimental diets that are supplemented with 0, 2, 4, and 6 g astaxanthins/kg diet, respectively, of the crude acetonic extract of Arthrospira platensis *NIOF17/003.*

Data are means ± SD and the n = 3. *Different letters in the same row are significantly different* ($p < 0.05$).

IBW, initial body weight (g); FBW, final body weight (g); WG, weight gain (g); SGR, specific growth rate (%/day); and FCR, feed conversion ratio.

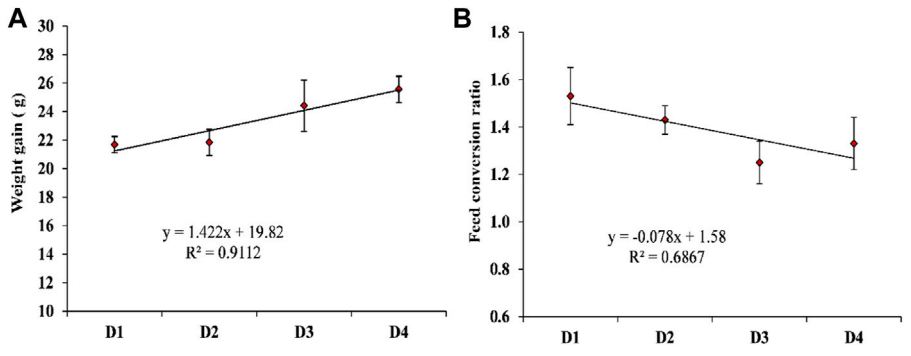


FIGURE 2 | The appropriate regression models of the increasing inclusion levels of dietary astaxanthin supplementation for the **(A)** weight gain; **(B)** food conversion ratio of *L. vannamei.* D_1, D_2, D_3, and D_4 are the experimental diets that are supplemented with 0, 2, 4, and 6 g astaxanthin/kg diet, respectively, of the natural astaxanthins of the crude acetonic extract of *Arthrospira platensis* NIOF17/003. Data are means ± SD and the *n* = 3.

content. While D_4 showed the highest significant ($p < 0.05$) impacts on fat and ash content (**Table 6**).

Bacterial Abundance Investigations

The effects of experimental diets supplemented with astaxanthin on both total heterotrophic bacteria (THB) and total *Vibrio* spp. count (TVC) in the water and intestine of *L. vannamei* are shown in **Table 7**. It was observed that the degradative heterotrophic bacteria were more abundant in the intestine than in water. Concerning pathogenic bacteria, the genus *Vibrio* spp. was chosen as an indicator for the pathogenicity of shrimp, and the count was lower than heterotrophic bacteria. However, when compared to the control, the THB count in the water and intestine decreased gradually as astaxanthin supplementation levels increased.

TABLE 6 | Proximate whole-body proximate analysis (% of wet weight) of Pacific white leg shrimp, *Litopenaeus vannamei,* fed experimental diets for 90 days.

Proximate composition indicators	Experimental diets[a]			
	D_1	D_2	D_3	D_4
Dry matter (%)	26.53 ± 0.10[a]	25.04 ± 0.04[b]	24.84 ± 0.12[c]	25.58 ± 0.07[b]
Protein (%)	23.12 ± 0.03[a]	21.77 ± 0.04[c]	21.56 ± 0.06[d]	22.19 ± 0.02[b]
Fat (%)	7.79 ± 0.01[d]	9.99 ± 0.02[c]	10.07 ± 0.04[b]	10.87 ± 0.03[a]
Ash (%)	1.60 ± 0.01[d]	1.99 ± 0.02[b]	1.92 ± 0.03[c]	2.08 ± 0.03[a]

[a]*D_1, D_2, D_3, and D_4 are the experimental diets that are supplemented with 0, 2, 4, and 6 g astaxanthins/kg diet, respectively, of the crude acetonic extract of* Arthrospira platensis *NIOF17/003.*

Data are means ± SD and the n = *3. Different letters in the same column are significantly different* ($p < 0.05$).

TABLE 7 | Bacterial abundance in the culture water and intestine of Pacific white leg shrimp, *Litopenaeus vannamei,* fed experimental diets for 90 days.

Bacterial count	Experimental diets[a]			
	D_1	D_2	D_3	D_4
Water				
THB (cfu mL^1 $x10^4$)	95.90 ± 0.04[d]	41.90 ± 0.03[c]	26.50 ± 0.06[b]	11.90 ± 0.66[a]
TVC (cfu mL^1 $x10^3$)	11.40 ± 0.05[d]	6.80 ± 0.02[c]	4.50 ± 0.03[b]	0.5 ± 0.04[a]
Intestine				
THB (cfu g^1 $x10^4$)	399.90 ± 0.04[d]	249.90 ± 0.03[c]	119.90 ± 0.04[b]	30.00 ± 0.03[a]
TVC (cfu g^1 $x10^3$)	111.20 ± 0.4[d]	47.60 ± 0.40[c]	28.20 ± 0.60[b]	6.70 ± 0.30[a]

[a]*D_1, D_2, D_3, and D_4 are the experimental diets that are supplemented with 0, 2, 4, and 6 g astaxanthins/kg diet, respectively, of the crude acetonic extract of* Arthrospira platensis *NIOF17/003.*

Data are means ± SD and the n = *9. Different letters in the same row are significantly different* ($p < 0.05$).

THB, total heterotrophic bacterial count; TVC, total Vibrio *spp. count.*

Immune-Related Gene Expressions

Dietary inclusion of astaxanthin enhanced the expression of all studied genes: *Bgp*, *Lys*, *proPO*, *TGases*, *Crus*, and *SOD* in the muscle tissue of *L. vannamei* at the end of the feeding trial (**Figure 3**). The expression of the *Bgp* gene was significantly increased in the dietary supplemented treatment with astaxanthin at a level of 4 g/kg compared to the control group and D_2 even though the expression in D_3 was higher than D4. Generally, gene expression of *Lys*, *proPO*, *TGases*, *Crus*, and *SOD* was significantly upregulated ($p < 0.05$) with increasing the concentration level of natural astaxanthins in the diet compared to the control. The *proPO* gene expression was the most upregulated gene among the other genes, and the relative fold change was 14.71 compared to the control group.

DISCUSSION

Recently, with the growing development of aquaculture, feed additives demand is globally increased, resulting in an enormous space for the industrial application of natural astaxanthin (Lu et al., 2021). Generally, natural astaxanthins are extracted from different aquatic organisms such as shrimp (Scurria et al., 2020), soft coral (Metwally et al., 2020), fish (Yu and Liu, 2020), seaweeds (Teramukai et al., 2020), and microalgae (Molino et al., 2018). Among all aquatic organisms, microalgae have the highest ability to produce astaxanthin than any aquatic animal (Gallego et al., 2019). Microalgae are high productive microorganisms, carbon captures, and oxygen producers. Therefore, the reliance on the production of microalgae-based astaxanthin has positive environmental impacts in mitigating the effects of global warming and creating environmental benefits (Lee and Ding, 1994; Wu et al., 2017).

In the present study, the final yield of crude extract, mainly astaxanthin, of *A. platensis* was 2.7%. Compared to other microalgal species, *Haematococcus pluvialis* is considered the most widespread species that produces natural astaxanthin with a high final yield ranging from 3.8%–4% (Lee and Ding, 1994; Aflalo et al., 2007; Ranga Rao et al., 2010). Meanwhile, the final yield of natural astaxanthin in the present study exceeded that reported in several microalgae species, such as *Chlorella zofingiensis*, *Neochloris wimmeri*, *Chlorococcum* sp., *Botryococcus braunii*, *Tetraselmis* sp., and *Scenedesmus obliquus*, which produced 0.68% (Orosa et al., 2001), 0.60% (Orosa et al., 2000), 0.20% (Zhang et al., 1997), 0.01% (Grung and Metzger, 1994), 0.23% (Lim et al., 2018), and 0.30% (Qin et al., 2008), respectively. The yields of astaxanthin among different microalgae species vary due to several reasons, such as strain, extraction solvent, and extraction conditions. Accordingly, *A. platensis* NIOF17/003 could be considered as a source for producing natural astaxanthin.

Astaxanthin has been used as a dietary supplementation for aquatic animals (Niu et al., 2009; Lim et al., 2018). In the current study, the FW and WG, were significantly increased with the increase of astaxanthin supplementation levels compared to the control. In accordance with the present findings, Flores et al. (2007) reported that the inclusion of 80 mg/kg of synthetic astaxanthin enhanced the growth, survival, and molting frequency of *P. vannamei*. In addition, the inclusion of natural astaxanthin extracted from *H. pluvialis* significantly improves the growth

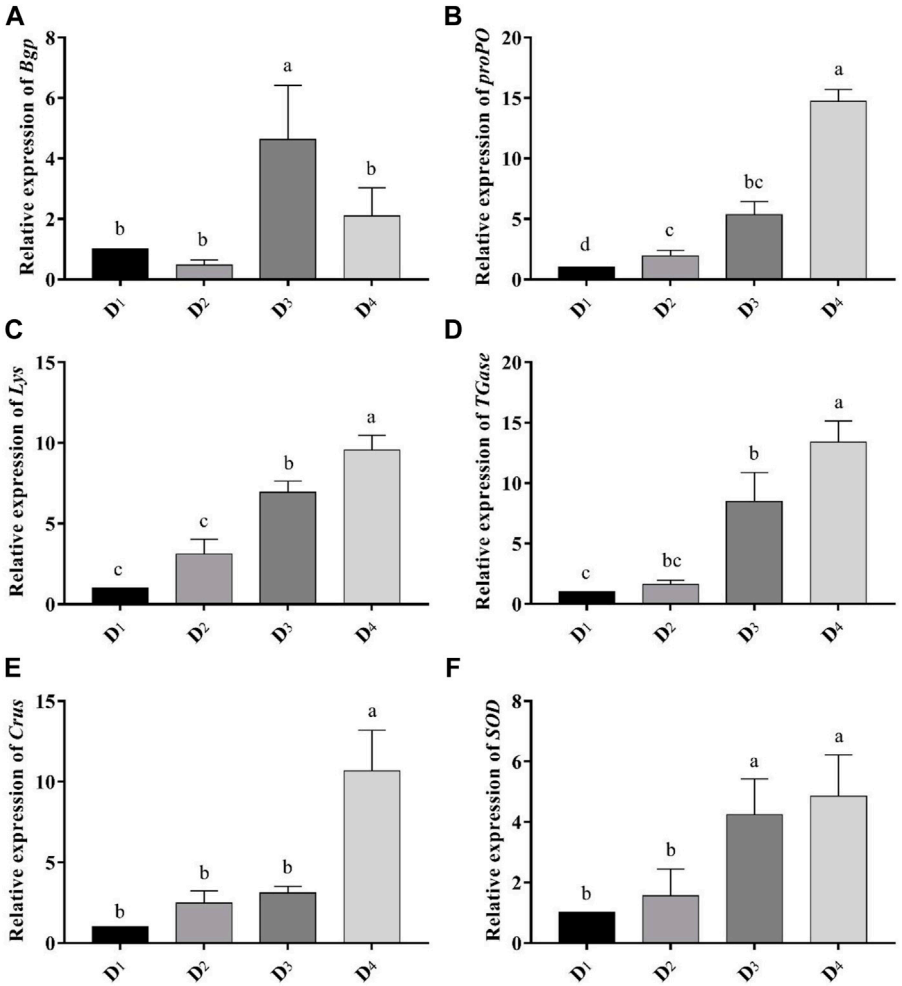


FIGURE 3 | Analysis of immune-related gene-expressions [*Bgp* **(A)**, *proPO* **(B)**, *Lys* **(C)**, *TGase* **(D)**, *Crus* **(E)**, and *SOD* **(F)**] comparing to the expression of *β-actin* gene in the different inclusion levels of astaxanthin dietary supplementation. D_1, D_2, D_3, and D_4 are the experimental diets that are supplemented with 0, 2, 4, and 6 g astaxanthins/kg diet, respectively, of the crude acetonic extract of *Arthrospira platensis* NIOF17/003. The data are means ± SD and the $n = 9$. Different letters for the same gene indicated a significantly differences ($p < 0.05$).

performance, pigmentation, and antioxidant capacity of the white prawn *Exopalaemon carinicauda* (Zhang et al., 2021). Astaxanthins extracted from *H. pluvialis* and *A. platensis* strain Pacifica improved shrimp kuruma and *M. Japonicus* growth and hypoxia stress resistance (Chien and Shiau, 2005).

The maintenance of high immune surveillance is one of the crucial measures of successful aquatic cultured animals (Mansour et al., 2018; Mansour et al., 2020). In crustaceans, the innate immune system is the main animal defence to any pathogen. This defence is mediated by several kinds of cells, enzymes, and antimicrobial peptides (Huang et al., 2020). The present finding revealed an upregulation of the *Bgp* gene in shrimp fed 4 and 6 g astaxanthin/kg diets, especially with the 4 g/kg diet. The *Bgp* gene works as a vital factor for activation of the proPO system, coagulation progression, and expression of antimicrobial peptides after recognizing the microbial components (Goncalves et al., 2012). The *Bgp* gene showed a delayed upregulation in *L. vannamei* fed immunostimulant b-1, 3-glucan from *Schizophyllum commune* daily for a 1-week feeding trial (Wang et al., 2008). This contrast with the present findings could be due to the short treatment period with b-1,3-glucan than astaxanthin and the different mode of action of both treatments.

In addition, the *proPO* gene expression was significantly increased in shrimp fed 4 and 6 g/kg astaxanthin supplemented diets compared to the control group, and it was approximately 14-fold higher than the control, in the present study. Furthermore, the expression of *proPO* is the highest among all studied immune-related genes in the present study with increasing the concentration of astaxanthin (6 g/kg diet). Whereas, one of the most important components of the shrimp immune system is prophenoloxidase (Sritunyalucksana and Söderhäll, 2000).

Lysozyme can hydrolyze bacterial cell walls and operates as a non-specific innate defense molecule against bacterial infections. It has been demonstrated that it activates in penaeid shrimp in response to *Vibrio*, and its gene has been cloned and characterized in *L. vannamei* and *M. japonicas* (Hikima et al.,

2003). In the present study, *Lys* gene expression in *L. vannamei* was increased gradually in the shrimps that fed with the three levels of astaxanthin supplemented diets. It was about 9-fold higher in the D_4 treatment (6 g/kg diet) than in the control group. Kuruma shrimp fed a diet supplemented with astaxanthin experienced higher lysozyme activity, and total hemocyte count and improved the survival of shrimp against low salinity levels (Wang et al., 2019). Transglutaminase is recognized as an invertebrate defense mechanism. *TGase* gene silencing has previously been demonstrated to make shrimp susceptible to both bacterial and viral infections, indicating that *TGase* is an important component of the shrimp immune system (Fagutao et al., 2012). In this study, supplemented diets with natural astaxanthin influenced *TGase* gene expression and showed a significant upregulation in the fish fed 6 g/kg astaxanthin supplemented diet compared to the control group. In the same manner, *Crust* gene expression was improved with dietary supplementation of astaxanthin in a dose-dependent manner. Crustin is one of the antimicrobial peptides in penaeid shrimps hemolymph. After oral treatment for 7 days with peptidoglycan, a significant increase in crustin mRNA levels in *M. japonicas* was reported (Rattanachai et al., 2004). In the same line, Pacific white shrimp *L. vannamei*, fed diet supplemented with 80 mg astaxanthin/kg diet significantly improved serum phenoloxidase activity serum bacteriolytic activity, total haemocyte counts, and phagocytic activity (Wang et al., 2015). In addition, the antioxidant prosperities of astaxanthin (**Figure 3F**) could directly participate in the immune enhancement in *L. vannamei*. However, the immune-stimulating activity of astaxanthin in crustaceans still needs more investigation to better understand its mode of action.

Superoxide dismutase (SOD) is one of the main antioxidant enzymes responsible for scavenging reactive oxygen species and is considered a safeguarding mechanism inside the tissue that could be damaged by oxidation processes and phagocytosis (Chien et al., 2003). Because of its unique chemical structure, astaxanthin has the potential to has antioxidant effects, including free radicals scavenging and activating the expression and activities of several antioxidant enzymes (Eren et al., 2019; Yu et al., 2021). In our study, the expression of *SOD* was significantly upregulated in shrimp fed astaxanthins at levels of 4 and 6 g/kg compared to the control group. In line with the current findings, astaxanthin supplementation increased the expression levels of *Cyt-Mn SOD*, *CAT*, and *GPx* genes (Liu et al., 2018) and SOD activity in *L. vannamei* (Chuchird et al., 2015). Whereas, astaxanthins as a carotenoids reported to protect white blood cells from oxidative damage, enhancing cell-mediated, and humoral immune responses of vertebrates and invertebrates (Song et al., 2020). This refers to the antioxidant activity of carotenoids that may be involved in the immunomodulatory action by quenching singlet oxygen and free radicals (Cvetkovic et al., 2013).

Generally, the contents of the gut microbiota have a strong influence on the health of aquatic organisms (Sharawy et al., 2020), such as digestion, nutrient absorption, immunity responses, and biological antibiosis (Li et al., 2018). The intestinal bacteria respond quickly to changes in food consumption, diet composition, and ingredients (Ringø et al., 2016). In the current study, the counts of THB and TVC were significantly ($p > 0.05$) decreased with increasing the inclusion levels of astaxanthin, compared to the control diet. Whereas, the bacterial abundance of *Vibrio* spp. was decreased in all astaxanthin supplemented diets compared to the control. Chuchird et al. (2015) reported an increase in the survival, growth, and resistance to *V. parahaemolyticus* of *L. vannamei* fed an astaxanthin supplemented diet. Furthermore, shrimp-fed diets supplemented with astaxanthin had significantly lower total intestinal bacteria and *Vibrio* spp. counts (Chuchird et al., 2015). The mode of action by which astaxanthin affected the bacterial population still not clear and could need more investigation. All these indications are in line with our findings, which revealed that astaxanthin is a promising substance for controlling the pathogenic bacteria load during the whole culture process of shrimp. These results were attributed to the high biological activities of acetonic extract of *A. platensis* NIOF17/003, mainly astaxanthin, which make it a wonderful, sustainable, and eco-friendly feed additive for aquaculture applications.

CONCLUSION

From the current findings, it could be concluded that the *Arthrospira platensis* NIOF17/003 strain (Accession GenBank number: MW396472) is a good source of astaxanthin with a high final yield of about 2.7%. Dietary supplementation with natural astaxanthin enhanced the growth, feed utilization, and chemical composition of Pacific white leg shrimp, *Litopenaeus vannamei*. In addition, astaxanthin proved a powerful immune stimulant, antioxidant, and antibacterial substance for *L. vanami*. More research is needed to determine the mechanism of astaxanthin's immunostimulant effects in shrimp, including cytokines mediated humoral and cellular innate immunity.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the authors upon reasonable request.

ETHICS STATEMENT

The animal study was reviewed and approved by the Research Committee of the National Institute of Oceanography and Fisheries, Egypt.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

This work was supported by the Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Saudi Arabia (Grant No. GRANT797).

REFERENCES

Abbas, E. M., Ali, F. S., Desouky, M. G., Ashour, M., El-Shafei, A., Maaty, M. M., et al. (2020). Novel Comprehensive Molecular and Ecological Study Introducing Coastal Mud Shrimp (*Solenocera Crassicornis*) Recorded at the Gulf of Suez, Egypt. *J. Mar. Sci. Eng.* 9 (1), 9. doi:10.3390/jmse9010009

Abo-Taleb, H., Ashour, M., El-Shafei, A., Alataway, A., and Maaty, M. M. (2020). Biodiversity of Calanoida Copepoda in Different Habitats of the North-Western Red Sea (Hurghada Shelf). *Water* 12 (3), 656. doi:10.3390/w12030656

Aflalo, C., Meshulam, Y., Zarka, A., and Boussiba, S. (2007). On the Relative Efficiency of Two- vs. One-Stage Production of Astaxanthin by the Green algaHaematococcus pluvialis. *Biotechnol. Bioeng.* 98 (1), 300–305. doi:10.1002/bit.21391

Alprol, A. E., Ashour, M., Mansour, A. T., Alzahrani, O. M., Mahmoud, S. F., and Gharib, S. M. (2021a). Assessment of Water Quality and Phytoplankton Structure of Eight Alexandria Beaches, Southeastern Mediterranean Sea, Egypt. *J. Mar. Sci. Eng.* 9 (12), 1328. doi:10.3390/jmse9121328

Alprol, A. E., Heneash, A. M. M., Ashour, M., Abualnaja, K. M., Alhashmialameer, D., Mansour, A. T., et al. (2021b). Potential Applications of *Arthrospira Platensis* Lipid-free Biomass in Bioremediation of Organic Dye from Industrial Textile Effluents and its Influence on Marine Rotifer (*Brachionus plicatilis*). *Materials* 14 (16), 4446. doi:10.3390/ma14164446

Alprol, A. E., Heneash, A. M. M., Soliman, A. M., Ashour, M., Alsanie, W. F., Gaber, A., et al. (2021c). Assessment of Water Quality, Eutrophication, and Zooplankton Community in Lake Burullus, Egypt. *Diversity* 13 (6), 268. doi:10.3390/d13060268

An, J., Gao, F., Ma, Q., Xiang, Y., Ren, D., and Lu, J. (2017). Screening for Enhanced Astaxanthin Accumulation Among *Spirulina Platensis* Mutants Generated by Atmospheric and Room Temperature Plasmas. *Algal Res.* 25, 464–472. doi:10.1016/j.algal.2017.06.006

AOAC (2003). *Official Methods of Analysis*. The Association of Official Analytical Chemists, Vol.I.17thed. Washington, DC, USA.

APHA (2005). *Standard Methods for the Examination of Water and Wastewater*. Washington, DC, USA: American Public Health Association.

Ashour, M., Mabrouk, M. M., Abo-Taleb, H. A., Sharawy, Z. Z., Ayoub, H. F., Van Doan, H., et al. (2021). A Liquid Seaweed Extract (TAM) Improves Aqueous Rearing Environment, Diversity of Zooplankton Community, whilst Enhancing Growth and Immune Response of Nile tilapia, *Oreochromis niloticus*, Challenged by Aeromonas Hydrophila. *Aquaculture* 543, 736915. doi:10.1016/j.aquaculture.2021.736915

Ashour, M., Mabrouk, M. M., Ayoub, H. F., El-Feky, M. M. M. M., Zaki, S. Z., Hoseinifar, S. H., et al. (2020). Effect of Dietary Seaweed Extract Supplementation on Growth, Feed Utilization, Hematological Indices, and Non-specific Immunity of Nile Tilapia, *Oreochromis niloticus* Challenged with *Aeromonas Hydrophila*. *J. Appl. Phycol.* 32 (5), 3467–3479. doi:10.1007/s10811-020-02178-1

Babin, A., Biard, C., and Moret, Y. (2010). Dietary Supplementation with Carotenoids Improves Immunity without Increasing its Cost in a Crustacean. *Am. Nat.* 176 (2), 234–241. doi:10.1086/653670

Boyd, C. E., and Tucker, C. S. (2012). *Pond Aquaculture Water Quality Management*. Springer Science & Business Media.

Cerenius, L., Lee, B. L., and Söderhäll, K. (2008). The proPO-System: Pros and Cons for its Role in Invertebrate Immunity. *Trends Immunol.* 29 (6), 263–271. doi:10.1016/j.it.2008.02.009

Chien, Y.-H., Pan, C.-H., and Hunter, B. (2003). The Resistance to Physical Stresses by *Penaeus monodon* Juveniles Fed Diets Supplemented with Astaxanthin. *Aquaculture* 216 (1-4), 177–191. doi:10.1016/S0044-8486(02)00056-X

Chien, Y.-H., and Shiau, W.-C. (2005). The Effects of Dietary Supplementation of Algae and Synthetic Astaxanthin on Body Astaxanthin, Survival, Growth, and Low Dissolved Oxygen Stress Resistance of Kuruma Prawn, *Marsupenaeus japonicus* Bate. *J. Exp. Mar. Biol. Ecol.* 318 (2), 201–211. doi:10.1016/j.jembe.2004.12.016

Chuchird, N., Rorkwiree, P., and Rairat, T. (2015). Effect of Dietary Formic Acid and Astaxanthin on the Survival and Growth of Pacific White Shrimp (*Litopenaeus Vannamei*) and Their Resistance to *Vibrio parahaemolyticus*. *SpringerPlus* 4 (1), 440–512. doi:10.1186/s40064-015-1234-x

Cornet, S., Biard, C., and Moret, Y. (2007). Is There a Role for Antioxidant Carotenoids in Limiting Self-Harming Immune Response in Invertebrates? *Biol. Lett.* 3 (3), 284–288. doi:10.1098/rsbl.2007.0003

Cvetkovic, D., Fiedor, L., Fiedor, J., Wiśniewska-Becker, A., and Markovic, D. (2013). *Molecular Base for Carotenoids Antioxidant Activity in Model and Biological Systems: The Health-Related Effects*. NY, USA: Nova Science Publishers: Hauppauge.

Díaz, A. C., Velurtas, S. M., Espino, M. L., and Fenucci, J. L. (2014). Effect of Dietary Astaxanthin on Free Radical Scavenging Capacity and Nitrite Stress Tolerance of Postlarvae Shrimp, *Pleoticus Muelleri*. *J. Agric. Food Chem.* 62 (51), 12326–12331. doi:10.1021/jf503754q

El-Shouny, W., Sharaf, M., Abomohra, A., and Abo-Eleneen, M. (2015). Production Enhancement of Some Valuable Compounds of *Arthrospira Platensis*. *J. Basic Environ. Sci.* 2, 74–83.

Elshobary, M. E., El-Shenody, R. A., Ashour, M., Zabed, H. M., and Qi, X. (2020). Antimicrobial and Antioxidant Characterization of Bioactive Components from *Chlorococcum Minutum*. *Food Biosci.* 35, 100567. doi:10.1016/j.fbio.2020.100567

Eren, B., Tuncay Tanrıverdi, S., Aydın Köse, F., and Özer, Ö. (2019). Antioxidant Properties Evaluation of Topical Astaxanthin Formulations as Anti-aging Products. *J. Cosmet. Dermatol* 18 (1), 242–250. doi:10.1111/jocd.12665

Fagutao, F. F., Maningas, M. B. B., Kondo, H., Aoki, T., and Hirono, I. (2012). Transglutaminase Regulates Immune-Related Genes in Shrimp. *Fish shellfish Immunol.* 32 (5), 711–715. doi:10.1016/j.fsi.2012.01.018

Flores, M., Díaz, F., Medina, R., Re, A. D., and Licea, A. (2007). Physiological, Metabolic and Haematological Responses in White Shrimp Litopenaeus Vannamei (Boone) Juveniles Fed Diets Supplemented with Astaxanthin Acclimated to Low-Salinity Water. *Aquac. Res.* 38 (7), 740–747. doi:10.1111/j.1365-2109.2007.01720.x

Gallego, R., Bueno, M., and Herrero, M. (2019). Sub- and Supercritical Fluid Extraction of Bioactive Compounds from Plants, Food-By-Products, Seaweeds and Microalgae - an Update. *TrAC Trends Anal. Chem.* 116, 198–213. doi:10.1016/j.trac.2019.04.030

Goncalves, P., Vernal, J., Rosa, R. D., Yepiz-Plascencia, G., de Souza, C. R. B., Barracco, M. A., et al. (2012). Evidence for a Novel Biological Role for the Multifunctional β-1,3-glucan Binding Protein in Shrimp. *Mol. Immunol.* 51 (3-4), 363–367. doi:10.1016/j.molimm.2012.03.032

Gouveia, L., Rema, P., Pereira, O., and Empis, J. (2003). Colouring Ornamental Fish (*Cyprinus carpio* and *Carassius auratus*) with Microalgal Biomass. *Aquac. Nutr.* 9 (2), 123–129. doi:10.1046/j.1365-2095.2003.00233.x

Grung, M., Metzger, P., and Liaaen-Jensen, S. v. (1994). Algal Carotenoids 53; Secondary Carotenoids of Algae 4; Secondary Carotenoids in the Green Alga *Botryococcus Braunii*, Race L, New Strain. *Biochem. Syst. Ecol.* 22 (1), 25–29. doi:10.1016/0305-1978(94)90111-2

Han, J., Lu, Y., Zheng, H., Liu, H., Deng, H., and Zhang, B. (2016). Differential Expression of CuZnSOD Gene under Low Temperature Stress in Noble Scallop Chlamys Nobilis with Different Carotenoid Content. *Fish shellfish Immunol.* 54, 30–39. doi:10.1016/j.fsi.2016.03.160

Hassan, S. M., Ashour, M., Sakai, N., Zhang, L., Hassanien, H. A., Gaber, A., et al. (2021). Impact of Seaweed Liquid Extract Biostimulant on Growth, Yield, and Chemical Composition of Cucumber (*Cucumis Sativus*). *Agriculture* 11 (4), 320. doi:10.3390/agriculture11040320

Higuera-Ciapara, I., Félix-Valenzuela, L., and Goycoolea, F. M. (2006). Astaxanthin: a Review of its Chemistry and Applications. *Crit. Rev. food Sci. Nutr.* 46 (2), 185–196. doi:10.1080/10408690590957188

Hikima, S., Hikima, J.-i., Rojtinnakorn, J., Hirono, I., and Aoki, T. (2003). Characterization and Function of Kuruma Shrimp Lysozyme Possessing Lytic Activity against *Vibrio* Species. *Gene* 316, 187–195. doi:10.1016/S0378-1119(03)00761-3

Huang, Z., Aweya, J. J., Zhu, C., Tran, N. T., Hong, Y., Li, S., et al. (2020). Modulation of Crustacean Innate Immune Response by Amino Acids and Their Metabolites: Inferences from Other Species. *Front. Immunol.* 11 (2812). doi:10.3389/fimmu.2020.574721

Jiravanichpaisal, P., Lee, B. L., and Söderhäll, K. (2006). Cell-mediated Immunity in Arthropods: Hematopoiesis, Coagulation, Melanization and Opsonization. *Immunobiology* 211 (4), 213–236. doi:10.1016/j.imbio.2005.10.015

Ju, Z. Y., Forster, I. P., and Dominy, W. G. (2009). Effects of Supplementing Two Species of Marine Algae or Their Fractions to a Formulated Diet on Growth,

Survival and Composition of Shrimp (*Litopenaeus Vannamei*). *Aquaculture* 292 (3-4), 237–243. doi:10.1016/j.aquaculture.2009.04.040

Lee, Y.-K., and Ding, S.-Y. (1994). Cell Cycle and Accumulation of Astaxanthin in Haematococcus Lacustris (Chlorophyta)1. *J. Phycol.* 30 (3), 445–449. doi:10.1111/j.0022-3646.1994.00445.x

Li, E., Xu, C., Wang, X., Wang, S., Zhao, Q., Zhang, M., et al. (2018). Gut Microbiota and its Modulation for Healthy Farming of Pacific White Shrimp *Litopenaeus Vannamei*. *Rev. Fish. Sci. Aquac.* 26 (3), 381–399. doi:10.1080/23308249.2018.1440530

Lim, K. C., Yusoff, F. M., Shariff, M., and Kamarudin, M. S. (2018). Astaxanthin as Feed Supplement in Aquatic Animals. *Rev. Aquacult* 10 (3), 738–773. doi:10.1111/raq.12200

Lin, Y.-C., Tayag, C. M., Huang, C.-L., Tsui, W.-C., and Chen, J.-C. (2010). White Shrimp *Litopenaeus Vannamei* that Had Received the Hot-Water Extract of *Spirulina Platensis* Showed Earlier Recovery in Immunity and Up-Regulation of Gene Expressions after pH Stress. *Fish shellfish Immunol.* 29 (6), 1092–1098. doi:10.1016/j.fsi.2010.09.002

Liñán-Cabello, M. A., Paniagua-Michel, J., and Zenteno-Savín, T. (2003). Carotenoids and Retinal Levels in Captive and Wild Shrimp,Litopenaeus Vannamei. *Aquac. Nutr.* 9 (6), 383–389. doi:10.1046/j.1365-2095.2003.00267.x

Liu, X., Wang, B., Li, Y., Wang, L., and Liu, J. (2018). Effects of Dietary Botanical and Synthetic Astaxanthin on E/Z and R/S Isomer Composition, Growth Performance, and Antioxidant Capacity of White Shrimp, *Litopenaeus Vannamei*, in the Nursery Phase. *Invertebr. Surviv. J.* 15 (1), 131–140. doi:10.25431/1824-307X/isj.v15i1.131-140

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods* 25 (4), 402–408. doi:10.1006/meth.2001.1262

Lu, Q., Li, H., Zou, Y., Liu, H., and Yang, L. (2021). Astaxanthin as a Microalgal Metabolite for Aquaculture: A Review on the Synthetic Mechanisms, Production Techniques, and Practical Application. *Algal Res.* 54, 102178–178. doi:10.1016/j.algal.2020.102178

Lukwambe, B., Nicholaus, R., Zhang, D., Yang, W., Zhu, J., and Zheng, Z. (2019). Successional Changes of Microalgae Community in Response to Commercial Probiotics in the Intensive Shrimp (*Litopenaeus Vannamei* Boone) Culture Systems. *Aquaculture* 511, 734257. doi:10.1016/j.aquaculture.2019.734257

Mabrouk, M. M., Ashour, M., Labena, A., Zaki, M. A. A., Abdelhamid, A. F., Gewaily, M. S., et al. (2021). Nanoparticles of Arthrospira Platensis Improves Growth, Antioxidative and Immunological Responses of Nile tilapia (*Oreochromis niloticus*) and its Resistance to Aeromonas Hydrophila. *Aquac. Res.* 53, 125–135. doi:10.1111/are.15558

Madkour, F. F., Kamil, A. E.-W., and Nasr, H. S. (2012). Production and Nutritive Value of *Spirulina Platensis* in Reduced Cost Media. *Egypt. J. Aquatic Res.* 38 (1), 51–57. doi:10.1016/j.ejar.2012.09.003

Mansour, A. T., Alsaqufi, A. S., Alkhamis, Y. A., Al-Gazar, F. F., Zaki, M. A., Nour, A. A. M., et al. (2021). The Evaluation of *Arthrospira Platensis* Bioactivity and Their Dietary Supplementation to Nile tilapia Vegetarian Diet on Growth Performance, Feed Utilization, Body Composition and Hemato-Biochemical Parameters. *Ann. Anim. Sci.* 21 (3), 1061–1080. doi:10.2478/aoas-2021-0003

Mansour, A. T., Ashour, M., Alprol, A. E., and Alsaqufi, A. S. (2022). Aquatic Plants and Aquatic Animals in the Context of Sustainability: Cultivation Techniques, Integration, and Blue Revolution. *Sustainability* 14, 3257. doi:10.3390/su14063257

Mansour, A. T., Espinosa, C., García-Beltrán, J. M., Miao, L., Ceballos Francisco, D. C., Alsaqufi, A. S., et al. (2020). Dietary Supplementation of Drumstick Tree, *Moringa Oleifera*, Improves Mucosal Immune Response in Skin and Gills of Seabream, *Sparus Aurata*, and Attenuates the Effect of Hydrogen Peroxide Exposure. *Fish. Physiol. Biochem.* 46 (3), 981–996. doi:10.1007/s10695-020-00763-2

Mansour, A. T., Miao, L., Espinosa, C., García-Beltrán, J. M., Ceballos Francisco, D. C., and Esteban, M. Á. (2018). Effects of Dietary Inclusion of *Moringa Oleifera* Leaves on Growth and Some Systemic and Mucosal Immune Parameters of Seabream. *Fish. Physiol. Biochem.* 44 (4), 1223–1240. doi:10.1007/s10695-018-0515-z

Mehrabi, Z., Firouzbakhsh, F., and Jafarpour, A. (2012). Effects of Dietary Supplementation of Synbiotic on Growth Performance, Serum Biochemical Parameters and Carcass Composition in Rainbow Trout (*Oncorhynchus mykiss*) Fingerlings. *J. Animal Physiology Animal Nutr.* 96 (3), 474–481. doi:10.1111/j.1439-0396.2011.01167.x

Metwally, A., El-Naggar, H., El-Damhougy, K., Bashar, M., Ashour, M., and Abo-Taleb, H. (2020). GC-MS Analysis of Bioactive Components in Six Different Crude Extracts from the Soft Coral (*Sinularia Maxim*) Collected from Ras Mohamed, Aqaba Gulf, Red Sea, Egypt. *Egypt. J. Aquatic Biolo. Fish.* 24 (6), 425–434. doi:10.21608/ejabf.2020.114293

Molino, A., Rimauro, J., Casella, P., Cerbone, A., Larocca, V., Chianese, S., et al. (2018). Extraction of Astaxanthin from Microalga *Haematococcus pluvialis* in Red Phase by Using Generally Recognized as Safe Solvents and Accelerated Extraction. *J. Biotechnol.* 283, 51–61. doi:10.1016/j.jbiotec.2018.07.010

Moradi, Z., Madadkar Haghjou, M., Zarei, M., Colville, L., and Raza, A. (2021). Synergy of Production of Value-Added Bioplastic, Astaxanthin and Phycobilin Co-products and Direct Green 6 Textile Dye Remediation in Spirulina Platensis. *Chemosphere* 280, 130920. doi:10.1016/j.chemosphere.2021.130920

Niu, J., Tian, L.-X., Liu, Y.-J., Yang, H.-J., Ye, C.-X., Gao, W., et al. (2009). Effect of Dietary Astaxanthin on Growth, Survival, and Stress Tolerance of Postlarval Shrimp, *Litopenaeus Vannamei*. *J. World Aquac. Soc.* 40 (6), 795–802. doi:10.1111/j.1749-7345.2009.00300.x

Orosa, M., Torres, E., Fidalgo, P., and Abalde, J. (2000). Production and Analysis of Secondary Carotenoids in Green Algae. *J. Appl. Phycol.* 12 (3), 553–556. doi:10.1023/A:1008173807143

Orosa, M., Valero, J. F., Herrero, C., and Abalde, J. (2001). Comparison of the Accumulation of Astaxanthin in *Haematococcus pluvialis* and Other Green Microalgae under N-Starvation and High Light Conditions. *Biotechnol. Lett.* 23 (13), 1079–1085. doi:10.1023/A:1010510508384

Osman, M. E. H., Abo-Shady, A. M., and El-Nagar, M. M. F. (2016). Cyanobacterial *Arthrospira (Spirulina Platensis*) as Safener against Harmful Effects of Fusilade Herbicide on Faba Bean Plant. *Rend. Fis. Acc. Lincei* 27 (3), 455–462. doi:10.1007/s12210-015-0498-y

Pakravan, S., Akbarzadeh, A., Sajjadi, M. M., Hajimoradloo, A., and Noori, F. (2017). Partial and Total Replacement of Fish Meal by Marine Microalga Spirulina Platensis in the Diet of Pacific White Shrimp Litopenaeus Vannamei: Growth, Digestive Enzyme Activities, Fatty Acid Composition and Responses to Ammonia and Hypoxia Stress. *Aquac. Res.* 48, 5576–5586. doi:10.1111/are.13379

Pei, S., Guan, Y., and Ma, Y. (2009). Effects of Dietary Supplementation of Astaxanthin on Growth, Survival and Antioxidant Capacity of Pacific White Shrimp (*Litopenaeus Vannamei*). *Fish. Sci. (Dalian)* 28 (3), 126–129.

Petit, H., Nègre-Sadargues, G., Castillo, R., and Trilles, J.-P. (1997). The Effects of Dietary Astaxanthin on Growth and Moulting Cycle of Postlarval Stages of the Prawn, *Penaeus Japonicus* (Crustacea, Decapoda). *Comp. Biochem. Physiology Part A Physiology* 117 (4), 539–544. doi:10.1016/S0300-9629(96)00431-8

Qin, S., Liu, G.-X., and Hu, Z.-Y. (2008). The Accumulation and Metabolism of Astaxanthin in *Scenedesmus Obliquus* (Chlorophyceae). *Process Biochem.* 43 (8), 795–802. doi:10.1016/j.procbio.2008.03.010

Ranga Rao, A., Raghunath Reddy, R. L., Baskaran, V., Sarada, R., and Ravishankar, G. A. (2010). Characterization of Microalgal Carotenoids by Mass Spectrometry and Their Bioavailability and Antioxidant Properties Elucidated in Rat Model. *J. Agric. Food Chem.* 58 (15), 8553–8559. doi:10.1021/jf101187k

Rattanachai, A., Hirono, I., Ohira, T., Takahashi, Y., and Aoki, T. (2004). Cloning of Kuruma Prawn *Marsupenaeus japonicus* Crustin-like Peptide cDNA and Analysis of its Expression. *Fish. Sci.* 70 (5), 765–771. doi:10.1111/j.1444-2906.2004.00869.x

Ringø, E., Zhou, Z., Vecino, J. L. G., Wadsworth, S., Romero, J., Krogdahl, Å., et al. (2016). Effect of Dietary Components on the Gut Microbiota of Aquatic Animals. A Never-ending Story? *Aquacult Nutr.* 22 (2), 219–282. doi:10.1111/anu.12346

Scurria, A., Fabiano Tixier, A.-S., Lino, C., Pagliaro, M., D'Agostino, F., Avellone, G., et al. (2020). High Yields of Shrimp Oil Rich in Omega-3 and Natural Astaxanthin from Shrimp Waste. *ACS omega* 5 (28), 17500–17505. doi:10.1021/acsomega.0c01978

Seabra, L. M. A. J., and Pedrosa, L. F. C. (2010). Astaxanthin: Structural and Functional Aspects. *Rev. Nutr.* 23 (6), 1041–1050. doi:10.1590/s1415-52732010000600010

Sharawy, Z. Z., Abbas, E. M., Abdelkhalek, N. K., Ashry, O. A., Abd El-Fattah, L. S., El-Sawy, M. A., et al. (2022). Effect of Organic Carbon Source and Stocking Densities on Growth Indices, Water Microflora, and Immune-Related Genes

Expression of *Litopenaeus Vannamei* Larvae in Intensive Culture. *Aquaculture* 546, 737397–397. doi:10.1016/j.aquaculture.2021.737397

Sharawy, Z. Z., Ashour, M., Abbas, E., Ashry, O., Helal, M., Nazmi, H., et al. (2020). Effects of Dietary Marine Microalgae, *Tetraselmis Suecica* , on Production, Gene Expression, Protein Markers and Bacterial Count of Pacific White Shrimp *Litopenaeus Vannamei*. *Aquac. Res.* 51 (6), 2216–2228. doi:10.1111/are.14566

Söderhäll, K., and Cerenius, L. (1992). Crustacean Immunity. *Annu. Rev. Fish Dis.* 2, 3–23. doi:10.1016/0959-8030(92)90053-Z

Song, L., Yuan, J., Ni, S., Zhou, Y., Wang, X., Chen, Y., et al. (2020). Enhancement of Adaptive Immune Responses of Aged Mice by Dietary Intake of β-glucans, with Special Emphasis on Anti-aging Activity. *Mol. Immunol.* 117, 160–167. doi:10.1016/j.molimm.2019.10.019

Sritunyalucksana, K., and Söderhäll, K. (2000). The proPO and Clotting System in Crustaceans. *Aquaculture* 191 (1), 53–70. doi:10.1016/s0044-8486(00)00411-7

Subhra Bikash, K. M., Bhattacharyya, S. B., and Mitra, A. (2015). Seaweed Incorporated Diet Improves Astaxanthin Content of Shrimp Muscle Tissue. *J Mar. Sci Res DevResearch Dev.* 05 (2), 1. doi:10.4172/2155-9910.1000161

Tayag, C. M., Lin, Y.-C., Li, C.-C., Liou, C.-H., and Chen, J.-C. (2010). Administration of the Hot-Water Extract of *Spirulina Platensis* Enhanced the Immune Response of White Shrimp *Litopenaeus Vannamei* and its Resistance against *Vibrio Alginolyticus*. *Fish shellfish Immunol.* 28 (5-6), 764–773. doi:10.1016/j.fsi.2010.01.023

Teramukai, K., Kakui, S., Beppu, F., Hosokawa, M., and Miyashita, K. (2020). Effective Extraction of Carotenoids from Brown Seaweeds and Vegetable Leaves with Edible Oils. *Innovative Food Sci. Emerg. Technol.* 60, 102302. doi:10.1016/j.ifset.2020.102302

Tomas, A. L., Sganga, D. E., Marciano, A., and López Greco, L. S. (2020). Effect of Diets on Carotenoid Content, Body Coloration, Biochemical Composition and Spermatophore Quality in the "red Cherry" shrimpNeocaridina davidi(Caridea, Atyidae). *Aquacult Nutr.* 26 (4), 1198–1210. doi:10.1111/anu.13076

Wade, N. M., Gabaudan, J., and Glencross, B. D. (2017). A Review of Carotenoid Utilisation and Function in Crustacean Aquaculture. *Rev. Aquacult* 9 (2), 141–156. doi:10.1111/raq.12109

Wang, H., Dai, A., Liu, F., and Guan, Y. (2015). Effects of Dietary Astaxanthin on the Immune Response, Resistance to White Spot Syndrome Virus and Transcription of Antioxidant Enzyme Genes in Pacific White Shrimp, *Litopenaeus Vannamei*. *Iran. J. Fish. Sci.* 14 (3), 699–718. doi:10.22092/IJFS.2018.114476

Wang, W., Ishikawa, M., Koshio, S., Yokoyama, S., Dawood, M. A. O., Hossain, M. S., et al. (2019). Effects of Dietary Astaxanthin and Vitamin E and Their Interactions on the Growth Performance, Pigmentation, Digestive Enzyme Activity of Kuruma Shrimp (*Marsupenaeus japonicus*). *Aquac. Res.* 50 (4), 1186–1197. doi:10.1111/are.13993

Wang, Y.-C., Chang, P.-S., and Chen, H.-Y. (2008). Differential Time-Series Expression of Immune-Related Genes of Pacific White Shrimp Litopenaeus Vannamei in Response to Dietary Inclusion of β-1,3-glucan. *Fish Shellfish Immunol.* 24 (1), 113–121. doi:10.1016/j.fsi.2007.09.008

Wu, W., Wang, P.-H., Lee, D.-J., and Chang, J.-S. (2017). Global Optimization of Microalgae-To-Biodiesel Chains with Integrated Cogasification Combined Cycle Systems Based on Greenhouse Gas Emissions Reductions. *Appl. Energy* 197, 63–82. doi:10.1016/j.apenergy.2017.03.117

Xie, J., Bu, L., Jin, S., Wang, X., Zhao, Q., Zhou, S., et al. (2020). Outbreak of Vibriosis Caused by Vibrio Harveyi and Vibrio Alginolyticus in Farmed Seahorse *Hippocampus kuda* in China. *Aquaculture* 523, 735168. doi:10.1016/j.aquaculture.2020.735168

Yang, S.-P., Wu, Z.-H., and Jian, J.-C. (2013). Effect of Marine Red Yeast Rhodosporidium Paludigenum on Antioxidant-Related Gene Expression in *Litopenaeus Vannamei*. *Israeli J. Aquaculture-Bamidgeh* 65, 20665.

Yu, W., and Liu, J. (2020). Astaxanthin Isomers: Selective Distribution and Isomerization in Aquatic Animals. *Aquaculture* 520, 734915. doi:10.1016/j.aquaculture.2019.734915

Yu, W., Zhang, M., Wang, B., Xu, R., Pang, T., and Liu, J. (2021). Dietary Haematococcus pluvialis Powder Supplementation Affect Carotenoid Content, Astaxanthin Isomer, Antioxidant Capacity and Immune-related Gene Expression in Pacific White Shrimp, Litopenaeus Vannamei. *Aquac. Res.* 52 (6), 2403–2414. doi:10.1111/are.15090

Zaki, M. A., Ashour, M., Heneash, A. M. M., Mabrouk, M. M., Alprol, A. E., Khairy, H. M., et al. (2021). Potential Applications of Native Cyanobacterium Isolate (*Arthrospira Platensis* NIOF17/003) for Biodiesel Production and Utilization of its Byproduct in Marine Rotifer (*Brachionus plicatilis*) Production. *Sustainability* 13 (4), 1769. doi:10.3390/su13041769

Zar, J. (1984). *Biostatistical Analysis*. 2nd edn. Englewood Cliffs. NJ, USA: Prentice-Hall.

Zhang, C., Jin, Y., Yu, Y., Xiang, J., and Li, F. (2021). Effects of Natural Astaxanthin from Microalgae and Chemically Synthetic Astaxanthin Supplementation on Two Different Varieties of the Ridgetail White Prawn (*Exopalaemon Carinicauda*). *Algal Res.* 57, 102347. doi:10.1016/j.algal.2021.102347

Zhang, D. H., Ng, Y. K., and Phang, S. M. (1997). Composition and Accumulation of Secondary Carotenoids in *Chlorococcum* Sp. *J. Appl. Phycol.* 9 (2), 147–155. doi:10.1023/A:1007926528388

Zhang, J., Liu, L.-X., Tian, G.-Y., Xu, D.-H., Liang, G. Y., Yue, Y. R., et al. (2013). Effects of Dietary Astaxanthin on Growth, Antioxidant Capacity and Gene Expression in Pacific White shrimpLitopenaeus Vannamei*i*. *Aquacult Nutr.* 19 (6), 917–927. doi:10.1111/anu.12037

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: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Marine Science

ORIGINAL RESEARCH
published: 19 July 2022
doi: 10.3389/fmars.2022.895386

Comparative Effects of Microalgal Species on Growth, Feeding, and Metabolism of Pearl Oysters, *Pinctada fucata martensii* and *Pinctada maxima*

Bingcong Ye[1,2†], *Zhifeng Gu*[1,2†], *Xingzhi Zhang*[2,3], *Yi Yang*[1,2], *Aimin Wang*[1,2] *and Chunsheng Liu*[1,2*]

[1]State Key Laboratory of Marine Resource Utilization in South China Sea, Hainan University, Haikou, China, [2]College of Marine Science, Hainan University, Haikou, China, [3]Guangxi Key Laboratory of Aquatic Genetic Breeding and Healthy Aquaculture, Guangxi Academy of Fisheries Sciences, Nanning, China

Pinctada fucata martensii and *P. maxima* are two main traditional pearl oyster species that can produce seawater pearls. Our previous study showed a higher clearance rate (CR) and growth performance in *P. f. martensii* than in *P. maxima* fed with *Isochrysis galbana*. In this study, the *P. f. martensii* and *P. maxima* juveniles of two sizes (large and small) were fed with six different microalgae diets [*I. galbana* (*I*), *Platymonas subcordiformis* (*P*), *Chaetoceros muelleri* I, I+P, I+C, and P+C] to evaluate the differences in growth, feeding, and metabolism between two pearl oyster species. After 60 d of the rearing period, *P. f. martensii* and *P. maxima* fed with mixed microalgae showed a significantly higher relative growth rate (RGR) than those fed with single microalgae ($P< 0.05$). The RGRs were significantly higher in *P. f. martensii* than those in *P. maxima* fed with the same diets ($P< 0.05$). The RGRs showed a decreasing tendency with the growth in both pearl oyster species. The CRs of pearl oysters fed with mixed microalgae were significantly higher than those fed with single microalgae ($P< 0.05$), and the CRs of *P. f. martensii* were significantly higher than those of *P. maxima* fed with the same diets ($P< 0.05$). Significantly lower respiration rates (RRs) were observed in small-size *P. f. martensii* groups fed with I, P, and I+P diets and all large *P. f. martensii* groups compared to *P. maxima* fed with the same diets ($P< 0.05$). Higher activities of amylase, cellulase, lipase, and pepsin in *P. f. martensii* were observed compared to *P. maxima* fed with the same diets at two sizes. The pepsin activities in *P. maxima* decreased with the growth, while there were no consistent pepsin activities of *P. f. martensii* with the growth. The carbonic anhydrase activities in *P. maxima* were significantly higher than those in *P. f. martensii* fed with the same diets ($P< 0.05$). The carbonic anhydrase activities were highest in the I+C diet group, followed by C+P and I+P, I, C, and P groups. Significant differences were observed among different diet groups in the same pearl oyster species ($P< 0.05$). Our results suggest that the lower CR and activities of digestive enzymes and higher RRs and activities of carbonic anhydrase may cause a lower growth rate of *P. maxima* compared to *P. f. martensii*.

Keywords: pearl oyster, microalgae, growth performance, physiological energetics, digestive enzymes, carbonic anhydrase

OPEN ACCESS

Edited by:
Mohamed Salah Azaza, National Institute of Marine S cience and Technology, Tunisia

Reviewed by:
Walid Medhioub, Institut National des Sciences et Technologies de la Mer, Tunisia
Sabrine Boucetta, University of Annaba, Algeria

***Correspondence:**
Chunsheng Liu
lcs5113@163.com

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Aquatic Physiology, a section of the journal Frontiers in Marine Science

Received: *13 March 2022*
Accepted: *20 June 2022*
Published: *19 July 2022*

Citation:
Ye B, Gu Z, Zhang X, Yang Y, Wang A and Liu C (2022) Comparative Effects of Microalgal Species on Growth, Feeding, and Metabolism of Pearl Oysters, Pinctada fucata martensii and Pinctada maxima. Front. Mar. Sci. 9:895386. doi: 10.3389/fmars.2022.895386

INTRODUCTION

Pearl oysters are farmed throughout the Indo-Pacific region (Southgate and Lucas, 2008; McDougall et al., 2016; Gu et al., 2020; He et al., 2021). Of these, *Pinctada fucata martensii* and *P. maxima* are the two main farmed species used for producing nucleated round pearls (Du et al., 2017; Hao et al., 2018; Massault et al., 2021; Zhang et al., 2021). The maximum shell height of *P. f. martensii* is 60–70 mm, while that of *P. maxima* ranges from 200 to 250 mm (Yukihira et al., 1998; Yang et al., 2018). *P. maxima* is primarily cultured for the high-value production of large-scale pearls, and *P. f. martensii* is cultured for small-scale pearls (Hao et al., 2018; Zhang et al., 2021). Both species are commercially important oyster species in pearl industries (Southgate and Lucas, 2008).

In China, *P. f. martensii* and *P. maxima* are the traditional pear oyster species for seawater pearl fishing and are cultured in the same offshore areas, mainly in Guangdong, Guangxi, and Hainan provinces (Hao et al., 2018; Zhang et al., 2021). However, mortality was observed highly in *P. maxima* farming, especially at the growth stage of 3–6 cm in shell height than *P. f. martensii* (Hamzah et al., 2011; Liang et al., 2016). Until now, the causes of the high mortality rate of *P. maxima* are ascribed to diseases, environmental degradation, micro-algal composition, and so on (Pass et al., 1987; Taylor et al., 2004; Kvingedal et al., 2010; Crockford and Jones, 2012; Deng et al., 2013a and Deng et al., 2013b). In our previous study, a higher clearance rate (CR) and growth rate in *P. f. martensii* were observed compared to *P. maxima* fed with *Isochrysis galbana* (Yang et al., 2021; Zhang et al., 2021). We inferred that the lower CR and feed conversion ratio (FCR) to microalgae caused a low growth rate in *P. maxima*.

An understanding of the changes in food intake and energy budget within an organism can help study the different growth performances in *P. f. martensii* and *P. maxima*. Several studies have shown that the higher growth rates of oysters are attributed to higher feeding rates and lower metabolic costs (Tamayo et al., 2014; Hao et al., 2018; Hall et al., 2020). Zhang et al. (2021) reported that the fast-growing selective strain of *P. f. martensii* showed a significantly higher CR and lower oxygen consumption and ammonia excretion rates (ARs) than unselected groups. In Pacific oyster (*Crassostrea gigas*), higher energy gain rates coupled with lower metabolic costs of growth were reported in fast growers (Tamayo et al., 2014). Similarly, a relatively higher food-processing capacity in faster-growing mussels and a generally higher metabolic cost of feeding and growth in slower-growing individuals were observed in green-lipped mussel (*Perna canaliculus*) (Ibarrola et al., 2017). In addition, changes in the activities of digestive enzymes have been considered reliable indicators of food digestive efficiency and nutritional status in bivalves (Palais et al., 2012; Ibarrola et al., 2017; Zhang et al., 2021). The amylase, lipase, and pepsin activities of the fasting-growing strain of *P. f. martensii* were significantly higher than those of a normal cultured population (Zhang et al., 2021). In bivalves, carbonic anhydrase is an important indicator of biological mineralization, which shows a positive relationship with food intake (Medakovi´c, 2000; Cardoso et al., 2019; Zhao et al., 2020). In black-lip pearl oyster *P. margaritifera*, oysters fed with the highest food level had thicker aragonite tables and a higher mineralization rate (Linard et al., 2011).

In this study, the growth performance, physiological energetics, and activities of the digestive enzymes and carbonic anhydrase of *P. f. martensii* and *P. maxima* fed with different microalgae diets were detected to test whether the food intake and digestion influences the growth rate of the oyster species.

MATERIALS AND METHODS

Microalgal Diet Culture

The microalgae used in this study were *I. galbana* (*I*, 5.51 ± 0.22 μm in cell diameter), *Platymonas subcordiformis* (*P*, 9.93 ± 0.94 μm in cell diameter), and *Chaetoceros muelleri* (*C*, 4.5 ± 0.25 μm in cell diameter). All microalgae were obtained from the Algal Species Laboratory of Hainan University, Haikou, Hainan province, China. Microalgae were cultured in 50-L white plastic buckets using a semi-continuous batch culturing system and Ningbo University #3 improvement solution (Chen et al., 2021). Air with 2% CO_2 was bubbled into the buckets to support growth. The algae were grown using a continuous culture regime under a light/dark cycle of 12/12 h at 24 ± 1°C for 7–10 d; the cell densities of *I*, *P*, and *C* were ~6×10^6, ~2×10^5, and ~3×10^6 cells/ml, respectively.

Proximate Composition of Microalgae

For biochemical analysis, each microalgal species was grown in triplicate 10-L flasks. Microalgae were centrifuged (5,000 rpm, 30 min) when cultures reached the late logarithmic growth phase. The sediments were washed with 0.5 M of ammonium formate and recentrifuged at 5,000 rpm for 30 min. Then, the microalgae concentrates were lyophilized at −60°C in a vacuum freeze dryer (Scientz-10 N, Ningbo, China). Dried microalgae were ground into powder using a tissue lyser (Tissuelyser-48, Shanghai Jingxin Industrial Development Co., Ltd., Shanghai, China). The crude protein content was determined by the Kjeldahl method using a fully automated Kjeldahl nitrogen/protein analyzer (FOSS-Soxtec 2050, Hoganäs, Sweden) after acid digestion in a Tecator digestor 2020. Crude lipid was extracted with petroleum ether (Sinopharm Chemical Reagent Co. LTD, Shanghai, China) as described by Li et al. (2020), and crude ash was determined gravimetrically in a muffle furnace by incineration at 550°C for 24 h.

Pearl Oyster Collection

On May 1, 2020, two pearl oyster species, *P. f. martensii* and *P. maxima*, were selected to conduct mass breeding experiments at a shellfish hatchery in Beihai, Guangxi province, China. Larvae were reared according to the procedures described by Zhang et al. (2021). After 30 d, juveniles from each species were transferred to coastal areas with a raft culture in Beihai (21°26′~21°55′ N, 108°50′~109°47′ E), Guangxi province, China.

In October 2020 and June 2021, 5-month [15.87 ± 0.84 mm (0.06 ± 0.02-g dry soft tissue weight) and 14.57 ± 1.04 mm

(0.04 ± 0.01-g dry soft tissue weight) in shell height for *P. f. martensii* and *P. maxima*, respectively] and 13-month [45.46 ± 2.34 mm (0.23 ± 0.03-g dry soft tissue weight) and 44.57 ± 2.64 mm (0.12 ± 0.02-g dry soft tissue weight) in shell height for *P. f. martensii* and *P. maxima*, respectively] pearl oysters were collected, transported to the laboratory, acclimatized for 1 week before the feeding experiment, and then maintained at 26 ± 2°C in 80 cm × 40 cm × 50 cm tanks containing filtered running seawater (with a salinity of 30 ± 2 ppt) with recirculating water systems. During the acclimatization period, these pearl oysters were fed with a moderate amount of a superfluous mixture of microalgae (*I*+*P*+*C*) twice a day (7:00 and 17:00), and a half volume of seawater was daily replaced.

Experimental Design

Five-month and 13-month *P. maxima* and *P. f. martensii* were fed with six different microalgae diets (*I*, *P*, *C*, *I*+*P*, *I*+*C*, and *P*+*C*). The culture experiment was maintained indoors for 60 d. For 5-month *P. maxima* and *P. f. martensii*, 540 pearl oysters of each species were randomly divided into six groups and fed with six different diets (*I*, *P*, *C*, *I*+*P*, *I*+*C*, and *P*+*C*). Oysters fed with the same diets were divided into three parallel groups and cultured in three 160-L tanks. The densities of six microalgae diets were 30×10^4 cells·ml^{-1} for *I*, 10×10^4 cells/ml for *P*, 30×10^4 cells·ml^{-1} for *C*, $(15\times10^4 + 5\times10^4)$ cells·ml^{-1} for *I*+*P*, $(15\times10^4 + 15\times10^4)$ cells·ml^{-1} for *I*+*C*, and $(5\times10^4 + 15\times10^4)$ cells·ml^{-1} for *P*+*C*, respectively. Before feeding, the concentration of each cultured microalgae was measured with a hemocytometer (Marienfeld, Devan Instrument Co., LTD, Wuxi, China) under a microscope. For 13-month *P. f. martensii* and *P. maxima*, 270 pearl oysters of each species were randomly divided into six groups and cultured as 5-month oysters. The culture conditions were as follows: pearl oysters were fed twice a day (7:00 and 17:00) with different microalgae. Half-volume seawater was changed daily, and all the water was changed once a week. The water temperature, salinity, and dissolved oxygen were 26 ± 2°C, 30 ± 2 ppt, and ≥ 4 mg·ml^{-1}, respectively.

After 60-day rearing, pearl oysters were starved for 24 h before sampling. For each tank, the remaining pearl oysters were counted, and the shell height of each pearl oyster was measured. Four pearl oysters of each tank were randomly sampled for physiological energetic analysis. The remained individuals of each tank were sacrificed, and the mantle and visceral mass of each individual were sampled and stored at -80°C.

Growth Performance

The shell height of all pearl oysters was measured at the beginning and end of the experiment. The shell height was measured as the vertical distance from the umbo to the ventral edge.

The survival rate (SR) and relative growth rate (RGR) were evaluated as follows:

$$SR, \% = \left(\begin{array}{l}Number\ of\ living\ individuals\\ at\ the\ end\ /\ Number\ of\\ individuals\ at\ the\ initial\ stage\end{array}\right)\times 100\%.$$

$$RGR, \% = \left[\left(\begin{array}{l}Final\ shell\ height\\ -Initial\ shell\ height\end{array}\right)/Initial\ shell\ height\right]\times 100\%.$$

Physiological Energetic Analysis

After 60-d rearing, four pearl oysters per tank were sampled for CR, respiration rate (RR), and AR analyses. The species and concentrations of microalgae used in each group were the same as in the rearing experiment. After physiological energetic analysis, the soft tissues of pearl oysters were sampled and dried at 65°C for 24 h to obtain their dry tissue weight.

Clearance Rate

For detecting the CR in each group, three pearl oysters were randomly collected and placed in 1-L beakers. Each treatment was replicated thrice. The microalgae species and concentration in each group were the same as in the rearing experiment. One blank beaker with no pearl oyster was set as the control. After 3 h, the concentration of algae was calculated under a microscope using a hemocytometer. The CR (L·h^{-1}·g^{-1}) was calculated according to the method by Wang et al. (2015), and the equation is given as follows:

$$CR = V\times ln\left[\left(C_T - C_0\right)\times Sd\right]/\left(N\times T\times DW\right)$$

where V is the volume beaker, and $C_T - C_0$ is the microalgae concentrations at the initial and T time. Sd is the variable coefficient of microalgae concentrations in the control group. N is the number of pearl oysters in the beaker, and T is the elapsed time (h). DW is the dry weight of soft tissues of pearl oysters.

Respiration Rate (VO_2)

The RR detection was performed after 3-h feeding. Each treatment was replicated thrice. Three pearl oysters were incubated in sealed 800-ml cylindrical chambers containing seawater with oxygen probes (YSI®5730). The oxygen concentration was recorded at regular intervals (∼1 h) until it dropped below 30% of the initial value. One parallel chamber without a pearl oyster was used as a control. The RR (mg·h^{-1}·g^{-1}) was calculated following the equation

$$VO_2 = V\times(CO_0 - CO_T)/\left(T\times DW\right)$$

where V is the volume of the cylindrical chamber, CO_0 and CO_T are the oxygen concentrations of seawater at initial and T time, and DW is the dry weight of soft tissues of pearl oysters.

Ammonia Excretion Rate (VNH_4-N)

The AR detection was performed after 3-h feeding. Each treatment was replicated thrice. AR was determined by placing individual pearl oysters in closed chambers containing 250 ml of air-saturated seawater filtered using 0.22-μm Millipore membranes. One chamber without pearl oysters was used as a control. After 60 min, the water samples were taken from each chamber and stored at −20°C until analysis according to the

phenol-hypochlorite method described by Solorzano (1969). The AR ($mg{\cdot}h^{-1}g^{-1}$) was calculated following the equation

$$VNH_4 - N = V \times (VNH_4 - N_T - VNH_4 - N_0) / (T \times DW)$$

where V is the volume of the chamber, VNH_4-N_T and VNH_4-N_0 are the ammonia concentrations of seawater at initial and T time, and DW is the dry weight of soft tissues of pearl oysters.

Detection of Digestive Enzyme Activities

The frozen visceral mass of pearl oysters was weighed, thawed at 4°C, and homogenized (5,000 rpm, 10 min) with 0.9% precooled and sterilized normal saline at a ratio of 1:9 (tissue: saline). These samples were centrifuged at 2,500 rpm for 10 min, and the supernatant was collected in a 1.5-ml Eppendorf tube for digestive enzyme activity analysis. The activities of amylase (Amylase Assay Kit, iodine-starch colorimetry), cellulase [Cellulase (CL) Test Kit, colorimetry], lipase (Lipase Assay Kit, colorimetry), and pepsin (Pepsin Assay Kit, colorimetry) were determined using the respective kits following the manufacturer's instructions (Jiancheng Biological Engineering Institute, Nanjing, China).

Detection of Carbonic Anhydrase Activities

The frozen mantle of each sampled pearl oyster was weighed and treated as the visceral mass mentioned above. Carbonic anhydrase activities were measured using the classical phenol red method (Roy et al., 2012). The samples were read spectrophotometrically at 450 nm, and results were expressed in units per milligram of protein (U g^{-1} protein).

Statistical Analysis

Data were presented as mean ± standard deviation (SD). All statistical analyses were conducted using Data Processing System (DPS) statistical software. The comparison among the proximate composition of three microalgae was made using one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) test. The data of growth performance, physiological energetics, digestive enzyme, and carbonic anhydrase activities were analyzed by two-way analysis of variance (ANOVA), and means were subsequently separated by Tukey's test. A value of $P< 0.05$ was considered statistically significant.

RESULTS

Proximate Compositions in Three Microalgal Diets

As shown in **Table 1**, there were significant variations in the proximate compositions among three microalgae ($P< 0.05$). The contents of crude protein in the *I* and *C* groups were 51.06% and 50.01% of dry weight, respectively, which showed significantly higher than those in the *P* group ($P< 0.05$). Similarly, the highest lipid content was observed in the *I* (12.77%) group, followed by *C* (10.96%) and *P* (8.42%) groups, and significant differences were found in the *I* vs. *P* group ($P< 0.05$).

Survival Rate and Growth Performance of *P. f. martensii* and *P. maxima* Fed With Different Microalgae

As shown in **Table 2**, the SR and RGR of pearl oysters of two sizes were significantly affected by diet and species ($P< 0.05$). For small-size pearl oysters, the growth of shell heights (GHs) of *P. f. martensii* and *P. maxima* fed with different microalgae ranged from 2.32 mm to 10.04 mm and from 5.69 mm to 11.04 mm, respectively, and the highest GHs in both *P. f. martensii* and *P. maxima* were fed with the *I*+*C* group (**Figure 1**). The RGRs of *P. f. martensii* and *P. maxima* fed with mixed microalgae were significantly higher than those of the same pearl oyster species fed with single microalgae ($P< 0.05$). The RGRs of *P. f. martensii* were significantly higher than those of *P. maxima* fed with the same diets ($P< 0.05$) (**Table 2**). The SRs of *P. f. martensii* and *P. maxima* fed with *P* were 66.67% and 50.00%, respectively, which were significantly lower than other groups ($P< 0.05$) (**Table 2**).

For large-size pearl oysters, although the GH, RGR, and SR among different microalgae showed a similar tendency to the small-size pearl oysters' experiment, obvious decreases in GH and RGR were observed (**Table 2**, **Figure 1**). The SRs of *P. maxima* fed with *P* and *C* were 44.44% and 66.67%, which showed an obvious decrease compared to small-size *P. maxima* fed with the same diets (**Table 2**).

Clearance Rate, Respiration Rate, and Ammonia Excretion Rate

The CRs of *P. f. martensii* and *P. maxima* fed with different diets are shown in **Table 3**. For small-size pearl oysters, the CRs of *P. f. martensii* and *P. maxima* were 0.58–1.82 $L{\cdot}h^{-1}{\cdot}g^{-1}$ and 0.25–1.62 $L{\cdot}h^{-1}{\cdot}g^{-1}$, respectively. Compared to the six different microalgae diets for *P. f. martensii* and *P. maxima*, the CRs were the highest

TABLE 1 | Proximate compositions of microalgae (% dry weight).

	Crude protein	Crude lipid	Ash
Isochrysis galbana	51.06 ± 1.57^{a}	12.77 ± 0.94^{a}	13.83 ± 1.20^{a}
Platymonas subcordiformis	42.11 ± 2.26^{b}	8.42 ± 1.03^{b}	12.63 ± 0.49^{a}
Chaetoceros muelleri	50.01 ± 1.73^{a}	10.96 ± 1.15^{ab}	10.87 ± 0.51^{b}

Data are mean ± S.D. (n=3). Values in column with different lowercase alphabets indicate significant differences (P< 0.05).

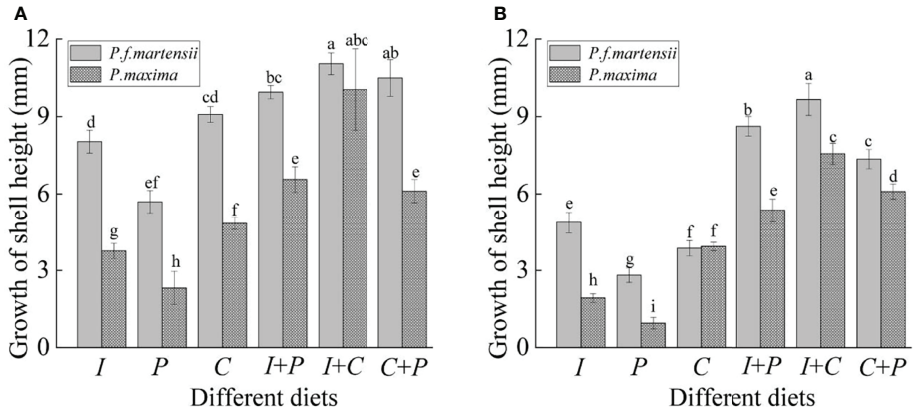


FIGURE 1 | Growth of shell height of *P. f. martensii* and *P. maxima* of different sizes [small **(A)** and large **(B)**] fed with different microalgae. Different letters represent significant differences ($P< 0.05$)

in *I*+*C* treatments (1.82 and 1.62 $L{\cdot}h^{-1}{\cdot}g^{-1}$, respectively). The CRs were significantly affected by pearl oyster species and diets ($P< 0.05$). The CRs of pearl oysters fed with mixed microalgae were significantly higher than those of pearl oysters fed with single microalgae ($P< 0.05$), and the CRs of *P. f. martensii* were significantly higher than those of *P. maxima* fed with the same diet ($P< 0.05$). The same tendency was also observed in large-size *P. f. martensii* and *P. maxima* groups.

The RRs of small-size *P. f. martensii* and *P. maxima* were 1.80–3.33 $mg{\cdot}h^{-1}g^{-1}$ and 2.46–4.76 $mg{\cdot}h^{-1}{\cdot}g^{-1}$, respectively, and were significantly affected by both pearl oyster species and diets ($P< 0.05$) (**Table 3**). The highest RRs were observed in the *P*-treated groups of *P. f. martensii* (3.65 $mg{\cdot}h^{-1}{\cdot}g^{-1}$) and *P. maxima* (4.76 $mg{\cdot}h^{-1}{\cdot}g^{-1}$), respectively, followed by the *I*+*P*-treated groups of these two pearl oyster species. In some microalgae-feeding groups (e.g., *I*, *P*, and *I*+*P*), significant differences in RRs were observed between *P. f. martensii* and *P. maxima* fed with the same diets ($P< 0.05$). For large-size pearl oysters, the RRs of *P. f. martensii* were significantly lower than those of *P. maxima* fed with the same diets (1.58–2.46 $mg{\cdot}h^{-1}{\cdot}g^{-1}$ and 2.14-3.14 $mg{\cdot}h^{-1}{\cdot}g^{-1}$ for *P. f. martensii* and *P. maxima*, respectively). Obvious decreases in RR were observed in both *P. f. martensii* and *P. maxima* than in small-size pearl oyster groups.

The ARs were not significantly affected by pearl oyster species in both small and large sizes ($P = 0.1741$ and 0.6448, respectively), while significant differences were observed among different diet groups ($P< 0.05$) (**Table 2**). In *P. f. martensii*, the ARs of small- and large-size oysters were

TABLE 2 | Growth performance of *P. f. martensii* and *P. maxima* of two sizes fed with different microalgae for 60 d.

		Treatments												**Two-way ANOVA (*P*-value)**		
		P. f. martensii						*P. maxima*						**Species**	**Diet**	**Diet*Species**
Group		*I*	*P*	*C*	*I*+*P*	*I*+*C*	*C*+*P*	*I*	*P*	*C*	*I*+*P*	*I*+*C*	*C*+*P*			
S	Initial SH (mm)	16.66 ± 0.67 ^{a}	15.83 ± 1.15^{a}	15.86 ± 0.85^{a}	14.83 ± 1.53^{a}	15.08 ± 1.54^{a}	15.62 ± 1.01^{a}	15.36 ± 2.61^{a}	14.43 ± 0.73^{a}	15.32 ± 1.08^{a}	14.69 ± 2.17^{a}	15.45 ± 2.15^{a}	14.56 ± 0.97^{a}	0.1876	0.7675	0.8913
	Final SH (mm)	24.69 ± 0.22^{a}	21.52 ± 1.32^{b}	24.94 ± 1.14^{a}	24.78 ± 1.42^{a}	26.12 ± 1.15^{a}	26.12 ± 0.91^{a}	19.12 ± 2.87^{bc}	16.75 ± 1.04^{c}	20.15 ± 1.20^{b}	21.25 ± 1.69^{b}	25.17 ± 3.31^{a}	20.67 ± 1.33^{b}	0.0000	0.0000	0.1918
	RGR	0.48 ± 0.05^{cd}	0.36 ± 0.03^{ef}	0.57 ± 0.02^{bc}	0.68 ± 0.08^{ab}	0.74 ± 0.11^{a}	0.67 ± 0.08^{ab}	0.25 ± 0.03^{gh}	0.16 ± 0.05^{h}	0.32 ± 0.02^{fg}	0.46 ± 0.11^{de}	0.65 ± 0.06^{ab}	0.42 ± 0.02^{def}	0.0000	0.0000	0.2159
	Survival rate (%)	81.11 ± 5.09^{ab}	66.67 ± 3.33^{c}	85.56 ± 5.09^{a}	85.56 ± 6.94^{a}	87.78 ± 6.94^{a}	85.56 ± 3.85^{a}	74.44 ± 6.94^{bc}	50.00 ± 8.82^{d}	74.44 ± 8.39^{bc}	78.89 ± 8.39^{ab}	87.78 ± 5.09^{a}	85.56 ± 5.09^{a}	0.0038	0.0000	0.2199
L	Initial SH (mm)	44.69 ± 4.68^{a}	43.71 ± 2.97^{a}	44.66 ± 2.98^{a}	45.46 ± 2.34^{a}	43.69 ± 2.20^{a}	45.03 ± 4.47^{a}	45.25 ± 2.73^{a}	45.81 ± 3.56^{a}	44.05 ± 1.67^{a}	45.47 ± 2.56^{a}	45.77 ± 3.42^{a}	44.42 ± 2.76^{a}	0.5809	0.9979	0.9299
	Final SH (mm)	49.56 ± 4.82^{abcd}	46.52 ± 2.89^{d}	48.52 ± 2.96^{bcd}	54.07 ± 2.73^{a}	53.33 ± 1.79^{ab}	52.36 ± 4.40^{abc}	47.18 ± 2.59^{cd}	46.76 ± 3.44^{d}	47.99 ± 1.79^{cd}	50.80 ± 2.85^{abcd}	53.30 ± 3.11^{ab}	50.48 ± 2.80^{abcd}	0.2218	0.0058	0.8855
	RGR	0.11 ± 0.01^{ef}	0.06 ± 0.01^{gh}	0.09 ± 0.01^{g}	0.19 ± 0.00^{b}	0.22 ± 0.02^{a}	0.16 ± 0.02^{c}	0.04 ± 0.01^{h}	0.02 ± 0.01^{i}	0.09 ± 0.00^{fg}	0.12 ± 0.01^{de}	0.17 ± 0.02^{c}	0.14 ± 0.01^{d}	0.0000	0.0000	0.0015
	Survival rate (%)	86.67 ± 6.67^{a}	68.89 ± 7.70^{bc}	82.22 ± 3.85^{a}	80.00 ± 6.67^{ab}	91.11 ± 3.85^{a}	82.22 ± 10.18^{a}	86.67 ± 6.67^{a}	44.44 ± 10.18^{d}	66.67 ± 6.67^{c}	86.67 ± 6.67^{a}	88.89 ± 3.85^{a}	84.44 ± 10.18^{a}	0.0313	0.0000	0.0088

Data are mean ± SD (n=3).

Different letters in the same column indicate significant difference (P< 0.05).

Initial SH, initial shell height; final SH, final shell height; RGR, relative growth rate.

TABLE 3 | Physiological determinations of *P. f. martensii* and *P. maxima* of two sizes fed with different microalgae.

		Treatments												Two-way ANOVA (*P*-value)		
		P. f. martensii						*P. maxima*						Species	Diet	Diet* Species
Group		*I*	*P*	*C*	*I+P*	*I+C*	*C+P*	*I*	*P*	*C*	*I+P*	*I+C*	*P+C*			
S	CR	1.46 ± 0.08^{c}	0.58 ± 0.11^{f}	1.52 ± 0.01^{c}	1.66 ± 0.01^{b}	1.82 ± 0.03^{a}	1.70 ± 0.02^{b}	0.76 ± 0.06^{ef}	0.25 ± 0.05^{g}	0.89 ± 0.01^{e}	1.25 ± 0.06^{d}	1.62 ± 0.05^{b}	1.14 ± 0.03^{d}	0.0001	0.0001	0.0000
	RR	1.80 ± 0.52^{e}	3.65 ± 0.09^{bc}	2.98 ± 0.12^{cd}	3.33 ± 0.19^{c}	3.06 ± 0.34^{cd}	3.23 ± 0.50^{c}	3.25 ± 0.46^{c}	4.76 ± 0.68^{a}	2.99 ± 0.43^{cd}	4.15 ± 0.60^{ab}	2.46 ± 0.35^{de}	3.68 ± 0.53^{bc}	0.0011	0.0000	0.0055
	AR	0.16 ± 0.05^{g}	0.38 ± 0.03^{bc}	0.34 ± 0.08^{bcd}	0.32 ± 0.07^{cde}	0.24 ± 0.02^{efg}	0.41 ± 0.11^{b}	0.39 ± 0.07^{bc}	0.55 ± 0.05^{a}	0.24 ± 0.02^{efg}	0.29 ± 0.02^{def}	0.21 ± 0.03^{fg}	0.33 ± 0.02^{bcd}	0.1747	0.0000	0.0000
L	CR	2.42 ± 0.03^{d}	1.75 ± 0.11^{ef}	2.07 ± 0.06^{e}	3.01 ± 0.11^{b}	3.60 ± 0.10^{a}	2.64 ± 0.05^{cd}	1.68 ± 0.04^{f}	1.00 ± 0.03^{g}	1.26 ± 0.10^{g}	2.47 ± 0.11^{d}	2.78 ± 0.12^{bc}	1.97 ± 0.07^{e}	0.0002	0.0001	0.0044
	RR	1.58 ± 0.32^{e}	2.26 ± 0.09^{c}	1.93 ± 0.17^{de}	1.91 ± 0.42^{de}	1.63 ± 0.12^{e}	2.25 ± 0.19^{cd}	2.14 ± 0.14^{cd}	2.73 ± 0.14^{b}	2.42 ± 0.10^{bc}	2.76 ± 0.12^{ab}	2.27 ± 0.11^{cd}	3.14 ± 0.43^{a}	0.0011	0.0001	0.0055
	AR	0.13 ± 0.02^{efg}	0.16 ± 0.01^{bcd}	0.13 ± 0.01^{efg}	0.19 ± 0.03^{ab}	0.11 ± 0.01^{g}	0.21 ± 0.01^{a}	0.16 ± 0.01^{cde}	0.21 ± 0.02^{a}	0.14 ± 0.03^{defg}	0.15 ± 0.02^{def}	0.12 ± 0.01^{fg}	0.18 ± 0.03^{abc}	0.6448	0.0000	0.0041

Data are mean ± SD (n=3).
Different letters in the same column indicate significant difference (P< 0.05).
CR, clearance rate; RR, respiration rate; AR, ammonia excretion rate.

0.16–0.41 mg·h^{-1}·g^{-1}and 0.11–0.21 mg·h^{-1}·g^{-1}, respectively. The ARs of *P. f. martensii* fed with *C+P* were the highest in both size groups. In *P. maxima*, the ARs of small- and large-size groups were 0.21–0.55 mg·h^{-1}·g^{-1} and 0.12–0.21 mg·h^{-1}·g^{-1}, respectively. The top three highest ARs of *P. maxima* of both sizes were fed with *P*, *I*, and *P+C*. Obvious decreases of ARs in both *P. f. martensii* and *P. maxima* were observed with the growth.

Digestive Enzyme Activities

The digestive enzyme activities of pearl oysters are shown in **Table 4**. The four digestive enzyme activities of pearl oysters were significantly affected by species and diets ($P< 0.05$). The four digestive enzyme activities in *P. f. martensii* and *P. maxima* fed with mixed microalgae almost showed an obvious increase compared to the same pearl oyster species fed with single microalgae. The amylase, cellulase, and pepsin activities in *P. f. martensii* and *P. maxima* fed with *P* were the lowest, and significant differences were observed when comparing *P*-treated pearl oysters with other diet groups ($P< 0.05$), except for the amylase activity of the *I* vs. *P* group in small-size *P. f. martensii*.

Obvious decreases in amylase activities in *P. f. martensii* and *P. maxima* were observed with their growth. In *P. maxima*, the lipase activities increased with the growth, while the opposite tendency of pepsin activities was observed. In *P. f. martensii*, no consistent lipase and pepsin activities were observed with their growth in six diet groups. Furthermore, higher activities of amylase, cellulase, lipase, and pepsin in *P. f. martensii* were observed compared to *P. maxima* fed with the same diets.

Carbonic Anhydrase Activities

As shown in **Figure 2**, the carbonic anhydrase activities in *P. maxima* were significantly higher than those in *P. f. martensii* fed with the same diets ($P< 0.05$). For small-size *P. f. martensii* and *P. maxima*, the carbonic anhydrase activities were highest in the *I+C* diet groups (0.31 and 0.36 U·g^{-1} prot for *P. f. martensii* and *P. maxima*, respectively), followed by *C+P* and *I+P*, *I*, *C*, and *P*, and significant differences were observed among different diet groups in the same pearl oyster species ($P< 0.05$). The same tendency of carbonic anhydrase activities was also observed in large-size *P. f. martensii* and *P. maxima* groups.

DISCUSSION

Microalgae are the main diet for bivalve mollusks in both wild and artificial culture conditions (Guedes and Malcata, 2012). Although two oyster species fed with six different diets showed significant increases in shell height, an obvious feeding preference for these two oyster species was observed in this study, which showed that mixed microalgae were better than the single ones, and the *I* or *C* group was better than the *P* group. Although many studies have reported suitable algae species in bivalve diets, a combination of flagellates and diatoms is better in an indoor culture (Galley et al., 2010; Hassan et al., 2021). Pacific oyster had better growth when fed with equal proportions of flagellates and diatoms compared to a high proportion of flagellates or diatoms (Rico-Villa et al., 2006). A mixed diet *(Isochrysis* sp. and *C. muelleri*) outperformed a monospecies diet in the blue mussel (*Mytilus edulis*) culture (Galley et al., 2010).

Bivalves have the ability to select their food in both natural and artificial conditions (Ward and Shumway, 2004). Many factors, such as cell size, cell surface, digestibility, toxic metabolite production, and gross biochemical composition, determine the suitability of microalgae as food for bivalves (Gouda et al., 2006; Rosa et al., 2017). Suitable particle sizes for *P. f. martensii* and *P. maxima* are 2.5–10 μm and >4 μm, respectively (Yukihira and Klumpp, 1999; Tomaru et al., 2002). Although the microalgae of all sizes used in this study could be easily ingested, small-size microalgae [*I. galbana* (*I*, ~5.51 μm) and *C. muelleri* (*C*, ~4.5 μm)] were more suitable for these two pearl oyster species

TABLE 4 | Digestive enzyme activities in intestinal tissues of *P. f. martensii* and *P. maxima* of two sizes fed with different microalgae.

		Treatments												**Two-way ANOVA (*P*-value)**		
		P. f. martensii						*P. maxima*						**Species**	**Diet**	**Diet* Species**
Group		*I*	*P*	*C*	*I+P*	*I+C*	*C+P*	*I*	*P*	*C*	*I+P*	*I+C*	*P+C*			
S	Amylase (U/mg prot)	0.80 ± 0.11^{cdef}	0.62 ± 0.06^{ef}	0.96 ± 0.10^{c}	0.81 ± 0.20^{cde}	1.57 ± 0.23^{a}	1.17 ± 0.15^{b}	0.61 ± 0.07^{f}	0.37 ± 0.07^{g}	0.68 ± 0.02^{ef}	0.79 ± 0.10^{cdef}	0.92 ± 0.11^{cd}	0.73 ± 0.05 ^{def}	0.0001	0.0001	0.0026
	Cellulase (U/mg prot)	1.84 ± 0.07^{e}	2.45 ± 0.10^{c}	1.97 ± 0.04^{de}	2.72 ± 0.15^{b}	2.40 ± 0.09^{c}	3.22 ± 0.07^{a}	1.32 ± 0.08^{g}	1.62 ± 0.05^{f}	1.43 ± 0.02^{g}	2.65 ± 0.12^{b}	2.04 ± 0.08^{d}	2.41 ± 0.10^{c}	0.0038	0.0080	0.0000
	Lipase (U/g prot)	6.71 ± 0.36^{bc}	3.31 ± 0.53^{e}	6.07 ± 1.18^{c}	6.83 ± 0.14^{bc}	7.92 ± 0.27^{a}	7.03 ± 0.16 ^{ab}	3.87 ± 0.54^{de}	1.97 ± 0.18^{f}	3.17 ± 0.18^{e}	4.26 ± 0.69^{d}	4.72 ± 0.85^{d}	4.55 ± 0.25^{d}	0.0002	0.0001	0.0976
	Pepsin (U/mg prot)	8.35 ± 0.54^{cd}	4.86 ± 0.64^{hi}	5.80 ± 0.51^{g}	8.84 ± 0.25^{c}	13.73 ± 0.16^{a}	10.66 ± 1.07^{b}	5.34 ± 1.04^{gh}	4.01 ± 0.74^{i}	6.78 ± 0.58^{fg}	7.15 ± 0.28^{ef}	8.12 ± 0.16^{cde}	7.34 ± 0.29 ^{def}	0.0004	0.000	0.0000
L	Amylase (U/mg prot)	0.51 ± 0.06^{de}	0.35 ± 0.04^{g}	0.52 ± 0.03^{d}	0.78 ± 0.07^{b}	1.03 ± 0.11^{a}	0.65 ± 0.05^{c}	0.43 ± 0.04^{ef}	0.22 ± 0.02^{h}	0.41 ± 0.04^{fg}	0.51 ± 0.01^{de}	0.56 ± 0.04^{cd}	0.48 ± 0.03^{def}	0.0008	0.0002	0.0001
	Cellulase (U/mg prot)	1.88 ± 0.06^{e}	2.55 ± 0.12$^{\mathbf{bc}}$	2.02 ± 0.04^{e}	3.09 ± 0.03^{a}	2.73 ± 0.09^{b}	3.25 ± 0.08^{a}	1.12 ± 0.07^{g}	1.66 ± 0.14^{f}	1.26 ± 0.04^{g}	2.61 ± 0.17^{b}	2.29 ± 0.16^{d}	2.41 ± 0.16^{cd}	0.0001	0.0001	0.0309
	Lipase (U/g prot)	6.41 ± 0.05^{e}	3.94 ± 0.08^{i}	5.66 ± 0.09^{g}	8.30 ± 0.14^{b}	9.68 ± 0.22^{a}	7.78 ± 0.18^{c}	5.72 ± 0.11^{g}	4.19 ± 0.06^{i}	4.58 ± 0.12^{h}	6.51 ± 0.02^{e}	7.23 ± 0.24^{d}	6.03 ± 0.09^{f}	0.0000	0.0000	0.0001
	Pepsin (U/mg prot)	7.68 ± 0.30^{d}	4.32 ± 0.12^{g}	6.02 ± 0.15^{e}	10.76 ± 0.22^{b}	13.64 ± 0.89^{a}	10.57 ± 0.27^{b}	5.08 ± 0.05^{f}	3.97 ± 0.48^{g}	5.43 ± 0.06^{f}	6.26 ± 0.14^{e}	7.83 ± 0.18^{d}	6.03 ± 0.05^{e}	0.0001	0.0000	0.0001

Data are mean ± SD (n=3).
Different letters in the same column indicate significant difference (P< 0.05).

compared to the large one [*P. subcordiformis* (*P*, ~9.93 μm)]. The nutritive quality of microalgae for bivalves is also determined by their biochemical composition (Martínez-Fernández et al., 2006). The biochemical composition of microalgae varies among species and according to culture conditions (Valenzuela-Espinoza et al., 2002; Martínez-Fernández et al., 2006). Protein is the largest organic component of microalgae. Lipid, especially polyunsaturated fatty acids, is also an essential nutrient. Previous studies showed that high dietary protein and lipid in microalgae were associated with a good growth of juvenile bivalves, such as blue mussel *M. edulis*, Pacific oyster *C. gigas*, Caribbean pearl oyster P. *aimbricata* (Leonardos and Lucas, 2000; Knuckey et al.,

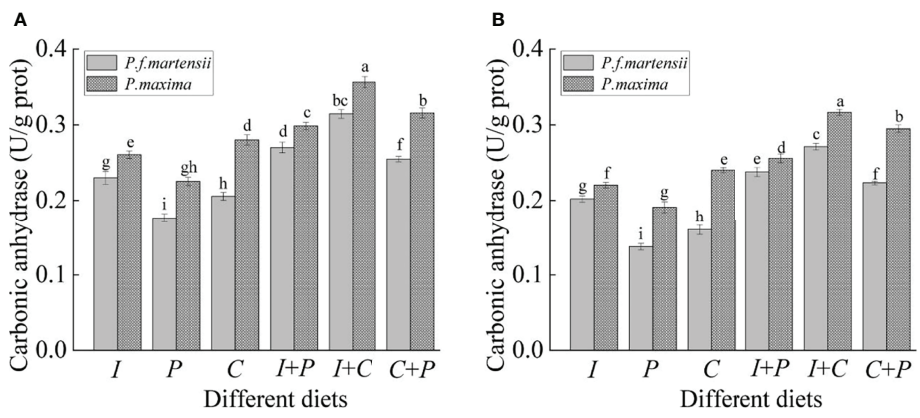


FIGURE 2 | Carbonic anhydrase activities of *P. f. martensii* and *P. maxima* of different sizes [small **(A)** and large **(B)**] fed with different microalgae. Means ± SD are presented (n = 6). Different letters represent significant differences (*P*< 0.05).

2002; Lodeiros et al., 2017). In our study, the crude protein and lipid in the *I* and *C* groups were higher than those in the *P* group, which indicated that more nutrients are obtained by pearl oysters from *I* and *C* treatments.

When comparing the differences in the growth rate and survival between *P. f. martensii* and *P. maxima*, significantly higher growth rates were observed in *P. f. martensii* at both two sizes compared to *P. maxima* fed with the same diets. Bivalve species have different strategies of feeding selection and energy budgets to adapt to different environments (Yukihira et al., 1998; Wang et al., 2000; Strohmeier et al., 2012). For example, Ibarrola et al. (2012) reported that the CR of mytilid mussel *M. chilensis* was the highest, followed by giant mussel *Choromytilus chorus* and ribbed mussel *Aulacomya ater*; the absorbed energy in *P. margaritifera* was significantly higher than that in *P. maxima* at 19°C, while the opposite results were observed at 28°C and 32°C (Yukihira et al., 2000). In our study, significantly higher CRs and lower RRs were observed in *P. f. martensii* than those in *P. maxima* fed with the same diets, although the ARs varied between these two oyster species. These results indicated more food intake and less energy consumption in *P. f. martensii* than in *P. maxima*. Furthermore, the activities of amylase, cellulase, lipase, and pepsin in *P. f. martensii* were also higher than those in *P. maxima*, which meant that *P. f. martensii* had a stronger digestive ability than *P. maxima*. Therefore, higher growth performances were detected in *P. f. martensii* than those in *P. maxima*.

The higher growth rates in *P. f. martensii* might be ascribed to the relatively higher adaptability to complex and volatile environments than those in *P. maxima*. *P. maxima* prefers to live in the oligotrophic tropical waters of coral reefs and atolls, while *P. f. martensii* prefers to live in coastal areas of higher latitudes and is more tolerant to extreme temperature and nutrient inputs (Yang et al., 2021; Zhang et al., 2021). The natural diets for pearl oysters were more complex than artificial diets. The higher SR in *P. f. martensii* was observed in coastal areas suspended on a raft compared to *P. maxima*, which showed the same tendency as our results (Hwang et al., 2007; Liang et al., 2016; Zhang et al., 2021). Therefore, the sensitivities to environmental changes at the juvenile stage are the main cause of the mortality of *P. maxima*.

The growth rate of bivalve species shows a decreasing tendency with their increased size, which is attributed to their energy budget changes at different life stages (Ibarrola et al., 2017; Hao et al., 2018; Liu et al., 2020). In our study, both the GH and the RGR of large-size *P. f. martensii* and *P. maxima* fed with the same microalgae significantly decreased than small-size groups. When comparing the energy budget parameters, the CRs of two pearl oyster species significantly increased, from 0.58–1.82 $L{\cdot}h^{-1}{\cdot}g^{-1}$ to 1.75–3.60 $L{\cdot}h^{-1}{\cdot}g^{-1}$ for *P. f. martensii* and from 0.25–1.62 $L{\cdot}h^{-1}{\cdot}g^{-1}$ to 1.00–2.78 $L{\cdot}h^{-1}{\cdot}g^{-1}$ for *P. maxima*, respectively. The CRs in *P. f. martensii* and *P. maxima* were almost the same as in the previous studies by Yukihira et al. (1998); Zhang et al. (2021), and Yang et al. (2021). Furthermore, the RRs and ARs of each pearl oyster species decreased with the increase in size, which was detected in other bivalve species, such as green-lipped mussel and pacific oyster (Tamayo et al., 2014; Ibarrola et al., 2017). The lower mass exponents for CR compared to RR and AR are the causes of the decrease in growth rate with the increase in body size (Rueda and Smaal, 2004; Ibarrola et al., 2017).

The feeding habits vary at different life stages in many marine organisms, and their digestive enzyme activities change because of their food preference (Kolkovski, 2001; Prudence et al., 2006; Ibarrola et al., 2012). In our study, the cellulase activities of *P. f. martensii* and *P. maxima* had no changes between the two sizes, while there was an obvious decrease in amylase activities in both two pearl oyster species, which indicated that carbohydrate requirements decreased with the increase in size in both *P. f. martensii* and *P. maxima*. Similar results have also been observed in *M. chilensis* of different sizes (Ibarrola et al., 2012). As for the lipase and pepsin activities, there were almost no changes in *P. f. martensii* with the increase in its size, while obvious increases for lipase and decreases for pepsin were observed in large groups of *P. maxima* compared to small-size groups. These obvious changes in lipase and pepsin activities indicated that the protein and lipid requirements of *P. maxima* changed dramatically with the size growth (Labarta et al., 2002; Zhang et al., 2021). However, the diets used in this study were unchanged; therefore, some microalgae groups, such as *P. subcordiformis* and *C. muelleri*, could not meet the nutritional requirements of large-size *P. maxima*, which led to a lower growth rate, even death.

The carbonic anhydrase activity changes markedly with different growth performances in the oyster mantle and is considered one of the important indicators of biological mineralization (Kawai, 1955; Ivanina et al., 2017). The carbonic anhydrase activity showed a similar tendency to the growth rate within the same pearl oyster species fed with different diets or at different life stages in this study. It is known that *P. maxima* has thicker shells than *P. f. martensii* (Gervis and Sims, 1992), which means that a higher proportion of total energy is used for the calcification of *P. maxima*. Significantly higher carbonic anhydrase activities were highly observed in *P. maxima* than those in *P. f. martensii*.

CONCLUSION

In conclusion, *P. f. martensii* and *P. maxima* fed with mixed microalgae showed higher growth and SR than those fed with single microalgae. Furthermore, the growth rate of these two pearl oyster species significantly decreased with the increased size. When comparing the growth performance between *P. f. martensii* and *P. maxima*, the RGR values in *P. f. martensii* were significantly higher than those in *P. maxima* fed with the same microalgae, which might be caused by the higher CR, lower RR values, and higher digestive enzyme activities in *P. f. martensii*.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by The Committee and Laboratory Animal Department of Hainan University.

AUTHOR CONTRIBUTIONS

BY performed the experiment and wrote the original draft. ZG worked with the software and supervision. XZ performed the experiment. YY wrote the paper. AW revised the paper. CL designed the experiment and wrote the paper. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported financially by the Key Research and Development Project of Hainan Province (ZDYF2021XDNY277, ZDYF2021SHFZ269) and the National Key Research and Development Program of China (2019YFD0901301, 2018YFD0900704).

REFERENCES

Cardoso, J. C., Ferreira, V., Zhang, X., Anjos, L., Félix, R. C. and Batista, F. M. (2019). Evolution and Diversity of Alpha-Carbonic Anhydrases in the Mantle of the Mediterranean Mussel (Mytilus Galloprovincialis). *Sci. Rep.* 9, 1–14. doi: 10.1038/s41598-019-46913-2

Chen, Q., Jiang, X. M., Han, Q. X., Sheng, P., Chai, Y. H., Peng, R. B., et al. (2021). Growth, Calcium Content, Proximate Composition, and Fatty Acid Composition of Triangle Sail Mussel (Hyriopsis Cumingii) Fed Five Different Microalgal Diets. *Aquaculture* 530, 735719. doi: 10.1016/j.aquaculture.2020.735719

Crockford, M. and Jones, B. (2012). *Aquatic Animal Health Subprogram: Investigation of Chlamydiales-Like Organisms in Pearl Oysters, Pinctada Maxima* (Government of Western Australian, Department of Fisheries). Perth.

Deng, Y., Fu, S., Liang, F., Du, X. and Xie, S. (2013a). Growth and Survival of Pearl Oyster Pinctada Maxima, Spat Reared Under Different Environmental Conditions. *J. Shellfish. Res.* 32 (3), 675–679. doi: 10.2983/035.032.0308

Deng, Y., Shao, F., Liang, F. and Xie, S. (2013b). Effects of Stocking Density, Diet, and Water Exchange on Growth and Survival of Pearl Oyster Pinctada Maxima Larvae. *Aquacult. Int.* 21, 1185–1194. doi: 10.1007/s10499-013-9622-0

Du, X. D., Fan, G. Y., Jiao, Y., Zhang, H., Guo, X. M., Huang, R. L., et al. (2017). The Pearl Oyster Pinctada Fucata Martensii Genome and Multi-Omic Analyses Provide Insights Into Biomineralization. *GigaScience.* 8, 1–12. doi: 10.1093/gigascience/gix059

Galley, T. H., Batista, F. M., Braithwaite, R., King, J. and Beaumont, A. R. (2010). Optimisation of Larval Culture of the Mussel Mytilus Edulis (L.). *Aquac. Int.* 18, 315–325. doi: 10.1007/s10499-009-9245-7

Gervis, M. H. and Sims, N. A. (1992). The Biology and Culture of Pearl Oysters (Bivalvia: Pteriidae). ICLARM Stud. Rev. Manila, Philippines. 49.

Gouda, R., Kenchington, E., Hatcher, B. and Vercaemer, B. (2006). Effects of Locally-Isolated Micro-Phytoplankton Diets on Growth and Survival of Sea Scallop (Placopecten Magellanicus) Larvae. *Aquaculture* 259 (1-4), 169–180. doi: 10.1016/j.aquaculture.2006.03.050

Guedes, A. C. and Malcata, F. X. (2012). Nutritional Value and Uses of Microalgae in Aquaculture. *Aquaculture* 10 (1516), 59–78. doi: 10.5772/30576

Gu, Z. F., Wei, H. J., Cheng, F., Wang, A. M. and Liu, C. S. (2020). Effects of Air Exposure Time and Temperature on Physiological Energetics and Oxidative Stress of Winged Pearl Oyster (Pteria Penguin). *Aquacult. Rep.* 17, 100384. doi: 10.1016/j.aqrep.2020.100384

Hall, S. A., Méthé, D., Stewart-Clark, S. E., Clark, K. F., and Tremblay, Fraser, T. (2020). Comparison of absorption efficiency and metabolic rate between wild and aquaculture oysters (Crassostrea virginica). *Aquacult. Rep.* 16, 100263. doi:10.1016/j.aqrep.2019.100263

Hamzah, M. S., Nababan, B. and Nababan, B. (2011). The Effect of Seasons and Depths on Growth and Survival Rate of Pearl Oyster (Pinctada Maxima) in Kodek Bay, North Lombok. *J. Ilmu. Teknol. Kelaut.* 3 (2), 48–61. doi: 10.29244/jitkt.v3i2.7821

Hao, R. J., Wang, Z. M., Yang, C. Y., Deng, Y. W., Zheng, Z., Wang, Q. H., et al. (2018). Metabolomic Responses of Juvenile Pearl Oyster Pinctada Maxima to Different Growth Performances. *Aquaculture* 491, 258–265. doi: 10.1016/j.aquaculture.2018.03.050

Hassan, M. M., Parks, V. and Laramore, S. (2021). Optimizing Microalgae Diets for Hard Clam, Mercenaria Mercenaria, Larvae Culture. *Aquacult. Rep.* 20, 100716. doi: 10.1016/j.aqrep.2021.100716

He, G., Liu, X., Xu, Y., Liang, J., Deng, Y., Zhang, Y., et al. (2021). Repeated Exposure to Simulated Marine Heatwaves Enhances the Thermal Tolerance in Pearl Oysters. *Aquat. Toxicol.* 239, 105959. doi: 10.1016/j.aquatox.2021.105959

Hwang, J. J., Yamakawa, T. and Aoki, I. (2007). Growth of Wild Pearl Oysters Pinctada Fucata, Pinctada Margaritifera and Pinctada Sugillata (Bivalvia: Pteriidae) in Taiwan. *Fish. Sci.* 73 (1), 132–141. doi: 10.1111/j.1444-2906.2007.01311.x

Ibarrola, I., Arambalza, U., Navarro, J. M., Urrutia, M. B., and Navarro, E. (2012). Allometric Relationships in Feeding and Digestion in the Chilean ytilids Mytilus chilensis (Hupé), Choromytilus chorus (Molina) and Aulacomya ater (Molina): A Comparative Study. *J. Exp. Mar. Biol. Ecol.* 426–427, 18–27. doi: 10.1016/j.jembe.2012.05.012

Ibarrola, I., Hilton, Z. and Ragg, N. L. (2017). Physiological Basis of Inter-Population, Inter-Familiar and Intra-Familiar Differences in Growth Rate in the Green-Lipped Mussel Perna Canaliculus. *Aquaculture* 479, 544–555. doi: 10.1016/j.aquaculture.2017.06.031

Ivanina, A. V., Falfushynska, H. I., Beniash, E., Piontkivska, H. and Sokolova, I. M. (2017). Biomineralization-Related Specialization of Hemocytes and Mantle Tissues of the Pacific Oyster Crassostrea Gigas. *J. Exp. Biol.* 220 (18), 3209–3221. doi: 10.1242/jeb.160861

Kawai, D. K. (1955). *Carbonic Anhydrase in Pearl Oyster-Ii. Changes of the Enzyme Activity in Relation to Growth and Seasons*, Publ. Seto Mar. Biol. Lab. 5, 89–94, Publications of the Seto Marine Biological Laboratory.

Knuckey, R. M., Brown, M. R., Barrett, S. M. and Hallegr, G. M. (2002). Effisolation of New Nanoplanktonic Diatom Strains and Their Evaluation as Diets for Juvenile Pacific Oysters (Crassostrea Gigas). *Aquaculture* 211 (1–4), 253–274. doi: 10.1016/S0044-8486(02)00010-8

Kolkovski, S. (2001). Digestive Enzymes in Fish Larvae and Juveniles—Implications and Applications to Formulated Diets. *Aquaculture* 200 (1-2), 181–201. doi: 10.1016/S0044-8486(01)00700-1

Kvingedal, R., Evans, B. S., Lind, C. E., Taylor, J. J., Dupont-Nivet, M. and Jerry, D. R. (2010). Population and Family Growth Response to Different Rearing Location, Heritability Estimates and Genotype×Environment Interaction in the Silver-Lip Pearl Oyster (Pinctada Maxima). *Aquaculture* 304 (1-4), 1–6. doi: 10.1016/j.aquaculture.2010.02.035

Labarta, U., Fernández-Reiriz, M. J., Navarro, J. M., and Velasco, A. (2002). Enzymatic Digestive Activity in Epifaunal (Mytilus Chilensis) and Infaunal (Mulinia Edulis) Bivalves in Response to Changes in Food Regimes in a Natural Environment. *Marine Biol.* 140, 669–676. doi: 10.1007/s00227-001-0742-0

Leonardos, N. and Lucas, I. A. N. (2000). The Nutritional Value of Algae Grown Under Different Culture Conditions for Mytilus Edulis L. Larvae. *Aquaculture* 182, 301–315. doi: 10.1016/S0044-8486(99)00269-0

Liang, F., Xie, S., Fu, S., Li, J. and Deng, Y. (2016). Growth Pattern and Biometric Relationship of Pearl Oyster Pinctada Maxima Cultured in Beibu Bay, China. *J. Appl. Aquacult.* 28 (2), 110–118. doi: 10.1080/10454438.2016.1172535

Li, C. L., Liu, E., Li, T. Y., Wang, A. M., Liu, C. S. and Gu, Z. F. (2020). Suitability of Three Seaweeds as Feed in Culturing Strawberry Conch Strombus Luhuanus. *Aquaculture* 519, 734–761. doi: 10.1016/j.aquaculture.2019.734761

Linard, C., Gueguen, Y., Moriceau, J., Soyez, C., Hui, B., Raoux, A., et al. (2011). Calcein Staining of Calcified Structures in Pearl Oyster Pinctada Margaritifera and the Effect of Food Resource Level on Shell Growth. *Aquaculture* 313, 149–155. doi: 10.1016/j.aquaculture.2011.01.008

Liu, C. S., Li, X. B., Wu, C. L., Wang, A. M. and Gu, Z. F. (2020). Effects of Three Light Intensities on the Survival, Growth Performance and Biochemical Composition of Two Size Giant Clams Tridacna Crocea in the Southern China Sea. *Aquaculture* 528, 735548. doi: 10.1016/j.aquaculture.2020.735548

Lodeiros, C. J., Freites, L., Márquez, A., Glem, M. E., Guevara, M. and Saucedo, P. E. (2017). Comparative Growth and Survival of Spat of the Caribbean Pearl Oyster, Pinctada Imbricata Cultivated Indoor With Microalgae Diets and Outdoor With Natural Diet. *Aquacult. Nutr.* 23 (3), 511–522. doi: 10.1111/anu.12419

Martínez-Fernández, E., Acosta-Salmón, H., and Southgate, P. C. (2006). The Nutritional Value of Seven Species of Tropical Microalgae for Black-Lip Pearl Oyster (Pinctada Margaritifera, L.) Larvae. *Aquaculture* 257, 491–503. doi:10.1016/j.aquaculture.2006.03.022

Massault, C., Jones, D. B., Zenger, K. R., Strugnell, J. M., Barnard, J. M. and Jerry, D. R. (2021). A SNP Parentage Assignment Panel for the Silver Lipped Pearl Oyster (Pinctada Maxima). *Aquacult. Rep.* 20, 100687. doi: 10.1016/j.aqrep.2021.100687

McDougall, C., Moase, P. and Degnan, B. M. (2016). Host and Donor Influence on Pearls Produced by the Silver-Lip Pearl Oyster, Pinctada Maxima. *Aquaculture* 450, 313–320. doi: 10.1016/j.aquaculture.2015.08.008

Medakovi´c, D. (2000). Carbonic Anhydrase Activity and Biomineralization Process in Embryos, Larvae and Adult Blue Mussels Mytilus Edulis L. *Helgol. Mar. Res.* 54, 1–6. doi: 10.1007/s101520050030

Palais, F., Dedourge, G. O., Beaudon, A., Pain, D. S., Trapp, J., Geffard, O., et al. (2012). One-Year Monitoring of Core Biomarker and Digestive Enzyme Responses in Transplanted Zebra Mussels (Dreissena Polymorpha). *Ecotoxicology* 21 (3), 888–905. doi: 10.1007/s10646-012-0851-1

Pass, D. A., Dybdahl, R. and Mannion, M. M. (1987). Investigations Into the Causes of Mortality of the Pearl Oyster, Pinctada Maxima (Jamson), in Western Australia. *Aquaculture* 65, 2, 149–169. doi: 10.1016/0044-8486(87)90259-6

Prudence, M., Moal, J., Boudry, P., Daniel, J. Y., Quere, C., Jeffroy, F., et al. (2006). An Amylase Gene Polymorphism Is Associated With Growth Differences in the Pacific Cupped Oyster Crassostrea Gigas. *Anim. Genet.* 37 (4), 348–351. doi: 10.1111/j.1365-2052.2006.01481.x

Rico-Villa, B., Le Coz, J. R., Mingant, C. and Robert, R. (2006). Influence of Phytoplankton Diet Mixtures on Microalgae Consumption, Larval Development and Settlement of the Pacific Oyster Crassostrea Gigas (Thunberg). *Aquaculture* 256, 377–388. doi: 10.1016/j.aquaculture.2006.02.015

Rosa, M., Ward, J. E., Holohan, B. A., Shumway, S. E. and Wikfors, G. H. (2017). Physicochemical Surface Properties of Microalgae and Their Combined Effects on Particle Selection by Suspension-Feeding Bivalve Molluscs. *J. Exp. Mar. Biol. Ecol.* 486, 59–68. doi: 10.1016/j.jembe.2016.09.007

Rueda, J. L. and Smaal, A. C. (2004). Variation of the Physiological Energetics of the Bivalve Spisula Subtruncata (Da Costa 1778) Within an Annual Cycle. *J. Exp. Mar. Biol. Ecol.* 301, 141–157. doi: 10.1016/j.jembe.2003.09.018

Solorzano, L. (1969). Determination of Ammonia in Natural Water by the Phenolhypochlorite Method. *Limnol. Oceanogr.* 14, 799–801. doi: 10.4319/lo.1969.14.5.0799

Southgate, P. and Lucas, J. (2008). *The Pearl Oyster* (Amsterdam: Elsevier Science).

Strohmeier, T., Strand, Ø., Alunno-Bruscia, M., Duinker, A. and Cranford, P. J. (2012). Variability in Particle Retention Efficiency by the Mussel Mytilus Edulis. *J. Exp. Mar. Biol. Ecol.* 412, 96–102. doi: 10.1016/j.jembe.2011.11.006

Tamayo, D., Ibarrola, I., Urrutxurtu, I. and Navarro, E. (2014). Physiological Basis of Extreme Growth Rate Differences in the Spat of Oyster (Crassostrea Gigas). *Mar. Biol.* 161, 1627–1637. doi: 10.1007/s00227-014-2447-1

Taylor, J. J., Southgate, P. C. and Rose, R. A. (2004). Effects of Salinity on Growth and Survival of Silver-Lip Pearl Oyster, Pinctada Maxima, Spat. *J. Shellfish. Res.* 23 (2), 375–378. doi: 61USC/12126121780002621

Tomaru, Y., Udaka, N., Kawabata, Z. I. and Nakano, S. I. (2002). Seasonal Change of Seston Size Distribution and Phytoplankton Composition in Bivalve Pearl Oyster Pinctada Fucata Martensii Culture Farm. *Hydrobiologia* 481, 181–185. doi: 10.1023/A:1021232832390

Valenzuela-Espinoza, E., Millan-Nunez, R. and Nunez-Cebrero, F. (2002). Protein Carbohydrate, Lipid and Chlorophyll a Content in Isochrysis Aff. Galbana (Clone T-Iso) Cultured With a Low Cost Alternative to the F/2 Medium. *Aquac. Eng.* 25, 207–216. doi: 10.1016/S0144-8609(01)00084-X

Wang, A., Deng, F., Zhang, X., Yan, B., and Zhang, M. (2000). RAPD Analysis on genetic diversity of Pinctada martensii Dunker. Wuhan University J. 46, 467−470.

Wang, Y. J., Li, L. S., Hu, M. H. and Lu, W. Q. (2015). Physiological Energetics of the Thick Shell Mussel Mytilus Coruscus Exposed to Seawater Acidification and Thermal Stress. *Sci. Total. Environ.* 514, 261–272. doi: 10.1016/j.scitotenv.2015.01.092

Ward, J. E. and Shumway, S. E. (2004). Separating the Grain From the Chaff: Particle Selection in Suspension- and Deposit-Feeding Bivalves. *J. Exp. Mar. Biol. Ecol.* 300, 83–130. doi: 10.1016/j.jembe.2004.03.002

Yang, S., Li, X., Vasquez, H. E., Wang, A., Shi, Y., Li, J., et al. (2021). Comparative Profiling of Survival, Growth and Intestinal Microbial Community of Pearl Oyster Pinctada Maxima Juvenile in the Industrial Farming: The Feasibility of Live Microalga Instead by Pray-Dried Microalgae Powder. *Front. Mar. Sci.* 8. doi: 10.3389/fmars.2021.800627

Yang, J. M., Luo, S. J., Li, J. H., Zheng, Z., Du, X. D. and Deng, Y. W. (2018). Transcriptome Analysis of Growth Heterosis in Pearl Oyster Pinctada Fucata Martensii. *FEBS. Open Bio.* 8, 1794–1803. doi: 10.1002/2211-5463.12502

Yukihira, H. and Klumpp, D. W. (1999). Feeding Adaptations of the Pearl Oysters, Pinctada Margaritifera and P. Maxima, to Variations in Natural Particulates. *Mar. Ecol. Prog. Series.* 182, 161–173. doi: 10.3354/meps182161

Yukihira, H., Klumpp, D. W. and Lucas, J. S. (1998). Comparative Effects of Microalgal Species and Food Concentration on Suspension Feeding and Energy Budgets of the Pearl Oysters Pinctada Margaritifera and P. Maxima (Bivalvia: Pteriidae). *Mar. Ecol. Prog. Ser.* 171, 71–84. doi: 10.3354/meps171071

Yukihira, H., Lucas, J. S. and Klumpp, D. W. (2000). Comparative Effects of Temperature on Suspension Feeding and Energy Budgets of the Pearl Oysters Pinctada Margaritifera and P. Maxima. *Mar. Ecol. Prog. Ser.* 195, 179–188. doi: 10.3354/meps195179

Zhang, X. Z., Ye, B. C., Gu, Z. F., Li, M., Yang, S. G., Wang, A. M., et al. (2021). Comparison in Growth, Feeding, and Metabolism Between a Fast-Growing Selective Strain and a Cultured Population of Pearl Oyster (Pinctada Fucata Martensii). *Front. Mar. Sci.* 8. doi: 10.3389/fmars.2021.770702

Zhao, L., Shirai, K., Tanaka, K., Milano, S., Higuchi, T., Murakami-Sugihara, N., et al. (2020). A Review of Transgenerational Effects of Ocean Acidification on Marine Bivalves and Their Implications for Sclerochronology. *Estuar. Coast. Shelf S.* 235, 106620. doi: 10.1016/j.ecss.2020.106620

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: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Marine Science

ORIGINAL RESEARCH
published: 22 July 2022
doi: 10.3389/fmars.2022.889778

A Screening Study on Effects Varying Dietary Macronutrient Composition on Gut Functions in Lumpfish *(Cyclopterus lumpus)*

Weiwen Zhou[1], Kristin Hamre[2], Elvis Chikwati[1], Katerina Kousoulaki[3], Ingrid Lein[4], Gerd Marit Berge[4], Øystein Sæle[2], Trond M. Kortner[1] and Åshild Krogdahl[1]*

[1]Department of Paraclinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences (NMBU), Ås, Norway, [2]Feed and Nutrition, Institute of Marine Research, Bergen, Norway, [3]Nofima AS, Department of Nutrition and Feed Technology, Bergen, Norway, [4]Nofima AS, Department of Aquaculture Production Technology, Sunndalsøra, Norway

OPEN ACCESS

Edited by:
Enric Gisbert,
Institute of Agrifood Research and Technology (IRTA), Spain

Reviewed by:
Chang'an Wang,
Chinese Academy of Fishery Sciences, China
Stefanie Colombo,
Dalhousie University, Canada

***Correspondence:**
Weiwen Zhou
weiwen.zhou@nmbu.no

Specialty section:
This article was submitted to Aquatic Physiology, a section of the journal Frontiers in Marine Science

Received: 04 March 2022
Accepted: 22 June 2022
Published: 22 July 2022

Citation:
Zhou W, Hamre K, Chikwati E, Kousoulaki K, Lein I, Berge GM, Sæle Ø, Kortner TM and Krogdahl Å (2022) A Screening Study on Effects Varying Dietary Macronutrient Composition on Gut Functions in Lumpfish (Cyclopterus lumpus). Front. Mar. Sci. 9:889778. doi: 10.3389/fmars.2022.889778

Cultivation of lumpfish (Cyclopterus lumpus) as lice cleaner fish for salmon is now expanding. For successful cultivation of a new species, understanding the basic biology of digestive functions is vital to facilitate and optimize diet formulation. This paper presents results from two experiments conducted to deepen our knowledge on lumpfish intestine physiology. Experiment 1 was a 42-day feeding trial in which lumpfish were fed twelve different diets in the following ranges of macronutrients: Protein 43-68%, lipid 4-17%, and carbohydrate 6-17%. Intestinal tissue, gut content and liver were sampled from 6 fish per tank. The results showed that with increasing lipid level and corresponding decrease in protein level, there was a linear decrease in several of the observed biomarkers, including activity of brush border membrane digestive enzymes, expression of genes related to nutrient digestion and transport, ion exchange, immune regulation, and cell remodeling. Increased intracellular accumulation of lipid (steatosis) was observed in gut and liver with increasing dietary lipid level. Fewer effects were observed for increased dietary carbohydrate and corresponding decreased protein level. Experiment 2 was a two-week feeding trial for estimation of macronutrient digestibility in which lumpfish were fed three diets, all containing 55% crude protein, with lipid to carbohydrate ratio of the low lipid diet of 7.5%/18.3%, the medium lipid diet of 13.8%/14.6%, and high lipid diet of 18.1%/9.5%. Fecal samples were collected as pooled samples per tank. These results showed that fatty acid digestibility's increased as dietary lipid level increased. Of note, starch digestibility decreased greatly as starch level increased, whereas protein digestibility did not change as lipid or starch level varied. Taken together, the present studies indicated that increasing lipid level in the diet with corresponding decrease in protein level affects digestion, absorption, and immune responses in the lumpfish intestine. Variation in dietary carbohydrate to protein level showed less effects, possibly due to low starch digestibility which makes the variation in available carbohydrates much less than the variation in the analysed level of dietary carbohydrates.

Keywords: cleaner fish, lumpfish, gut functions, macronutrient requirement, digestibility

1 INTRODUCTION

Cultivation of lumpfish (*Cyclopterus lumpus*) expanded rapidly during the past decade, i.e. approximate 34 million individual fish for delousing of Atlantic salmon were produced in Norway in 2020 (statistics by Norwegian Directorate of Fisheries, 2021), and in Scotland the number of lumpfish produced was 6.6 million (Munro, 2020). For successful cultivation of a new species detailed knowledge of several physiological aspects is necessary, particularly regarding digestive physiology and nutrient requirement. Powell et al. stated that one third of lumpfish die of starvation within a few weeks in salmon cages (Powell et al., 2018), indicating supplementary feed is necessary for lumpfish after deployment. This may be associated with its feeding preference. Lumpfish is an opportunistic, omnivorous feeder that not only feed on sea lice after deployment (Powell et al., 2018). Naturally, lumpfish seem to predominantly prey on crustacean, followed by molluscs and small fish (Ingolfsson and Kristjansson, 2002; Imsland et al., 2015). A diet of high protein content seems to be optimal in terms of its natural preference. Although plant ingredient tolerance has been tested for lumpfish (Willora et al., 2020; Willora et al., 2021), studies investigating nutrient requirement of lumpfish are rare. No relevant scientific information is available currently for the feed producers. Present commercial diets seem to be produced based on knowledge from other fish species as well as experience generated from inhouse, unpublished trials and commercial production of lumpfish.

Present knowledge of digestive physiology in lumpfish is very limited. Zhukova and Stroganov (Zhukova and Stroganov, 2021) described the anatomical structure of the alimentary tract in lumpfish, with focus on esophagus and the stomach. The oesophagus is reported to be relatively short with a thick muscular wall whose thickness may increase more than two folds during contraction. The stomach was observed with well-developed gastric glands and distinguishably divided into cardiac, fundus, and pyloric parts. Intestinal length was estimated to be 1.2 times of the standard body length, and the histological structure found the same throughout its entire length. Some studies on lumpfish employed histopathological evaluation of intestine as an indicator of inflammatory responses after changes in diet composition or feeding strategy (Imsland et al., 2018; Imsland et al., 2019a; Imsland et al., 2019b). The observed responses, however, were mild or none.

The gut is primarily an organ for digestion and absorption of nutrients. For fish, it also serves as an important organ for osmoregulation (Grosell, 2006; Gregorio and Fuentes, 2018). As the intestinal epithelial surface is constantly exposed to the elements of the external environment, it therefore must also provide defence, which herein refers to the physical barrier and immune response, against alien components and organisms (Zhu et al., 2013; Garcia-Hernandez et al., 2017). Studies in other fish species have clearly demonstrated how diet composition can influence nearly all aspects of the intestine, i.e., digestion and absorption (Krogdahl et al., 1999; Silva et al., 2010; Gu et al., 2014), osmoregulation (Taylor and Grosell, 2006), physical barrier (Hu et al., 2016) and immune regulation (Krogdahl et al., 2015). The present study was therefore conducted to strengthen knowledge of effects of variation in macronutrient diet composition on gut function and thereby to gain information necessary for successful cultivation of lumpfish.

The results presented herein are based on investigation of samples collected in two feeding experiments. The main part of the results stems from a feeding experiment (E1) conducted to estimate optimal balance between protein, lipid, and carbohydrates in diets for lumpfish of 15-50 g, based on observations of growth and feed conversion ratio, physiological characteristics of the intestine, mucosal and systemic immune responses. Results regarding growth performance, with more than three times increase in weight, body composition, welfare scores, and systemic immune responses are published in the paper of Hamre et al. (Hamre et al., 2022) with the following conclusion: The best growth and welfare were obtained in fish fed a diet with 55% protein, 17% lipid and 6% carbohydrates. However, to meet the wish from farmers of reducing growth, diets for lumpfish weighing from 10-50 g body weight should contain approximately 55% protein, minimum 10% lipid and maximum 10% carbohydrate. The work presented in the former paper also stated that the latter composition would be suitable also for lumpfish of 1.7-10g.

The present study comprises results regarding characteristics of the intestinal tract based on samples from E1. However, as the E1 experiment did not give sufficient fecal sample material for estimation of nutrient digestibility, an additional experiment (E2) was conducted addressing specifically macronutrient digestibility, the results of which are also included in the present paper. In addition to the macronutrient digestibility analyses, the lumpfish intestine was characterized in detail by histomorphological evaluations, digestive enzyme activity and bile acid assays, as well as by expression profiling of genes involved in nutrient digestion and transport, ion exchange, immune regulation, and cell remodeling. Liver structure and function was also evaluated to provide information on nutrient turnover and integrated metabolism.

2 MATERIALS AND METHODS

The feeding trials were conducted in accordance with Norwegian laws and regulations concerning experiments with live animals. Experiments were overseen by the Norwegian Food Safety Authority.

2.1 Experimental Fish and Feed

2.1.1 Fish and Feed for Experiment 1

This experiment evaluated effects of diet composition on digestive functions in lumpfish with an initial body weight of 15 g growing to 50 g. The experiment was conducted based on a three-component mixture design (Cornell, 2011), set up with Design Expert ver. 8.0.4. (Stat-Ease Inc. MN, USA). As it is a special type of response surface methodology for experimental design, for which replicates are not necessary (Hamre et al., 2003; Hamre and Mangor-Jensen, 2006; Hamre et al., 2013), we set one tank for most diets. In total, twelve different diets were

formulated and fed to fish in one tank for each one of 11 diets. The 12th diet was fed to fish in 3 tanks to obtain a measure of tank variation. The experimental diets were produced at the Aquafeed Technology Centre of Nofima in Bergen, Norway, in the same production series, using a Wenger TX-52 co-rotating twin-screw extruder with 150 kg/h capacity. The dietary oil was added in the different feed mixes prior to extrusion. The settings of the extruder were "normal"; i.e., the production can be upscaled to a feed factory (extruder settings considered: screw configuration (D), die opening (1.5 mm), knife speed (2671-3108 rpm), feed rate (110-150 kg/h), at the DDC the amount of added steam was 10-12 kg/h, and water 0.16-0.18 kg/min, and in the extruder there was added 0.24-0.45 kg/min water and no steam. The ingoing temperature of the feed mass in the extruder was 79-84°C and the outgoing 103-118°C. The produced pellets were air-dried in a carousel dryer (Model 200.2, Paul Klöckner GmbH, Nisteral, Germany) at 85°C for 10-12 min to a final moisture level between 6.36 and 8.26%. The size of the dried pellets was approx.: 1:5 – 1:7 × approx.: 2:3mm with bulk density between 443 and 582 g/L. Diet recipes and proximate composition are shown in **Table 1**, and **Figure 1** illustrates the dietary design of experiment E1. The following ranges of macronutrients were covered: Protein 43-68%, lipid 4-17%, and carbohydrate 6-17%. For most observed biomarkers, samples from all treatments were investigated. For some, due to resource restrictions, it was possible to investigate only selected macronutrient combinations.

Details of the experimental conditions are published in (Hamre et al., 2022). In brief, the fish were produced by a commercial hatchery and transported to Nofima's research facility at Sunndalsøra, Norway. Fourteen 150 L conical tanks were used, each with 90 fish. Dead fish were removed daily, counted, and weighed. Feed was distributed continuously (15 sec feeding every 5 minutes) using small belt automatic feeders above each tank. The fish were fed to satiation, and the feed rations increased from 30 to 68 g/tank in tanks according to appetite. The fish were fed 1-1.6mm pellets until 20 g size and 1.6-2.3mm until the end of the experimental period. Due to the small pellet sizes, feed intake could not be recorded by our system. The temperature was set to a mean of 9.8°C (min 8.9°C, max 10.6°C). The water flow was set to 4 l/min and oxygen was adjusted to 80-100% by adding oxygen to the water holding tank when needed. Temperature was recorded daily, and oxygen was measured and adjusted 2-3 times per week. As the trial was run in a flow-through system, ammonia nitrogen and nitrate nitrogen were not recorded.

TABLE 1 | Diet formulation and proximate composition for experiment 1 (E1).

Diet no.	1	2	3	4	5	6	7	8	9	10	11	12
Basic mix:												
Fish meal[1]	24.5	24.5	24.5	24.5	24.5	24.5	24.5	24.5	24.5	24.5	24.5	24.5
Krill hydrolysate[2]	3	3	3	3	3	3	3	3	3	3	3	3
Vitamin mix[3]	2.2435	2.2435	2.2435	2.2435	2.2435	2.2435	2.2435	2.2435	2.2435	2.2435	2.2435	2.2435
Krill oil[4]	1	1	1	1	1	1	1	1	1	1	1	1
Mineral mix[5]	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84
Lys[6]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Choline chloride[7]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Cholesterol[8]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Aquate[9]	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Biomoss[9]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Taurine[10]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Carop. Pink[11]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Yttrium oxide[12]	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Varying ingredients:												
Cod muscle meal[13]	30.5	26.1	23.3	28.4	24	21.2	23.4	19.1	16.3	20	15.6	12.9
Fish oil[1]	0.05	7.9	12.85	0.1	7.95	12.9	0.35	8.15	13	0.5	8.25	13.1
Wheat gluten[14]	30.5	26.1	23.33	28.38	23.98	21.2	23.43	19.08	16.3	19.95	15.58	12.85
Wheat meal[15]	1	1.5	1.8	5.1	5.6	5.9	14.5	15.05	15.4	21.15	21.7	22.05
NaH_2PO_4[16]	4.2	4.65	4.95	4.3	4.75	5.05	4.55	4.9	5.25	4.7	5.15	5.4
Analysed composition %												
Crude protein	68	62	58	65	58	55	59	52	48	54	48	45
Total lipid	4	11.8	16.4	4.1	11.9	16.9	4.2	12	16.5	4.5	12.3	17.3
Carbohydrates	5.9	6	5.8	8.3	8.2	8.1	13.7	13.6	13.7	16.9	17.5	17.7
Ash	11.6	11.2	11.2	11.2	11.4	11.4	11.3	11.1	11.2	11.2	11.1	10.9
Choline	0.503	–	0.473	–	0.483	–	–	–	–	–	–	0.446
Dry matter	92	93	93	92	92	92	92	92	92	91	92	93

Values are in %. [1]Norsildmel, Egersund, Norway; [2]Approx. 60% dry matter, Olympic, Herøy, Norway; [3]Provides in the final feed 3000 IU vit D3, 410 mg/kg vit E, 20 mg/kg vit K3, 700 mg/kg vit C, 20 mg/kg B1, 30 mg/kg vit B2, 25 mg/kg B6, 0.05 mg/kg vit 12, 60 mg/kg pantothenic acid, 10 mg/kg folic acid, 200 mg/kg niacin, and 1 mg/kg biotin; [4]Aker Biomarine, Oslo, Norway; [5]Provides in the final diet 50 mg/kg Mn, 750 mg/kg Mg, 150 mg/kg Fe, 120 mg/kg Zn, 10 mg/kg Cu, 800 mg/kg Na, 0.2 mg/kg Se and 0.2 mg/kg Co; [6]L-lysine, delivered by Vilomix, Hønefoss, Norway; [7]Choline chloride, delivered by Vilomix, Hønefoss, Norway; [8]Cholesterol, Carbogen Amics B.V., Veenendaal, The Netherlands; [9]Aquate and Biomos, Alltech Norway AS, Førde. Norway; [10]Taurine, VWR, Oslo, Norway; [11]Taurine, VWR, Oslo, Norway; [12]Yttrium oxide, Y2O3 (99.9%), delivered by Vilomix, Hønefoss, Norway; [13]Seagarden, Karmøy, Norway; [14]Wheat gluten, Amytex 100, Tereos Syral, Aalst, Belgium; [15]Wheat meal, Norgesmøllene AS, Bergen, Norway; [16]MSP (26% P), Mono sodiumphosphate, delivered by Vilomix, Hønefoss, Norway.

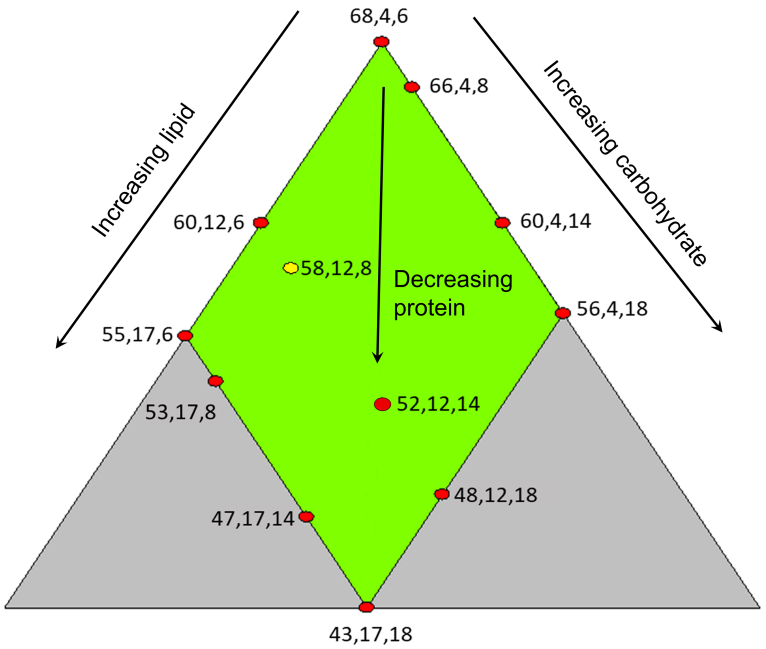


FIGURE 1 | Illustration of feed design. Each feed is indicated with a red dot and numbers beside the dot presenting macronutrient composition in the following sequence, from left to right, level of crude protein, crude lipid and total carbohydrates. The yellow dot represents diet was fed to fish in three tanks.

2.1.2 Fish and Feed for Experiment 2

An additional experiment, E2, was conducted to gain knowledge on macronutrient digestibility. Starch digestibility was of particular interest as great variations have been observed between other species (Krogdahl et al., 2004). Feed formulations are shown in **Table 2**. The diets were formulated to contain 55% crude protein with low (Diet 1), medium (Diet 2) and high (Diet 3) levels of lipid, and with carbohydrate level of contrary variation to lipid level. The same ingredients were used for all three diets, and the different lipid and carbohydrate levels were balanced by differing the dietary inclusion levels of lipid and wheat meal, respectively. The diets were produced at Aquafeed Technology Centre of Nofima in Bergen, Norway. Each of the three diets were fed to fish in triplicate tanks for a period of 14 days.

Lumpfish with an average body weight of around 100 g were distributed to 9 tanks, 100 individuals per tank, providing a biomass of around 10 kg in each tank. The tanks were cylindrical, with flat bottom and black inner surface, and an efficient water volume of 350 L. All tanks were equipped with automatic belt feeders for distribution of feed, and a separate light source mounted on each tank. Inlet water was run through a 10 μm filter and UV treated. Water flow was set at 12 L/min and oxygen saturation was kept within 80-100%. Average water temperature was 11.6°C (10.9-12.4°C).

2.2 Sampling

2.2.1 Sampling for E1

The trial was terminated after 42 days. At termination of the experiment, 6 fish from each tank were sampled at random, weighed, and intestinal tissues collected for analyses. The intestine was cleaned of mesenteric fat and divided into three segments, i.e., pyloric intestine including the pyloric caeca (PI), mid intestine (MI) and distal intestine (DI) (See **Supplementary Figure S1**). Each segment was opened longitudinally. Limited by fish size, gut content was only collected from MI, where the amount was enough for analysis. The collected gut content of MI was snap frozen in liquid nitrogen and stored at -80°C before further processing and analysis of activity of digestive enzymes and concentration of bile salts. Samples of tissue from all sections, located in the middle of the sections were collected for RNA extraction (submerged in RNAlater solution, incubated at 4°C for 24 h and stored at -20°C) and histomorphological evaluation (fixed in 4% phosphate-buffered formaldehyde solution for 24 h and transferred to 70% ethanol for storage). The remaining tissue was collected for brush border digestive enzyme assessment, snap frozen in liquid nitrogen and stored at -80°C.

2.2.2 Sampling for E2

The trial was terminated after 14 days, when fecal samples were collected as pooled samples per tank. Because of the very short length of DI of lumpfish, it cannot be stripped for feces like salmonids, therefore fish were euthanized by an overdose of anesthetic (Tricaine mesylate, MS-222) and the total content of the DI was collected by dissecting the intestine.

2.3 Analytical Procedures

2.3.1 Activity of Digestive Enzymes and Bile Salt Concentration

The intestinal tissue samples were thawed and homogenized (1:20 w/v) in ice-cold 2 mM Tris/50 mM mannitol, pH 7.1, containing

TABLE 2 | Diet formulation and proximate composition for experiment 2 (E2), g/kg.

	1	2	3
Ingredients:			
Fish meal[1]	366.2	362.9	387.6
Wheat gluten[2]	183.1	181.5	193.8
Wheat meal[3]	188	125	40
Fish oil[4]	5	73	120.9
Codfish powder[5]	100	100	100
Krill hydrolysate[6]	20	20	20
Krill meal[7]	40	40	40
Krill oil[8]	10	10	10
Vitamin mix[9]	30	30	30
MSP[10]	24	24	24
Mineral mix[11]	8.4	8.4	8.4
Biomos[12]	4	4	4
Cholesterol[13]	5	5	5
Choline cloride[14]	5	5	5
Lysine[15]	6	6	6
Taurine[16]	2	2	2
Stay-C[17]	2.2	2.2	2.2
Carphyll pink[18]	1	1	1
Yttrium oxide[19]	0.1	0.1	0.1
Analysed content, %:			
Dry Matter	89.4	92	93.2
Lipid	7.5	13.8	18.1
Carbohydrates	18.3	14.6	9.5
Starch	16	11.7	6.6
Protein	55.3	55.3	57.3
Ash	8.2	8.3	8.4
Choline	0.521	0.516	0.54
Energy, MJ/kg	19.9	20.9	22.2

[1] Fishmeal, Norse-LT, Vedda AS, Langevåg, Norway,; [2] Wheat gluten, Amytex 100, Tereos Syral, Aalst, Belgium [3] Wheat meal, Norgesmøllene AS, Bergen, Norway; [4] Fish Oil, NorSalmOil, Pelagia, Egersund, Norway; [5] Codfish powder, Seagarden AS, Avaldsnes, Norway; [6] Krill hydrolysat, Rimfrost AS, Ålesund, Norway; [7] Krill meal, Rimfrost AS, Ålesund, Norway; [8] Krill oil, Aker BioMarine, Lysaker, Norway; [9] Vitamin premix, Nofima vitamin premix, Vilomix, Hønefoss, Norway [10] MSP (26% P), Mono sodiumphosphate, delivered by Vilomix, Hønefoss, Norway [11] Mineral premix, Nofima mineral premix, Vilomix, Hønefoss, Norway [12] Biomos, Alltech Norway AS, Førde. Norway; [13] Cholesterol, Carbogen Amics B.V., Veenendaal, The Netherlands [14] Choline chloride, delivered by Vilomix, Hønefoss, Norway [15] L-lysine, delivered by Vilomix, Hønefoss, Norway; [16] Taurine, VWR, Oslo, Norway;[17] Stay-C 35%, delivered by Vilomix, Hønefoss, Norway; [18] Carophyll pink (10% astaxanthin), delivered by Vilomix, Hønefoss, Norway [19] Yttrium oxide, Y_2O_3 (99.9%), delivered by Vilomix, Hønefoss, Norway.

phenyl-methyl-sulphonyl fluoride (P-7626, Sigma, Norway) as serine protease inhibitor. The homogenates were sonicated, aliquoted and stored at -80°C until analysis.

Leucine aminopeptidase (LAP) and maltase were the brush border digestive enzymes assessed. The activity of LAP was measured employing the Sigma procedure no. 251 (Krogdahl et al., 2003). L-leucyl-b-naphthylamide is used as the substrate and is reacted with diluted homogenates at 37 °C for 1 h. The reaction is terminated by HCl. A subsequent color reaction is done by adding sodium nitrite, ammonium sulfate and *N*-(1-Naphthyl)ethylenediamine dihydrochloride in order at room temperature. A standard curve is made for calculation by measuring serial diluted 2-Naphthylamine. The color absorbance is read at 580nm. To measure maltase activity, the method described by Dahlqvist (Dahlqvist, 1970) was applied. Maltose is used as substrate and is reacted with diluted homogenates at 37 °C for 1 h. The reaction is terminated and colorized by TGO solution (mixture of Trizma buffer, detergent, o-dianisidine and peroxidase). A standard curve is made for calculation by measuring serial glucose dilution. The color absorbance is read at 480nm.

Activity of trypsin, amylase and lipase, and total bile salt level were measured in pooled freeze-dried content from MI. Trypsin activity was determined colorimetrically (Kakade et al., 1973). Benzoylarginine p-nitroanilide (Sigma no. B-4875, Sigma Chemical Co., St. Louis, MO) is used as substrate and is reacted to freeze-dried content solution at 37 °C for 10 min. The reaction is terminated by 30% acetic acid. The color absorbance is read at 410nm. Absorbance variance between sample and blank is defined as enzyme unit instead of international enzyme unit. Lipase activity was determined as described in (Brockman, 1981). 4-Nitrophenyl myristate (Sigma 70124, Sigma Chemical Co., St. Louis, MO) is used as substrate and the reaction temperature is 37 °C. The absorbance is read at 405nm every 30 sec for 7 times. The enzyme unit is defined as absorbance variance per minute. Amylase was determined as described by (Froystad et al., 2006), using a Randox amylase assay kit (AY3805, Randox Laboratories Ltd., Crumlin, UK). The kit employs Ethylidene PNPG7 method (Kruse-Jarres et al., 1989) for α-amylase measurement. Ethylidene-blocked p-nitrophenyl-maltoheptaoside is the substrate in the enzyme assay reagent and reaction temperature is 25°C. Absorbance is read at 405nm after 20, 30 and 40 min of reaction and the enzyme unit is defined as absorbance variance per minute. Bile salt level was determined using the enzyme cycling amplification/Thio – NAD method (Inverness Medical, Cheshire, UK) in the ADVIA®1650 Chemistry System (Siemens Healthcare Diagnostics Inc.) at the Central Laboratory of the Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Oslo. The assay measures total 3αOH of cholic acid, whereas the reported results indicate the corresponding level of bile salt as taurocholate.

2.3.2 Quantitative Real-Time PCR

Total RNA was extracted from tissue samples of PI, MI, DI, and liver. (~20 mg) of all fish using Trizol reagent (PureLink™ RNA Mini Kit, Thermo Fisher Scientific). RNA was purified by an on-column DNase kit (PureLink™ DNase Set, Thermo Fisher Scientific) according to the manufacturer's protocol. RNA purity and concentration were measured using the Epoch Microplate Spectrophotometer (BioTeK Instruments, Winooski, USA). The RNA integrity was verified by the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) in combination with RNA Nano Chip (Agilent Technologies, Santa Clara, CA, USA). First-strand complementary DNA (cDNA) was synthesized using 1.0 µg RNA from two fish of the same tank, namely 0.5 µg RNA of each two fish were combined as a unit of RNA sample for cDNA synthesis. The reaction volume was 20 µl, including 4 µl mastermix of from the kit SuperScript™ IV VILO™ Master Mix (Thermo Fisher Scientific). Negative controls were performed in parallel by omitting RNA or enzyme.

Lumpfish mRNA sequences were derived from NCBI database. The selected genes include elongation factor 1-alpha (*ef1a*), beta-actin (*bactin*), hypoxanthine-guanine phosphoribosyltransferase

1 (*hprt1*), tubulin beta chain (*tubb*), sucrase-isomaltase (*si*), solute carrier family 27 member 4 (*slc27a4*), solute carrier family 15 member 1 (*slc15a1*), niemann-pick C1-like 1 (*npc1l1*), solute carrier family 12 member 1 (*slc12a1*), tight junction protein 1a (*tjp1a1*), occludin (*occludin*), cyclooxygenase-2 (*cox2*), immunogobulin M (*igm*), inhibitor of nuclear factor kappa B kinase subunit beta (*ikbkb*), complement component 5 (*c5*), chemokine (C-X-C motif) ligand 19 (*cxcl19*), tumor necrosis factor alpha (*tnfa*), nuclear factor kappa-light-chain-enhancer of activated B cells (*nfkb*), transcription factor p65 (*rela*), major histocompatibility complex II (*MHCII*), matrix metallopeptidase 13 (*mmp13*) and proliferating cell nuclear antigen (*pcna*). The selected genes cover the functions of disaccharide digestion (*si*), nutrient transport *(slc27a4*, *slc15a1*, *npc1l1*), ion-exchange (*slc12a1*), tight junction forming (*tjp1a1*, *occludin*), immune regulation (*cox2*, *igm*, *ikbkb*, *c5*, *cxcl19*, *tnfa*, *nfkb*, *rela*, *MHCII*, *mmp13*) and cell proliferation (*pcna*). Reference gene candidates include *ef1a*, *tubb*, *hprt1* and *bactin*.

Primer information are shown in **Supplementary Table S2**. The qPCR primers were designed using the Primer-BLAST tool of NCBI. All primer pairs were first used in gradient reactions to determine optimal annealing temperatures. To confirm amplification specificity, the PCR products from each primer pair were subjected to melting curve analysis and visual inspection of the PCR products by agarose gel electrophoresis. PCR efficiency for each gene assay was determined using 2-fold serial dilutions of randomly pooled complementary DNA.

Expression of target genes were analyzed using the LightCycler 96 (Roche Diagnostics, Basel, Switzerland) with a 10-µl DNA amplification reaction. Each 10-µl DNA amplification reaction contained 2 µl PCR grade water, 2 µl of 1:10 diluted cDNA template, 5 µl LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics) and 0.5 µl (10 µM) of each forward and reverse primer. Each sample was assayed in duplicate, including a no-template control. The three-step qPCR run included an enzyme activation step at 95°C (5 min), 40 cycles at 95°C (10 s), annealing temperature (10 s), and 72°C (15 s) and a melting curve step. The mean normalized expression of the target genes was calculated from raw Cq values (Muller et al., 2002). Reference genes selected was done in terms of its stability among different fish (Kortner et al., 2011). The chosen reference genes are: *ef1a*, *tubb* and *hprt1* for PI and liver, *ef1a*, *bactin* and *hprt1* for MI and *tubb*, *bactin* and *hprt1* for DI.

2.3.3 Histological Evaluation

The samples fixed for histological evaluation from all intestinal segments collected, i.e., PI, MI, and DI, were processed using standard histological methods and stained with hematoxylin and eosin (H&E). The histological sections were estimated to describe the general structure as well as assessing for any morphological changes in the intestinal mucosa such as inflammation. Since the relative weights (OSI) of intestinal sections and liver seemed to vary more specifically to dietary lipid content, histological examination was conducted for the six sampled fish fed the following four diets spanning from low to high lipid content: i.e., 6, 21, 28, and 38 g lipid/100g protein (Diet 1, 5, 3 and 12, respectively). The evaluated morphological characteristics included changes in cellularity of submucosa and lamina propria, enterocyte supranuclear vacuolization and numbers of intra-epithelial lymphocytes. The degree of changes was graded as normal, mild, moderate, marked and severe, and was scored from 0 to 4, respectively.

2.3.4 Macronutrients and Yttrium in Feed and Feces

Samples of the feed and feces were analyzed at the Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, Ås, Norway. Dry matter was determined by oven drying at 105°C, 16–18 h, to constant weight. Nitrogen was determined using the Kjeldahl method (Kjeltech Auto Analyser, Tecator, Höganäs, Sweden) (crude protein: N×6.25), fatty acid composition by FAME analysis (O'Fallon et al., 2007) in a Trace GC Ultra with auto injector (Thermo Scientific), and starch was measured as glucose after hydrolysis by α-amylase and amyloglucosidase, followed by glucose determination by the 'Glut-DH method'. Yttrium was analyzed by dissolution of ashed samples with hydrochloric acid and nitric acid by heating, then dissolved in 5% nitric acid. Yttrium was then detected with an ICP-AEF, Optima 3000 V (Perkin Elmer, USA).

2.4 Calculations and Statistics

Organosomatic indices of the intestinal sections = tissue weight/body weight × 100.

Apparent digestibility coefficient (referred to as digestibility in the text) =

$100 - 100 \times (\%\ \text{nutrient in feces}/\%\ \text{nutrient in diet}) \times (\%\ Y_2O_3\ \text{in diet}/\%\ Y_2O_3\ \text{in feces})$

Models describing the effects of macronutrient composition on the different responses were calculated using the software Design Expert ver. 8.0.4. (Stat-Ease Inc. MN, USA). Different models were fitted to the data and the recommended model with the best fit was chosen. When no model had a significant fit to the response data, only mean and standard deviation of the total dataset are given. Models and coefficients were considered significant at $p < 0.05$. Models where $0.05 < p < 0.1$ were considered a trend and presented.

Differences in histological scores for the various evaluated morphological characteristics were analyzed using ordinal logistic regression. When score differences were only 2 levels, statistical significance was assessed using the Fisher exact test. *Post hoc* analysis for significant test results was conducted using the chi-square test (chisq.post.hoc function of Fifer package in R). Differences were considered significant at $p < 0.05$.

3 RESULTS

3.1 Digestive Enzyme Activities in Gut Tissues and Digesta, and Nutrient Digestibility

As the present study is among the very first studies investigating the physiology of the intestinal tract of lumpfish, some summary statements regarding the general characteristics are given: The average OSI of the three intestinal sections, PI, MI, and DI, was 2.1, 1.6, and 0.6% of body weight and they comprised 49,

38, and 13% of the intestinal weight, respectively. Of total LAP capacity, i.e., units per kg fish, 34, 50, and 16% were observed in PI, MI, and DI, respectively, and of maltase capacity, 48, 40, and 12%. Digestive enzyme activities in chyme, results only available from MI, were as follows: trypsin activity averaged 25 U/mg DM, amylase 2 U/mg DM, and lipase 0.27 U/mg DM. Bile salt concentration in chyme from MI was 49 mg/g DM. Average digestibility, as measured in collected feces, was for protein: 83%, sum of fatty acids: 95%, and starch: 64%.

3.2 Effects of Macronutrient Composition on Intestinal and Liver Functions

In the following content we have chosen to present the results and comments as follows: For results showing linear relationship to the macronutrient levels, effects of decreasing protein level are presented first, in light of possible effects of protein deficiency at low dietary levels. Thereafter effects of increasing level of lipid are presented and finally effects of increasing level of carbohydrates. For results showing more complicated relationships, the comments are presented as found most suitable for the outcome of the data evaluation. Linear relationships were found for most of the observed variables, but for those explicitly mentioned in the text showing no relationship with diet macronutrient composition or following a model other than a linear one. To check model parameters, one can refer to **Tables 3**, **4**, and **Supplementary Tables S3**, **S4** listing the coefficients derived from the model equation.

3.2.1 Organosomatic Indices

Variation of diet composition significantly affected the intestinal weight of PI and MI (**Figure 2** and **Table 3**). In PI, OSI increased with decreasing protein level, increased with increasing dietary lipid level, and had no response with carbohydrate variation. In MI, OSI increased with decreasing protein level, increased with increasing dietary lipid level, and increased with increasing carbohydrate level.

3.2.2 Brush Border Digestive Enzyme Activities

Specific activity of both LAP and maltase was highest in MI, lowest in the PI, and intermediate in DI. Also, the value for LAP capacity, i.e., per kg of fish, was highest in the MI, whereas for maltase, the highest value was observed for PI. The DI showed the lowest capacity values for both LAP and maltase.

In PI, specific activity of LAP and maltase showed significant relationships with diet macronutrient composition ($p = 0.032$ and 0.034, **Table 3** and **Figure 3**). The specific LAP and maltase activities (U/mg protein) decreased with decreasing protein level, decreased with increasing dietary lipid level and, to lesser extent, increased with increasing carbohydrate level. On the other hand, the enzyme capacity (U/kg fish) of LAP and maltase ($p = 0.090$ and $p > 0.10$, **Table 3** and **Figure 3**) showed no significant correlations with diet macronutrient composition in this section.

In MI, both specific activity and capacity of LAP and maltase showed clear relationships with diet composition ($p = 0.005$, 0.016, 0.001 and 0.002, **Table 3** and **Figure 3**), i.e., a decrease with decreasing protein level, a decrease with increasing dietary lipid level, and an increase with increasing carbohydrate level.

In DI, there were no significant effects for either specific activity or capacity and the analysis by software cannot illustrate a clear trend neither ($p = 0.080$, 0.1, 0.060 and 0.070, **Table 3**).

3.2.3 Enzyme Activities and Bile Salt Level in MI Digesta

Lipase activity in digesta from MI showed a significant, linear, relationship with dietary protein level, decreasing with decreasing protein level ($p = 0.020$, **Table 3** and **Figure 3**). Lipase activity decreased with increasing lipid level as well as with increasing carbohydrate level. The trypsin and amylase activities in digesta from MI did not show significant relationships ($p > 0.10$, **Table 3**) with diet macronutrient composition. Bile salt concentration showed a tendency towards a similar response pattern as for the lipase ($p = 0.077$, **Table 3** and **Figure 3**), i.e., decrease with decreasing protein, increase with increasing lipid and carbohydrate level.

3.2.4 Gene Expression in Intestinal Tissue

In PI, *si* and *slc15a1*, coding for a disaccharidase and a peptide transporter, respectively, were the two genes showing significant responses to diet macronutrient composition ($p = 0.039$ and 0.050, respectively, **Table 4** and **Figure 4**). Their relationships

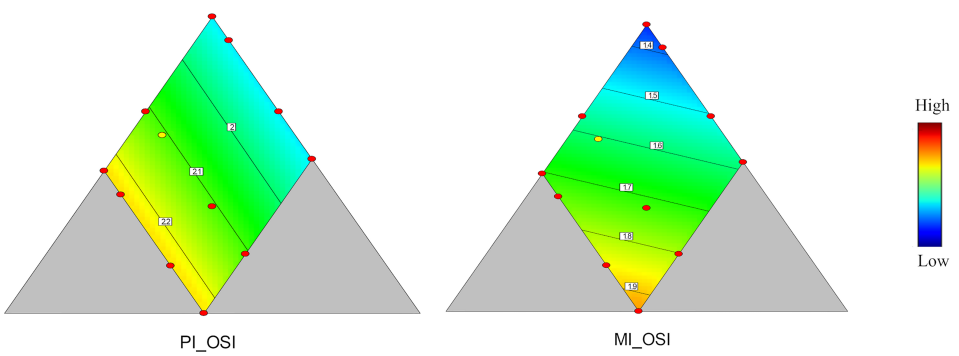


FIGURE 2 | Organosomatic indices of PI and MI. Color scale from blue to red indicates value changes from low to high. The yellow dot represents diet was fed to three tanks. Macronutrient composition of each dot can refer to **Figure 1**.

TABLE 3 | Result summary: Organ indices and digestive enzymes.

Response	Minimum	Maximum	Mean	Std. Dev.	Ratio	Model	P model
PI_OSI	1.76	2.37	2.09	0.16	1.3	Linear	**0.004**
MI_OSI	1.32	2.04	1.64	0.20	1.5	Linear	**0.002**
DI_OSI	0.41	0.69	0.57	0.07	1.7	Mean	
PI_LAPprot	35	79	52	13	2.2	Linear	**0.032**
PI_LAPkg	31	65	44	9	2.1	Linear	0.090
PI_MALTprot	8.7	17	11	3	2.0	Linear	**0.034**
PI_MALTkg	5.8	14	9.3	1.9	2.4	Mean	
MI_LAPprot	88	188	131	35	2.1	Linear	**0.005**
MI_LAPkg	51	80	66	10	1.5	Linear	**0.016**
MI_MALTprot	9.9	27	16	5	2.7	Linear	**0.001**
MI_MALTkg	5.8	11	7.8	1.51	1.9	Linear	**0.002**
DI_LAPprot	61	182	115	37	3.0	Linear	0.080
DI_LAPkg	11	32	21	6	2.8	Linear	0.1
DI_MALTprot	7.1	21	12	4	2.9	Linear	0.060
DI_MALTkg	1.2	3.6	2.3	0.7	2.9	Linear	0.070
MI_Lipase	0.067	0.42	0.27	0.11	6.3	Linear	**0.020**
MI_Bile salt conc.	32	68	49	10	2.1	Linear	0.077
MI_Amylase	0.5	6	2.0	1.5	11	Mean	
MI_Trypsin	13	36	25	7	2.7	Mean	

Data series minimum and maximum values, means and standard deviations, the ratio between max and min, the model that fitted the data best and probability that the data distribution is random (different from mean). The names of responses comprise intestinal sections and biomarkers tested. PI, proximal intestine. MI, mid intestine. DI, distal intestine. OSI, organosomatic index (%). LAPprot, LAP specific activity (U/mg protein). LAPkg, LAP capacity (U/kg fish). MALTprot, maltase specific activity (U/mg protein). MALTkg, maltase capacity (U/kg fish). Lipase, lipase activity (U/mg dry matter). Bile salt conc, bile salt concentration (mg/g dry matter). Amylase, amylase activity (U/mg dry matter). Trypsin, trypsin activity (U/mg dry matter). Bold value indicates significance (p model < 0.05).

with diet composition were similar, i.e., decreasing in value with decreasing protein content, decreasing with increasing lipid level and increasing, although only slightly, with increasing carbohydrate. Expression of *rela*, *igm* and *cxcl19*, all involved in immune functions, tended to vary with diet composition ($p = 0.080$, 0.073 and 0.094, respectively, **Table 4** and **Figure 4**). The trend observed for expression of *rela* was a decrease with decreasing protein level, decrease with increasing lipid level and increase with carbohydrate level, while *igm* expression decreased with decreasing protein level, decreased with increasing lipid level, but increased slightly with increasing carbohydrate level. The trend of *cxcl19* expression fitted a quadratic model, where the minimum was at medium protein, high lipid, and low carbohydrate level, and the maximum was at medium protein and lipid level, and high carbohydrate.

In MI, more genes responded significantly to variation in diet composition. As in PI, significant response was observed for *si* and *slc15a*, and the pattern of responses was quite similar, with a decrease with decreasing dietary protein, a decrease with increasing lipid and an increase with dietary carbohydrate level ($p = 0.026$ and 0.001, **Table 4** and **Figure 5**). Also, *slc12a1*, the gene involved in ion exchange expression decreased with decreasing protein level, decreased with increasing lipid ($p = 0.031$, **Table 4** and **Figure 5**) and increased with increasing carbohydrate level. Regarding genes involved in immune functions, linear effects of diet composition were seen for *igm and mmp13*, and quadratic responses were seen for *rela* and *cxcl19* (for p values see **Table 4** and **Figure 5**). For the former four, expression decreased with decreasing protein level, most pronounced for *mmp13*, decreased with increasing lipid level as well as with increasing carbohydrate level.

For *rela*, which fitted a quadratic model, the maximum expression was found near the center of the design, i.e., at medium levels of all the three macronutrients. Two low values were seen, i.e., at medium protein, high lipid, and low carbohydrates, and at low protein, high lipid, and high carbohydrates. The highest value for *cxcl19* was observed at the highest protein level, at which both lipid and carbohydrates were low. Lowest value was found at medium protein and lipid level and high carbohydrate level. For some genes showing fewer clear relationships with diet composition, trends ($0.05 < p < 0.10$) were indicated, i.e., for *npc1l1*, *cox2*, and *pcna* (for p values see **Table 4**). The relationship of expression of *npc1l1*, the cholesterol transporter, showed decreased level with decreasing protein level, decreased level with increasing lipid level and increased level with increasing carbohydrate level. The expression *cox2* and the cell proliferating related gene *pcna* fitted quadratic models. The maximum expression of *cox2* was at high protein, low lipid, and low carbohydrate level, with a lower peak at low protein level, high lipid, and high carbohydrate level. The minimum was shown at medium protein level, medium lipid, and high carbohydrate level. Maximum expression of *pcna* was at medium lipid and protein level and low carbohydrate level.

In DI, only expression of the tight junction related gene *occludin* and the immune related gene *igm* were significantly influenced by diet composition ($p = 0.003$ and 0.004 respectively, **Table 4**). Expression of *igm* was best explained by a quadratic model (**Figure 6**), with two maxima, one at medium protein, high lipid, and low carbohydrate level, and the other at medium protein level, low lipid, and high carbohydrate level. The minimum was observed at low protein, high lipid, and high carbohydrate level. The *occludin* expression, following a linear

TABLE 4 | Result summary: Expression of selected genes in liver and intestine.

Response	Minimum	Maximum	Mean	Std. Dev.	Ratio	Model	P model
L_*ikbkb*	0.020	0.037	0.027	0.005	1.8	Mean	
L_*rela*	0.176	0.386	0.271	0.065	2.2	Mean	
L_*igm*	0.0022	0.0043	0.0032	0.0007	2.0	Mean	
L_*cox2*	0.0025	0.0042	0.0033	0.0006	1.7	Mean	
L_*tjp1a1*	0.034	0.052	0.045	0.005	1.5	Mean	
L_*pcna*	0.210	0.412	0.313	0.055	2.0	Quadratic	**0.020**
L_*c5*	0.645	1.171	0.845	0.144	1.8	Linear	**0.025**
L_*mmp13*	0.021	0.048	0.034	0.008	2.3	Mean	
L_*MHCII*	0.025	0.052	0.038	0.009	2.1	Mean	
L_*cxcl19*	0.002	0.019	0.006	0.005	9.5	Special Cubic	0.179
L_*tnfa*	0.0005	0.0018	0.0012	0.0004	3.2	Mean	
L_*nfkb*	0.139	0.237	0.172	0.030	1.7	Mean	
L_*npc1l1*	0.041	0.089	0.068	0.016	2.2	Mean	
PI_*ikbkb*	0.060	0.085	0.073	0.009	1.4	Mean	
PI_*rela*	0.271	0.478	0.370	0.066	1.8	Linear	0.080
PI_*igm*	0.0009	0.0028	0.0016	0.0005	3.2	Linear	0.073
PI_*cox2*	0.0039	0.0097	0.0065	0.0016	2.5	Mean	
PI_*tjp1a1*	0.054	0.096	0.068	0.010	1.8	Mean	
PI_*pcna*	0.313	0.386	0.351	0.021	1.2	Mean	
PI_*c5*	0.007	0.017	0.010	0.002	2.4	Mean	
PI_*mmp13*	0.013	0.033	0.025	0.005	2.5	Mean	
PI_*MHCII*	0.256	0.638	0.376	0.097	2.5	Mean	
PI_*slc27a4*	0.160	0.303	0.216	0.034	1.9	Mean	
PI_*slc12a1*	0.205	0.335	0.253	0.040	1.6	Mean	
PI_*occludin*	0.020	0.032	0.025	0.004	1.6	Mean	
PI_*slc15a1*	0.144	0.243	0.185	0.031	1.7	Linear	**0.050**
PI_*cxcl19*	0.0024	0.0207	0.0082	0.0046	8.7	Quadratic	0.094
PI_*tnfa*	0.0023	0.0069	0.0042	0.0012	3.0	Mean	
PI_*nfkb*	0.181	0.276	0.224	0.028	1.5	Mean	
PI_*npc1l1*	0.147	0.280	0.205	0.039	1.9	Mean	
PI_*si*	0.275	0.431	0.343	0.052	1.6	Linear	**0.039**
MI_*ikbkb*	0.046	0.060	0.051	0.005	1.3	Mean	
MI_*rela*	0.208	0.279	0.245	0.020	1.3	Quadratic	**0.005**
MI_*igm*	0.00067	0.00162	0.00104	0.00031	2.4	Linear	**0.010**
MI_*cox2*	0.0038	0.0070	0.0048	0.0008	1.9	Quadratic	0.066
MI_*tjp1a1*	0.034	0.045	0.039	0.003	1.3	Mean	
MI_*pcna*	0.266	0.353	0.310	0.022	1.3	Quadratic	0.062
MI_*c5*	0.011	0.017	0.014	0.002	1.5	Mean	
MI_*mmp13*	0.009	0.020	0.013	0.004	2.2	Linear	**0.004**
MI_*MHCII*	0.059	0.124	0.080	0.017	2.1	Mean	
MI_*slc27a4*	0.134	0.189	0.160	0.017	1.4	Mean	
MI_*slc12a1*	0.057	0.094	0.078	0.012	1.7	Linear	**0.031**
MI_*occludin*	0.016	0.021	0.018	0.001	1.3	Mean	
MI_*slc15a1*	0.076	0.133	0.099	0.018	1.8	Linear	**0.001**
MI_*cxcl19*	0.0021	0.0063	0.0041	0.0015	3.0	Quadratic	**0.045**
MI_*tnfa*	0.0009	0.0018	0.0014	0.0003	2.1	Mean	
MI_*nfkb*	0.103	0.154	0.124	0.013	1.5	Special Cubic	0.181
MI_*npc1l1*	0.121	0.208	0.164	0.021	1.7	Linear	0.098
MI_*si*	0.205	0.325	0.267	0.034	1.6	Linear	**0.026**
DI_*ikbkb*	0.061	0.093	0.076	0.009	1.5	Mean	
DI_*rela*	0.196	0.287	0.251	0.028	1.5	Mean	
DI_*igm*	0.0014	0.0030	0.0020	0.0005	2.1	Quadratic	**0.003**
DI_*cox2*	0.0039	0.0065	0.0050	0.0006	1.7	Mean	
DI_*tjp1a1*	0.033	0.049	0.038	0.003	1.5	Mean	
DI_*pcna*	0.283	0.352	0.312	0.022	1.2	Mean	
DI_*c5*	0.0035	0.0080	0.0054	0.0012	2.3	Mean	
DI_*mmp13*	0.013	0.026	0.019	0.004	2.0	Special Cubic	0.119
DI_*MCHII*	0.066	0.153	0.102	0.030	2.3	Mean	
DI_*slc27a4*	0.059	0.102	0.077	0.014	1.7	Mean	
DI_*slc12a1*	0.106	0.188	0.137	0.026	1.8	Mean	
DI_*occludin*	0.013	0.021	0.016	0.003	1.6	Linear	**0.004**
DI_*slc15a1*	0.039	0.076	0.056	0.013	2.0	Mean	
DI_*cxcl19*	0.0031	0.0072	0.0045	0.0013	2.3	Quadratic	0.104

(Continued)

TABLE 4 | Continued

Response	Minimum	Maximum	Mean	Std. Dev.	Ratio	Model	P model
DI_*tnfa*	0.0014	0.0027	0.0020	0.0005	2.0	Mean	
DI_*nfkb*	0.095	0.149	0.120	0.015	1.6	Mean	
DI_*npc1l1*	0.084	0.130	0.100	0.016	1.5	Mean	
DI_*si*	0.295	0.474	0.393	0.053	1.6	Mean	

The names of responses comprise intestinal sections and genes measured. L, liver. PI, proximal intestine. MI, mid intestine. DI, distal intestine. ikbkb, inhibitor of nuclear factor kappa B kinase subunit beta. rela, transcription factor p65. igm, immunogobulin M. cox2, cyclooxygenase-2. tjp1a1, tight junction protein 1a. pcna, proliferating cell nuclear antigen. c5, complement component 5. mmp13, matrix metallopeptidase 13. MHCII, major histocompatibility complex II. cxcl19, chemokine (C-X-C motif) ligand 19. tnfa, tumor necrosis factor alpha. nfkb, nuclear factor kappa-light-chain-enhancer of activated B cells. npc1l, niemann-pick C1-like 1. slc27a4, solute carrier family 27 member 4. slc12a1, solute carrier family 12 member 1. occludin, Occludin protein. slc15a1, solute carrier family 15 member 1. si, sucrase-isomaltase. Bold value indicates significance (p model < 0.05). Data series minimum and maximum values, means and standard deviations, the ratio between max and min, the model that fitted the data best and probability that the data distribution is random (different from mean).

model, showed highest values at high protein a low lipid level, with minor, but positive dependency on carbohydrate level.

3.2.5 Gene Expression in the Liver

Among the genes observed in the liver, only *c5* and *pcna*, coding for proteins participating in immune regulation and cell proliferation, respectively, showed significant responses to the dietary variation (p = 0.020 and 0.025, respectively, **Table 4**). The expression of *c5* was affected only to a minor degree by protein level, decreased with increasing lipid, and increased with increasing carbohydrate level (**Figure 7**). The *pcna* expression data fitted a quadratic model (**Figure 7**) with two maxima, one at medium protein, high lipid, and low carbohydrate level, the other at low protein, high lipid, and high carbohydrate level. One minimum was apparent, i.e., at high protein, low lipid and medium carbohydrate and high protein level.

3.2.6 Histology of the Alimentary Tract and Liver

The general histological appearance of the gut mucosa is presented in **Figures 8A–D**. The stomach structure was quite similar to that observed in other fish species with simple columnar epithelium, gastric glands, and a thick smooth muscle layer with two layers, the inner circular and the outer longitudinal (**Figure 8A**). The structure of the pyloric caeca (**Figure 8B**) also appeared similar to what has been observed in other fish species. The caeca were surrounded by pancreatic tissue and were of thin wall with simple unbranched mucosal folds. Lamina propria and submucosa were thin. Fused caeca, due to branching of pyloric caeca were a unique finding for the lumpfish with shared muscular and serosal wall (**Figure 8B**, blue arrow). The mid intestine showed structures comparable to those observed in Atlantic salmon with short mucosal folds and short branching, thin

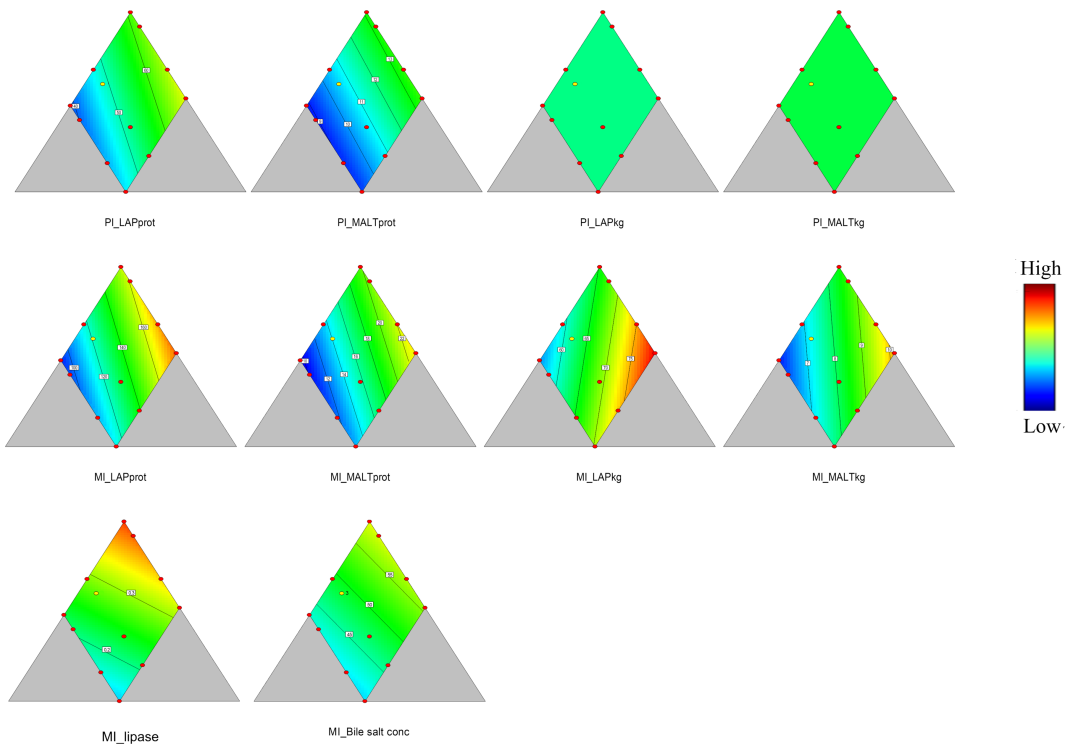


FIGURE 3 | Leucine aminopeptidase (LAP) and maltase specific activity and capacity in proximal intestine (PI) and mid intestine (MI), and lipase activity (units/mg dry matter) and bile salt concentration (mg/g dry matter) in digesta from MI. LAPprot and MALTprot, LAP and maltase specific activity (units/mg protein). LAPkg and MALT kg, LAP and maltase capacity (units/kg fish). Lipase, lipase activity (units/mg dry matter). Bile salt conc, bile salt concentration (mg/g dry matter). Color scale from blue to red indicates value changes from low to high. The yellow dot represents diet was fed to three tanks. Macronutrient composition of each dot can refer to **Figure 1**.

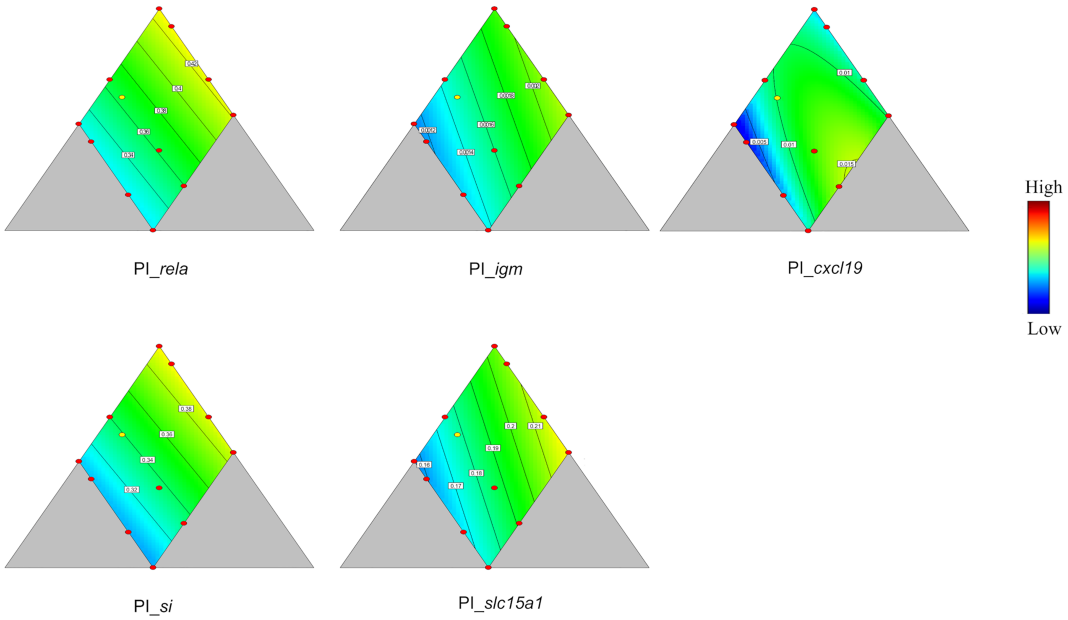


FIGURE 4 | Genes showing significant responses ($p < 0.05$) or trend ($0.05 < p < 0.1$) in proximal intestine (PI). *rela,* nuclear factor NF-kappa-B p65 subunit; *igm,* immunoglobulin M; *cxcl19,* chemokine ligand 19; *si*, sucrase-isomaltase; *slc15a1*, peptide transporter 1. Color scale from blue to red indicates value changes from low to high. The yellow dot represents diet was fed to three tanks. Macronutrient composition of each dot can refer to **Figure 1**.

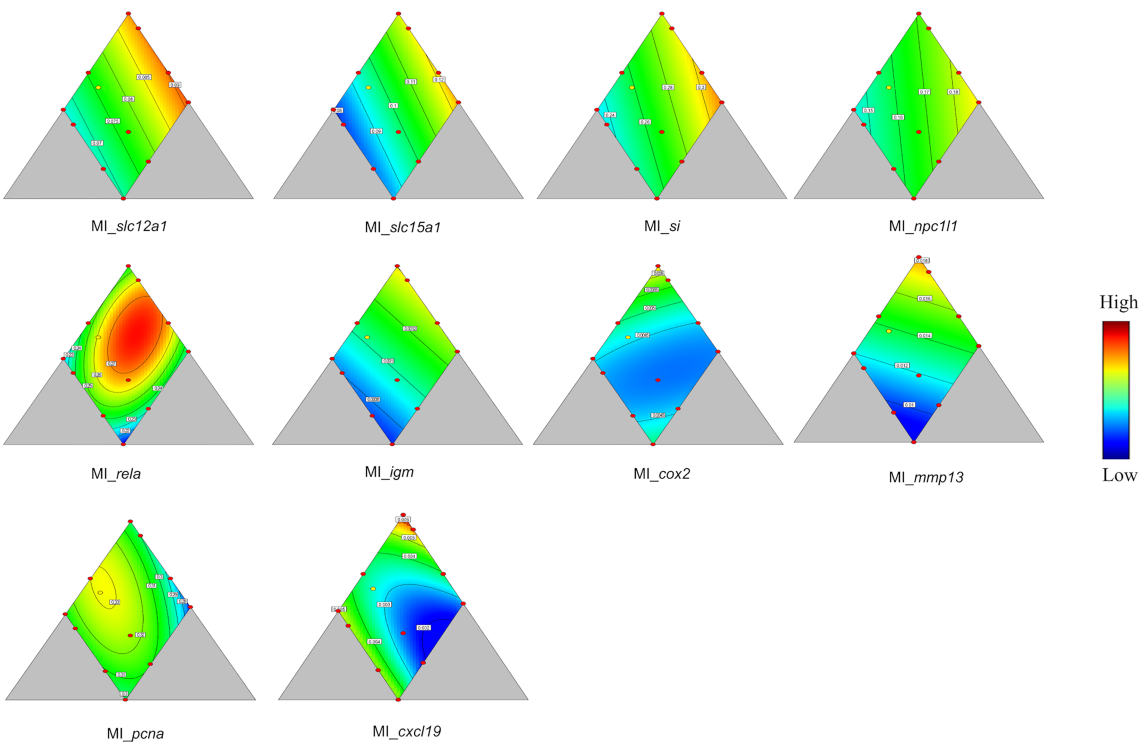


FIGURE 5 | Genes showing significant responses ($p < 0.05$) or trend ($0.05 < p < 0.1$) in mid intestine (MI). *slc12a1,* Na-K-Cl cotransporter; *slc15a1,* peptide transporter 1; *si*, sucrase-isomaltase; *npc1l1*, Niemann-pick C1-like 1; *rela*, nuclear factor NF-kappa-B p65 subunit; *igm*, immunoglobulin M; *cox2*, cyclooxygenase 2; *mmp13*, matrix metallopeptidase 13; *pcna*, proliferating cell nuclear antigen; *cxcl19*, chemokin ligand 19. Color scale from blue to red indicates value changes from low to high. The yellow dot represents diet was fed to three tanks. Macronutrient composition of each dot can refer to **Figure 1**.

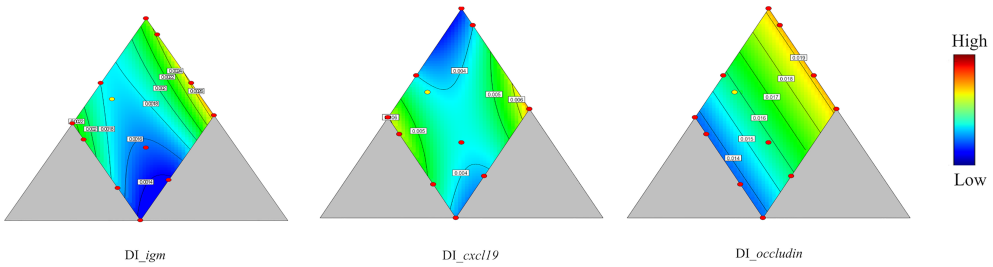


FIGURE 6 | Genes showing significant responses ($p < 0.05$) or trend ($0.05 < p < 0.1$) in distal intestine (DI). *igm*, immunoglobulin M; *cxcl19*, chemokine ligand 19; *occludin*, an integral membrane protein at the tight junctions. Color scale from blue to red indicates value changes from low to high. The yellow dot represents diet was fed to three tanks. Macronutrient composition of each dot can refer to **Figure 1**.

lamina propria and submucosa with sparse cellularity, but thick muscle layer (**Figure 8C**). The structure of the distal intestine (**Figure 8D**) appeared similar to that in Atlantic salmon with simple (Baeverfjord and Krogdahl, 1996; Knudsen et al., 2007), and complex (branched) mucosal folds which were taller than in mid intestine, and lamina propria and submucosa with fibrous tissue and little cellular composition. The enterocytes showed little or no vacuolization, a feature which may distinguish the lumpfish from Atlantic salmon which, in the fed state, show high vacuolation of the distal intestinal enterocytes.

Figures 8E–H show histological features of the cells of the PI and liver in fish fed diets with high protein and low lipid and low protein high lipid levels. Hyper-vacuolization, a symptom of excessive lipid accumulation, so-called steatosis, was observed in both PI and liver. The steatosis level was scored and analyzed for PI, MI, and liver (**Figure 9**), clearly showing increasing steatosis with increasing lipid level.

3.2.7 Digestibility

The results of the additional digestibility experiment, E2, are illustrated in **Figure 10**, showing effects of dietary lipid level on starch, protein, and fatty acid digestibility. Protein digestibility did not change as lipid or starch level varied. Fatty acid digestibility increased as dietary lipid level increased. For saturated fatty acids this effect was not significant, but the results showed the same trend as for other fatty acids ($p = 0.06$). The digestibility of ω-6 fatty acids was relatively low, between 60% and 68%. Noticeably, starch digestibility decreased greatly as starch level increased, from 84.3% at 6.6% inclusion to 50% at 16% inclusion. This means a mean partial digestibility of the starch increment from 6.6% to 16.0% of about 27% and increase in available starch level from 55-80 g/kg diet.

4 DISCUSSION

Lumpfish belongs to the family *Cyclopteridae* in which only lumpfish is commercially produced. It means gut characteristics of evolutionarily relevant species of lumpfish is lacking. In this context, comparing to other fish species of similar feeding habit is a better way to understand our results in lumpfish. Despite of shorter intestinal length to body length compared to lumpfish, Atlantic salmon is also a carnivorous fish with stomach, with available well-defined gut characteristic. Therefore, Atlantic salmon is chosen as a major species for comparison in the following discussion.

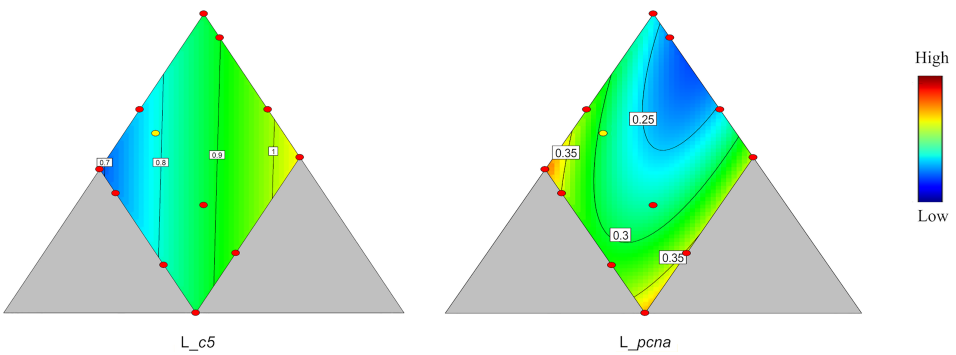


FIGURE 7 | Genes showing significant response ($p < 0.05$) responses in liver. *c5*, complement component 5; *pcna*, proliferating cell nuclear antigen. Color scale from blue to red indicates value changes from low to high. The yellow dot represents diet was fed to three tanks. Macronutrient composition of each dot can refer to **Figure 1**.

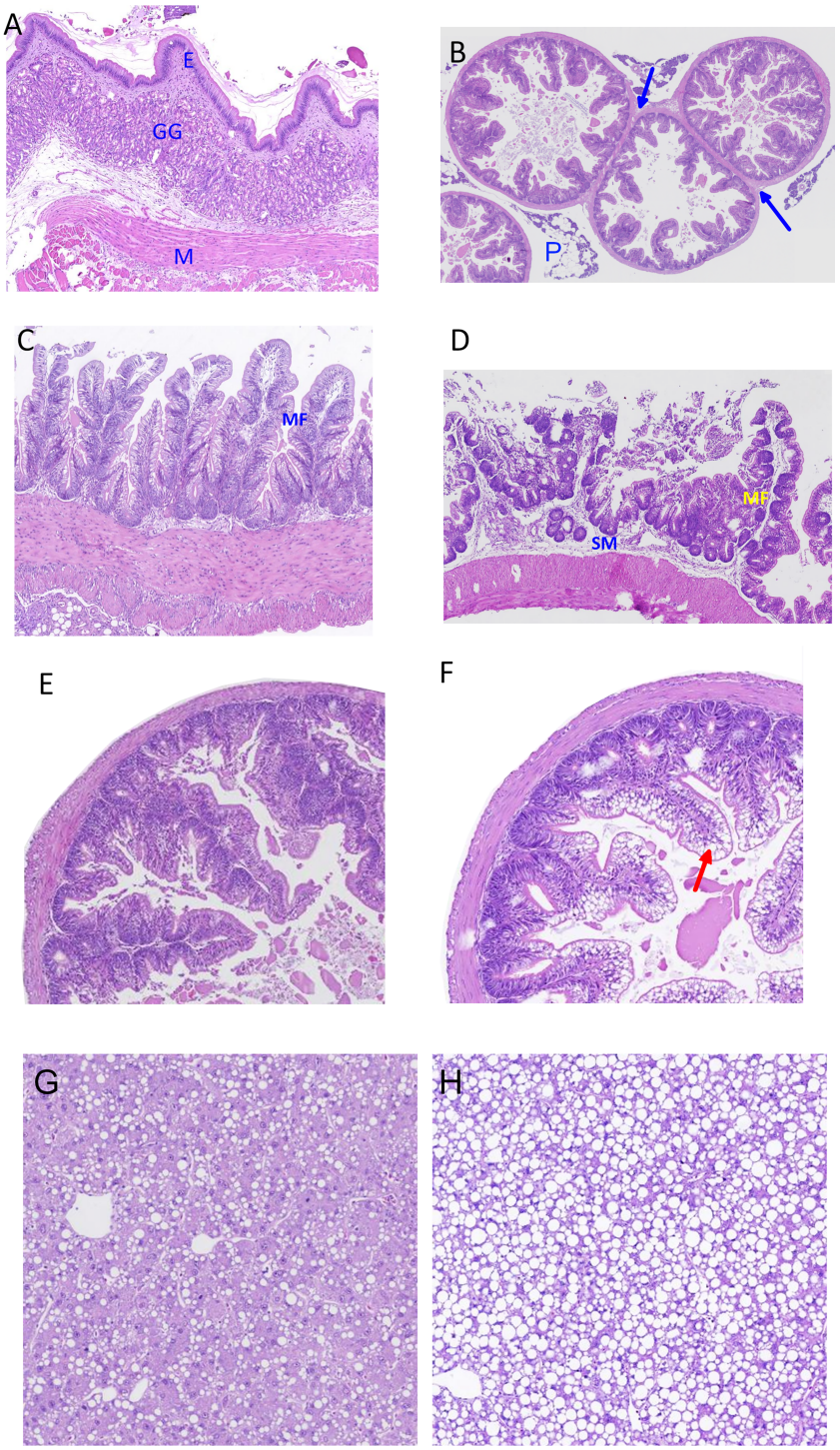


FIGURE 8 | Images of histological characteristics of the mucosa in the stomach **(A)**, pyloric intestine **(B)**, mid intestine **(C)** and distal intestine **(D)** of lumpfish, as indicated by H&E staining, and images of pyloric caeca with normal appearance **(E)** and with clear signs of hypervacuolation/steatosis **(F)**, and images of liver with normal appearance **(G)** and with clear signs of hypervacuolation/steatosis **(H)**. Image A: E, simple columnar epithelium; GG, gastric glands; M, smooth muscle layer with inner circular and outer longitudinal indicated. Image B: blue arrows indicate the unique feature of fused caeca, the share of muscular and serosal wall, and indicates a point of branching in pyloric caeca; P, pancreatic tissue. Image C: MF, short mucosal folds with short branching and with thin lamina propria and submucosa with sparse cellularity, and a thick muscle layer are apparent. Image D: MF, simple and branched mucosal folds (MF) with enterocytes of no vacuolation; SM, submucosa with and little cellularity. Image F, red arrow indicates vacuolation in enterocytes which is considered a sign of steatosis

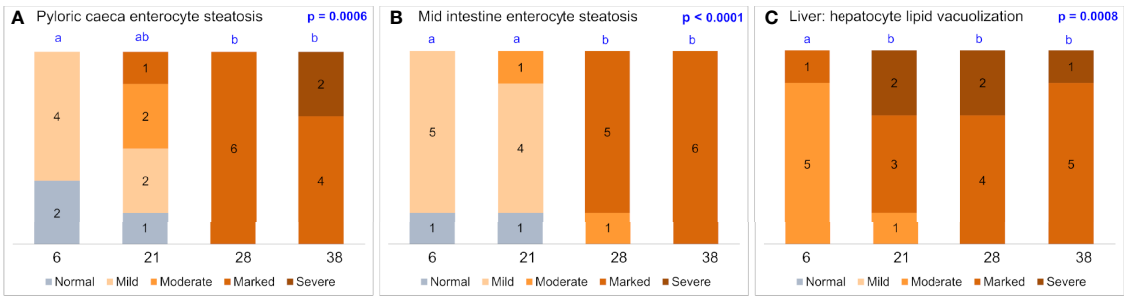


FIGURE 9 | Results of histological scoring of vacuolation in the enterocytes of the pyloric ceaca **(A)**, mid intestine **(B)** and hepatocytes **(C)**. The numbers of the x-axis indicate level of lipid in the diets, i.e. (Protein (P)/Lipid (L)/Carbohydrates (C): Diet 1: 68/4.3/5.9%; Diet 5: 58/11.9/8.2%; Diet 3: 58/16.4/5.8% and Diet 12: 45/17.3/17.7%, respectively. The x-values 6, 21, 28, and 38 represent the ratio lipid to 100g protein in the diets. Stack bars with different letters above indicate significant difference.

4.1 General Features of Lumpfish Intestine

The present study greatly improves general knowledge regarding characteristics of the lumpfish intestine and makes comparison with other fish species possible. As studies of similar characteristics of the intestinal tract of Atlantic salmon, employing the same methods and assays as in the present work is available, the present lumpfish results are compared to similarities and differences relative to Atlantic salmon. The weight of the pyloric intestine of lumpfish comprised a much lower proportion of the weight of the intestinal tract than observed in salmon,

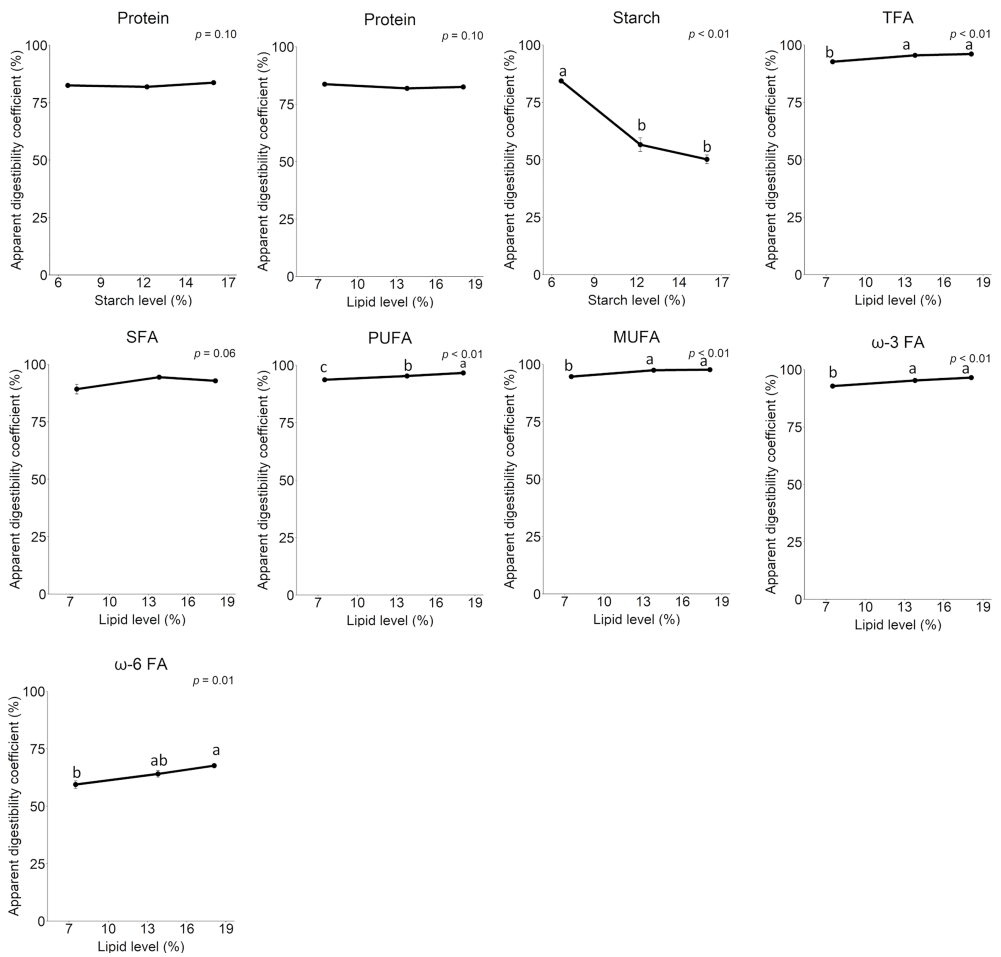
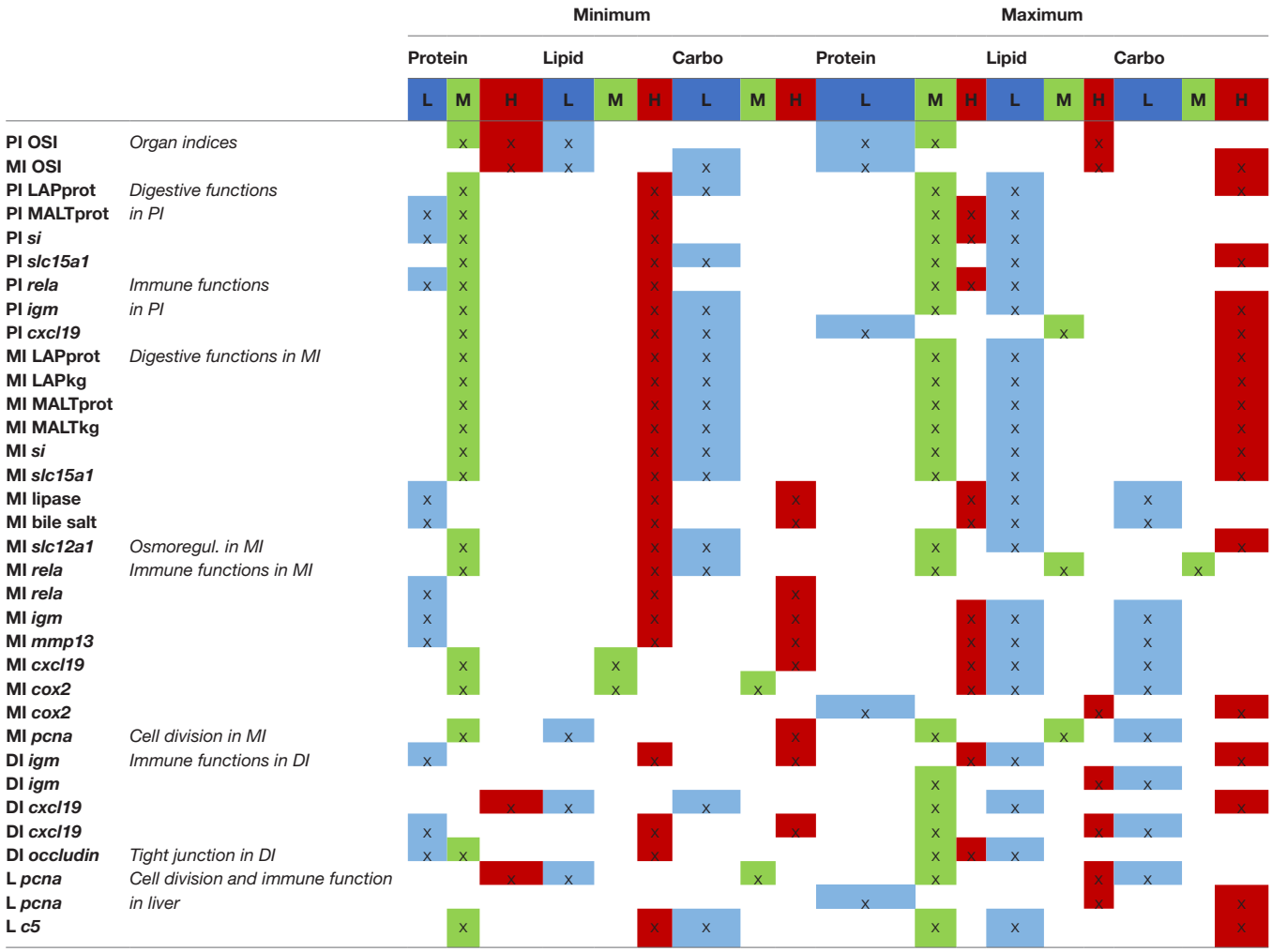


FIGURE 10 | Apparent digestibility coefficients of protein, starch, and total fatty acids. Starch digestibility is related to dietary starch level, total fatty acid digestibility is related to dietary lipid level, and protein digestibility is related to both starch and lipid level. TFAs, total fatty acids; SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; ω-3 FA, omega-3 fatty acids; ω-6 FA, omega-6 fatty acids.

TABLE 5 | Indication of position in the triangle of minimum and maximum for the biomarkers which showed apparent relationship with macronutrient content of the diets*.



PI OSI	*Organ indices*
MI OSI	
PI LAPprot	*Digestive functions*
PI MALTprot	*in PI*
PI *si*	
PI *slc15a1*	
PI *rela*	*Immune functions*
PI *igm*	*in PI*
PI *cxcl19*	
MI LAPprot	*Digestive functions in MI*
MI LAPkg	
MI MALTprot	
MI MALTkg	
MI *si*	
MI *slc15a1*	
MI lipase	
MI bile salt	
MI *slc12a1*	*Osmoregul. in MI*
MI *rela*	*Immune functions in MI*
MI *rela*	
MI *igm*	
MI *mmp13*	
MI *cxcl19*	
MI *cox2*	
MI *cox2*	
MI *pcna*	*Cell division in MI*
DI *igm*	*Immune functions in DI*
DI *igm*	
DI *cxcl19*	
DI *cxcl19*	
DI *occludin*	*Tight junction in DI*
L *pcna*	*Cell division and immune function*
L *pcna*	*in liver*
L *c5*	

The indictors are categorized as low (L), medium (M) and high (H) as interpreted from the respective graphs shown in* ***Figures 3–7**. Background colors represent low (blue), medium (green) and high (red) for each nutrient.*

about 50% compared to about 75% in salmon (Kraugerud et al., 2007). On the other hand, the MI was much larger in lumpfish, about 40% compared to about 5% in salmon, whereas the DI comprised a small part of the intestine of both species, about 10 and 20%, respectively. In the salmon, the enzyme capacities of these intestinal sections reflect the weigh differences, with 70 – 80% of the total capacity (U/kg fish) in the PI (Bakke-McKellep et al., 2008). In the lumpfish, however, of total LAP and maltase capacity, the PI and MI showed similar values, in the range 40 to 50%. The activity (U/mg digesta dry matter) of the pancreatic enzymes, trypsin, amylase, and lipase, measured in content from MI, showed values very different from values observed in Atlantic salmon, in particular for trypsin and lipase. In lumpfish the trypsin activity was 1/6 and lipase 1/10, of values often observed in salmon (Chikwati et al., 2013). Amylase showed values quite similar to those observed in salmon (Froystad et al., 2006). The species differences observed for these pancreatic enzymes may be related to differences in diet composition between the experiments. However, the high activities of trypsin and lipase in the salmon, may also be a result of the breeding program steady improving growth rate and capacity for feed intake and utilization. The similarity of the observed amylase activities in lumpfish and salmon is remarkable, as salmon amylase is known to be rather ineffective. The salmon amylase has at least two amino acid sequence modifications greatly reducing its specific activity, compared, for example, to the amylase of rainbow trout (*Oncorhynchus mykiss*) which shows values 10 times higher than observed for the Atlantic salmon and herein for lumpfish (Froystad et al., 2006). Our results suggest that similar limitations as in the Atlantic salmon may be present also in the amylase of the lumpfish. Further evidence supporting this is that the starch digestibility decreased to around 50% when dietary starch level increased to 16%, indicating the starch was not efficiently digested and absorbed at levels above 6%.

The decreasing digestibility of starch with increasing dietary inclusion level implicates that the variation in available

carbohydrates of the diets was much less than the variation in analyzed level of carbohydrates, in both the E1 and E2 experiment. Variation in analyzed carbohydrates in E1 was from 6 to 18%, whereas available carbohydrate, based on estimates from E2, varied from about 5.5 to 8.1% (almost 30%). This means that the quantitative aspects of effects of analyzed and available carbohydrate levels on the relationships between diet composition and the observed biomarkers might differ substantially. For protein and lipid, the analyzed and available levels differed much less. The impact of variation in these nutrients on the various observed biomarkers would therefore be expected to be quite similar whether based on chemically analyzed values or as levels of digestible nutrients.

Although the amylase activity was not affected by dietary starch, it seemed that the disaccharidase activities were induced at the transcriptional level (*si* expression) as well as on the enzymatic level (maltase specific activity and capacity) in MI, shown as slight positive relationship with carbohydrate level. Similar relationships have been observed also in Atlantic salmon and rainbow trout (Krogdahl et al., 2004). The mechanisms underlying such effects cannot, however, be found based on the available results in the present study. These effects may be related to the increased amount of digested starch (5.6, 6.6 and 8.1 g/100g diet in E2) when dietary starch increased as the digested starch provided substrates, i.e., maltose, which may have induced disaccharidase expression and activity. Besides this, bacteria inhabiting the intestine of fish and the metabolites produced by these, may influence host intestinal metabolism and homeostasis, i.e. macronutrient and cholesterol metabolism (Le Roy et al., 2019), as well as immune regulations (Zhang et al., 2017). Therefore, the undigested starch remaining in the lumen might influence intestinal metabolism and immune regulations in lumpfish by altering intestinal microbiota composition. This suggestion is in line the results recently reported from experiments with the pompano (*Trachinotus ovatus)* (Zhao et al., 2020) and largemouth bass (*Micropterus salmoides)* (Zhang et al., 2020). Gut microbiota were not investigated in the present study but deserves attention in further studies. All in all, it can be concluded that the diet for lumpfish should not contain high level of dietary starch, as this will not be utilized by the fish, and instead increase environmental pollution, and may also harm production and health of the lumpfish.

4.2 Lipid Accumulation in the Lumpfish Intestine

The diet effects observed for OSI of PI and MI in E1, with a positive, linear relationship with dietary lipid level, indicate increased lipid accumulation in the intestinal tissue, and were confirmed by the histologically apparent elevation of the number of vacuoles in enterocytes of PI and MI. These changes may result from either excessive dietary lipid content, or deficiency of substances essential for lipid transport and metabolism. Enterocyte lipid accumulation has been observed in studies of Atlantic cod (*Gadus morhua*) (Kjaer et al., 2009), pikeperch (*Sander lucioperca*) (Kowalska et al., 2011) and Atlantic salmon (Penn, 2011) in which lipid accumulation was observed as an increase in the presence of lipid droplet in enterocytes of fish fed high-fat diets. The cause of the lipid accumulation in these fish may be deficient supply of essential nutrients necessary for efficient transport of lipid through the enterocytes. Deficiency of essential fatty acids have been observed to cause lipid accumulation in the enterocytes of Atlantic salmon (Olsen et al., 1999; Olsen et al., 2003; Bou et al., 2017).Choline is another nutrient recently shown to be essential for Atlantic salmon and signs of deficiency comprise similar symptoms (Hansen et al., 2020). Whether deficient supply of essential fatty acids, choline, or some other essential nutrient involved in lipid transport and metabolism are causing the histological alterations observed in the present study, cannot be concluded upon due to lack of knowledge. Great efforts are needed to define nutrient requirement of lumpfish, necessary to secure good health and welfare.

Among the genes investigated in the present work, two are involved in lipid transport, i.e., *slc27a4*, coding for the principal long chain fatty acid transporter (Krammer et al., 2011; Mitchell et al., 2011), and *npc1l1*, a cholesterol transporter and key actor in absorption of cholesterol by enterocytes and hepatocytes (Davis et al., 2004; Betters and Yu, 2010). The independence of expression of *slc27a4* in PI and MI of the lumpfish on dietary lipid and choline level, is in line with results from Atlantic salmon (Hansen et al., 2020). The trend of decreasing expression of *npc1l1* with increasing lipid level and decreasing choline level indicates that this might be a compensatory mechanism related to the increasing dietary cholesterol. This is in line with the results from salmon fed a choline deficient diet (Kortner et al., 2014; Krogdahl et al., 2020).

4.3 Macronutrient Composition Affected Immune Responses in Lumpfish Intestine

The results observed for the genes, *mmp13*, *rela*, *igm* and *cxcl19*, all involved in regulation of immune responses, suggest that the immune functions located in MI are more responsive to macronutrient variation than those located in PI and DI. The decrease in expression of *mmp13* and *igm*, with increasing lipid level indicate responses in the activity of B cells (*igm*), and lymphocyte recruitment (*mmp13*) (Xu et al., 2018). These responses may be related to the increasing level of ω3-PUFAs with increasing dietary lipid level, i.e., increased levels of fish oil. Earlier studies have shown that ω3-PUFAs, mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), may inhibit expression of immune relevant genes (Teitelbaum and Walker, 2001; Calder, 2008). Also results of *in vitro* studies have shown suppression of B cells by EPA and DHA (Wang et al., 2011; Yang et al., 2016).

The results which showed effects of carbohydrate level on the intestinal immune regulation, indicated by altered expression of *rela*, *cox2*, *mmp13*, *cxcl19*, and *pcna* in MI, must be interpreted with caution, as the digestibility, and therefore the availability of the starch, decreased greatly with increasing dietary inclusion level. This fact makes interpretation of the results of the four genes *rela*, *cox2*, *cxcl19*, and *pcna* particularly challenging as they showed quadratic responses and with different maxima and minima. Further studies are needed before the results regarding

effects of variation in carbohydrate level can be discussed and concluded upon.

Although certain effects of diet composition on immune related genes were observed, the histological observations did not show important symptoms of inflammation. The diet effects on expression of immune genes can therefore merely be reflections of normal adjustments to variation in diet composition. It is also possible, however, that they indicate altered resistance to pathogens such as bacteria or virus, for the better or worse. Challenge tests should therefore be conducted to clarify such possible effects.

4.4 Indication of Optimum Balance Between the Dietary Macronutrients

To get an overview of the present results, they are summarized in **Table 5** with indication of the nutrient level (low, medium or high) which gives minimum and maximum for the variables which showed a statistically significant relationship with diet composition and visually apparent relationship with dietary level of macronutrients in **Figures 3–10**. The MI seemed to show the clearest picture, whereas the pictures for DI and the liver were less clear. Based on the results for MI, the maximum results for the biomarkers of digestive functions were observed for diets with medium to high protein level. For the indicators of protein digestion (LAP and *slc15a1*), the highest value was observed at medium protein, low lipid, and high carbohydrate level, for carbohydrate digestion (MALT and *si*) the highest results were found for medium protein, high carbohydrate, and low lipid level, whereas for lipid digestion (lipase) the highest results were observed for diets with high protein, low lipid, and low carbohydrate level. For the biomarkers of immune functions in MI, high values were found at low lipid and carbohydrate levels for most of the biomarkers. The minimum results were found at low to medium level of protein and high level of lipid and carbohydrates. As the activity of lipase (Krogdahl and Sell, 1989) and maltase, as well as expression of a number of immune related genes showed the lowest values at low protein level, the results indicate that the diets with low protein level were protein deficient. It is well known that protein deficiency severely affects the immune functions and disease resistance in animals including humans (Carrillo et al., 2014). It should be kept in mind that the gut mucosa is among the most dynamic tissues in an animal body and has a great capacity to adapt to diet composition (Olli et al., 1994). Therefore, the finding that level of LAP was low at low protein level may reflect low production of peptidases at low dietary protein level rather than protein deficiency, in accordance with the need for efficient protein digestion. The effects of carbohydrate level were less clear regarding indication of minimum and maximum of the biomarkers. This may be related to the fact that the range of available carbohydrates among the diets was rather small, resulting in less clear relationships. Based on the present results, it is not possible to conclude firmly regarding optimal macronutrient balance in lumpfish diets.

As mentioned in the introduction, our previously published results from E1, indicated that peak performance of lumpfish weighing between 15 and 50g, based on growth, body composition, welfare score and immune responses in isolated head kidney cells, would be obtained with a diet containing 55% protein, minimum 17% lipid and maximum 6% carbohydrate (Hamre et al., 2022). The present results, showing very low starch digestibility, suggest that dietary starch level should be limited to about 6%. The intestinal immune related biomarkers showed a linear relationship with the dietary protein/lipid ratio at low carbohydrate, with the highest response at maximum protein level. Since it is not certain if strong immune responses in unchallenged tissues is positive or negative for the fish, a recommendation on the optimal macronutrient composition cannot be given based on the present results regarding immune function. However, according to Hamre et al. (2022), head kidney cells from fish fed the diet with the maximum level of protein, showed a suboptimal immune response when challenged with lipopolysaccharide (LPS). Fish fed the diet with the highest growth (55, 17, 6) had a normal immune response. In line with this, the present results do not suggest alteration in the conclusion presented in our previous paper, i.e., that for peak performance a diet with high lipid, minimum carbohydrate and medium protein is optimal for lumpfish of 10-50g.

5 CONCLUSIONS

The current study improved knowledge regarding responses to dietary macronutrient composition in lumpfish intestine *via* a three-component mixture design. The intestinal functions were broadly impacted by increasing lipid level in the diet with corresponding decrease in protein level, including digestion of polypeptides, maltose and lipid, absorption of fatty acids and peptides, and immune regulations that involve *rela*, *igm* and *cxcl19* and *mmp13*. Elevated lipid digestibility by dietary lipid level was also observed. Although alteration by dietary carbohydrate to protein level was observed on multiple parameters, such as the expression of *igm* and *mmp13*, we should be careful considering the influence of carbohydrate variation because of low starch digestibility when dietary starch level was above 6%. In all, a diet of 55% protein, 17% lipid and 6% carbohydrate is recommended for lumpfish.

Among the many questions to which answers are urgently needed for the feed industry to be able to produce nutritionally well-balanced feeds for lumpfish, the present study answers only a few. Further information is urgently needed and should be addressed in future research. Examples of questions which need answers regard optimal inclusion of available macronutrients, vitamin and minerals, for growth, function as lice cleaners and, not at least, immune functions and disease resistance.

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 Norwegian Food Safety Authority.

AUTHOR CONTRIBUTIONS

WZ analyzed samples and data processing, and drafting and completing of the manuscript. ÅK and TK overviewed the analyses, joined the data processing and writing of the manuscript. KK, KH, ØS, IL and GB participated in planning of experimental design and feed formulation. KK was in charge of feed production. GB was in charge of the feeding trial. EC performed histological analysis and related statistics. KH conducted statistics related to the mixture design. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by the Norwegian Seafood Research Fund (FHF) as part of the project CleanFeed, research no. 901331.

ACKNOWLEDGMENTS

The authors acknowledge the contributions of the laboratory collaborators from IMR (Institute of Marine Research in Norway) and Nofima AS BioLab and Aqualab, for the execution of chemical analyses in feeds, the staff of the Feed Technology Center of Nofima in Bergen for the experimental feed production, the staff of MOWI and Nofima Aquaculture Research Center Sunndalsøra for the execution of the fish feeding trials, and the technicians of the Nutrition and health group at Faculty of Veterinary Medicine, University of Life Sciences (NMBU) for skillful assistance of the assays.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.889778/full#supplementary-material

REFERENCES

Baeverfjord, G. and Krogdahl, A. (1996). Development and Regression of Soybean Meal Induced Enteritis in Atlantic Salmon, Salmo Salar L, Distal Intestine: A Comparison With the Intestines of Fasted Fish. *J. Fish. Dis.* 19 (5), 375–387. doi: 10.1111/j.1365-2761.1996.tb00376.x

Bakke-McKellep, A. M., Sanden, M., Danieli, A., Acierno, R., Hemre, G. I., Maffia, M., et al. (2008). Atlantic Salmon (Salmo Salar L.) Parr Fed Genetically Modified Soybeans and Maize: Histological, Digestive, Metabolic, and Immunological Investigations. *Res. In Vet. Sci.* 84 (3), 395–408. doi: 10.1016/j.rvsc.2007.06.008

Betters, J. L. and Yu, L. Q. (2010). NPC1L1 and Cholesterol Transport. *FEBS Lett.* 584 (13), 2740–2747. doi: 10.1016/j.febslet.2010.03.030

Bou, M., Berge, G. M., Baeverfjord, G., Sigholt, T., Ostbye, T. K., Romarheim, O. H., et al. (2017). Requirements of N-3 Very Long-Chain PUFA in Atlantic Salmon (Salmo Salar L): Effects of Different Dietary Levels of EPA and DHA on Fish Performance and Tissue Composition and Integrity. *Br. J. Nutr.* 117 (1), 30–47. doi: 10.1017/S0007114516004396

Brockman, H. L. (1981). [72] Triglyceride Lipase From Porcine Pancreas: EC 3.1. 1.3 Triacylglycerol Acylhydrolase. *Methods Enzymol.* 71, 619–627. doi: 10.1016/0076-6879(81)71074-7

Calder, P. C. (2008). The Relationship Between the Fatty Acid Composition of Immune Cells and Their Function. *Prostaglandins Leukotrienes Essential Fatty Acids* 79 (3-5), 101–108. doi: 10.1016/j.plefa.2008.09.016

Carrillo, E., Jimenez, M. A., Sanchez, C., Cunha, J., Martins, C. M., Seva, A. D., et al. (2014). Protein Malnutrition Impairs the Immune Response and Influences the Severity of Infection in a Hamster Model of Chronic Visceral Leishmaniasis. *PLoS One* 9 (2). e89412. doi: 10.1371/journal.pone.0089412

Chikwati, E., Sahlmann, C., Holm, H., Penn, H., Krogdahl, A. and Bakke, A. (2013). Alterations in Digestive Enzyme Activities During the Development of Diet-Induced Enteritis in Atlantic Salmon, Salmo Salar L. *Aquaculture* 402, 28–37. doi: 10.1016/j.aquaculture.2013.03.023

Cornell, J. A. (2011). *Experiments With Mixtures: Designs, Models, and the Analysis of Mixture Data* (New York: John Wiley & Sons Inc).

Dahlqvist, A. (1970). Assay of Intestinal Disaccharidases. *Enzymol. Biol. Clin.* 11, 52–66. doi: 10.1159/000458348

Davis, H. R., Zhu, L. J., Hoos, L. M., Tetzloff, G., Maguire, M., Liu, J. J., et al. (2004). Niemann-Pick C1 Like 1 (NPC1L1) is the Intestinal Phytosterol and Cholesterol Transporter and a Key Modulator of Whole-Body Cholesterol Homeostasis. *J. Biol. Chem.* 279 (32), 33586–33592. doi: 10.1074/jbc.M405817200

Froystad, M. K., Lilleeng, E., Sundby, A. and Krogdahl, Å. (2006). Cloning and Characterization of Alpha-Amylase From Atlantic Salmon (Salmo Salar L.). *Comp. Biochem. Physiol. a-Molecular Integr. Physiol.* 145 (4), 479–492. doi: 10.1016/j.cbpa.2006.08.003

Garcia-Hernandez, V., Quiros, M. and Nusrat, A. (2017). Intestinal Epithelial Claudins: Expression and Regulation in Homeostasis and Inflammation. *Annu. NY Acad. Sci.* 1397 (1), 66–79. doi: 10.1111/nyas.13360

Gregorio, S. F. and Fuentes, J. (2018). Regulation of Bicarbonate Secretion in Marine Fish Intestine by the Calcium-Sensing Receptor. *Int. J. Mol. Sci.* 19 (4), 14. doi: 10.3390/ijms19041072

Grosell, M. (2006). Intestinal Anion Exchange in Marine Fish Osmoregulation. *J. Exp. Biol.* 209 (15), 2813–2827. doi: 10.1242/jeb.02345

Gu, M., Kortner, T. M., Penn, M., Hansen, A. K. and Krogdahl, A. (2014). Effects of Dietary Plant Meal and Soya-Saponin Supplementation on Intestinal and Hepatic Lipid Droplet Accumulation and Lipoprotein and Sterol Metabolism in Atlantic Salmon (Salmo Salar L.). *Br. J. Nutr.* 111 (3), 432–444. doi: 10.1017/S0007114513002717

Hamre, K., Berge, G. M., Saele, Ø., Holen, E., Kousoulaki, K., Remo, S. C., et al. (2022). Optimization of the Balance Between Protein, Lipid and Carbohydrate in Diets for Lumpfish (Cyclopterus Lumpus). *Aquacult. Nutr.* 2022, 1–15. doi: 10.1155/2022/1155989

Hamre, K. and Mangor-Jensen, A. (2006). A Multivariate Approach to Optimization of Macronutrient Composition in Weaning Diets for Cod (Gadus Morhua). *Aquacult. Nutr.* 12 (1), 15–24. doi: 10.1111/j.1365-2095.2006.00377.x

Hamre, K., Nordgreen, A., Grotan, E. and Breck, O. (2013). A Holistic Approach to Development of Diets for Ballan Wrasse (Labrus Berggylta) - a New Species in Aquaculture. *PeerJ* 1, e99. doi: 10.7717/peerj.99

Hamre, K., Ofsti, A., Naess, T., Nortvedt, R. and Holm, J. C. (2003). Macronutrient Composition of Formulated Diets for Atlantic Halibut (Hippoglossus Hippoglossus, L.) Juveniles. *Aquaculture* 227 (1-4), 233–244. doi: 10.1016/s0044-8486(03)00506-4

Hansen, A. K. G., Kortner, T. M., Krasnov, A., Bjorkhem, I., Penn, M. and Krogdahl, Å. (2020). Choline Supplementation Prevents Diet Induced Gut Mucosa Lipid Accumulation in Post-Smolt Atlantic Salmon (Salmo Salar L.). *BMC Vet. Res.* 16 (1). 1–15. doi: 10.1186/s12917-020-2252-7

Hu, H. B., Kortner, T. M., Gajardo, K., Chikwati, E., Tinsley, J. and Krogdahl, A. (2016). Intestinal Fluid Permeability in Atlantic Salmon (Salmo Salar L.) Is Affected by Dietary Protein Source. *PLoS One* 11 (12). e0167515. doi: 10.1371/journal.pone.0167515

Imsland, A. K., Reynolds, P., Eliassen, G., Hangstad, T. A., Nytro, A. V., Foss, A., et al. (2015). Feeding Preferences of Lumpfish (Cyclopterus Lumpus L.) Maintained in Open Net-Pens With Atlantic Salmon (Salmo Salar L.). *Aquaculture* 436, 47–51. doi: 10.1016/j.aquaculture.2014.10.048

Imsland, A. K. D., Reynolds, P., Jonassen, T. M., Hangstad, T. A., Adron, J., Elvegard, T. A., et al. (2019a). Comparison of Diet Composition, Feeding, Growth and Health of Lumpfish (Cyclopterus Lumpus L.) Fed Either Feed Blocks or Pelleted Commercial Feed. *Aquacult. Res.* 50 (7), 1952–1963. doi: 10.1111/are.14083

Imsland, A. K. D., Reynolds, P., Jonassen, T. M., Hangstad, T. A., Elvegard, T. A., Urskog, T. C., et al. (2018). Effects of Three Commercial Diets on Growth, Cataract Development and Histopathology of Lumpfish (Cyclopterus Lumpus L.). *Aquacult. Res.* 49 (9), 3131–3141. doi: 10.1111/are.13776

Imsland, A. K. D., Reynolds, P., Jonassen, T. M., Hangstad, T. A., Elvegard, T. A., Urskog, T. C., et al. (2019b). Effects of Different Feeding Frequencies on Growth, Cataract Development and Histopathology of Lumpfish (Cyclopterus Lumpus L.). *Aquaculture* 501, 161–168. doi: 10.1016/j.aquaculture.2018.11.026

Ingolfsson, A. and Kristjansson, B. K. (2002). Diet of Juvenile Lumpsucker Cyclopterus Lumpus (Cyclopteridae) in Floating Seaweed: Effects of Ontogeny and Prey Availability. *Copeia* 2, 472–476. doi: 10.1643/0045-8511(2002)002[0472:Dojlcl]2.0.Co;2

Kakade, M. L., Hoffa, D. E. and Liener, I. E. (1973). Contribution of Trypsin-Inhibitors to Deleterious Effects of Unheated Soybeans Fed to Rats. *J. Nutr.* 103 (12), 1772–1778. doi: 10.1093/jn/103.12.1772

Kjaer, M. A., Vegusdal, A., Berge, G. M., Galloway, T. F., Hillestad, M., Krogdahl, Å., et al. (2009). Characterisation of Lipid Transport in Atlantic Cod (Gadus Morhua) When Fasted and Fed High or Low Fat Diets. *Aquaculture* 288 (3-4), 325–336. doi: 10.1016/j.aquaculture.2008.12.022

Knudsen, D., Uran, P., Arnous, A., Koppe, W. and Frokiaer, H. (2007). Saponin-Containing Subfractions of Soybean Molasses Induce Enteritis in the Distal Intestine of Atlantic Salmon. *J. Agric. Food Chem.* 55 (6), 2261–2267. doi: 10.1021/jf0626967

Kortner, T. M., Bjorkhem, I., Krasnov, A., Timmerhaus, G. and Krogdahl, A. (2014). Dietary Cholesterol Supplementation to a Plant-Based Diet Suppresses the Complete Pathway of Cholesterol Synthesis and Induces Bile Acid Production in Atlantic Salmon (Salmo Salar L.). *Br. J. Nutr.* 111 (12), 2089–2103. doi: 10.1017/S0007114514000373

Kortner, T. M., Valen, E. C., Kortner, H., Marjara, I. S., Krogdahl, A. and Bakke, A. M. (2011). Candidate Reference Genes for Quantitative Real-Time PCR (qPCR) Assays During Development of a Diet-Related Enteropathy in Atlantic Salmon (Salmo Salar L.) and the Potential Pitfalls of Uncritical Use of Normalization Software Tools. *Aquaculture* 318 (3-4), 355–363. doi: 10.1016/j.aquaculture.2011.05.038

Kowalska, A., Zakes, Z., Jankowska, B. and Demska-Zakes, K. (2011). Effect of Different Dietary Lipid Levels on Growth Performance, Slaughter Yield, Chemical Composition, and Histology of Liver and Intestine of Pikeperch, Sander Lucioperca. *Czech J. Anim. Sci.* 56 (3), 136–149. doi: 10.17221/1298-Cjas

Krammer, J., Digel, M., Ehehalt, F., Stremmel, W., Fullekrug, J. and Ehehalt, R. (2011). Overexpression of CD36 and Acyl-CoA Synthetases FATP2, FATP4 and ACSL1 Increases Fatty Acid Uptake in Human Hepatoma Cells. *Int. J. Med. Sci.* 8 (7), 599–614. doi: 10.7150/ijms.8.599

Kraugerud, O. F., Penn, M., Storebakken, T., Refstie, S., Krogdahl, A. and Svihus, B. (2007). Nutrient Digestibilities and Gut Function in Atlantic Salmon (Salmo Salar) Fed Diets With Cellulose or non-Starch Polysaccharides From Soy. *Aquaculture* 273 (1), 96–107. doi: 10.1016/j.aquaculture.2007.09.013

Krogdahl, A., Bakke-McKellep, A. M. and Baeverfjord, G. (2003). Effects of Graded Levels of Standard Soybean Meal on Intestinal Structure, Mucosal Enzyme Activities, and Pancreatic Response in Atlantic Salmon (Salmo Salar L.). *Aquacult. Nutr.* 9 (6), 361–371. doi: 10.1046/j.1365-2095.2003.00264.x

Krogdahl, A., Gajardo, K., Kortner, T. M., Penn, M., Gu, M., Berge, G. M., et al. (2015). Soya Saponins Induce Enteritis in Atlantic Salmon (Salmo Salar L.). *J. Agric. Food Chem.* 63 (15), 3887–3902. doi: 10.1021/jf506242t

Krogdahl, Å., Hansen, A. K. G., Kortner, T. M., Björkhem, I., Krasnov, A., Berge, G. M., et al. (2020). Choline and Phosphatidylcholine, But Not Methionine, Cysteine, Taurine and Taurocholate, Eliminate Excessive Gut Mucosal Lipid Accumulation in Atlantic Salmon (Salmo Salar L). *Aquaculture* 528. 735552. doi: 10.1016/j.aquaculture.2020.735552

Krogdahl, Å., Nordrum, S., Sørensen, M., Brudeseth, L. and Røsjø, C. (1999). Effects of Diet Composition on Apparent Nutrient Absorption Along the Intestinal Tract and of Subsequent Fasting on Mucosal Disaccharidase Activities and Plasma Nutrient Concentration in Atlantic Salmon, Salmo Salar L. *Aquacult. Nutr.* 5 (2), 121–133. doi: 10.1046/j.1365-2095.1999.00095.x

Krogdahl, Å. and Sell, J. L. (1989). Influence of Age on Lipase, Amylase, and Protease Activities in Pancreatic Tissue and Intestinal Contents of Young Turkeys. *Poultry Sci.* 68 (11), 1561–1568. doi: 10.3382/ps.0681561

Krogdahl, Å., Sundby, A. and Olli, J. J. (2004). Atlantic Salmon (Salmo Salar) and Rainbow Trout (Oncorhynchus Mykiss) Digest and Metabolize Nutrients Differently. Effects of Water Salinity and Dietary Starch Level. *Aquaculture* 229 (1-4), 335–360. doi: 10.1016/S0044-8486(03)00396-X

Kruse-Jarres, J. D., Kaiser, C., Hafkenscheid, J. C. M., Hohenwallner, W., Stein, W., Bohner, J., et al. (1989). Evaluation of a New Alpha-Amylase Assay Using 4.6-Ethylidene-(G7)-1-4-Nitrophenyl-(G1)-Alpha-D-Maltoheptaoside as Substrate. *J. Clin. Chem. Clin. Biochem.* 27 (2), 103–113. doi: 10.1515/cclm.1989.27.2.103

Le Roy, T., Lecuyer, E., Chassaing, B., Rhimi, M., Lhomme, M., Boudebbouze, S., et al. (2019). The Intestinal Microbiota Regulates Host Cholesterol Homeostasis. *BMC Biol.* 17 (1). 1–18. doi: 10.1186/s12915-019-0715-8

Mitchell, R. W., On, N. H., Del Bigio, M. R., Miller, D. W. and Hatch, G. M. (2011). Fatty Acid Transport Protein Expression in Human Brain and Potential Role in Fatty Acid Transport Across Human Brain Microvessel Endothelial Cells. *J. Neurochem.* 117 (4), 735–746. doi: 10.1111/j.1471-4159.2011.07245.x

Muller, P. Y., Janovjak, H., Miserez, A. R. and Dobbie, Z. (2002). Processing of Gene Expression Data Generated by Quantitative Real-Time RT-PCR. *Biotechniques* 32 (6), 1372.

Munro, L. A. (2020). *"Scottish Fish Farm Production Survey 2019"* (Scotland: The Scottish Government). https://www.fiskeridir.no/English/Aquaculture/Statistics/Cleanerfish-Lumpfish-and-Wrasse

Norwegian Directorate of Fisheries. (2021). Available at: https://www.fiskeridir.no/English/Aquaculture/Statistics/Cleanerfish-Lumpfish-and-Wrasse

O'Fallon, J. V., Busboom, J. R., Nelson, M. L. and Gaskins, C. T. (2007). A Direct Method for Fatty Acid Methyl Ester Synthesis: Application to Wet Meat Tissues, Oils, and Feedstuffs. *J. Anim. Sci.* 85 (6), 1511–1521. doi: 10.2527/jas.2006-491

Olli, J. J., Hjelmeland, K. and Krogdahl, Å. (1994). Soybean Trypsin Inhibitors in Diets for Atlantic Salmon (Salmo Salar, L.) - Effects on Nutrient Digestibilities and Trypsin in Pyloric Ceca Homogenate and Intestinal Content. *Comp. Biochem. Physiol. a-Physiol.* 109 (4), 923–928. doi: 10.1016/0300-9629(94)90240-2

Olsen, R. E., Dragnes, B. T., Myklebust, R. and Ringo, E. (2003). Effect of Soybean Oil and Soybean Lecithin on Intestinal Lipid Composition and Lipid Droplet Accumulation of Rainbow Trout, Oncorhynchus Mykiss Walbaum. *Fish. Physiol. Biochem.* 29 (3), 181–192. doi: 10.1023/B:Fish.0000045708.67760.43

Olsen, R. E., Myklebust, R., Kaino, T. and Ringo, E. (1999). Lipid Digestibility and Ultrastructural Changes in the Enterocytes of Arctic Char (Salvelinus Alpinus L.) Fed Linseed Oil and Soybean Lecithin. *Fish. Physiol. Biochem.* 21 (1), 35–44. doi: 10.1023/A:1007726615889

Penn, M. H. (2011). ""Lipid Malabsorption in Atlantic Salmon – the Recurring Problem of Floating Feces,"" in *Annual Report on Fish Health* (Norwegian Institute of Veterinary Medicine).

Powell, A., Treasurer, J. W., Pooley, C. L., Keay, A. J., Lloyd, R., Imsland, A. K., et al. (2018). Use of Lumpfish for Sea-Lice Control in Salmon Farming: Challenges and Opportunities. *Rev. Aquacult.* 10 (3), 683–702. doi: 10.1111/raq.12194

Silva, F. C. P., Nicoli, J. R., Zambonino-Infante, J. L., Le Gall, M. M., Kaushik, S. and Gatesoupe, F. J. (2010). Influence of Partial Substitution of Dietary Fish Meal on the Activity of Digestive Enzymes in the Intestinal Brush Border Membrane of Gilthead Sea Bream, Sparus Aurata and Goldfish, Carassius Auratus. *Aquaculture* 306 (1-4), 233–237. doi: 10.1016/j.aquaculture.2010.05.018

Taylor, J. R. and Grosell, M. (2006). Feeding and Osmoregulation: Dual Function of the Marine Teleost Intestine. *J. Exp. Biol.* 209 (Pt 15), 2939–2951. doi: 10.1242/jeb.02342

Teitelbaum, J. E. and Walker, W. A. (2001). Review: The Role of Omega 3 Fatty Acids in Intestinal Inflammation. *J. Nutr. Biochem.* 12 (1), 21–32. doi: 10.1016/S0955-2863(00)00141-8

Wang, Y. B., Yang, X. J., Qin, D. K., Feng, Y., Guo, Y. M. and Yao, J. H. (2011). Effects of Eicosapentaenoic Acid and Docosahexaenoic Acid on Responses of LPS-Stimulated Intestinal B Lymphocytes From Broiler Chickens Studied *In Vitro*. *Eur. Food Res. Technol.* 233 (4), 677–683. doi: 10.1007/s00217-011-1554-1

Willora, F. P., Gronevik, B., Liu, C., Palihawadana, A., Sorensen, M. and Hagen, O. (2021). Total Replacement of Marine Oil by Rapeseed Oil in Plant Protein Rich Diets of Juvenile Lumpfish (Cyclopterus Lumpus): Effects on Growth Performance, Chemical and Fatty Acid Composition. *Aquacult. Rep.* 19. 100560. doi: 10.1016/j.aqrep.2020.100560

Willora, F. P., Nadanasabesan, N., Knutsen, H. R., Liu, C., Sorensen, M. and Hagen, O. (2020). Growth Performance, Fast Muscle Development and Chemical Composition of Juvenile Lumpfish (Cyclopterus Lumpus) Fed Diets Incorporating Soy and Pea Protein Concentrates. *Aquacult. Rep.* 17. 100352. doi: 10.1016/j.aqrep.2020.100352

Xu, S. S., Webb, S. E., Lau, T. C. K. and Cheng, S. H. (2018). Matrix Metalloproteinases (MMPs) Mediate Leukocyte Recruitment During the Inflammatory Phase of Zebrafish Heart Regeneration. *Sci. Rep.* 8. 1–14. doi: 10.1038/s41598-018-25490-w

Yang, X., Wang, Y. B., Yao, J. H. and Yang, X. J. (2016). Mechanistic Study of Signalling Pathways in Intestinal B Lymphocytes Using Eicosapentaenoic and Docosahexaenoic Acid in Broiler Chickens. *Food Agric. Immunol.* 27 (3), 329–337. doi: 10.1080/09540105.2015.1104652

Zhang, M., Sun, K. J., Wu, Y. J., Yang, Y., Tso, P. and Wu, Z. L. (2017). Interactions Between Intestinal Microbiota and Host Immune Response in Inflammatory Bowel Disease. *Front. Immunol.* 8. doi: 10.3389/fimmu.2017.00942

Zhang, Y. M., Xie, S. W., Guo, T. Y., Liu, Z. L., Fang, H. H., Zheng, L., et al. (2020). High Dietary Starch Inclusion Impairs Growth and Antioxidant Status, and Alters Liver Organization and Intestinal Microbiota in Largemouth Bass Micropterus Salmoides. *Aquacult. Nutr.* 26 (5), 1806–1821. doi: 10.1111/anu.13131

Zhao, W., Xie, J. J., Fang, H. H., Liu, Y. J., Tian, L. X. and Niu, J. (2020). Effects of Corn Starch Level on Growth Performance, Antioxidant Capacity, Gut Morphology and Intestinal Microflora of Juvenile Golden Pompano, Trachinotus Ovatus. *Aquaculture* 524. 735197. doi: 10.1016/j.aquaculture.2020.735197

Zhukova, K., and Stroganov, A. N. (2021). Anatomy of the digestive system of lumpfish (Cyclopterus lumpus) as an adaptation to puffing behavior. *The Anatomical Record*. doi: 10.1002/ar.24799

Zhu, L. Y., Nie, L., Zhu, G., Xiang, L. X. and Shao, J. Z. (2013). Advances in Research of Fish Immune-Relevant Genes: A Comparative Overview of Innate and Adaptive Immunity in Teleosts. *Dev. Comp. Immunol.* 39 (1-2), 39–62. doi: 10.1016/j.dci.2012.04.001

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: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Marine Science

TYPE Original Research
PUBLISHED 28 July 2022
DOI 10.3389/fmars.2022.960746

OPEN ACCESS

EDITED BY
Mohamed Salah Azaza,
National Institute of Marine Science and Technology, Tunisia

REVIEWED BY
Saichiro Yokoyama,
Kagoshima University, Japan
Neila Hamza,
Institut National des Sciences et Technologies de la Mer, Tunisia

*CORRESPONDENCE
Carmen Navarro-Guillén
carmen.navarro@csic.es

SPECIALTY SECTION
This article was submitted to Aquatic Physiology, a section of the journal Frontiers in Marine Science

RECEIVED 03 June 2022
ACCEPTED 05 July 2022
PUBLISHED 28 July 2022

CITATION
Navarro-Guillén C, Yúfera M and Perera E (2022) Biochemical features and modulation of digestive enzymes by environmental temperature in the greater amberjack, *Seriola dumerili*. *Front. Mar. Sci.* 9:960746. doi: 10.3389/fmars.2022.960746

Biochemical features and modulation of digestive enzymes by environmental temperature in the greater amberjack, *Seriola dumerili*

Carmen Navarro-Guillén*, Manuel Yúfera and Erick Perera

Dept. Biología Marina y Acuicultura, Instituto de Ciencias Marinas de Andalucia (ICMAN-CSIC), Campus Universitatio Rio San Pedro, Puerto Real, Spain

The study of fish digestive biochemistry is essential to understand factors that affect the net efficiency of food transformation and growth, and therefore aquaculture profitability. The aim of the present study was to assess the activity and functional characteristics of key digestive enzymes in juveniles of greater amberjack (*Seriola dumerili*), as well as the possible modulation of their relative importance by water temperature. For that, a combination of biochemical assays and substrate-SDS-PAGE were used. Under physiological conditions pepsin activity was negligible. Chymotrypsin was the most active enzyme in the digestive tract of the greater amberjack, while lipase was the enzyme with lower activity, though both enzymes in addition to trypsin were responsive to water temperature as revealed by discriminant analysis. *Seriola dumerili* showed to have pH-sensitive and, except for chymotrypsin, thermally robust proteases. Inhibition assays showed the major importance of serine proteases and revealed inverse trypsin and chymotrypsin responses to environmental temperature, with higher trypsin contribution in 26°C-fish while higher chymotrypsin contribution in 18°C-fish. Zymograms revealed three isotrypsin and three isochymotrypsin enzymes, with no variation in the presence of particular isoforms among rearing temperatures. However, they confirmed the role of chymotrypsin activity in providing digestive plasticity, with one of the isoforms being more active at lower temperatures. Thus, results indicate that variation in the relative contribution of chymotrypsin isoenzymes to a particular environmental temperature occurs due to different physic-chemical features of isoforms as a source of functional flexibility. This study assessed for the first time the effects of rearing temperature on greater amberjack digestive enzymes, increasing the knowledge on its digestive biochemistry, and aiding in the improvement of management practices for this species industrialization.

KEYWORDS

chymotrypsin, digestive enzymes, enzyme characterization, functional flexibility, isoforms, *Seriola dumerili*, water temperature

Introduction

Temperature is likely the major physical environmental factor affecting the fish physiology through its influence on overall metabolism and energy balance, including significant effects on the first steps of energy acquisition such as ingestion rate, activity of digestive enzymes, digestibility, absorption and evacuation rate (Volkoff and Rønnestad, 2020). Therefore, temperature is one of the main factors driving evolutionary adaptations (Nitzan et al., 2019). The tissue-specific responses to stressful temperatures have been addressed in different fish species such as zebrafish (*Danio rerio*) and tilapia (*Oreochromis niloticus*) (Hu et al., 2016). Within species, differences in tolerance to low temperature have been also revealed, involving metabolic pathways such as glycolysis and gluconeogenesis in the gills, and biosynthesis of aminoacids in the liver (Nitzan et al., 2019).

Within the temperature tolerance range, many studies have been also performed on the effects of temperature on growth, physiological responses, and health of farmed fish, including the effects of seasonal thermal changes (Pelusio et al., 2021). However, for relatively novel species for the aquaculture industry this information is rather limited. That is the case of the greater amberjack, *Seriola dumerili*, a pelagic teleost with great interest for the diversification of marine fish aquaculture due to its high grow rates and flesh quality (Sicuro and Luzzana, 2016; Navarro-Guillén et al., 2019; Monge-Ortiz et al., 2020). There is scarce information related to temperature effects on the physiology of *S. dumerili*. The optimal rearing temperature for the species has been determined to be 26°C, with a valid range down to 22°C without affecting growth or feed utilization. The minimum temperature was set at 17°C, in which deleterious effects on growth and shape were observed (Fernández-Montero et al., 2018; Fernández-Montero et al., 2020). Nevertheless, fry and juveniles seeded in cages for on-growing often face seawater temperatures which, in the case of the Mediterranean Sea, range between 15-28°C, approximately. This may have seasonal effects on feeding and growth efficiencies, with relevant economic and environmental impacts.

The study of fish digestive biochemistry is essential to understand one of the key factors affecting the net efficiency of food utilization and growth, and therefore aquaculture profitability. In this context, the types and functional features of the digestive enzymes involved in the hydrolysis of the main nutrients are basic information to understand digestive capacities of species of interest. To the best of our knowledge, digestive enzymes in *Seriola dumerili* have been not characterized, with the exception of the study published by Oliveira et al. (2017) analyzing trypsin and chymotrypsin activities in digestive viscera for industrial purposes. In related species, information is only available for trypsin in the Japanese amberjack (*Seriola quinqueradiata*) (Kishimura et al., 2006). The relationship between digestive enzymes and temperature in *Seriola* spp. have been also poorly studied, despite this allow a better understanding of its phenotypic/digestive flexibility in response to an environmental factor of ecological and aquaculture relevance. In the Japanese amberjack and yellowtail kingfish (*Seriola lalandi*), intestinal enzymes activity levels were higher at lower temperatures, possibly in response to the increased gut transit time at reduced water temperatures (Kofuji et al., 2005; Miegel et al., 2010).

Moreover, while most studies on digestive biochemistry of fish have reported several isoenzymes for different enzymes, only a handful of studies have investigated the adaptive value of this isoenzyme richness. The occurrence of multiple isoforms for digestive proteases is thought to provide an adaptive advantage for insects feeding on plants containing inhibitors (Díaz-Mendoza et al., 2005), for crustaceans feeding on a broad diet (Saborowski et al., 2012; Perera et al., 2015), and as an adaptation to environmental temperature in only few fish species (Gelman et al., 2008; Sandholt et al., 2019). In the Atlantic salmon (*Salmo salar*), trypsin isoenzymes have been thoroughly studied (Torrissen, 1987; Rungruangsak-Torrissen, 2012) and related to differences in the absorption and transport of free amino acids (Torrissen et al., 1994; Rungruangsak-Torrissen and Male, 2000). However, digestive and growth efficiencies are not always related to trypsin-specific activity or isoenzyme composition, but to the activity ratio of trypsin to chymotrypsin (Rungruangsak-Torrissen, 2012).

Therefore, the specific aims of the present study were to assess *i*) the activity and functional characteristics of key digestive enzymes in greater amberjack juveniles (i.e., pepsin, trypsin, chymotrypsin, leucine aminopeptidase and lipase), *ii*) their possible modulation by water temperature, and *iii*) the adaptive value of protease isoenzymes with respect to environmental temperature. The overall objective is to aid in the improvement of management practices for this species industrialization.

Materials and methods

Fish rearing and sampling

Greater amberjack juveniles were supplied by Futuna Blue España S.L. (Puerto de Santa María, Cádiz, Spain) and transferred to the Institute of Marine Sciences of Andalusia (Puerto Real, Cádiz, Spain) facilities. Juveniles were randomly distributed in three 1 m^3 cylindroconical tanks (8 fish $tank^{-1}$), each tank belonging to an independent recirculating aquaculture system (RAS), and acclimated to final experimental temperatures during one week. During the period of acclimatation water temperature in one tank decreased at a rate of 0.5°C per day up to 18°C, in other tank it increased at a rate of 0.5°C per day up to 26°C, while

in the remaining tank was kept constant at 22°C. Acclimated juveniles with 60.9 ± 19.6 g of mean wet body weight were reared under a light/dark cycle (12 h light: 12 h dark) at the three temperatures. Water salinity was 34.1 ± 1.2 g L^{-1}, oxygen level was above 90%, pH was8.0 ± 0.1 and NH_4< 0.25 mg L^{-1}. The fish were fed a commercial diet (Skretting, Burgos, Spain) to satiety by hand five times a day (09:00, 10:30, 12:30, 14:00 and 16:00h, local time). After 2 weeks under these conditions, fish were sampled at 16:00h to ensure all fish were in the same feeding status (chyme present in all digestive tract sections), as previous results revealed a slowing down of intestinal transit time at low water temperatures. During sampling, fish were euthanized with overdose of 2-phenoxyethanol (Sigma-Aldrich, Merck, Germany) and the whole digestive tract was removed and separated in sections: stomach, pyloric caeca, and anterior, middle and posterior intestine (Figure 1). Dissected digestive tracts were stored at -20°C until analysis.

All experimental procedures complied with the Guidelines of the European Union Council (2010/63/EU) for the use and experimentation of laboratory animals and were reviewed and approved by the Spanish National Research Council (CSIC) bioethical committee for project THERMODIGEST (RTI2018-096134-B-I00).

Preparation of extracts and enzymatic assays

Samples were mechanically homogenized in distilled water (1:5 v:w) using an Ultra-Turrax® Homogenizer (IKA®-Werke, Germany) and centrifuged for 20 min at 10000 g at 4°C (Eppendorf 5417R, Germany). The supernatants from stomach and pyloric caeca samples were used as source of enzymes for pepsin and alkaline enzymes characterization, respectively, for which enzymatic assays were performed at 37°C. However, to evaluate the distribution of digestive enzyme activity through the digestive tract and the effect of temperature on activity levels, the supernatants from all digestive sections were used and the enzymatic reactions were performed at the temperature the fish providing each sample was adapted to (18, 22 or 26°C).

Pepsin activity in stomach extracts was determined by the method of Anson (1938): 15 µL of extract was mixed to 0.8 mL of 0.5% acid denatured bovine hemoglobin diluted in 0.2 M HCl-glycine buffer pH 3. After incubation for 30 min, the enzymatic reaction was stopped by adding 0.3 mL of 20% trichloroacetic acid (TCA), cooled at 4°C for 15 min and then centrifuged at 8000 g for 15 min. The absorbance of resulting supernatant was measured at 280 nm. Blanks were constructed by adding the enzyme extracts just after TCA to the reaction mixture. A standard curve was prepared with increasing concentrations of tyrosine to determine the molar extinction coefficient of tyrosine at 280 nm. One unit of activity was defined as the amount of enzyme required to produce 1 µg of tyrosine per minute.

Trypsin and chymotrypsin assays were performed as described before (Perera et al., 2008). Trypsin activity was assayed using 1.25 mM N-benzoyl-DL-arginine*p*-nitroanilide (BA*p*NA) in 200 mM Tris–HCl, 20 mM $CaCl_2$, 0.3M NaCl, pH 7.5 (referred hence-forth as the assay buffer). Chymotrypsin activity was measured with 0.1 mM Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide (SA*p*NA) in the same buffer. Substrate stock solutions of BA*p*NA and SA*p*NA were prepared in DMSO and diluted up to working concentration with buffer prior the assays. Lipase and leucine aminopeptidase were measured as before (Perera and Yúfera, 2017). Lipase activity was measured using 1 mM 4-nitrophenyl palmitate (4NPP) in assay buffer containing 0.3% Triton X-100. Substrate stock solution was prepared in 1:4 v:v acetonitrile/isopropanol. Leucine aminopeptidase activity was assessed by the hydrolysis of 0.5 mM L-Leucine-*p*-nitroanilide (Leu*p*NA) in assay buffer. Substrate stock solution was prepared in methanol. For all these alkaline enzymes,

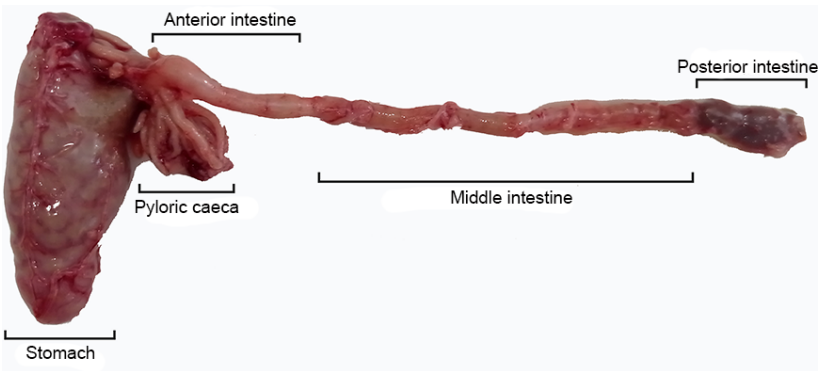


FIGURE 1
Digestive tract of a *Seriola dumerili* juvenile indicating the dissected regions for digestive enzymes analysis.

enzymatic reactions started in 96-well microplates by the addition of 10 µL of the enzyme extract to 200 µL of the respective substrate in buffer, and then, the liberation of *p*-nitroaniline and *p*-nitrophenol were kinetically followed at 405 nm in a microplate reader (ELx808 BioTek, BioTek Instrument, Inc.).

Effect of ionic strength and calcium on enzymatic activity

The effect of NaCl and $CaCl_2$ on the activity of the digestive enzymes was examined by using different concentrations of NaCl (0, 0.1, 0.5, 1 and 1.5 M) and $CaCl_2$ (0, 20, 50, 100 and 200mM) in the assay buffer. The optimum concentrations of NaCl or $CaCl_2$ were always used in subsequent assays.

Effects of pH and temperature on the activity and stability of digestive enzymes

The effect of pH on enzyme activities was evaluated using the following buffer solutions: 50 mM glycine-HCl (pH 2-4), 50 mM sodium cacodylate (pH 5-6), 50 mM Tris-HCl (pH 7-8) and 50 mM glycine-NaOH (pH 9-10). Excluding the assay buffer, enzyme activities were measured as described above. Optimal temperature for each enzyme was determined by evaluating the activity of the enzymes at the optimum pH over the range 10°C to 80°C. The effects of pH and temperature on the stability of the digestive enzymes were assessed by pre-incubating the enzyme extracts at different pH and temperature for 15, 30 and 60 min prior to the enzyme assays at optimum pH and 37°C. The stability results were expressed as residual activity (% of the maximum activity).

Effect of inhibitors on proteolytic enzymes

Classes of alkaline proteases in the digestive tract of fish, using the pyloric caeca as the source of enzymes, were characterized by assessing the effect of protease inhibitors on caseinolytic activity (see section 3.5. Effect of protease inhibitors on caseinolytic activity). Inhibitors employed were soybean trypsin inhibitor (SBTI) for serine-proteases, EDTA for metalloproteases, Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK) for trypsin, and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) for chymotrypsin. In addition, the specific inhibitors TLCK and TPCK were used to inhibit trypsin and chymotrypsin activities on BA*p*NA and SA*p*NA, respectively. For the assays, enzymatic extracts were incubated with the inhibitors for 60 min at 37°C and then assayed using the appropriate substrate. A preliminary trial revealed no increase on inhibitory capacity at concentrations higher than those used in this work. Enzyme extracts incubated with buffer instead of inhibitors were used as controls and referred to as 100% of enzyme activity.

Zymograms of digestive proteases

Substrate-SDS-PAGE (5% stacking gel, 13% separating gel) was used to determine the composition of proteases in the digestive tract (Perera et al., 2008). Gels were run at 30 mA constant current and 4°C in a vertical electrophoresis device (Mini-PROTEAN 3, Bio-Rad, USA). The gel was then immersed in a 3% casein solution for 30 min at 4°C to allow the diffusion of casein into the gel and incubated at 37°C for 45 min to allow the proteases to digest gel-embedded casein. The gel was washed with tap water and stained with 0.1% Coomassie Brilliant Blue in 45% methanol with 10% acetic acid and finally distained in 10% methanol with 10% acetic acid solution. The gel stains blue because of the presence of casein except in areas with proteolytic activity. Unreduced molecular weight markers (14.4 - 116kDa, Thermo Scientific, USA) were used to determine apparent molecular weights. For protease classification, the same substrate-SDS-PAGE procedure was used, but enzyme extracts were incubated with inhibitors for 60 min at 37°C before electrophoresis. Inhibitors used were TLCK, TPCK, PMSF, SBTI and EDTA. The absence of bands in the presence of specific inhibitors indicates a specific type of protease. Given the limited efficiency of some inhibitors to completely abrogate the activity of fish enzymes, reduced intensity of band was also taken as indicative of type of protease, as the same amount of protein were always loaded into the gel (4.5 µg of protein).

Q_{10} value and activation energy (E_a)

To gain more insights on the effects of temperature on digestive efficiency, and the putative contribution of different isoenzymes in response to differences in rearing temperature, the thermal sensitivity (Q_{10} value) and activation energy (E_a) were calculated. Q_{10} values were determined using the van 't Hoff equation as follows:

$$Q10 = \left(\frac{A2}{A1}\right)^{\frac{10}{T2-T1}}$$

where A1 and A2 are activity levels at temperatures T1 and T2 (when T1< T2). The activation energy (E_a) was obtained from slopes in Arrhenius plots:

$$slope = -\frac{Ea}{R}$$

where R is the gas constant (8.314 J K^{-1} mol^{-1}). The values were calculated for the range from 30 to 50°C for Q_{10} and from

30 to 60°C for E_a of lipase and pepsin and from 10 to 60°C for E_a of trypsin, chymotrypsin and leucine aminopeptidase.

Statistical analyses

All descriptive statistics are expressed as mean ± standard deviation of the mean (SD). For each enzyme, statistical differences in total activity among temperatures were assessed through one-way ANOVA and, whenever significant differences were identified, means were compared by the *Post hoc* multiple comparisons Tukey's test. Differences among temperatures were considered significant at $P< 0.05$. Before analyses, the ANOVA assumptions of normality and homogeneity of variance were tested using the Shapiro-Wilk and Levene's tests, respectively.

A forward stepwise discriminant analysis using the activity of all enzymes studied at the different regions of the digestive tract was performed. This analysis was carried out to understand the combination of variables that better explain the differences in digestive biochemistry among fish reared at different temperatures. For the analysis, it was considered F to enter as 0.01, F to remove at 0.0, and tolerance of 0.01. After significant functions development, the relative importance of the original variables was gauged by standardized values. All analyses were performed with SPSS 26 software (IBM, New York, USA).

Results

Activity and distribution of digestive enzymes

Total enzyme secretion was assessed by performing the analyses at the optimal pH for each enzyme and 37°C. Chymotrypsin was the most abundant enzyme and lipase the enzyme with the lowest activity (Figure 2A). Significant differences in total activity levels were found for all enzymes among the rearing temperatures, with fish reared at 22 and 26°C showing higher activity levels than at 18°C for all intestinal enzymes except for chymotrypsin, for which activity levels were only statistically different between fish reared at 26 and 18°C (Figure 2A). Total pepsin activity levels were higher in fish reared at 22 and 26°C than at 18°C (Supplementary Table 1). Under real conditions of temperature (18, 22 or 26°C) and physiological pH, the general activity pattern in pyloric caeca was similar but activity levels much lower (Supplementary Table 1). This was particularly noticeable for pepsin and lipase. Lipase activity was undetectable in extracts from fish reared at 22 and 18°C, and pepsin activity was not detected in any extract, independently of rearing temperature (Supplementary Table 1).

Extracts from fish reared at 22°C were chosen to describe the overall distribution of activities along the digestive tract. At the moment of sampling (after last meal), all digestive enzymes analyzed were more abundant in the middle and posterior intestine (Figure 2B). Interestingly, the transit of the enzymes along the tract was not identical, as most chymotrypsin activity was already in the posterior intestine, while for trypsin a significant amount of activity was still in the middle intestine at this time (Figure 2B).

Extracts from fish reared at all the three temperatures (18, 22, and 26°C) were further used to depict the effect of rearing temperature on the relative relevance of the enzymes at particular sections of the tract, by performing a discriminant analysis. Two significant discriminant functions could be developed from the activity of digestive enzymes in all the analyzed sections. These functions collectively accounted for 100% of the total variance (Table 1). The activity of lipase in pyloric caeca and posterior intestine, total lipase activity, trypsin in pyloric caeca, and chymotrypsin in almost all segments of the tract were the variables with the highest relevance in the first discriminant function (Table 1), which accounted for 78.3% of the total variance (Table 1). In this function, 18/22°C and 26°C

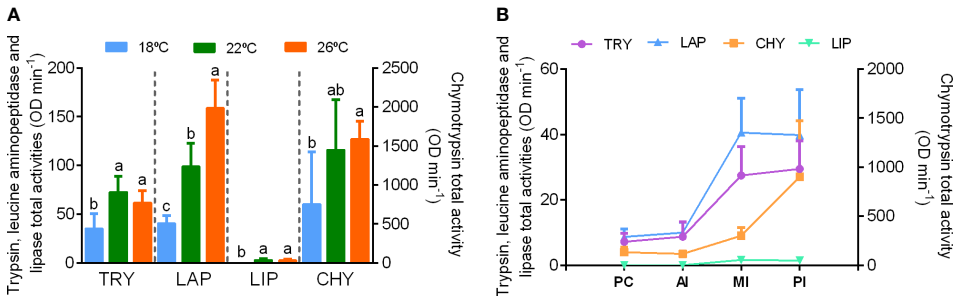


FIGURE 2
(A) Total activity of digestive enzymes in the intestinal tract of *Seriola dumerili* juveniles reared at 18 (blue), 22 (green) and 26°C (orange). **(B)** Total activity of digestive enzymes present in the gut sections of *Seriola dumerili* reared at 22°C. PC – pyloric caeca, AI – anterior intestine, MI – middle intestine, PI – posterior intestine. Chymotrypsin activity is represented in the right Y axis. TRY, trypsin; CHY, chymotrypsin; LAP, leucine aminopeptidase; LIP, lipase. Data is presented as means ± SD (n = 8). Different letters indicate significant differences for each enzyme among rearing temperatures ($P< 0.05$).

adapted fish showed opposed central values (Figure 3, axis x). However, the second discriminant function, although explained only 21.7% of the total variance, discriminated among all the three temperatures (Figure 3, axis y), with chymotrypsin in the pyloric caeca being by far the variable with the highest relevance, although trypsin in the pyloric caeca and chymotrypsin in the middle intestine also significantly contributed (Table 1). Conversely, their activity in other sections of the tract and their total activities had poor importance in this function, as well as the activity of the other enzymes studied.

Optimal conditions for activity

Optimal concentrations of NaCl and $CaCl_2$ for the activity of the enzymes studied were assessed (Table 2). Notably, serine proteases such as trypsin and chymotrypsin required 10 times more $CaCl_2$ for optimal activity than leucine aminopeptidase and lipase (Table 2). Optimal pH and temperature for the activity of the enzymes studied were similar among fishes adapted to different temperatures. Optimal pH was 2.5 for pepsin, 10 for trypsin, 9 for chymotrypsin, 7.5-8 for leucine aminopeptidase, and 11 for lipase. Optimal temperature was 60-70°C for pepsin, greater than or equal to 70°C for trypsin and chymotrypsin, 50-60°C for leucine aminopeptidase, and 65-75°C for lipase (Table 2). However, slight differences were noted such as higher optimal temperature for pepsin and leucine aminopeptidase in extracts coming from fish adapted to the higher temperature (i.e., 26°C), and higher optimal temperature for lipase in extracts coming from fish adapted to 22°C (Table 2).

pH stability of digestive enzymes

Enzymatic activities shared a similar pattern of pH stability, being affected by all pH values assayed even after short incubation time (Figure 4). The most notable difference among enzymes is that lipase activity was less stable facing all pH assayed, losing more than 50% of activity after 15 min at physiological pH (i.e., pH8) and with pH 3 completely abrogating the activity after this incubation time (Figure 4H). Pepsin activity was stable for 1 h in the range of pH 3 to 8 (Figure 4I).

Regarding the effect of rearing temperatures on pH stability of fish enzymes, differences were observed only for trypsin and chymotrypsin. For instance, while for all fish most alkaline enzymes studied (trypsin, chymotrypsin, leucine aminopeptidase, and lipase) lost about a 50%of activity after 15 min at all pH values assayed (pH 3, 5, 8, 11), trypsin activity was stable at pH 11 for 1h only in extracts from 18°C-adapted fish (Figure 4A). In addition,

TABLE 1 (A) Summary of the canonical discrimination analysis for discriminant functions used to identified differences in digestive biochemistry among *Seriola dumerili* juveniles reared at different temperatures. (B) Standardized function coefficients for each of the significant discriminant functions used to identify differences in digestive biochemistry.

(A)

Function	Eigenvalue	% of variance	Cumulative %	Canonical correlation
1	496.527	78.3	78.3	0.999
2	137.376	21.7	100.0	0.996

(B)

	Function	
	1	2
TRY_PC	-3.194	3.711
TRY_AI	-0.674	-0.302
TRY_MI	0.389	-1.228
TRY_PI	-0.654	1.889
CHY_PC	3.349	-6.363
CHY_AI	3.796	0.837
CHY_MI	-4.285	3.827
CHY_PI	-0.177	-1.230
LEU_PC	2.403	-0.921
LEU_MI	1.771	0.886
LEU_PI	1.716	1.028
LEU_total	-0.593	-2.101
LIP_PC	3.849	-0.444
LIP_AI	1.434	0.268
LIP_PI	-3.652	1.191
LIP_total	3.163	0.59

10.3389/fmars.2022.960746

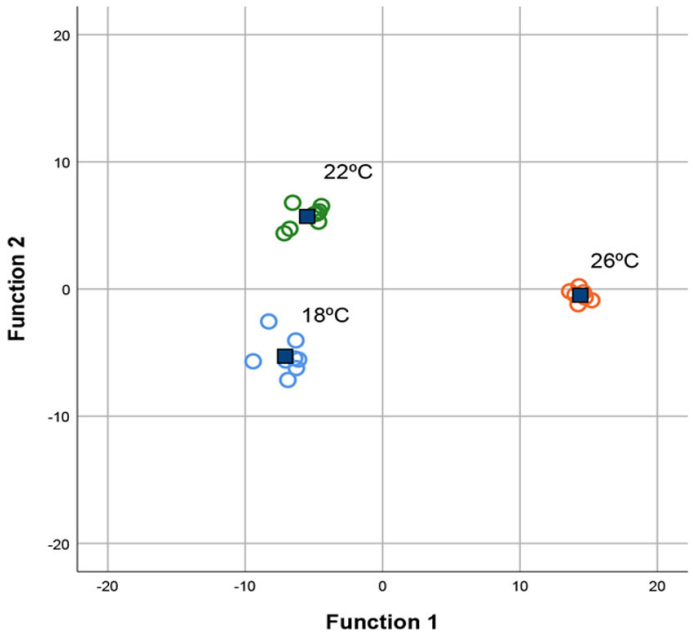


FIGURE 3
Plot of the two axes from the forward stepwise discriminant function analysis of digestive enzymes total activities in *Seriola dumerili* juveniles reared at 18 (blue), 22 (green) and 26°C (orange). Group centroids are represented by blue squares.

TABLE 2 Optimal pH, temperature, ionic strength and calcium for digestive enzymes activities in the greater amberjack (*Seriola dumerili*) adapted to different rearing temperatures (18, 22 and 26°C).

Enzyme	Optimal pH	Optimal T (°C)	Optimal NaCl (M)	Optimal $CaCl_2$ (M)
Pepsin			–	–
18°C	2.5	60		
22°C	2.5	60		
26°C	2.5	70		
Trypsin			0.1	0.2
18°C	10	≥70		
22°C	10	≥70		
26°C	10	≥70		
Chymotrypsin			1	0.2
18°C	9	≥70		
22°C	9	≥70		
26°C	9	≥70		
Leucine aminopeptidase			0.1	0.02
18°C	8	50		
22°C	7.5	50		
26°C	7.5	60		
Lipase			0.5	0.05
18°C	11	65		
22°C	11	75		
26°C	11	65		

trypsin activity in extracts from 26°C-adapted fish was the most affected by pH 3, losing about 80% of activity after 30 min, while in extracts from fish from the other two water temperatures, trypsin activity remained above 40% at this time (Figures 4A-C). Also, chymotrypsin activity was more stable in the range pH 5 to 11 in extracts coming from 26°C-adapted fish than in those from the other two groups (Figure 4F). Conversely, stability patterns of leucine aminopeptidase, lipase, and pepsin activities were nearly identical in fish adapted to the three experimental temperatures (Figures 4G-I).

Temperature stability of digestive enzymes

Differences were evident in the thermal stability of studied enzymes (Figure 5). The most stable enzyme was trypsin, whose activity was stable up to 60°C for at least 1 h (Figure 5G). The next more stable enzyme was leucine aminopeptidase, which was fairly stable at 40°C for 1 h and kept more than 50% of activity after this period at 60°C (Figures 5A-C). Pepsin was also stable at 40°C for 1 h, although its activity decreased more than 50% after 30 min at 60°C (Figure 5I). Conversely, chymotrypsin activity was thermally sensitive, losing 20% of activity at 40°C and more than 80% of activity at 60°C after just 15 min (Figure 5H). However, the most thermally sensitive enzyme was lipase; 60°C was able to completely abrogate activity after just 15 min (Figures 5D–F).

Regarding the influence of rearing temperature on the thermal stability of fish digestive enzymes, variations for leucine aminopeptidase and lipase were observed. Leucine aminopeptidase activity was more stable in extracts from fish adapted to 22°C (Figure 5B), retaining 80% of activity after 1 h at 60°C, whereas only 40-60% of activity was maintained under

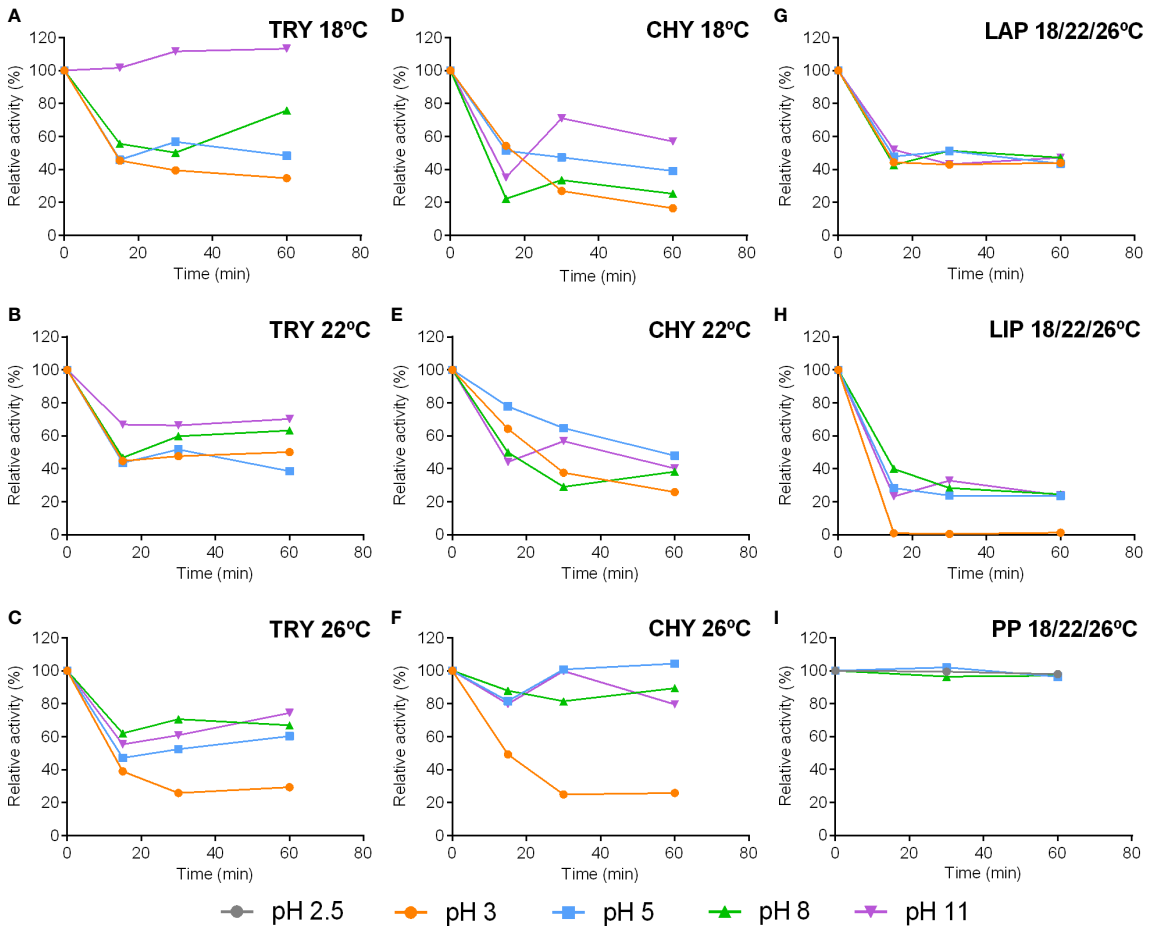


FIGURE 4
Stability of trypsin (TRY), chymotrypsin (CHY), leucine aminopeptidase (LAP), lipase (LIP) and pepsin (PP) activities from pyloric caeca and stomach of *S. dumerili* incubated at different pH. Leucine aminopeptidase, lipase and pepsin graphs represent mean values from all treatments. Individual graphs of pH stability for each enzyme in extracts coming from the different rearing temperatures are shown in the Supplementary Figure 1.

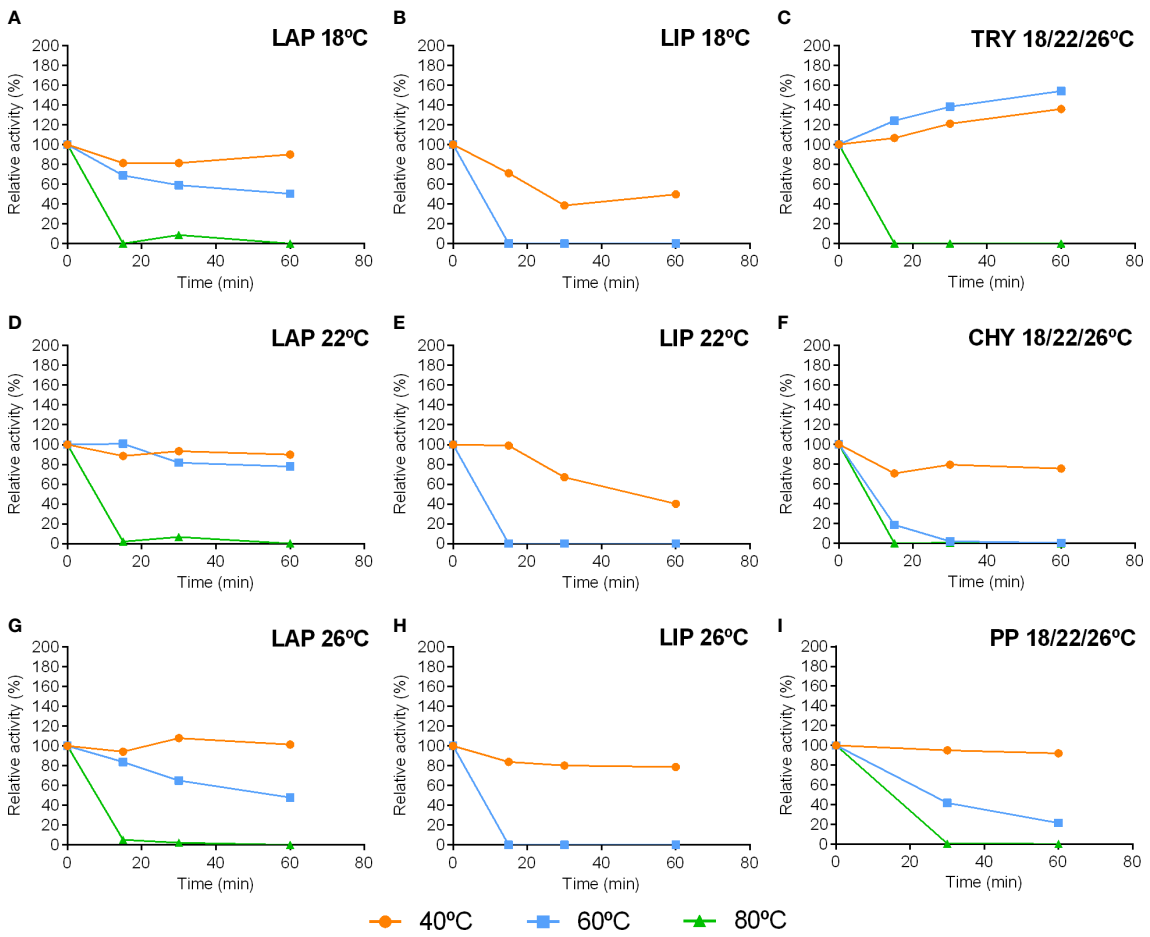


FIGURE 5
Stability of leucine aminopeptidase (LAP), lipase (LIP), trypsin (TRY), chymotrypsin (CHY) and pepsin (PP) activities from pyloric caeca and stomach of *S. dumerili* incubated at different temperatures. Trypsin, chymotrypsin and pepsin graphs represent mean values from all treatments. Individual graphs of thermal stability for each enzyme in extracts coming from the different rearing temperatures are shown in the Supplementary Figure 2.

these conditions in extracts from fish from 18°C or 26°C (Figures 5A, C). Likewise, lipase activity in extracts from 26°C-adapted fish was more stable than that from fish maintained at lower temperatures (Figure 5F). Conversely, thermal stability pattern of trypsin, chymotrypsin, and pepsin activities was nearly identical among extracts from fish adapted to the different temperatures (Figures 5G–I).

Effect of protease inhibitors on caseinolytic activity

Given the key role of serine proteases in protein digestion, classes of proteases in the extracts were further assessed by using specific protease inhibitors to inhibit casein hydrolysis. The serine protease inhibitor SBTI abrogated 37-47% of caseinolytic activity in the extracts (Table 3). The trypsin inhibitor TLCK inhibited 20-38% of the activity, while 13-28% of proteolysis was abrogated by the chymotrypsin inhibitor TPCK (Table 3). Interestingly, the chelating agents EDTA inhibited caseinolytic activity by 17-23% (Table 3).

Regarding differences among fish rearing temperatures on the relative contribution of the different types of proteolytic enzymes to overall protein digestion, we found a reverse trend with trypsin and chymotrypsin inhibitors (Table 3). Concomitantly with water temperature increase, inhibition of caseinolytic activity by TLCK increased while inhibition by TPCK decreased (Table 3).

Effect of protease inhibitors on trypsin and chymotrypsin activities

To further evaluate the relative contribution of trypsin and chymotrypsin in fish reared at different temperatures, we used

TABLE 3 Effect of proteinase inhibitors on caseinolytic activity of pyloric caeca of *S. dumerili* adapted to different rearing temperatures (18, 22 and 26°C).

Target	Protease inhibitor	Concentration	T (°C)	% of inhibition
Serine proteases	STBI	50 μM		
			18°C	37.16
			22°C	42.30
			26°C	46.79
Trypsin	TLCK	0.5 mM		
			18°C	19.62
			22°C	25.03
			26°C	38.15
Chymotrypsin	TPCK	0.3 mM		
			18°C	27.83
			22°C	21.46
			26°C	12.90
Metallo-proteases	EDTA	50 mM		
			18°C	17.11
			22°C	21.51
			26°C	22.93

the same inhibitors (TLCK and TPCK) to inhibit trypsin and chymotrypsin activities, this time through the use of specific substrates (BA*p*NA and SA*p*NA, respectively). TLCK inhibited 80% of trypsin activity on BA*p*NA irrespective of the thermal origin of fish (Figure 6A), whereas TPCK inhibition pattern was reverse to water temperature. In extracts coming from 18°C-adapted fish, TPCK inhibited 80% of chymotrypsin activity, while only 40% of the activity was inhibited in fish kept at the highest temperature (26°C) (Figure 6B).

Enzyme zymograms

Zymograms revealed 6 active zones of caseinolytic activity (Figure 7A, control lane). All bands were inhibited by SBTI and EDTA, indicating they are serine proteases depending on divalent cations, presumably calcium, for their activity (Figure 7A). Conversely, PMSF, which is other serine protease inhibitor, did not inhibit activity bands (Figure 7A). Enzymes with the lowest and the highest electrophoretic mobility, and that of about 30 kDa were total or partially inhibited by TLCK in addition to SBTI, revealing that these bands correspond to trypsin enzymes (Figure 7A). Three very active bands of similar molecular weight (around 18 kDa) were inhibited by SBTI but not by TLCK, thus being more likely chymotrypsins, although not affected by TPCK (Figure 7A).

We further studied the variations in isoenzymes composition and/or activity in extracts coming from fish adapted to different temperatures (Figure 7B). When incubation was performed at a standard high temperature (37°C), no differences in activity or

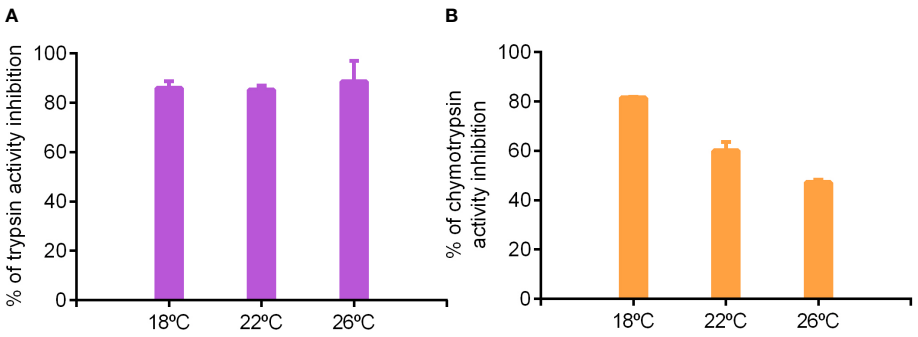


FIGURE 6
Effect of TLCK **(A)** and TPCK **(B)** inhibitors on trypsin and chymotrypsin activities, respectively, of pyloric caeca of *S. dumerili* adapted to different rearing temperatures (18, 22 and 26°C).

isoenzymes composition were noticed among extracts from fish adapted to the different temperatures (Figure 7B). However, when incubations were done at the temperature in which the fish were reared, clear differences were observed. Activity levels, inferred from band intensity, decreased as incubation temperature did for all bands except for the chymotrypsin of the higher electrophoretic mobility. This band exhibited a high activity at 18°C, similar to that observed when incubated at higher temperatures (Figure 7B), revealing differences in temperature dependence for activity among chymotrypsin isoforms.

Q_{10} value and activation energy (E_a)

Q_{10} values were similar for all enzymes studied and no major differences were observed among extracts coming from fish reared at different temperatures (Table 4). No break point in the Arrhenius plots for the studied enzymes was found. However, values of E_a for trypsin, chymotrypsin and leucine aminopeptidase activities decreased as fish rearing temperature increased (Table 4). For pepsin and lipase E_a values were more variable, with the maximum values observed in fish at 18 for

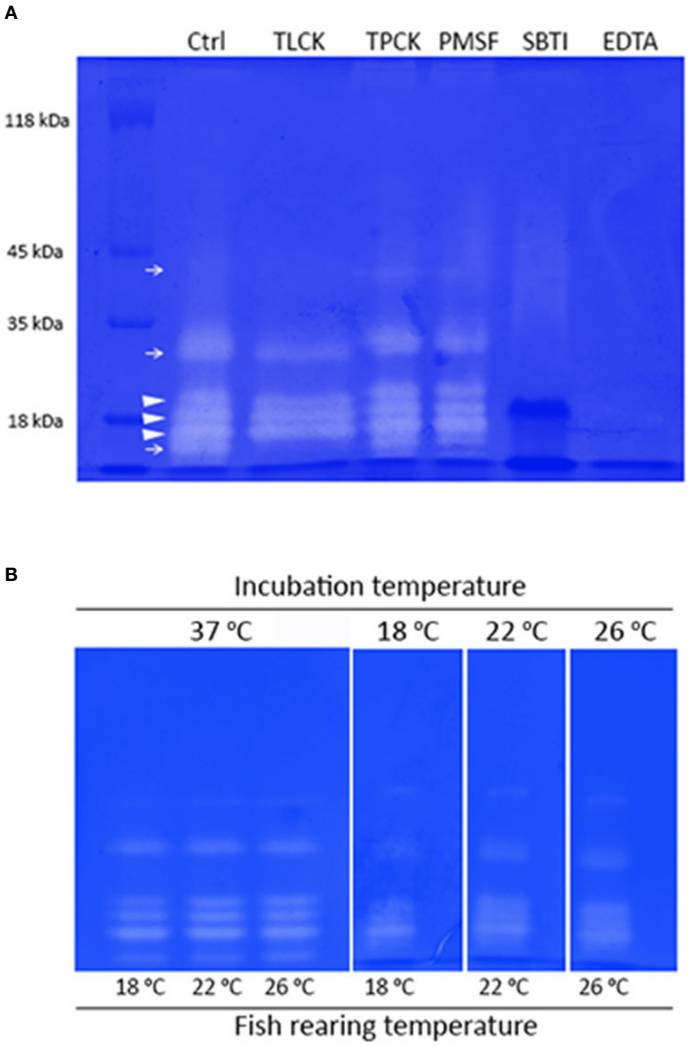


FIGURE 7
(A) 13% substrate SDS-PAGE showing caseinolytic activity bands in a pool containing pyloric caeca extracts of *S. dumerili* adapted to different rearing temperatures (18, 22 and 26°C) (Ctrl) and inhibition by specific inhibitors for trypsin (TLCK), chymotrypsin (TPCK), serine proteinases (PMSF and SBTI) and metalloproteases (EDTA). Type of proteases is indicated in the control lane as follows: trypsin like proteinases (→) and chymotrypsin like proteinases (►). **(B)** 13% substrate SDS-PAGE showing caseinolytic activity bands in independent pools of pyloric caeca extracts of *S. dumerili* from each rearing temperature (18, 22 and 26°C) and incubated at 37°C or at the temperature the fish providing each sample was adapted to (18, 22 and 26°C).

both enzymes, while the minimum values were recorded in fish at 26 and 22°C for pepsin and lipase, respectively (Table 4).

4 Discussion

High chymotrypsin activity and responsiveness to temperature are key features of the greater amberjack digestive biochemistry

Chymotrypsin was by far the most abundant enzyme in the digestive tract. The contribution of trypsin and chymotrypsin activities in fish is rather variable. For example, in scaleless carp (*Gymnocypris przewalskii*) the activity of trypsin is much higher than that of chymotrypsin (Tian et al., 2019), while in the Pacific bluefin tuna (*Thunnus orientalis*) chymotrypsin activity contributes more to digestion than trypsin activity (de la Parra et al., 2007). Even so, our observations in general agree with the notion that fish with carnivorous preferences show high proteolytic enzyme activities to digest high-protein animals, while herbivorous species exhibit higher carbohydrase activities to digest the storage carbohydrates of macroalgae (German et al., 2004). Indeed, no detectable amylase activity in any segment of the tract was found in the present study, using a colorimetric method that was suited to measure this activity in other fish species such as gilthead seabream (*Sparus aurata*) (Perera and Yúfera, 2017). This result is in line with observations in other carnivore fish regarding low or undetectable amylase activity. A previous study assessing amylase activity in fish with different feeding habits linked low amylase activity to carnivorous fish species with a stomach, whereas stomachless omnivorous fish trend to show higher amylase activity levels (Hidalgo et al., 1999). These authors reported low amylase activity levels for gilthead seabream, rainbow trout (*Oncorhynchus mykiss*) and European eel (*Anguilla anguilla*). Likewise, almost undetectable levels of amylase activity have been reported for the carnivorous fish *Glyptosternum maculatum* (Xiong et al., 2011). On the other hand, total pepsin activity, measured at the enzyme optimal pH and 37°C, is a good estimation for the availability of pepsinogen, but not for active pepsin. pH in the fish stomach does not always reach the levels required for pepsin activation, explaining the negligible pepsin activity levels observed in the present study when measured at physiological conditions. A relatively poor acidification capacity, and thus, limited pepsin activation, have been previously reported for gilthead seabream juveniles (Gilannejad et al., 2018; Gilannejad et al., 2021), Atlantic salmon (Krogdahl et al., 2015) and Senegalese sole juveniles (Yúfera and Darias, 2007), suggesting that functional pepsin activity is frequently over estimated.

Temperature is well documented to significantly modify transit time in fish (Yúfera et al., 2019; Volkoff and Rønnestad, 2020). The observed overall distribution of enzymes along the

TABLE 4 Q_{10} value and activation energy (E_a) for digestive enzymes of *Seriola dumerili* at different rearing temperatures.

Enzyme/T (°C)	Q_{10}	E_a (kJ mol^{-1})
Pepsin		
18°C	1.01	12.56
22°C	1.13	10.06
26°C	1.13	6.27
Trypsin		
18°C	1.23	8.57
22°C	1.22	8.49
26°C	1.21	7.83
Chymotrypsin		
18°C	1.04	7.72
22°C	1.02	7.28
26°C	1.03	6.63
Aminopeptidase		
18°C	1.42	9.63
22°C	1.44	9.41
26°C	1.28	7.13
Lipase		
18°C	1.33	8.93
22°C	1.09	3.12
26°C	1.30	6.92

The values were calculated for the range from 30 to 50°C for Q_{10} and from 30 to 60°C for E_a of lipase and pepsin and from 10 to 60°C for E_a of trypsin, chymotrypsin and leucine aminopeptidase.

digestive tract, with higher activities toward the posterior segments, seems to be a result of the time of sampling after feeding, and corroborates the results of previous studies using inert markers on the rapid transit time in this species under the same feeding regimen (Navarro-Guillén et al., 2021). The results of the discriminant analysis revealed a clear effect of rearing temperature on the digestive function of the greater amberjack. For instance, lipase and chymotrypsin activities through nearly all segments of the tract are responsive to high environmental temperature, suggesting a shift in the digestion capacity from 18/22°C to 26°C. In fact, in a previous trial, greater amberjack reared at 26°C showed higher growth rate and feed efficiency than fish reared at 22 and 18°C (Soriano, 2021), and also in consonance with the study by Fernández-Montero et al. (2018). However, it is interesting that both, trypsin and chymotrypsin activities in pyloric caeca are the better discriminants of the environmental temperature the fish are acclimated to. The fact that this occurs well after feeding, and not as a response to recent feed ingestion, strongly suggests an adaptation to ambient temperature also at the level of synthesis and secretion of enzymes, likely as preparation for next feeding, and/or different retention time of these enzymes in the pyloric caeca. Indeed, in yellowtail kingfish (*Seriola lalandi*), a greater proportion of nutrient digestion occurred during winter in the stomach, pyloric caeca and anterior intestine, while it occurred in the posterior intestine in summer, probably due to the slower gut motility during winter (Miegel et al., 2010). Globally, our multivariate analysis supports that fish digestion plasticity regarding environmental temperature largely relies on trypsin and chymotrypsin activities, being chymotrypsin the most responsive enzyme, though lipid digestion seems also to be under temperature control.

Most of the digestive enzymes of greater amberjack share optimal conditions with those from other fish species (Kishimura et al., 2006; de la Parra et al., 2007; Candiotto et al., 2018) and crustaceans (Perera et al., 2008; Chávez-Rodríguez et al, 2020; Muhlia-Almazán and Fernández-Gimenez, 2022), pointing out the relative high thermal optimum of alkaline proteases, although a previous study described lower thermal optimum for trypsin and chymotrypsin of greater amberjack (Oliveira et al., 2017). Also, the enzymes studied are all susceptible to acidic conditions, with lipase being the most sensitive enzyme, probably as a result of a more complex tridimensional architecture and structural requirements for activity as reported for other lipases (Perera et al., 2008). Nevertheless, while proteases such as pepsin, trypsin and leucine aminopeptidase of greater amberjack are thermally robust, chymotrypsin activity is thermally sensitive, indicating a clear difference in the effects of temperature over chymotrypsin and all other proteases. This finding also points to chymotrypsin enzymes as putative drivers of the effects of temperature on the digestive process. Similar results of chymotrypsin thermal stability have been described for other fish species such as the Brazilian flounder (*Paralichthys orbignyanus*) and gilthead seabream (Alarcón et al., 1998; Liang et al., 2011; Candiotto et al., 2018).

Stability patters suggest different physic-chemical features of isoforms

The fact that the activity of most enzymes decreased up to a certain level and then remained relatively stable over time strongly suggests the presence of isoforms with differences in susceptibility to the assayed conditions. Moreover, while thermal stability patterns were nearly identical among most proteases studied regardless of rearing temperature (i.e., trypsin, chymotrypsin, pepsin), stability of trypsin and chymotrypsin under varied pH conditions suggests differences among rearing temperatures in isoforms composition or contribution of particular isoenzymes. Trypsin activity was more stable at highly alkaline pH values in extracts from 18°C-adapted fish, while the same enzyme was more affected by acidic conditions in extracts from 26°C-adapted fish. On the other hand, chymotrypsin activity was more stable in the range pH 5 to 11 in extracts coming from 26°C-adapted fish than in extracts coming from fish reared at 18/22°C. Isoforms for digestive proteases in fish often differ in biochemical features (Klomklao et al., 2009), thus our results were not unexpected in this regard, but the changes in the relative contribution of those isoforms driven by differences among fish rearing temperatures have not been reported before.

Inhibition studies revealed digestive plasticity regarding ambient temperature and the key role of chymotrypsin

Our results on the inhibition of caseinolytic activity, as indirect indicative of overall protein digestion capacity, corroborate the key role of serine proteases in digestion, particularly trypsin and chymotrypsin activities, as occur in other fish species (Natalia et al., 2004; Candiotto et al., 2018). Moreover, we found a clear dependence of these enzymes on divalent cations, in agreement with that described for other fish species (Kishimura et al., 2006; Klomklao et al., 2009).

Results on the effects of trypsin and chymotrypsin inhibitors on caseinolytic activity suggest a shift in the contribution of these activities as the rearing temperature of fish varies. The higher inhibition of overall proteolysis by TPCK in extracts from fish adapted to 18°C suggests that chymotrypsin may have a more important role at lower temperatures. In Atlantic salmon (*Salmo salar*), the role of chymotrypsin in low temperature adaptation is well documented. Chymotrypsin activity increased when there was a reduction in growth rate because of external factors such as low temperature, thus fish compensated to a certain extent by

lowering the trypsin to chymotrypsin (T/C) ratio (Rungruangsak-Torrissen et al., 2006).

We further addressed this issue by using specific substrates for these enzymes and observed that the contribution of activities changed for chymotrypsin but not for trypsin. Inhibition of chymotrypsin by TPCK showed a reverse pattern with water temperature, with inhibition increasing from 40% to 80% as fish rearing temperature decreased. The most plausible explanation for these results is a change in the relative contribution of chymotrypsin isoenzymes, with an increase in the contribution of particular chymotrypsin isoenzymes at lower temperatures that are more sensitive to TPCK. Unfortunately, while trypsin isoenzymes were clearly identified in activity gels, with apparent molecular weights similar to those reported for other fish trypsins (Natalia et al., 2004; Kurtovic et al., 2006; Klomklao et al., 2009), our attempts to identify chymotrypsin isoenzymes in the zymogram gels were unsuccessful using TPCK as done in the tube assays. Other chymotrypsin inhibitor was used, carbobenzoxy-Phe chloromethyl ketone (ZPCK, data not shown), but it did not work either. Problems to identify fish chymotrypsins in gels are recurrent in the literature. For gilthead seabream and common dentex (*Dentex dentex*), no inhibitory effect was observed for TPCK in gels, though ZPCK partially deleted two bands in zymograms from both species (Alarcón et al., 1998). Similarly, in the Pacific red snapper (*Lutjanus peru*), TPCK had no inhibitory effect in zymograms of alkaline proteases (Peña-Marín et al., 2021). However, in the present study, three bands were strongly inhibited by SBTI but not by TLCK, thus assuming to be chymotrypsins.

When casein zymograms were performed using pools of extracts coming from fishes adapted to the different temperatures, or with extracts from individual fishes (Supplementary Figure 3), no variation in the presence/absence of these three isoforms was noticed among rearing temperatures. Only a small individual variation was observed, likely due to low-frequency individual polymorphism, with one of these isoforms (the one of intermediate electrophoretic mobility) being absent or less represented in few individuals irrespective of rearing temperature. Thus, results obtained in this study regarding the changing role of chymotrypsin activity at low temperature could not be explained by a shift in particular isoforms. Therefore, a feasible hypothesis is that variation in the relative contribution of chymotrypsin isoenzymes to a particular environmental temperature occurs due to different physic-chemical features among isoforms as suggested by results from our stability assays.

To gain insight on the *in vivo* contribution of chymotrypsin isoenzymes under different ambient temperatures, we further analyzed by zymograms the changes in chymotrypsin isoenzymes pattern and activity in extracts from fish adapted to different temperatures and revealed the activity in gels at 37°C and at the corresponding temperatures. Activity of isoforms at 37°C, inferred from band intensity, was identical for extracts from fishes adapted to the different temperatures, corroborating that the amount of enzyme loaded into the gel was the same among treatments, and that there are no changes in synthesis and secretion of particular isoforms among temperatures. However, activity decreased as incubation temperature did for all bands (including the three trypsins and two chymotrypsin isoforms) except for the chymotrypsin of the higher electrophoretic mobility. This chymotrypsin isoenzyme exhibited high activity levels at 18°C, being similar to that observed when incubated at higher temperatures (Figure 7B). Thus, it is demonstrated that the isoenzyme richness and the biochemical differences among isoforms revealed in this study, lead to different digestion performance under realistic temperature conditions. Although in only few species, there is some evidence of the impact of protease isoforms with different biochemical features on fish performance at different temperatures (Rungruangsak-Torrissen et al., 1998; Rungruangsak-Torrissen and Male, 2000; Gelman et al., 2008). However, whether the presence of this chymotrypsin isoenzyme in the greater amberjack compensates for decrease activity of other proteases, and to what extent, remains unknown. This will be difficult to assess *in vivo* as all individuals hold this isoenzyme and thus, no performance comparisons can be made at low temperatures between individuals having or not the isoenzyme, as assessed in the Atlantic salmon (Rungruangsak-Torrissen et al., 1998; Rungruangsak-Torrissen and Male, 2000). Yet, the fact that all individuals analyzed presented this isoform may suggest that it has an adaptive value for the species. The analysis of a higher number of individuals and from different origins, to broad the genetic variability of the population studied, would shed some light on this issue.

Activation energy reveals digestive enzymes adaptation to a wide temperature range

On average, Q_{10} and E_a values were similar for all studied enzymes of greater amberjack. The E_a found for the studied enzymes were lower than those described for silver carp, common carp, sheatfish, gilthead seabream, redfish and turbot (Jónás et al., 1983; Munilla-Morán and Saborido-Rey, 1996; Alarcón et al., 1998), though this last study used a proteinaseous substrate for alkaline activity, while specific substrates were used in the current work for individual enzymes. In any case, enzymes of the greater amberjack seem to be more efficient that those of the above-mentioned fish, as the lower the E_a value the higher the efficiency of the enzyme.

Moreover, we found no break point in the Arrhenius plots for the studied enzymes, which is interpreted as an adaptation to a wide temperature range, as described for redfish and turbot (Munilla-Morán and Saborido-Rey, 1996). In other fish species, break points are evident, indicating changes in digestion efficiency. For gilthead seabream, Munilla-Morán and Saborido-Rey (1996) described a break point at 20°C for gastric acid proteases, being E_a below this temperature about six times higher than above the break point,

indicating that this species seems to have a more efficient acidic digestion of protein in a warm environment. By contrast, a later study on the same species did not find such a break point for acid proteases (Alarcón et al., 1998).

In addition to the absence of break points, we observed a slight decrease in E_a for proteases (trypsin, chymotrypsin and leucine aminopeptidase) as rearing temperature increased, with lower values at 26°C. These observations agree with the notion of different biochemical features among isoenzymes. Increased efficiency at higher environmental temperature would aid the fish to deal with an increased feed intake and/or counteract an increase in transit time. By contrast, lipase E_a pattern was not linked to temperature gradient. The lowest E_a for lipase was found at 22°C (standard temperature for the species culture), while the highest was at 18°C. The more reasonable hypothesis for these results is again the presence of isoforms. However, due to the technical difficulty of performing zymograms for lipases (Kurtovic et al., 2010), we are not able for the moment to confirm this assumption.

In conclusion, the present study describes the functional characteristics of greater amberjack digestive enzymes, showing that they have biochemical features similar to those of other fish species. It is of particular interest that some specific enzymes and isoforms, particularly chymotrypsins, may play a key role in providing certain phenotypic flexibility regarding environmental temperature, being this study one of the few providing experimental evidence of an adaptive value of digestive enzyme polymorphism in farmed fish. More investigation is needed on this issue since a better understanding of the balance among the different factors affecting digestion efficiency in the greater amberjack would lead to optimized feeding protocols depending on the environmental temperature, wherever in sea cages or in-land facilities.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by the Spanish National Research Council Bioethical Committee.

Author contributions

CN-G: conceptualization, methodology, formal analysis, writing—original draft preparation. MY: conceptualization, writing—review and editing, project administration, and funding acquisition. EP: conceptualization, methodology, formal analysis, supervision, writing—original draft preparation, writing—review and editing. All authors contributed to the article and approved the submitted version.

Funding

This research was funded by the project RTI208-096134-B-I00 from the Spanish Ministry of Science, Innovation and Universities (MCIU), the State Research Agency (AEI) and the European Regional Development Fund (FEDER/ERDF) granted to MY. CN-G was supported by the researcher contract DOC_01203 funded by the Andalusian Plan for Research Development and Innovation (PAIDI 2020).

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

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.960746/full#supplementary-material

References

Alarcón, F. J., Díaz, M., Moyano, F. J., and Abellán, E. (1998). Characterization and functional properties of digestive proteases in two sparids; gilthead seabream (*Sparus aurata*) and common dentex (*Dentex dentex*). *Fish Physiol. Biochem.* 19 (3), 257–267. doi: 10.1023/A:1007717708491

Anson, M. L. (1938). The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *J. Gen. Physiol.* 22 (1), 79–89. doi: 10.1085/jgp.22.1.79

Candiotto, F. B., Freitas-Júnior, A. C. V., Neri, R. C. A., Bezerra, R. S., Rodrigues, R. V., Sampaio, L. A., et al. (2018). Characterization of digestive enzymes from captive Brazilian flounder *Paralichthys orbignyanus*. *Braz. J. Biol.* 78 (2), 281–288. doi: 10.1590/1519-6984.06616

Chávez-Rodríguez, L., Rodríguez-Viera, L., Montero-Alejo, V., Perdomo-Morales, R., Mancera, J. M., and Perera, E. (2020). A very sctive α-amylase and

an inhibitor-based control of proteinases are key features of digestive biochemistry of the omnivorous Caribbean king crab *Maguimithrax spinosissimus*. *J. Evol. Biochem. Physiol.* 56 (6), 550–564. doi: 10.1134/S0022093020060083

de la Parra, A. M., Rosas, A., Lazo, J. P., and Viana, M. T. (2007). Partial characterization of the digestive enzymes of pacific bluefin tuna *Thunnus orientalis* under culture conditions. *Fish Physiol. Biochem.* 33 (3), 223–231. doi: 10.1007/s10695-007-9134-9

Díaz-Mendoza, M., Ortego, F., García de Lacoba, M., Magaña, C., de la Poza, M., Farinós, G. P., et al. (2005). Diversity of trypsins in the Mediterranean corn borer *Sesamia nonagrioides* (Lepidoptera: Noctuidae), revealed by nucleic acid sequences and enzyme purification. *Insect Biochem. Mol. Biol.* 35, 1005–1020. doi: 10.1016/j.ibmb.2005.04.003

Fernández-Montero, A., Caballero, M. J., Torrecillas, S., Tuset, V. M., Lombarte, A., Ginés, R., et al. (2018). Effect of temperature on growth performance of greater amberjack (*Seriola dumerili* risso 1810) juveniles. *Aquac. Res.* 49, 908–918. doi: 10.1111/are.13537

Fernández-Montero, A., Torrecillas, S., Tort, L., Ginés, R., Acosta, F., Izquierdo, M. S., et al. (2020). Stress response and skin mucus production of greater amberjack (*Seriola dumerili*) under different rearing conditions. *Aquaculture* 520, 735005. doi: 10.1016/j.aquaculture.2020.735005

Gelman, A., Kuz'mina, V., Drabkin, V., and Glatman, L. (2008). "Temperature adaptation of digestive enzymes in fish," in *In feeding and digestive functions in fishes*. Eds. J. E. P. Cyrino, D. P. Bureau and B. Kapoor (Enfield, NH, USA: Science Publishers), 155–225.

German, D. P., Horn, M. H., and Gawlicka, A. (2004). Digestive enzyme activities in herbivorous and carnivorous prickleback fishes (Teleostei: Stichaeidae): ontogenetic, dietary, and phylogenetic effects. *Physiol. Biochem. Zool.* 77 (5), 789–804. doi: 10.1086/422228

Gilannejad, N., Martínez-Rodríguez, G., Yúfera, M., and Moyano, F. J. (2018). Modelling digestive hydrolysis of nutrients in fish using factorial designs and desirability function. *PloS One* 13 (11), e0206556. doi: 10.1371/journal.pone.0206556

Gilannejad, N., Moyano, F. J., Martínez-Rodríguez, G., and Yúfera, M. (2021). The digestive function of gilthead seabream juveniles in relation to feeding frequency. *Aquaculture* 531, 735867. doi: 10.1016/j.aquaculture.2020.735867

Hidalgo, M. C., Urea, E., and Sanz, A. (1999). Comparative study of digestive enzymes in fish with different nutritional habits. proteolytic and amylase activities. *Aquaculture* 170 (3), 267–283. doi: 10.1016/S0044-8486(98)00413-X

Hu, P., Liu, M., Liu, Y., Wang, J., Zhang, D., Niu, H., et al. (2016). Transcriptome comparison reveals a genetic network regulating the lower temperature limit in fish. *Sci. Rep.* 6, 28952. doi: 10.1038/srep28952

Jónás, E., Rágyanszki, M., Oláh, J., and Boross, L. (1983). Proteolytic digestive enzymes of carnivorous (*Silurus glanis* l.), herbivorous (*Hypophthalmichthys molitrix* val.) and omnivorous (*Cyprinus carpio* l.) fishes. *Aquaculture* 30, 145–154. doi: 10.1016/0044-8486(83)90158-8

Kishimura, H., Hayashi, K., Miyashita, Y., and Nonami, Y. (2006). Characteristics of trypsins from the viscera of true sardine (*Sardinops melanostictus*) and the pyloric ceca of arabesque greenling (*Pleuroprammus azonus*). *Food Chem.* 97 (1), 65–70. doi: 10.1016/j.foodchem.2005.03.008

Klomklao, S., Kishimura, H., Nonami, Y., and Benjakul, S. (2009). Biochemical properties of two isoforms of trypsin purified from the intestine of skipjack tuna (*Katsuwonus pelamis*). *Food Chem.* 115 (1), 155–162. doi: 10.1016/j.foodchem.2008.11.087

Kofuji, P. Y. M., Akimoto, A., Hosokawa, H., and Masumoto, T. (2005). Seasonal changes in proteolytic enzymes of yellowtail *Seriola quinqueradiata* (Temminck & schlegel; carangidae) fed extruded diets containing different protein and energy levels. *Aquac. Res.* 36 (7), 696–703. doi: 10.1111/j.1365-2109.2005.01276.x

Krogdahl, Å., Sundby, A., and Holm, H. (2015). Characteristics of digestive processes in Atlantic salmon (*Salmo salar*). Enzyme pH optima, chyme pH, and enzyme activities. *Aquaculture* 449, 27–36. doi: 10.1016/j.aquaculture.2015.02.032

Kurtovic, I., Marshall, S. N., and Simpson, B. K. (2006). Isolation and characterization of a trypsin fraction from the pyloric ceca of chinook salmon (*Oncorhynchus tshawytscha*). *Comp. Biochem. Phys. B* 143 (4), 432–440. doi: 10.1016/j.cbpb.2005.12.020

Kurtovic, I., Marshall, S. N., Zhao, X., and Simpson, B. K. (2010). Purification and properties of digestive lipases from Chinook salmon (*Oncorhynchus tshawytscha*) and new Zealand hoki (*Macruronus novaezelandiae*). *Fish Physiol. Biochem.* 36 (4), 1041–1060. doi: 10.1007/s10695-010-9382-y

Liang, Z., Suzanne, M. B., Abdel, E. G., Marianne, S. B., and Deepika, D. (2011). Extraction, purification and characterization of fish chymotrypsin: a review. *Am. J. Biochem. Biotechnol.* 7 (3), 104–123. doi: 10.3844/ajbbsp.2011.104.123

Miegel, R. P., Pain, S. J., Wettere, W. H. E. J., Howarth, G. S., and Stone, D. A. J. (2010). Effect of water temperature on gut transit time, digestive enzyme activity and nutrient digestibility in yellowtail kingfish (*Seriola lalandi*). *Aquaculture* 308 (3/4), 145–151. doi: 10.1016/j.aquaculture.2010.07.036

Monge-Ortiz, R., Martínez-Llorens, S., Lemos-Neto, M. J., Falcó-Giaccaglia, S. L., Pagán, M. J., Godoy-Olmos, S., et al. (2020). Growth, sensory and chemical characterization of Mediterranean yellowtail (*Seriola dumerili*) fed diets with partial replacement of fish meal by other protein sources. *Aquac. Rep.* 18, 100455. doi: 10.1016/j.aqrep.2020.10046

Muhlia-Almazán, A. T., and Fernández-Gimenez, A. V. (2022). Understanding the digestive peptidases from crustaceans: from their biochemical basis and classical perspective to the biotechnological approach. *Mar. Biotechnol.* 18, 480–491. doi: 10.1007/s10126-022-10122-2

Munilla-Morán, R., and Saborido-Rey, F. (1996). Digestive enzymes in marine species. i. proteinase activities in gut from redfish (Sebastes mentella), seabream (Sparus aurata) and turbot (*Scophthalmus maximus*). *Comp. Biochem. Phys. B* 113, 395–402. doi: 10.1016/0305-0491(95)02057-8

Natalia, Y., Hashim, R., Ali, A., and Chong, A. (2004). Characterization of digestive enzymes in a carnivorous ornamental fish, the Asian bony tongue *Scleropages formosus* (Osteoglossidae). *Aquaculture* 233 (1), 305–320. doi: 10.1016/j.aquaculture.2003.08.012

Navarro-Guillén, C., Conceição, L. E. C., Pinto, W., Siguero, I., Urrutia, P., Moyano, F. J., et al. (2019). Fast growing greater amberjack post-larvae require a high energy-high protein weaning diet. *Aquaculture* 499, 195–202. doi: 10.1016/j.aquaculture.2018.09.037

Navarro-Guillén, C., Gilannejad, N., Martínez-Rodríguez, G., and Yúfera, M. (2021). Water temperature affects feeding behavior and gut transit time in greater amberjack juveniles (*Seriola dumerili*) fed multiple daily meals. *Eur. Aquac. Soc.* 867–868

Nitzan, T., Kokou, F., Doron-Faigenboim, A., Slosman, T., Biran, J., Mizrahi, I., et al. (2019). Transcriptome analysis reveals common and differential response to low temperature exposure between tolerant and sensitive blue tilapia (*Oreochromis aureus*). *Front. Genet.* 10. doi: 10.3389/fgene.2019.00100

Oliveira, V., Nascimento, T. P., Assis, C. R. D., Bezerra, R. S., and Porto, A. L. F. (2017). Study on enzymes of industrial interest in digestive viscera: Greater amberjack (*Seriola dumerili*). *J. Coast. Life Med.* 5, 233–238. doi: 10.12980/jclm.5.2017J6-300

Pelusio, N. F., Scicchitano, D., Parma, L., Dondi, F., Brini, E., D'Amico, F., et al. (2021). Interaction between dietary lipid level and seasonal temperature changes in gilthead sea bream *Sparus aurata*: effects on growth, fat deposition, plasma biochemistry, digestive enzyme activity, and gut bacterial community. *Front. Mar. Sci.* 8. doi: 10.3389/fmars.2021.664701

Peña-Marín, E. S., Ibarra-Castro, L., Martínez-Brown, J. M., Hernández-López, I. A., Tovar-Ramírez, D., Pérez-Urbiola, J. C., et al. (2021). Partial characterization of digestive proteases in pacific red snapper *Lutjanus peru* nichols & murphy 1922 (Perciformes: Lutjanidae). *Lat. Am. J. Aquat. Res.* 49, 442–450. doi: 10.3856/vol49-issue3-fulltext-2624

Perera, E., Moyano, F. J., Díaz, M., Perdomo-Morales, R., Montero-Alejo, V., Alonso, E., et al. (2008). Polymorphism and partial characterization of digestive enzymes in the spiny lobster *Panulirus argus*. comp. *Biochem. Phys. B* 150 (3), 247–254. doi: 10.1016/j.cbpb.2008.03.009

Perera, E., Rodríguez-Viera, L., Perdomo-Morales, R., Montero-Alejo, V., Moyano, F. J., Martínez-Rodríguez, G., et al. (2015). Trypsin isozymes in the lobster *Panulirus argus* (Latreille 1804): from molecules to physiology. *J. Comp. Physiol. B.* 185 (1), 17–35. doi: 10.1007/s00360-014-0851-y

Perera, E., and Yúfera, M. (2017). Effects of soybean meal on digestive enzymes activity, expression of inflammation-related genes, and chromatin modifications in marine fish (*Sparus aurata* l.) larvae. *Fish Physiol. Biochem.* 43 (2), 563–578. doi: 10.1007/s10695-016-0310-7

Rungruangsak-Torrissen, K. (2012). "Trypsin and its implementations for growth, maturation, and dietary quality assessment," in *Trypsin: structure, biosynthesis and functions*. Eds. K. Weaver and C. Kelley (New York: Nova Science Publishers, Inc.), 1–59.

Rungruangsak-Torrissen, K., and Male, R. (2000). "Trypsin isozymes: development, digestion and structure," in *Seafood enzymes, utilization and influence on postharvest seafood quality*. Eds. N. F. Haard and B. K. Simpson (New York: Marcel Dekker, Inc.).

Rungruangsak-Torrissen, K., Moss, R., Andresen, L. H., Berg, A., and Waagbø, R. (2006). Different expressions of trypsin and chymotrypsin in relation to growth in Atlantic salmon (*Salmo salar* l.). *Fish Physiol. Biochem.* 32 (1), 7–23. doi: 10.1007/s10695-005-0630-5

Rungruangsak-Torrissen, K., Pringle, G. M., Moss, R., and Houlihan, D. F. (1998). Effects of varying rearing temperatures on expression of different trypsin isozymes, feed conversion efficiency and growth in Atlantic salmon (*Salmo salar* l.). *Fish Physiol. Biochem.* 19, 247–255. doi: 10.1023/A:1007731717021

Saborowski, R., Schatte, J., and Gimenez, L. (2012). Catalytic properties and polymorphism of serine endopeptidases from the midgut gland of the brown

shrimp *Crangon crangon* (Decapoda, caridea) mar. *Biol* 159 (5), 1107–1118. doi: 10.1007/s00227-012-1890-0

Sandholt, G. B., Stefansson, B., Scheving, R., and Gudmundsdottir, Á. (2019). Biochemical characterization of a native group III trypsin ZT from Atlantic cod (*Gadus morhua*). *Int. J. Biol. Macromol.* 15, 847–855. doi: 10.1016/j.ijbiomac.2018.12.099

Sicuro, B., and Luzzana, U. (2016). The state of seriola spp. other than yellowtail (*S. quinqueradiata*) farming in the world. *Rev. Fish. Sci. Aquac.* 24, 314–325. doi: 10.1080/23308249.2016.1187583

Soriano, P. (2021). *Influencia de la temperatura sobre el crecimiento y metabolismo de la seriola (Seriola dumerili). [bachelor thesis]* (Spain: University of Cádiz).

Tian, H., Meng, Y., Li, C., Zhang, L., Xu, G., Shi, Y., et al. (2019). A study of the digestive enzyme activities in scaleless carp (*Gymnocypris przewalskii*) on the qinghai-Tibetan plateau. *Aquac. Rep.* 13, 100174. doi: 10.1016/j.aqrep.2018.10.002

Torrissen, K. R. (1987). Genetic variation of trypsin-like isozymes correlated to fish size of Atlantic salmon (*Salmo salar*). *Aquaculture* 62, 1–10. doi: 10.1016/0044-8486(87)90180-3

Torrissen, K. R., Lied, E., and Espe, M. (1994). Differences in digestion and absorption of dietary protein in Atlantic salmon (*Salmo salar*) with genetically different trypsin isozymes. *J. Fish Biol.* 45, 1087–1104. doi: 10.1111/j.1095-8649.1994.tb01075.x

Volkoff, H., and Rønnestad, I. (2020). Effects of temperature on feeding and digestive processes in fish. *Temperature* 7, 307–320. doi: 10.1080/23328940.2020.1765950

Xiong, D. M., Xie, C. X., Zhang, H. J., and Liu, H. P. (2011). Digestive enzymes along digestive tract of a carnivorous fish *Glyptosternum maculatum* (Sisoridae, siluriformes). *J. Anim. Physiol. Anim. Nutr.* 95 (1), 56–64. doi: 10.1111/j.1439-0396.2009.00984.x

Yúfera, M., and Darias, M. J. (2007). Changes in the gastrointestinal pH from larvae to adult in Senegal sole (*Solea senegalensis*). *Aquaculture* 267, 94–99. doi: 10.1016/j.aquaculture.2007.02.009

Yúfera, M., Nguyen, M. V., Navarro-Guillén, C., Moyano, F. J., Jordal, A. E. O., Espe, M., et al. (2019). Effect of increased rearing temperature on digestive function in cobia early juvenile. *Comp. Biochem. Physiol. A* 230, 71–80. doi: 10.1016/j.cbpa.2019.01.007

frontiers | Frontiers in Physiology

ORIGINAL RESEARCH
published: 05 August 2022
doi: 10.3389/fphys.2022.927819

Effects of Different Viscous Guar Gums on Growth, Apparent Nutrient Digestibility, Intestinal Development and Morphology in Juvenile Largemouth Bass, *Micropterus salmoides*

Yu Liu[1,2,3], *Yumeng Zhang*[1,2,3], *Jiongting Fan*[1,2,3], *Hang Zhou*[1,2,3], *Huajing Huang*[1,2,3], *Yixiong Cao*[1,2,3], *Wen Jiang*[1,2,3], *Wei Zhang*[1,2,3], *Junming Deng*[1,2,3]* and *Beiping Tan*[1,2,3]*

[1]College of Fisheries, Guangdong Ocean University, Zhanjiang, China, [2]Aquatic Animals Precision Nutrition and High-Efficiency Feed Engineering Research Centre of Guangdong Province, Zhanjiang, China, [3]Key Laboratory of Aquatic, Livestock and Poultry Feed Science and Technology in South China, Ministry of Agriculture, Zhanjiang, China

OPEN ACCESS

Edited by:
Mohamed Salah Azaza, National Institute of Marine Science and Technology, Tunisia

Reviewed by:
Fethi Mensi, Institut National des Sciences et Technologies de la Mer, Tunisia
Claudia Ivette Maytorena Verdugo, Universidad Juárez Autónoma de Tabasco, Mexico

***Correspondence:**
Junming Deng
djunming@163.com
Beiping Tan
bptan@126.com

Specialty section:
This article was submitted to Aquatic Physiology, a section of the journal Frontiers in Physiology

Received: 25 April 2022
Accepted: 30 May 2022
Published: 05 August 2022

Citation:
Liu Y, Zhang Y, Fan J, Zhou H, Huang H, Cao Y, Jiang W, Zhang W, Deng J and Tan B (2022) Effects of Different Viscous Guar Gums on Growth, Apparent Nutrient Digestibility, Intestinal Development and Morphology in Juvenile Largemouth Bass, Micropterus salmoides. Front. Physiol. 13:927819. doi: 10.3389/fphys.2022.927819

An 8-week feeding trial was conducted to investigate the effects of different viscous guar gums on the growth performance, apparent nutrient digestibility, intestinal development and morphology of juvenile largemouth bass. Four isoproteic and isolipidic diets (crude protein 42.5%, crude lipid 13.7%) were formulated to contain 8% cellulose (Control group), 8% low viscous guar gum with 2,500 mPa s (Lvs-GG group), 8% medium viscous guar gum with 5,200 mPa s (Mvs-GG group) and 8% high viscous guar gum with 6,000 mPa s (Hvs-GG group), respectively. Each diet was fed to quadruplicate groups of 40 fish (6.00 ± 0.01 g) per repetition. Dietary guar gum inclusion significantly decreased the weight gain rate, specific growth rate, protein efficiency ratio, protein productive value and lipid deposition rate, and these parameters decreased considerably with increasing guar gum viscous and were lowest in the Hvs-GG group. Dietary guar gum inclusion significantly decreased the apparent digestibility of dry matter, crude protein and crude lipid, and these parameters decreased considerably with increasing guar gum viscous and were lowest in the Hvs-GG group. Intestinal protease, lipase and creatine kinase activities in the guar gum groups were significantly lower than those in the control group, and intestinal protease and lipase activities decreased considerably with increased guar gum viscous. Intestinal alkaline phosphatase activity in the Hvs-GG group and intestinal Na^+/K^+-ATPase activity in the Mvs-GG and Hvs-GG groups were significantly lower than those in the Lvs-GG and control groups. Serum high-density lipoprotein, total cholesterol and triglyceride concentrations and superoxide dismutase activity in the guar gum groups were significantly lower than those in the control group. Intestinal villus height and muscular thickness in the guar gum groups were considerably higher than those in the control group, whereas the goblet cell relative number in the Mvs-GG and Hvs-GG groups and the microvillus height in the Lvs-GG and Hvs-GG groups were significantly lower than those in the control group. The expression level of IGF-1 in the guar gum groups and the expression

level of GLP-2 in the Mvs-GG and Hvs-GG groups were significantly higher than those in the control group. These results indicated that guar gum diets adversely affected intestinal morphology, decreased intestinal digestive and absorptive enzyme activities, and caused poor nutrient digestibility and growth performance in juvenile largemouth bass. Moreover, the adverse effects of guar gum are closely related to its viscous, and high viscous guar gum produces more extreme negative impacts on juvenile largemouth bass.

Keywords: guar gum, viscous, growth performance, apparent digestibility, intestinal morphology, *Micropterus salmoides*

INTRODUCTION

Limited fishmeal production and a rapid development of aquaculture have resulted in increasingly high fishmeal prices, which has largely increased farming costs and limited the further development of aquaculture (Yannis et al., 2018; Zhang et al., 2018; Zhang et al., 2019; Yin et al., 2021). The search for feasible alternatives to fishmeal has attracted widespread attention from researchers and considerable progress has been made in the last few decades. Multiple protein sources, including plant-based proteins, animal by-products and single-cell proteins have been tested as feasible alternatives to fishmeal (Zeng et al., 2018). In particular, plant proteins are considered the most promising fishmeal substitutes due to their wide availability, high yield, stable quality and low price (Deng et al., 2017). However, the presence of anti-nutritional factors (ANFs) limited the use of plant proteins in aquafeeds (Collins et al., 2013; Azeredo et al., 2017). ANFs, such as non-starch polysaccharides (NSPs), trypsin inhibitors, antigenic proteins and saponins have been shown to be detrimental to fish growth and health (Takii et al., 2008; Merrifield et al., 2009; He et al., 2020; Deng et al., 2021). NSPs are major components of the plant cell wall, and are abundant in plant feed ingredients (10–70%, varies with plant species), including cellulose, hemi-cellulose and pectin, which can be divided into insoluble and soluble NSPs (Sinha et al., 2011; Choct, 2015). Therefore, the use of plant proteins in aquafeeds increases the level of NSPs in fish diets (Deng et al., 2021). Fish cannot directly utilize dietary NSPs due to the lack of endogenous NSPs-degrading enzymes (Kuz Mina, 1996); thus, dietary NSPs usually remain in the digestive tract and produce different physiological effects. In recent years, an increasing number of studies have begun to investigate the physiological effects of dietary NSPs on fish (Amirkolaie et al., 2005; Kraugerud et al., 2007; Glencross et al., 2012; Cai et al., 2019; Ren et al., 2020; Deng et al., 2021), and these studies showed that dietary NSPs exert beneficial or detrimental physiological effects on fish were type-dependent, due to the difference in their physicochemical characteristics. Generally, SNSP is considered to have more substantial anti-nutritional effects than INSP, and high-dose dietary SNSP even impairs intestinal or hepatic health in fish (Sinha et al., 2011; Cai et al., 2019; Deng et al., 2021). Studies have also indicated that the adverse effects of dietary SNSP mainly derived from its viscosity (Sinha et al., 2011; Cai et al., 2019; Deng et al., 2021), but few studies have covered this issue.

Guar gum is mainly galactomannan (hemi-cellulose), a viscous SNSP derived from the seeds of *Cyamopsis tetragonolobus* with massive yield and excellent bonding properties (Kazuhiko et al., 1993; Yu et al., 2015). Moreover, the bonding properties (viscosity) of guar gum can be regulated by modifying pH-responsive functional groups such as $-CH_3$, -COOH, $-SO_3H$, and $-CONH_2$ (George et al., 2019). Nowadays, guar gum is widely used as a stabilizer and thickener in food, pharmaceutical and cosmetic processing to improve product quality (Theocharidou et al., 2022). More recently, guar gum has attracted increasing attention as a feasible aquafeeds binder due to its binding properties, showing the ability to improve feed quality (water stability, hardness), reducing nutrient loss and water pollution (Thombare et al., 2016; Gao et al., 2019; Karim et al., 2022). Moreover, dietary guar gum can produce various physiological effects on animals. In mammals, guar gum has been reported to increase digesta viscosity and short-chain fatty acid concentration, reduce serum cholesterol and fecal pH (Jenkins et al., 1975; Kazuhiko et al., 1993; Seal and Mathers, 2001). In fish, studies have shown that dietary guar gum decrease nutrient digestibility and fish growth (Storebakken, 1985; Amirkolaie et al., 2005; Ramos et al., 2015; Gao et al., 2019). Some of these studies pointed out that dietary guar gum exerts beneficial or detrimental effects on fish growth was dose-dependent, dietary supplementation with high doses of guar gum generally exhibiting inhibitory effects on fish growth (Ramos et al., 2015; Gao et al., 2019). Increasing dietary guar gum levels leads to an increase in dietary viscosity (Casas et al., 2000; Ramos et al., 2015), so it can be assumed that the adverse effects of high doses of guar gum on fish growth may be induced by high viscosity. However, the association between guar gum viscosity and its anti-nutritional effect remains unclear.

Largemouth bass *Micropterus salmoides*, a typical freshwater carnivorous fish that is widely cultured due to its strong adaptability and fast growth rate (Li et al., 2007; Li et al., 2010; Hu et al., 2019; Liu et al., 2022). In 2019, the cultural production of this species in China reached 477,808 tons (Xu et al., 2022), which generate considerable economic benefits. With aquaculture development, more plant ingredients will inevitably be used in aquafeeds (Kokou and Fountoulaki, 2018), which poses more severe dietary NSPs challenges for farmed fish. The natural diet of carnivorous fish does not contain NSPs, so that dietary NSPs may have more extreme physiological effects on carnivorous fish. However, there are limited studies related to dietary NSPs on carnivorous fish, including largemouth bass.

TABLE 1 | The formulation and approximately composition of experimental diets (%).

Group	C	Lvs-GG	Mvs-GG	Hvs-GG
Ingredients				
Fish meal[a]	45.00	45.00	45.00	45.00
Corn gluten meal	10.00	10.00	10.00	10.00
Soy protein isolate	15.00	15.00	15.00	15.00
Fish oil	4.50	4.50	4.50	4.50
Soy oil	3.40	3.40	3.40	3.40
Soy lecithin	1.00	1.00	1.00	1.00
Starch	10.00	10.00	10.00	10.00
Cellulose	8.00			
Lvs-GG[b]		8.00		
Mvs-GG[b]			8.00	
Hvs-GG[b]				8.00
$Ca(H_2PO_4)_2$	1.00	1.00	1.00	1.00
NaCl	0.20	0.20	0.20	0.20
Choline chloride	0.30	0.30	0.30	0.30
Vitamin C	0.03	0.03	0.03	0.03
Vitamin and Mineral premix[c]	1.50	1.50	1.50	1.50
Ethoxyquin[a]	0.02	0.02	0.02	0.02
Yttrium (III) oxide	0.05	0.05	0.05	0.05
Proximate composition				
Moisture	10.69	10.76	10.73	10.80
Crude protein	42.59	42.53	42.50	42.54
Crude lipid	13.75	13.70	13.69	13.72
Ash	9.70	9.62	9.68	9.71
Viscosity (mPa•s)	5.14	102.26	221.75	385.80

[a]*Supplied by Zhanjiang Haibao Feed Co., Ltd. (Zhanjiang, China); fish meal, 65.81% crude protein, 7.69% crude lipid.*
[b]*Supplied by Guangrao Liuhe Chemical Co., Ltd. (Dongying, China).*
[c]*Supplied by Qingdao Master Biotech (Qingdao, China).*

Therefore, this study will investigate the effects of different viscous guar gum on growth, apparent nutrient digestibility, intestinal development and morphology in juvenile largemouth bass, so as to reveal the association between guar gum viscosity and its anti-nutritional effects in carnivorous fish. Moreover, these results will provide data support for the application of guar gum as a binder for aquafeed in the future.

MATERIALS AND METHODS

Experimental Diets

Four isoproteic and isolipidic diets were formulated (crude protein 42.5%, crude lipid 13.7%) to contain 8% cellulose (Control group), 8% low viscous guar gum with 2,500 mPa s (Lvs-GG group), 8% middle viscous guar gum with 5,200 mPa s (Mvs-GG group) and 8% high viscous guar gum with 6,000 mPa s (Hvs-GG group), respectively. All ingredients were ground into powder and passed through a 0.30 mm diameter sieve, then accurately weighed and mixed evenly with a Hobart-type mixer (JS-14, Zhejiang Zhengtai Elecric Co., Ltd., China). Then, fish oil, soybean oil, soybean lecithin and pure water were added to make a dough. The dough was extruded through a 2.0 mm diameter die using a double-helix extruder (F-75, South China University of Technology, China) to obtain experimental feeds. Finally, the feeds were air-dried at room temperature and stored at -20°C until used. The formulation and proximate compositions of experimental diets are shown in **Table 1**.

Fish and Feeding Trial

Largemouth bass juveniles were obtained from the freshwater aquaculture base of Guangdong Ocean University. Largemouth bass juveniles have fasted for 24 h before grouping, and 640 individuals with a healthy physique, no disease or injury and a uniform size (6.00 ± 0.01 g) were selected as experimental fish. These fish were randomly divided into four groups with four replicates per group, and each net cage had 40 fish. All net cages have a uniform size (1.2 m × 0.8 m × 1.0 m) and they are placed in the same cement pool. During the feeding period, the water temperature was controlled at 28–31°C with ammonia nitrogen <0.02 mg/L through adjusting flow rate and water change, and the dissolved oxygen was controlled at > 6.0 mg/L by continuous oxygenation with an oxygenator (SC-150DX, Ningbo Yinzhou Hengxi Saier Electric Factory, China). The feeding trial lasted for 8 weeks, and the test fish were fed twice daily until they were satiated (07:00 and 17:00). The number and body weight of fish mortalities and feed consumption in each cage were recorded.

Digestibility Trial

Digestibility trial was performed during the feeding trial according to the method described by He et al. (2021). Briefly, yttrium trioxide (99.9%, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) was used as an indicator in the experimental diets. Fecal collection was performed after the test fish had been accustomed to the experimental diets for 2 weeks. Specifically, feces were collected daily from the bottom of the net cages using a 200-mesh brail after the test fish had been fed for 5–7 h. Subsequently, complete feces were selected and dried at 65°C for 6 h, and then stored at −20°C for until use. Fecal collection lasts for 6 weeks to ensure that the fecal samples meet the test requirements. The determination of yttrium content in fecal and diets was carried out by inductively coupled plasma mass spectrometry Briefly, a 100–200 mg sample was first digested by adding 6 ml of nitric acid and 1 ml of hydrogen peroxide with a microwave digestion apparatus (Anton Paar Multiwave PRO 41HVT56, Austria), and the digested solution was subsequently taken for determination using a mass spectrometry (Agilent 7500cx, United States).

Sampling Strategy

At the end of the feeding trial, fish were fasted for 24 h. The ultimate fish in each net cage was accurately weighed and counted to calculate the growth indices. Before sampling, the test fish were anesthetized with 0.1 g/L of MS-222 (Sigma, United States). Four fish were randomly selected from each cage, and the total length of the fish was measured, weighed and then dissected; the visceral mass, liver and intestine were sequentially weighed, and the length of the intestine was measured. Another four fish were randomly selected from each cage, and the blood was drawn from the tail vein using a 1-ml syringe. The blood samples were transferred to EP tubes, left at 4°C overnight and centrifuged at 3,500 r/min for 10 min. Finally, the supernatant was taken as a

serum sample and stored at −80°C for subsequent analysis. Another two fish were randomly selected from each cage for dissection, and their intestines were taken out and placed in EP tubes with RNAlater, snap-frozen in liquid nitrogen and then stored at −80°C for subsequent analysis. Another three fish were randomly selected from each cage and frozen at −20°C for whole-body composition analysis.

Morphological Observation of Hindgut

One fish was randomly selected from each cage for dissection; the hindgut (1 cm) was taken out and fixed with 4% formaldehyde, and stood for 24 h. The tissue samples were dehydrated in a series of graded ethanol, and embedded in paraffin after dehydration. After the paraffin was solidified, the intestinal paraffin was cut into slices with a thickness of 5 μm with a microtome, followed by hematoxylin-eosin staining, and encapsulated to make sections. The prepared sections were observed with a Nikon Ni-U microscope imaging system (Nikon Ni-U, Japan), and the intestinal villus height and width, crypt depth, muscular thickness, and the number of goblet cells were counted according to the method described in a previous study (Huang et al., 2022).

Another fish was randomly selected from each cage in the control, Lvs-GG and Hvs-GG groups for dissection, and the hindgut tissue was fixed with 2.5% glutaraldehyde. After 24 h fixation, the tissues were transferred into phosphate-buffered saline containing 2% osmium tetroxide, and then the tissues were dehydrated with a series of graded ethanol. After dehydration, the tissue was embedded in epoxy resin 812 and then cut into ultrathin sections using an ultra-microtome (Leica EM UC7, Japan) for uranyl acetate and lead citrate staining. Finally, the morphology of the intestinal microvilli and intestinal epithelial cells was observed using a transmission electron microscope (HITACHI HT7600, Japan).

Chemical Analysis

The chemical composition analysis of the experimental diets, whole-body and feces refer to the standard method (AOAC, 2005). The samples were dried at 105°C to constant weight to measure the moisture content, and crude protein was determined using the Kjeldahl method (N × 6.25); crude lipid was determined using the Soxhlet extraction method; crude ash was determined by burning in a muffle furnace at 550°C for 16 h. The viscosity of experimental diets was measured using a viscometer (LV-SSR, Shanghai Fang Rui Instrument Co., Ltd., Shanghai, China). Briefly, the feed was first crushed to pass through an 80 mesh sieve, then 1 g of feed powder was mixed with 10 ml of deionized water, incubated at 25°C for 30 min, followed by centrifugation at 10,000 g for 10 min. Finally, the supernatant was used for viscosity determination.

Intestinal Enzyme Activity Analysis

Sample preparation: wet intestine tissues were accurately weighted and plus ninefold volume of ice-cold phosphate buffer (PBS, pH 7.4), then homogenized using a homogenizer and centrifuged for 15 min at 3,000 rpm/min. Finally, the supernatant was used for digestive enzyme activity analysis. The activities of intestinal lipase, protease, amylase, creatine kinase (CK), Na^+/K^+-ATPase and alkaline phosphatase (AKP) were measured by commercial kits (ELISA, Shanghai Enzyme Link Biotechnology Co., Ltd.) following the kit instructions (No. ML036371, No. ML036449, No. ML652041, No. ML036438, No. ML036470 and No. ML556611, respectively).

Serum Biochemical Indices Analysis

The concentration of total cholesterol (T-CHO), low-density lipoprotein (LDL-C), high-density lipoprotein (HDL-C), triglyceride (TG), malondialdehyde (MDA) and the activity of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the serum were measured using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the kit instructions (No. A111-1-1, No. A113-1-1, No. A112-1-1, No. A003-1-2, No. A110-1-1, No. A001-3-2, No. A084-2-1, No. A007-1-1, No. C010-2-1 and No. C009-2-1, respectively).

Real-Time Quantitative PCR Assay

Total intestinal RNA was extracted with Trizol kit (Quanjin Biology, Beijing, China), and its purity and concentration were tested. Subsequently, qualified total RNA samples were used as templates to synthesize complementary DNA (cDNA) with a reverse transcription kit (Accurate Biology, China), and stored at −20°C until use. The primer sequences used for fluorescence quantification are shown in **Table 2**, and the primers were synthesized by Shanghai Sangon Biotech Co., Ltd., (Shanghai, China). The mRNA expression levels were detected using a high-throughput fluorescent quantitative PCR instrument (480II) (Light Cycler480II, Thermo) under a 10 μL SYBR® Green Premix Pro Taq HS qPCR Kit II (Accurate Biology, China) reaction system. Four replicate assays were performed in each sample, and melting curve analysis was performed after each reaction to check product specificity. The mRNA expression of all genes was calculated by the $2^{-\Delta\Delta CT}$ method, and the mRNA expression level of the target gene was normalized with the *ef1α* mRNA expression of the control group as the standard.

Statistical Analysis

Data in this study were presented as mean ± standard error (Means ± SEM). All data were subjected to one-way analysis of variance (ANOVA) with SPSS (v22.0) software, and Turkey's multiple range test was performed when the difference was significant. $p < 0.05$ represents significant differences.

RESULTS

Growth Performance and Feed Utilization

The survival rate (SR) of juvenile largemouth bass was not significantly affected by the experimental diets ($p < 0.05$; **Table 3**). The final body weight (FBW), weight gain rate (WGR), specific growth rate (SGR), protein efficiency ratio (PER), protein deposition rate (PDR) and lipid deposition rate (LDR) in the guar gum groups were significantly lower than those

TABLE 2 | Primer sequences for real-time PCR.

Gene	Primer type	Sequence 5′-3′	E-value (%)	Tm (°C)	Accession no.
Ef1α	F[a]	TGCTGCTGGTGTTGGTGAGTT	97.99	61.2	XM_038724777.1
	R[a]	TTCTGGCTGTAAGGGGGCTC			
IGF1	F	CTTCAAGAGTGCGATGTGC	97.96	59.3	XM_038736342.1
	R	GCCATAGCCTGTTGGTTTACTG			
GLP-2	F	CCGAGCAACACTGGTACTGA	115.63	56.6	XM_038738328.1
	R	GCTGAGAGTGAGGTTGACGA			

F[a], forward primer; R[a], reverse primer.
IGF1, insulin-like growth factors -1; GLP-2, Glucagon-like peptide 2.

TABLE 3 | Effects of different viscous guar gum on growth performance and feed utilization of juvenile largemouth bass.

Group	Control	Lvs-GG	Mvs-GG	Hvs-GG
FBW (g)	67.23 ± 1.26[c]	56.40 ± 1.42[b]	49.60 ± 0.97[a]	50.96 ± 1.49[a]
SR[a] (%)	98.75 ± 1.25	98.13 ± 1.20	99.38 ± 0.63	99.38 ± 0.63
WGR[b] (%)	1118.50 ± 20.53[c]	942.42 ± 24.59[b]	852.14 ± 28.32[a]	848.99 ± 24.87[a]
SGR[c] (%/d)	4.31 ± 0.03[c]	4.00 ± 0.05[b]	3.82 ± 0.06[a]	3.82 ± 0.05[a]
FI[d] (% BW/d)	2.85 ± 0.05[a]	3.34 ± 0.08[b]	3.69 ± 0.14[c]	3.65 ± 0.09[c]
FCR[e]	0.95 ± 0.02[a]	1.16 ± 0.03[b]	1.31 ± 0.06[c]	1.30 ± 0.04[c]
PER[f]	2.46 ± 0.05[c]	2.04 ± 0.06[b]	1.74 ± 0.06[a]	1.82 ± 0.06[a]
PDR[g] (%)	38.57 ± 0.77[c]	31.42 ± 0.89[b]	27.21 ± 0.93[b]	27.53 ± 0.92[a]
LDR[h] (%)	67.91 ± 1.29[c]	44.41 ± 1.21[b]	31.87 ± 1.08[b]	29.57 ± 0.97[a]

FBW, final body weight; SR, survival rate; WGR, weight gain rate; SGR, specific growth rate; FI, feed intake; FCR, feed conversion ratio; PER, protein efficiency ratio; PDR, protein deposition rate; LDR, lipid deposition rate.
*Values are presented as means ± S.E.M (*n *= 4). Different superscript letters in the same row means there was a significant difference between data (*p *< 0.05).*
[a]SR (%) = 100 × the final fish number/the initial fish number.
[b]WGR (%) = 100 × (final body weight—initial body weight)/initial body weight.
[c]SGR (%/d) = 100 × ((ln final body weight)—(ln initial body weight))/d.
[d]FI (% BW/d) = 100 × final body weight/[(final body weight + initial body weight)/2 × d].
[e]FCR, total feed intake (dry matter)/(final biomass—initial biomass + biomass of dead fish).
[f]PER = (final body weight—initial body weight)/total protein intake.
[g]PDR (%) = 100 × (final body weight × final body protein—initial body weight × initial body protein)/total protein intake.
[h]LDR (%) = 100 ×final body weight × final body lipid—initial body weight × initial body lipid)/total lipid intake.

TABLE 4 | Effects of different viscous guar gum on morphological parameters of juvenile largemouth bass.

Group	Control	Lvs-GG	Mvs-GG	Hvs-GG
Morphological parameters				
CF[a] (g/cm^3)	2.20 ± 0.04	2.12 ± 0.04	2.12 ± 0.03	2.10 ± 0.03
OI[b] (%)	8.04 ± 0.13[b]	7.64 ± 0.12[a,b]	7.60 ± 0.22[a,b]	7.39 ± 0.12[a]
HSI[c] (%)	1.86 ± 0.06[c]	0.98 ± 0.08[b]	0.86 ± 0.04[a,b]	0.76 ± 0.03[a]
VSI[d] (%)	0.69 ± 0.02[a]	1.09 ± 0.03[b]	1.21 ± 0.03[c]	1.26 ± 0.03[d]
ILI[e] (%)	0.86 ± 0.01[a]	0.96 ± 0.14[b]	0.94 ± 0.01[b]	0.95 ± 0.02[b]
Body composition, %				
Moisture	72.01 ± 1.06	72.59 ± 0.92	72.70 ± 1.12	73.73 ± 1.27
Crude protein	15.66 ± 0.20	15.29 ± 0.18	15.73 ± 0.17	15.79 ± 0.04
Crude lipid	8.53 ± 0.09[c]	7.39 ± 0.08[b]	7.33 ± 0.20[b]	6.68 ± 0.03[a]
Ash	4.06 ± 0.20	3.97 ± 0.12	4.10 ± 0.15	4.10 ± 0.12

CF, condition factor; OI: organ index; HSI, hepasomatic index; VSI, viserosomatic index; ILI, intestinal length index.
*Values are presented as means ± S.E.M (*n *= 4). Different superscript letters in the same row means there was a significant difference between data (*p *< 0.05).*
[a]CF (g/cm^3) = body weight/(body length)^3.
[b]OI (%) = 100 × visceral weight/body weight.
[c]HSI (%) = 100 × hepatic weight/body weight.
[d]VSI (%) = 100 × intestinal weight/body weight.
[e]ILI (%) = 100 × intestinal length/body length.

in the control group; these parameters were also significantly decreased with increased guar gum viscous ($p < 0.05$). Conversely, the feed intake (FI) and feed conversion ratio (FCR) in the guar gum groups were significantly higher than those in the control group; these parameters were also increased dramatically with increased guar gum viscous ($p < 0.05$).

TABLE 5 | Effects of different viscous guar gum on nutrient apparent digestibility of juvenile largemouth bass.

Group	Control	Lvs-GG	Mvs-GG	Hvs-GG
Dry matter (%)	85.52 ± 1.24^{c}	80.32 ± 0.60^{b}	74.42 ± 0.26^{a}	73.98 ± 0.45^{a}
Crude protein (%)	91.32 ± 0.36^{c}	84.36 ± 1.07^{b}	80.10 ± 0.25^{a}	80.65 ± 0.75^{a}
Crude lipid (%)	90.88 ± 0.2^{c}	82.66 ± 0.48^{b}	71.54 ± 0.40^{a}	72.05 ± 0.56^{a}

*Values are presented as means ± S.E.M (*n *= 4). Different superscript letters in the same row means there was a significant difference between data (*p *< 0.05).*
Apparent digestibility coefficient of dry matter (%) = 100 × [1– (dietary Y_2O_3 level/feces Y_2O_3 level)].
Apparent digestibility coefficient of nutrients (%) = 100 × [1– (dietary Y_2O_3 level/feces Y_2O_3 level) × (feces nutrient level/dietary nutrient level)].

TABLE 6 | Effects of different viscous guar gum on intestinal digestive and absorptive enzyme activities.

Group	Control	Lvs-GG	Mvs-GG	Hvs-GG
Protease (U/g protein)	4.55 ± 0.14^{c}	4.09 ± 0.13^{b}	3.53 ± 0.26^{a}	3.37 ± 0.17^{a}
Lipase (U/g protein)	0.85 ± 0.04^{c}	0.77 ± 0.05^{bc}	0.71 ± 0.03^{b}	0.53 ± 0.02^{a}
Amylase (U/g protein)	0.33 ± 0.04	0.35 ± 0.04	0.32 ± 0.03	0.28 ± 0.03
Creatine kinase (U/mg protein)	0.16 ± 0.02^{b}	0.12 ± 0.02^{a}	0.12 ± 0.01^{a}	0.11 ± 0.01^{a}
Na^+/K^+-ATPase (U/mg protein)	24.37 ± 1.44^{b}	22.54 ± 0.72^{b}	22.11 ± 0.81^{ab}	19.42 ± 0.37^{a}
Alkaline phosphatase (U/g protein)	145.63 ± 5.69^{b}	158.22 ± 5.29^{b}	133.04 ± 3.01^{a}	132.11 ± 5.01^{a}

*Values are presented as means ± S.E.M (*n *= 4). Different superscript letters in the same row means there was a significant difference between data (*p *< 0.05).*

Morphological Parameters

The condition factors (CF), organ index (OI), and the moisture, crude protein and ash concentrations of juvenile largemouth bass were not significantly affected by the experimental diets ($p > 0.05$; **Table 4**). The hepasomatic index (HSI) in the guar gum groups was considerably lower than that in the control group, and this parameter was markedly decreased with increased guar gum viscous ($p < 0.05$). The viserosomatic index (VSI) and intestinal length index (ILI) in the guar gum groups were significantly higher than that in the control group, and the VSI was increased dramatically with increased guar gum viscous ($p < 0.05$). The crude lipid concentration in the guar gum groups was considerably lower than that in the control group, and decreased substantially with increased guar gum viscous ($p < 0.05$).

Apparent Digestibility

The apparent digestibility of dry matter, crude protein and crude lipid in the guar gum groups were considerably lower than those in the control group, and these parameters decreased substantially with increased guar gum viscous ($p < 0.05$; **Table 5**).

Intestinal Digestive and Absorptive Enzyme Activity

Intestinal amylase activity was not significantly affected by the experimental diets ($p < 0.05$; **Table 6**). Intestinal protease, lipase and creatine kinase (CK) activities in the guar gum groups were significantly lower than those in the control group, and intestinal protease and lipase activities decreased considerably with increased guar gum viscous ($p < 0.05$). Intestinal alkaline phosphatase (AKP) activity in the Hvs-GG group was considerably lower than that in the Lvs-GG and control groups ($p < 0.05$). Intestinal Na^+/K^+-ATPase activity in the Mvs-GG and Hvs-GG groups was considerably lower than in the Lvs-GG and control groups ($p < 0.05$).

Serum Biochemical Parameters

Serum LDL-C concentration and AST, CAT and POD activities were not significantly affected by the experimental diets ($p > 0.05$; **Table 7**). Serum HDL-C, T-CHO, TG) concentrations and SOD activity in the guar gum groups were significantly lower than those in the control group, and serum TG concentration decreased considerably with increased guar gum viscous ($p < 0.05$). Serum MDA concentration in the Mvs-GG and Hvs-GG groups was significantly higher than that in the Lvs-GG and control groups ($p < 0.05$). Serum ALT activity in the Hvs-GG group was considerably higher than that in other groups ($p < 0.05$).

Intestinal Morphology

The morphological observations of the hindgut are shown in **Figure 1** and **Figure 2**. Intestinal villus width and crypt depth were not significantly affected by the experimental diets ($p > 0.05$; **Table 8**). Intestinal villus height and muscular thickness in the guar gum groups were considerably higher than those in the control group ($p < 0.05$). The goblet cell relative number in the Mvs-GG and Hvs-GG groups was considerably lower than in the Lvs-GG and control groups ($p < 0.05$). The microvillus height in the Lvs-GG and Hvs-GG groups was considerably lower than in the control group ($p < 0.05$).

Intestinal Development-Related Gene Expression

The expression level of GLP-2 in the Lvs-GG group was significantly lower than that in the control group, while increased significantly in the Mvs-GG and Hvs-GG groups ($p < 0.05$; **Figure 3**). The expression level of IGF-1 in the guar

TABLE 7 | Effects of different viscous guar gum on serum biochemical parameters of juvenile largemouth bass.

Group	Control	Lvs-GG	Mvs-GG	Hvs-GG
HDL-C (mmol/L)	5.11 ± 0.63^{b}	3.66 ± 0.37^{a}	3.50 ± 0.23^{a}	3.40 ± 0.19^{a}
LDL-C (mmol/L)	3.53 ± 0.23	3.24 ± 0.34	3.26 ± 0.06	3.26 ± 0.41
T-CHO (mmol/L)	10.75 ± 0.60^{b}	7.41 ± 0.54^{a}	7.26 ± 0.29^{a}	7.53 ± 0.50^{a}
TG (mmol/L)	10.17 ± 0.75^{c}	8.88 ± 0.34^{b}	7.18 ± 0.17^{a}	6.94 ± 0.23^{a}
ALT (U/L)	3.84 ± 0.15^{a}	4.40 ± 0.42^{a}	3.77 ± 0.37^{a}	6.64 ± 0.34^{b}
AST (U/L)	15.75 ± 0.40	15.68 ± 1.09	14.90 ± 1.84	15.79 ± 0.97
SOD (U/mL)	217.72 ± 10.52^{b}	174.30 ± 6.55^{a}	180.47 ± 5.28^{a}	169.16 ± 6.50^{a}
MDA (nmol/ml)	19.23 ± 1.20^{a}	19.37 ± 1.62^{a}	35.14 ± 1.56^{b}	34.23 ± 0.90^{b}
CAT(U/ml)	6.23 ± 0.26	6.61 ± 0.25	6.46 ± 0.28	6.79 ± 0.25
POD (U/ml)	1.31 ± 0.04	1.29 ± 0.04	1.25 ± 0.03	1.30 ± 0.03

HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein; T-CHO, total cholesterol; TG, triglyceride; MDA, malondialdehyde: ALT, alanine aminotransferase; AST, aspartate aminotransferase; POD, peroxidase; CAT, catalase; SOD, superoxide dismutase.

Values are presented as means ± S.E.M (n = 4). Different superscript letters in the same row means there was a significant difference between data ($p < 0.05$).

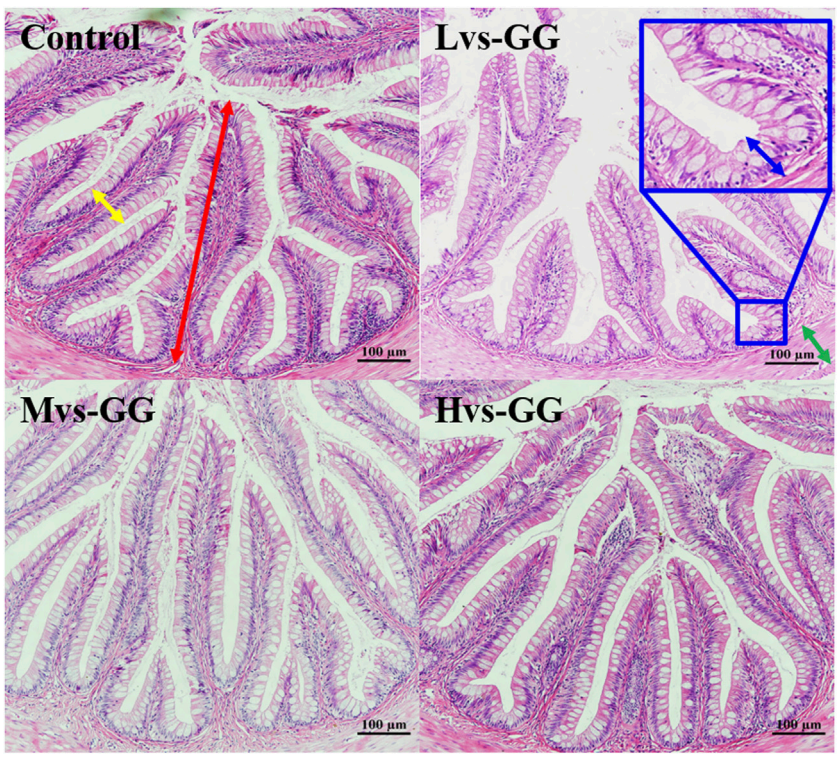


FIGURE 1 | Effects of increasing dietary guar gum viscous on hindgut morphology of juvenile largemouth bass (H&E staining, magnification ×200). Yellow double-side arrow: villus width; Red double-side arrow: villus height; green double-side arrow: muscularis thickness; blue double-side arrow: crypt depth; blue arrow: goblet cell.

gum groups were significantly higher than that in the control group ($p < 0.05$).

DISCUSSION

This study was the first attempt to evaluate the effects of different viscous guar gum on growth, apparent nutrient digestibility, intestinal development and morphology in largemouth bass. In early studies, guar gum has been reported to have the ability to lower serum cholesterol concentrations, control body weight and alleviate obesity in human (Jenkins et al., 1975; Tuomilehto et al., 1980; Casas et al., 2000; Butt et al., 2007). Although these features may benefit humans, for farmed animals, such as fish, it means a decrease in growth performance, which is detrimental to farming profitability. Recently, guar gum (galactomannan) has attracted increasing attention as an anti-nutritional factor or feed binder in fish (Brinker, 2009; Brinker and Friedrich, 2012; Thombare et al., 2016; Gao et al., 2019), and several studies have shown that dietary supplementation with guar gum is beneficial for

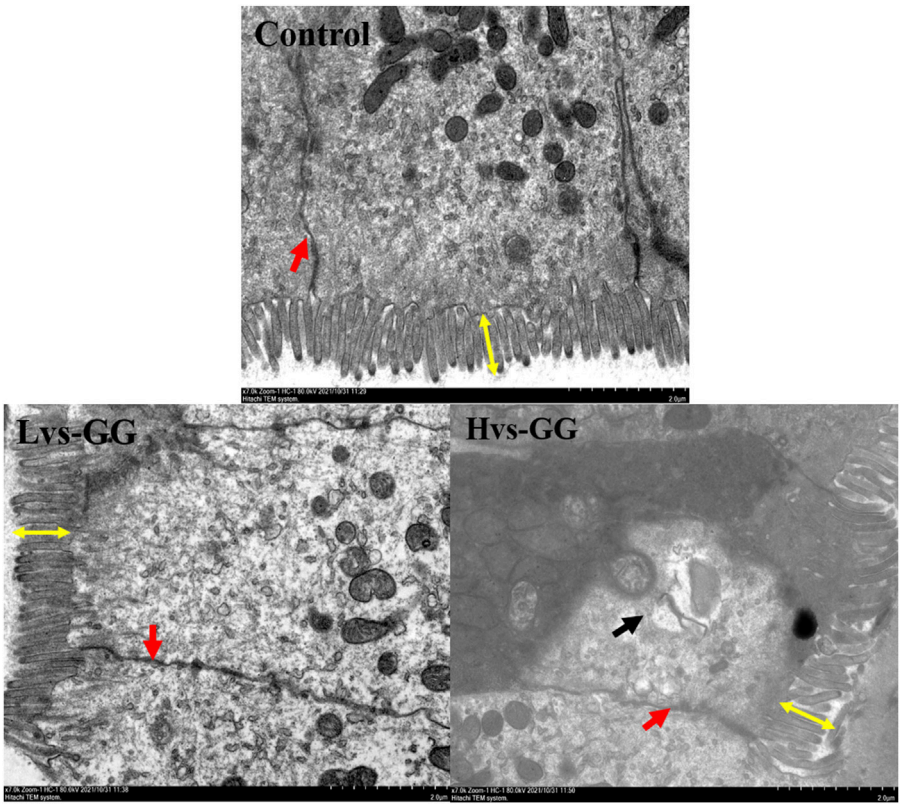


FIGURE 2 | Effects of increasing dietary guar gum viscous on hindgut morphology of juvenile largemouth bass (Transmission electron microscope observation, magnification ×7,000). Yellow double-side arrow: microvillus height; black arrow: epithelial cell death; red arrow: epithelial cell interval.

TABLE 8 | Effects of different viscous guar gum on hindgut morphology of juvenile largemouth bass.

Group	Control	Lvs-GG	Mvs-GG	Hvs-GG
Villus height (μm)	518.35 ± 24.40^{a}	646.97 ± 18.27^{b}	625.79 ± 26.57^{b}	629.12 ± 22.00^{b}
Villus width (μm)	103.45 ± 13.28	98.47 ± 5.42	99.60 ± 10.54	107.94 ± 7.05
crypt depth (μm)	25.59 ± 3.14	23.69 ± 1.62	23.26 ± 1.58	23.89 ± 1.50
Muscularis thickness (μm)	110.06 ± 6.48^{a}	132.71 ± 6.07^{b}	147.71 ± 7.88^{b}	133.82 ± 4.45^{b}
Goblet cell relative number (per 100 μm)	17.00 ± 0.50^{b}	16.29 ± 0.72^{b}	12.50 ± 0.65^{a}	13.20 ± 0.49^{a}
Microvillus height (μm)	1.31 ± 0.03^{b}	1.16 ± 0.04^{a}	-	1.22 ± 0.05^{a}

Values are presented as means ± S.E.M (n = 4). Different superscript letters in the same row means there was a significant difference between data ($p < 0.05$).

improving feed quality but not for fish growth (Tran-Tu et al., 2018; Gao et al., 2019). In the present study, dietary guar gum significantly inhibited the growth performance of largemouth bass, and fish fed with high viscous guar gum had the poorest growth performance, suggesting that high viscous guar gum is more detrimental to the growth of juvenile largemouth bass. In a previous study, increasing dietary guar gum levels also decreased the growth performance of gibel carp (*Carassius gibelio*) due to increased dietary guar gum levels increased dietary viscous (Gao et al., 2019). This evidence demonstrated that viscous is the prominent anti-nutritional feature of guar gum, and high viscous diets are detrimental to fish growth. Generally, increasing dietary viscous delays intestinal evacuation and thus adversely affects feed intake (Leenhouwers et al., 2006; Sinha et al., 2011). In a previous study, increasing dietary guar gum levels from 1% to 5% resulted in a significantly decreased FI of gibel carp (Gao et al., 2019). However, changes in FI in this study showed that increasing dietary viscosity promotes fish feeding. Similarly, the FI of rainbow trout (*Oncorhynchus mykiss*) fed SNSP (viscous) diets was significantly increased compared with fish fed a normal diet (Deng et al., 2021). This evidence suggests that the effects of increasing dietary viscosity on FI in fish remain controversial, and the difference may be species-related, which requires further study.

Digestive and absorptive enzymes are essential for fish to utilize feed nutrients and play an important role in fish growth (Li et al., 2019; Lin S et al., 2020). Also, the activity of digestive and absorptive enzymes determines the digestibility and

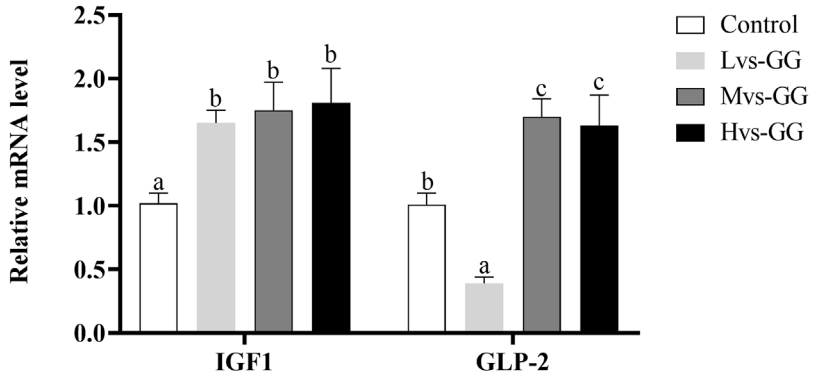


FIGURE 3 | Effects of increasing dietary guar gum viscous on the expression levels of intestinal IGF1 and GLP-2 in juvenile largemouth bass fed different viscous guar gum. Bars represent the mean ± SEM (n = 4), and different superscript letters represent significant differences between treatments ($p < 0.05$).

utilization of feed nutrients by fish. Meanwhile, the characteristics and quantities of dietary components will inevitably affect the activity of digestive and absorptive enzymes in fish (Hu et al., 2018; Zhang et al., 2021; Mardones et al., 2022). In a previous study, high dietary viscous has been reported to inhibit feed utilization by altering the mixing process between digestive enzymes and substrates, or hindering the effective interaction of digestive enzymes on the intestinal mucosal surface (Sinha et al., 2011). In this study, the activity of protease, lipase, CK, Na^+/K^+-ATPase and AKP were all shown to have a decreased trend with increased dietary guar gum viscous, and most of these parameters were lowest in the Hvs-GG group, indicating that high viscous guar gum is more detrimental to the digestive and absorptive enzyme activities in juvenile largemouth bass. Similarly, increasing dietary viscosity decreased the activity of chymotrypsin and AKP in gibel carp (Gao et al., 2019), and reduced the intestinal trypsin activity in rainbow trout (Deng et al., 2021). This evidence suggest that high viscous diets may also inhibit the utilization of feed nutrients on fish by directly reducing intestinal digestive and absorptive enzyme activities. Although the nutrient content of feces may be lost through contact with water, making it impossible to provide precise nutrient digestibility of the experimental diet in this study, the near-visual values of nutrient digestibility are still valid to illustrate the effect of different viscous guar gum on nutrient digestibility. In this study, we noticed that a significant decrease in apparent nutrient digestibility with increasing guar gum viscosity, which is consistent with a decrease in intestinal digestive and absorptive enzyme activity. Therefore, the decrease in digestive and absorptive enzyme activities may reasonably explain the reduction of apparent digestibility of dry matter, crude protein and crude lipid in fish fed on guar gum diets. Furthermore, an increase in dietary viscosity has been reported to cause mucin efflux with the digesta, resulting in endogenous nitrogen loss and reduced apparent protein digestibility (Piel et al., 2005; Sinha et al., 2011). Hence, the significant reduction of apparent protein digestibility in fish fed guar gum diets may also be associated with endogenous nitrogen loss.

Previous studies have confirmed that the structure of guar gum (galactomannan) has a large number of hydroxyl units (Ebringerová, 2005). These hydroxyl units can bind to mineral elements, causing mineral element loss. In fish, several studies have demonstrated that dietary NSPs inhibit the utilization of mineral elements (e.g., Na, K, Zn, Mg, Ca, P, Cu, and Fe), as well as an increase in the mineral elements' concentration of the feces (Leenhouwers et al., 2007a; Leenhouwers et al., 2007b; Hansen and Storebakken, 2007). Noteworthy, some mineral elements are essential for the activity of digestive and absorptive enzyme activities. For instance, Ca act as an activator of proteases (Li et al., 2017), and Na and P as substrates can effectively regulate Na^+/K^+-ATPase activity (Gal-Garber et al., 2003). Therefore, the decrease in digestive and absorptive enzyme activities in fish fed guar gum diets may also be associated with the loss of mineral elements.

It is well known that the liver and gastrointestinal tract constitute the primary digestive system of fish, where dietary nutrients are absorbed by the intestine and then transferred to the liver for storage or utilization. Therefore, fish slaughter parameters (OI, HSI, VSI and ILI) are commonly used to assess nutritional status (Knutsen et al., 2019; Shen et al., 2021). Generally, HSI can effectively reflect the accumulation of nutrients, while VSI and ILI were assumed to evaluate the status of digestion and absorption abilities in fish. In this study, HSI was significantly decreased in fish fed guar gum diets, and this parameter was significantly reduced with increased dietary guar gum viscous. The decrease in HSI may be associated with poor apparent digestibility of feed nutrients, due to the reduced nutrient intake reduce the accumulation of nutrients in the liver. In previous studies, increasing dietary SNSP levels has been reported to increase the length and size of digestive organs in monogastric animals (including fish), due to the increased viscosity (Sinha et al., 2011). In this study, VSI and ILI were also significantly increased in fish fed guar gum diets, accompanied by a significant upregulation of intestinal development-related genes, including IGF1 and GLP-2. These results suggest that increasing dietary viscosity may prolong intestinal length via upregulation of intestinal development-related genes, but the mechanism remains unclear.

Similarly, dietary supplementation with guar gum significantly increased the gastrointestinal tract weight of African catfish (*Clarias gariepinus*) (Leenhouwers et al., 2006). Combined with lower digestive and absorptive enzyme activities and poor apparent nutrient digestibility, it can be speculated that prolonging intestinal length may be a strategy for juvenile largemouth bass to cope with high viscous guar gum diets, by increasing digestion and absorption area to obtain more nutrients. Moreover, poor lipid digestibility ultimately leads to a significant reduction in serum HDL-C, T-CHO, TG and whole-body crude lipid concentrations in fish fed guar gum diets.

AST and ALT are enzymes involved in amino acid metabolism and are mainly distributed in hepatocytes, released into the blood when hepatocytes are damaged. Therefore, the activity of AST and ALT are commonly used to assess the liver health status of fish (Deng et al., 2017). In the present study, changes in ALT activity indicate that high viscous guar gum induced liver damage in juvenile largemouth bass. In addition, fish fed high viscous guar gum diets also showed a significant decrease in serum SOD activity and an increase in MDA concentration, suggesting that high viscous guar gum reduces the antioxidant capacity of juvenile largemouth bass. More importantly, a decrease in antioxidant capacity usually leads to increased radicals, thereby inducing oxidative damage (Lin et al., 2018). Hence, liver damage caused by a high viscous diet may be associated with decreased antioxidant capacity. Similarly, high SNSP diets also induced liver impairment in yellow catfish (*Pelteobagrus fulvidraco*) (Cai et al., 2019).

Dietary components inevitably affect the intestinal morphology of fish; hence, intestinal morphology is commonly used to evaluate the effects of diets on fish intestines (Hartviksen et al., 2014; Huang et al., 2022). More importantly, the intestinal physiological functions are closely associated with the morphology (Fang et al., 2019; Li et al., 2020). For instance, changes in villus height, folds and goblet cell numbers can affect the digestive and absorptive functions of the intestine (Lin A et al., 2020; Liu et al., 2021). In general, factors that increase the digestive and absorption area are conducive to promote digestion and absorption in the intestinal tract. In this study, the microvillus height was significantly decreased in fish fed guar gum diets, suggesting that increasing dietary viscosity is detrimental to intestinal digestive and absorption functions. Conversely, the villus height and muscular thickness were significantly increased in fish fed guar gum diets. Combined with poor nutrient digestibility, we tend to believe that the increase in villus height is an adaptation to the adverse effects of high viscous diets. Intestinal muscular thickness can represent intestinal peristaltic capacity (Huang et al., 2022). Increasing dietary viscous has been associated with increased digesta viscous Leenhouwers et al., 2007a), delayed gastric emptying and decreased intestinal internal oxygen tension, which providing high-quality conditions for anaerobic microbial proliferation (Choct, 1997; Sinha et al., 2011). More importantly, the expansion of some anaerobic microbial may increase the concentrations of toxic metabolites (Carré et al., 1995), which are detrimental to intestinal health. In this case, increased intestinal motility may provide benefits in alleviate the negative effects of high viscous diets. Thus, it can be speculated the increase of intestinal muscularis thickness is an adaptive development of high viscous diets.

CONCLUSION

In conclusion, guar gum diets adversely affected intestinal morphology, decreased intestinal digestive and absorptive enzyme activities, and caused poor nutrient digestibility and growth performance in juvenile largemouth bass. Moreover, the adverse effects of guar gum are closely related to its viscous, and high viscous guar gum produces more extreme negative impacts on juvenile largemouth bass. Therefore, the application of guar gum as an aquafeed binder requires consideration of its viscous.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

The feeding trial and sample analysis were performed by YL, HZ, JF, HH, YZ, YC, and WJ, with the help of WZ, JD, and BT. The manuscript was written or revised by all authors.

FUNDING

This work was supported by the Program for Scientific Research Startup Funds of Guangdong Ocean University (060302022007), the National Natural Science Foundation of China (31760761), the National Key R&D Program of China (2019YFD0900200), Postgraduate Education Innovation Project of Guangdong Ocean University (202246) and Zhanjiang Innovation and Entrepreneurship Team Cultivation "Pilot Program".

ACKNOWLEDGMENTS

The authors would like to thank Professor Dong Xiaohui and Zhang Shuang for their help in raw material procurement, and also to Dr Huangyang, Long Shuisheng, Xu Jia, and Yin Bin, Lu Bingneng, Xiao Yousang, Wei Zhangbo, She Xiaoqi, Sun Mengya, and Liu Yongyin for their help during the feeding period.

REFERENCES

Amirkolaie, A. K., Leenhouwers, J. I., Verreth, J. A. J., and Schrama, J. W. (2005). Type of Dietary Fibre (Soluble Versus Insoluble) Influences Digestion, Faeces Characteristics and Faecal Waste Production in Nile Tilapia (*Oreochromis Niloticus L*). *Aquac. Res.* 36, 1157–1166. doi:10.1111/j.1365-2109.2005.01330.x

AOAC (2005). *Official Methods of Analysis of Aoac International*. 18th ed. Gaithersburg, MD, USA: Association of Official Analytical Chemists.

Azeredo, R., Machado, M., Guardiola, F. A., Cerezuela, R., Afonso, A., Peres, H., et al. (2017). Local Immune Response of Two Mucosal Surfaces of the European Seabass, *Dicentrarchus Labrax,* Fed Tryptophan- or Methionine-Supplemented Diets. *Fish. Shellfish Immunol.* 70, 76–86. doi:10.1016/j.fsi.2017.09.016

Brinker, A., and Friedrich, C. (2012). Fish Meal Replacement by Plant Protein Substitution and Guar Gum Addition in Trout Feed. Part II: Effects on Faeces Stability and Rheology. *Biorheology* 49, 27–48. doi:10.3233/BIR-2012-0605

Brinker, A. (2009). Improving the Mechanical Characteristics of Faecal Waste in Rainbow Trout: The Influence of Fish Size and Treatment with a Non-Starch Polysaccharide (Guar Gum). *Aquac. Nutr.* 15, 229–240. doi:10.1111/j.1365-2095.2008.00587.x

Butt, M. S., Shahzadi, N., Sharif, M. K., and Nasir, M. (2007). Guar Gum: A Miracle Therapy for Hypercholesterolemia, Hyperglycemia and Obesity. *Crit. Rev. Food Sci. Nutr.* 47, 389–396. doi:10.1080/10408390600846267

Cai, C., Ren, S., Cui, G., Ni, Q., Li, X., Meng, Y., et al. (2019). Short-Term Stress Due to Dietary Pectin Induces Cholestasis, and Chronic Stress Induces Hepatic Steatosis and Fibrosis in Yellow Catfish, *Pelteobagrus Fulvidraco*. *Aquaculture* 516, 734607. doi:10.1016/j.aquaculture.2019.734607

Carré, B., Gomez, J., and Chagneau, A. M. (1995). Contribution of Oligosaccharide and Polysaccharide Digestion, and Excreta Losses of Lactic Acid and Short Chain Fatty Acids, to Dietary Metabolisable Energy Values in Broiler Chickens and Adult Cockerels. *Br. Poult. Sci.* 36, 611–629. doi:10.1080/00071669508417807

Casas, J., Mohedano, A., and García-Ochoa, F. (2000). Viscosity of Guar Gum and Xanthan/Guar Gum Mixture Solutions. *J. Sci. Food Agric.* 80, 1722–1727. doi:10.1002/1097-0010(20000915)80:12<1722::aid-jsfa708>3.0.co;2-x

Choct, M. (1997). Feed Non-Starch Polysaccharides: Chemical Structures and Nutritional Significance. *Feed Milling Int.* 191, 13–26.

Choct, M. (2015). Feed Non-Starch Polysaccharides for Monogastric Animals: Classification and Function. *Anim. Prod. Sci.* 55, 1360. doi:10.1071/AN15276

Collins, S. A., Mansfield, G. S., Desai, A. R., Van Kessel, A. G., Hill, J. E., and Drew, M. D. (2013). Structural Equation Modeling of Antinutrients in Rainbow Trout Diets and their Impact on Feed Intake and Growth. *Aquaculture* 416-417, 219–227. doi:10.1016/j.aquaculture.2013.09.020

Deng, J. M., Wang, Y., Chen, L. Q., Mai, K. S., Wang, Z., and Zhang, X. (2017). Effects of Replacing Plant Proteins with Rubber Seed Meal on Growth, Nutrient Utilization and Blood Biochemical Parameters of Tilapia (*Oreochromis Niloticus* × *O. Aureus*). *Aquac. Nutr.* 23, 30–39. doi:10.1111/anu.12355

Deng, J., Zhang, X., Sun, Y., Mi, H., and Zhang, L. (2021). Effects of Different Types of Non-Starch Polysaccharides on Growth, Digestive Enzyme Activity, Intestinal Barrier Function and Antioxidant Activity of Rainbow Trout (*Oncorhynchus Mykiss*). *Aquac. Rep.* 21, 100864. doi:10.1016/j.aqrep.2021.100864

Ebringerová, A. (2005). Structural Diversity and Application Potential of Hemicelluloses. *Macromol. Symp.* 232, 1–12. doi:10.1002/masy.200551401

Fang, H., Xie, J., Liao, S., Tian, Y., Guo, S., Liu, Y., et al. (2019). Effects of Dietary Inclusion of Shrimp Paste on Growth Performance, Digestive Enzymes Activities, Antioxidant and Immunological Status and Intestinal Morphology of Hybrid Snakehead (*Channa Maculata* ♀ × *Channa Argus* ♂). *Front. Physiol.* 10, 1027. doi:10.3389/fphys.2019.01027

Gal-Garber, O., Mabjeesh, S., Sklan, D., and Uni, Z. (2003). Nutrient Transport in the Small Intestine: Na^+, K^+-Atpase Expression and Activity in the Small Intestine of the Chicken as Influenced by Dietary Sodium. *Poult. Sci.* 82, 1127–1133. doi:10.1093/ps/82.7.1127

Gao, S., Han, D., Zhu, X., Yang, Y., Liu, H., Xie, S., et al. (2019). Effects of Guar Gum on the Growth Performance and Intestinal Histology of Gibel Carp (*Carassius Gibelio*). *Aquaculture* 501, 90–96. doi:10.1016/j.aquaculture.2018.11.009

George, A., Shah, P. A., and Shrivastav, P. S. (2019). Guar Gum: Versatile Natural Polymer for Drug Delivery Applications. *Eur. Polym. J.* 112, 722–735. doi:10.1016/j.eurpolymj.2018.10.042

Glencross, B., Rutherford, N., and Bourne, N. (2012). The Influence of Various Starch and Non-Starch Polysaccharides on the Digestibility of Diets Fed to Rainbow Trout (*Oncorhynchus Mykiss*). *Aquaculture* 356–357, 141–146. doi:10.1016/j.aquaculture.2012.05.023

Hansen, J. Ø., and Storebakken, T. (2007). Effects of Dietary Cellulose Level on Pellet Quality and Nutrient Digestibilities in Rainbow Trout (*Oncorhynchus Mykiss*). *Aquaculture* 272, 458–465. doi:10.1016/j.aquaculture.2007.09.005

Hartviksen, M., Vecino, J. L. G., Ringø, E., Bakke, A. M., Wadsworth, S., Krogdahl, A., et al. (2014). Alternative Dietary Protein Sources for Atlantic Salmon (*Salmo Salar L.*) Effect on Intestinal Microbiota, Intestinal and Liver Histology and Growth. *Aquac. Nutr.* 20, 381–398. doi:10.1111/anu.12087

He, Y., Ye, G., Chi, S., Tan, B., Dong, X., Yang, Q., et al. (2020). Integrative Transcriptomic and Small RNA Sequencing Reveals Immune-Related Mirna-Mrna Regulation Network for Soybean Meal-Induced Enteritis in Hybrid Grouper, *Epinephelus Fuscoguttatus*♀ × *Epinephelus Lanceolatus*♂. *Front. Immunol.* 11, 1502. doi:10.3389/fimmu.2020.01502

He, Y., Guo, X., Tan, B., Dong, X., Yang, Q., Liu, H., et al. (2021). Replacing Fish Meal with Fermented Rice Protein in Diets for Hybrid Groupers (*Epinephelus Fuscoguttatus*♀× *Epinephelus Lanceolatus*♂): Effects on Growth, Digestive and Absorption Capacities, Inflammatory-Related Gene Expression, and Intestinal Microbiota. *Aquac. Rep.* 19, 100603. doi:10.1016/j.aqrep.2021.100603

Hu, P., Ge, Y., Yi, G., Liu, W., and Tang, W. (2018). Evaluation of Amino Acid Content, Immunity and Digestibility of *Fenneropenaeus Chinensis* Juveniles for Stock Enhancement. *J. Guangdong Ocean Univ.* 38, 90–95. doi:10.3969/j.issn.1673-9159.2018.06.014

Hu, B., Song, L., Mao, S., and Xu, P. (2019). Effects of Four Chinese Herbal Preparations on Growth Performance and Antioxidant Activity in Juvenile *Micropterus Salmoides*. *J. Guangdong Ocean Univ.* 39, 101–107. doi:10.3969/j.issn.1673-9159.2019.04.015

Huang, B., Zhang, S., Dong, X., Chi, S., Yang, Q., Liu, H., et al. (2022). Effects Of Fishmeal Replacement by Black Soldier Fly on Growth Performance, Digestive Enzyme Activity, Intestine Morphology, Intestinal Flora and Immune Response of Pearl Gentian Grouper (*Epinephelus Fuscoguttatus* ♀ × *Epinephelus Lanceolatus* ♂). *Fish. Shellfish Immunol.* 120, 497–506. doi:10.1016/j.fsi.2021.12.027

Jenkins, D. A., Newton, C., Leeds, A., and Cummings, J. (1975). Effect of Pectin, Guar Gum, and Wheat Fibre on Serum-Cholesterol. *Lancet* 305, 1116–1117. doi:10.1016/S0140-6736(75)92503-9

Karim, A., Naila, B., Khwaja, S., Hussain, S. I., and Ghafar, M. (2022). Evaluation of Different Starch Binders on Physical Quality of Fish Feed Pellets. *Braz. J. Biol.* 84, e256242. doi:10.1590/1519-6984.256242

Kazuhiko, Y., Keiji, S., Hiroyuki, Y., and Ichikawa, T. (1993). Effects of Partially Hydrolyzed Guar Gum on Postprandial Plasma Glucose and Lipid Levels in Humans. *Nippon. Shokuryo Gakkaishi* 58, 1364–1369. doi:10.1271/bbb.58.1364

Knutsen, H. R., Johnsen, I. H., Keizer, S., Sørensen, M., Roques, J. A. C., Heden, I., et al. (2019). Fish Welfare, Fast Muscle Cellularity, Fatty Acid and Body-Composition of Juvenile Spotted Wolffish (*Anarhichas Minor*) Fed a Combination of Plant Proteins and Microalgae (*Nannochloropsis Oceanica*). *Aquaculture* 506, 212–223. doi:10.1016/j.aquaculture.2019.03.043

Kokou, F., and Fountoulaki, E. (2018). Aquaculture Waste Production Associated with Antinutrient Presence in Common Fish Feed Plant Ingredients. *Aquaculture* 495, 295–310. doi:10.1016/j.aquaculture.2018.06.003

Kraugerud, O. F., Penn, M., Storebakken, T., Refstie, S., Krogdahl, Å., and Svihus, B. (2007). Nutrient Digestibilities and Gut Function in Atlantic Salmon (*Salmo Salar*) Fed Diets with Cellulose or Non-Starch Polysaccharides from Soy. *Aquaculture* 273, 96–107. doi:10.1016/j.aquaculture.2007.09.013

Kuz Mina, V. V. (1996). Influence of Age on Digestive Enzyme Activity in Some Freshwater Teleosts. *Aquaculture* 148, 25–37. doi:10.1016/S0044-8486(96)01370-1

Leenhouwers, J. I., Adjei-Boateng, D., Verreth, J. A. J., and Schrama, J. W. (2006). Digesta Viscosity, Nutrient Digestibility and Organ Weights in African Catfish (*Clarias Gariepinus*) Fed Diets Supplemented with Different Levels of a Soluble Non-Starch Polysaccharide. *Aquac. Nutr.* 12, 111–116. doi:10.1111/j.1365-2095.2006.00389.x

Leenhouwers, J. I., Ortega, R. C., Verreth, J. A. J., and Schrama, J. W. (2007a). Digesta Characteristics in Relation to Nutrient Digestibility and Mineral Absorption in Nile Tilapia (*Oreochromis Niloticus L.*) Fed Cereal Grains of Increasing Viscosity. *Aquaculture* 273, 556–565. doi:10.1016/j.aquaculture.2007.10.044

Leenhouwers, J. I., Veld, M. T., Verreth, J., and Schrama, J. W. (2007b). Digesta Characteristiscs and Performance of African Catfish (*Clarias Gariepinus*) Fed Cereal Grains that Differ in Viscosity. *Aquaculture* 264, 330–341. doi:10.1016/j.aquaculture.2007.01.003

Li, S., Bai, J., Ye, X., Lao, H., and Jian, Q. (2007). Cloning and Sequence Analysis of CDNA Encoding Growth Hormone and Insulin-Like Growth Factor I in Largemouth Bass (*Micropterus Salmoides*). *J. Guangdong Ocean Univ.* 27, 1–5.

Li, R., Bai, J., Li, S., Lu, J., Li, X., and Liu, C. (2010). Analysis on Genetic Structure of Selected Population of Largemouth Bass by Microsatellite DNA Markers. *J. Guangdong Ocean Univ.* 30, 11–15.

Li, M., Jin, D., Zhang, J., Feng, Y., and Zheng, R. (2017). Activate Condition Optimization of Trypsin and its Application in Animal Cell Culture. *J. Food Sci. Biotechnol.* 36, 1304–1310. doi:10.3969/j.issn.1673-1689.2017.12.012

Li, J., Wang, C., Wang, L., Xu, Q., Zhao, Z., Luo, L., et al. (2019). Effects of Glutamate Supplementation in Low Phosphorus Diets on Intestinal Digestive Enzyme Activities and Intestinal Morphology of Juvenile Songpu Mirror Carp (Cyprinus Carpio L). *J. Guangdong Ocean Univ.* 39, 20–26. doi:10.3969/j.issn.1673-9159.2019.04.004

Li, J., Wang, C., Zhang, Y., Wu, D., and Fan, Z. (2020). Effect of Arginine Supplementation in High Starch Diets on Intestinal Digestive Enzyme Activities and Intestinal Morphology of Songpu Mirror Carp (*Cyprinus Carpio L*). *J. Guangdong Ocean Univ.* 41, 39–46. doi:10.3969/j.issn.1673-9159.2021.01.006

Lin A, A., Wang, W., Chen, G., Zhang, J., Huang, J., Pan, C., et al. (2020). Effects of Two Lactic Acid Bacteria on Growth Performance and Activities of Digestive and Non-Specific Immune Enzymes of Juvenile Cobia (*Rachycentroncanadum*). *J. Guangdong Ocean Univ.* 40, 112–117. doi:10.3969/j.issn.1673-9159.2020.05.014

Lin, S., Shi, C., Mu, M., Chen, Y., and Luo, L. (2018). Effect of High Dietary Starch Levels on Growth, Hepatic Glucose Metabolism, Oxidative Status and Immune Response of Juvenile Largemouth Bass, *Micropterus Salmoides*. *Fish. Shellfish Immunol.* 78, 121–126. doi:10.1016/j.fsi.2018.04.046

Lin S, S., Zhou, X., Zhou, Y., Kuang, W., Chen, Y., Luo, L., et al. (2020). Intestinal Morphology, Immunity and Microbiota Response to Dietary Fibers in Largemouth Bass, *Micropterus Salmoide*. *Fish. Shellfish Immunol.* 103, 135–142. doi:10.1016/j.fsi.2020.04.070

Liu, H., Li, L., Ayiku, S., Tang, Z., Fan, W., Tan, B., et al. (2021). Effects of Dietary Yeast Culture Supplementation on Growth, Intestinal Morphology, Immunity, and Disease Resistance in *Epinephelus Fuscoguttatus*♀×*Epinephelus Lanceolatus*♂. *J. Guangdong Ocean Univ.* 41, 1–11. doi:10.3969/j.issn.1673-9159.2021.03.001

Liu, Y., Huang, H., Fan, J., Zhou, H., Zhang, Y., Cao, Y., et al. (2022). Effects of Dietary Non-Starch Polysaccharides Level on the Growth, Intestinal Flora and Intestinal Health of Juvenile Largemouth Bass *Micropterus Salmoides*. *Aquaculture* 557, 738343. doi:10.1016/j.aquaculture.2022.738343

Mardones, O., Oyarzún-Salazar, R., Labbé, B. S., Miguez, J. M., Vargas-Chacoff, L., and Munoz, J. L. P. (2022). Intestinal Variation of Serotonin, Melatonin, and Digestive Enzymes Activities Along Food Passage Time Through Git in Salmo Salar Fed with Supplemented Diets with Tryptophan and Melatonin. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 266, 111159. doi:10.1016/j.cbpa.2022.111159

Merrifield, D. L., Dimitroglou, A., Bradley, G., Baker, R. T. M., and Davies, S. J. (2009). Soybean Meal Alters Autochthonous Microbial Populations, Microvilli Morphology and Compromises Intestinal Enterocyte Integrity of Rainbow Trout, *Oncorhynchus Mykiss* (*Walbaum*). *J. Fish. Dis.* 32, 755–766. doi:10.1111/j.1365-2761.2009.01052.x

Piel, C., Montagne, L., Sève, B., and Lallès, J. (2005). Increasing Digesta Viscosity Using Carboxymethylcellulose in Weaned Piglets Stimulates Ileal Goblet Cell Numbers and Maturation. *J. Nutr.* 135, 86–91. doi:10.1093/jn/135.1.86

Ramos, L. R. V., Romano, L. A., Monserrat, J. M., Abreu, P. C., Verde, P. E., and Tesser, M. (2015). Biological Responses in Mullet Mugil Liza Juveniles Fed with Guar Gum Supplemented Diets. *Animal Feed Sci. Technol.* 205, 98–106. doi:10.1016/j.anifeedsci.2015.04.004

Ren, S., Cai, C., Cui, G., Ni, Q., Ye, Y., Su, X., et al. (2020). High Dosages of Pectin and Cellulose Cause Different Degrees of Damage to the Livers and Intestines of *Pelteobagrus Fulvidraco*. *Aquaculture* 514, 734445. doi:10.1016/j.aquaculture.2019.734445

Seal, C. J., and Mathers, J. C. (2001). Comparative Gastrointestinal and Plasma Cholesterol Responses of Rats Fed on Cholesterol-Free Diets Supplemented with Guar Gum and Sodium Alginate. *Br. J. Nutr.* 85, 317–324. doi:10.1079/BJN2000250

Shen, Y., Li, H., Zhao, J., Tang, S., Zhao, Y., Bi, Y., et al. (2021). The Digestive System of Mandarin Fish (*Siniperca Chuatsi*) can Adapt to Domestication by Feeding with Artificial Diet. *Aquaculture* 538, 736546. doi:10.1016/J.Aquaculture.2021.736546

Sinha, A. K., Kumar, V., Makkar, H. P. S., De Boeck, G., and Becker, K. (2011). Non-Starch Polysaccharides and their Role in Fish Nutrition-A Review. *Food Chem.* 127, 1409–1426. doi:10.1016/j.foodchem.2011.02.042

Storebakken, T. (1985). Binders in Fish Feeds: I. Effect of Alginate and Guar Gum on Growth, Digestibility, Feed Intake and Passage through the Gastrointestinal Tract of Rainbow Trout. *Aquaculture* 47, 11–26. doi:10.1016/0044-8486(85)90004-3

Takii, K., Nakamura, M., Urakawa, K., Miyashita, S., Kumai, H., Kubo, Y., et al. (2008). Soybean Trypsin Inhibitors Inhibit Trypsin-Like and Basic Proteinase Activities of Cultured-Fishes. *Fish. Sci.* 64, 935–938. doi:10.2331/fishsci.64.935

Theocharidou, A., Mourtzinos, I., and Ritzoulis, C. (2022). The Role of Guar Gum on Sensory Perception, on Food Function, and on the Development of Dysphagia Supplements-A Review. *Food Hydrocoll. Health* 2, 100053. doi:10.1016/j.fhfh.2022.100053

Thombare, N., Jha, U., Mishra, S., and Siddiqui, M. Z. (2016). Guar Gum as a Promising Starting Material for Diverse Applications: A Review. *Int. J. Biol. Macromol.* 88, 361–372. doi:10.1016/j.ijbiomac.2016.04.001

Tran-Tu, L. C., Hien, T. T. T., Bosma, R. H., Heinsbroek, L. T. N., Verreth, J. A. J., and Schrama, J. (2018). Effect of Ingredient Particle Sizes and Dietary Viscosity on Digestion and Faecal Waste of Striped Catfish (*Pangasianodon Hypophthalmus*). *Aquac. Nutr.* 24, 961–969. doi:10.1111/anu.12632

Tuomilehto, J., Voutilainen, E., Huttunen, J., Vinni, S., and Homan, K. (1980). Effect of Guar Gum on Body Weight and Serum Lipids in Hypercholesterolemic Females. *Acta Med. Scand.* 208, 45–48. doi:10.1111/j.0954-6820.1980.tb01148.x

Xu, X., Yang, H., Zhang, C., Bian, Y., Yao, W., Xu, Z., et al. (2022). Effects Of Replacing Fishmeal with Cottonseed Protein Concentrate on Growth Performance, Flesh Quality and Gossypol Deposition of Largemouth Bass (*Micropterus Salmoides*). *Aquaculture* 548, 737551. doi:10.1016/j.aquaculture.2021.737551

Yannis, K., Emmanouil, K., Vassiliki, I., John, H., Nikos, P., Papanna, K., et al. (2018). Effects of High Level Fishmeal Replacement by Plant Proteins Supplemented with Different Levels of Lysine on Growth Performance and Incidence of Systemic Noninfectious Granulomatosis in Meagre (*Argyrosomus Regius*). *Aquacult. Nutr.* 24, 1738–1751. doi:10.1111/anu.12814

Yin, B., Liu, H., Tan, B., Dong, X., Zhang, S., Yang, Q., et al. (2021). MHC II-PI_3K/Akt/mTOR Signaling Pathway Regulates Intestinal Immune Response Induced by Soy Glycinin in Hybrid Grouper: Protective Effects of Sodium Butyrate. *Front. Immunol.* 11, 615980. doi:10.3389/fimmu.2020.615980

Yu, J., Liu, S., and Wang, C. (2015). *Advances in Feed Research of Guar Bean by-Products*. China Feed, 30–32.

Zeng, Q., Rahimnejad, S., Wang, L., Song, K., Lu, K., and Zhang, C. X. (2018). Effects of Guanidinoacetic Acid Supplementation in All-Plant Protein Diets on Growth, Antioxidant Capacity and Muscle Energy Metabolism of Bullfrog Rana (*Lithobates*) Catesbeiana. *Aquac. Res.* 49, 748–756. doi:10.1111/are.13505

Zhang, H., Pu, X., Yang, Q., Tan, B., Dong, X. H., Chi, S., et al. (2018). Effects of Replacing Fish Meal with High-Protein Cottonseed Meal on Growth Performance, Non-Specific Immune Index and Disease Resistance for *Litopenaeus Vannamei*. *J. Guangdong Ocean Univ.* 038, 20–26. doi:10.3969/j.issn.1673-9159.2018.04.003

Zhang, Y., Chen, P., Liang, X. F., Han, J., Wu, X. F., Yang, Y. H., et al. (2019). Metabolic Disorder Induces Fatty Liver in Japanese Seabass, *Lateolabrax Japonicas* Fed a Full Plant Protein Diet and Regulated by cAMP-JNK/NF-KB-Caspase Signal Pathway. *Fish. Shellfish Immunol.* 90, 223–234. doi:10.1016/j.fsi.2019.04.060

Zhang, M., Pan, L., Fan, D., He, J., Su, C., Gao, S., et al. (2021). Study of Fermented Feed by Mixed Strains and their Effects on the Survival, Growth, Digestive Enzyme Activity and Intestinal Flora of *Penaeus Vannamei*. *Aquaculture* 530, 735703. doi:10.1016/j.aquaculture.2020.735703

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: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Marine Science

TYPE Original Research
PUBLISHED 12 August 2022
DOI 10.3389/fmars.2022.945417

OPEN ACCESS

EDITED BY
Helena Peres,
University of Porto, Portugal

REVIEWED BY
Hongliang Zuo,
Sun Yat-sen University, China
Hongwei Shan,
Ocean University of Chinainstitution
Qingdao, China
Chengbo Sun,
Guangdong Ocean University, China

*CORRESPONDENCE
Wen Huang
huangwen549@126.com

SPECIALTY SECTION
This article was submitted to
Aquatic Physiology,
a section of the journal
Frontiers in Marine Science

RECEIVED 16 May 2022
ACCEPTED 21 July 2022
PUBLISHED 12 August 2022

CITATION
Peng K, Chen X, Lu H, Zhao J, Chen Y, Li C, Li H and Huang W (2022) Effect of dietary soybean meal on growth performance, apparent digestibility, intestinal digestive enzyme activity, and muscle growth–related gene expression of *Litopenaeus vannamei*. *Front. Mar. Sci.* 9:945417.
doi: 10.3389/fmars.2022.945417

Effect of dietary soybean meal on growth performance, apparent digestibility, intestinal digestive enzyme activity, and muscle growth–related gene expression of *Litopenaeus vannamei*

Kai Peng[1], Xiaoying Chen[1], Huijie Lu[1], Jichen Zhao[1], Yihong Chen[2], Chaozheng Li[3,4], Huo Li[5] and Wen Huang[1,4]*

[1]Institute of Animal Science, Guangdong Academy of Agricultural Sciences, Key Laboratory of Animal Nutrition and Feed Science in South China, Ministry of Agriculture and Rural Affairs, Guangdong Provincial Key Laboratory of Animal Breeding and Nutrition, Guangzhou, China, [2]School of Life Science, South China Normal University, Guangzhou, China, [3]State Key Laboratory of Biocontrol, Institute of Aquatic Economic Animals and the Guangdong Province Key Laboratory for Aquatic Economic Animals, Sun Yat-Sen University, Guangzhou, China, [4]Maoming Branch Center of Guangdong Provincial Laboratory of Lingnan Modern Agricultural Science and Technology, Maoming, China, [5]Guangdong Provincial Engineering and Technology Research Center, Guangdong Jinyang Biotechnology Co. Ltd., Maoming, China

Soybean meal is one of the major components of aquatic animal diets, whereas little information is available about the evaluation of soybean meal growth suppression mode of action. A 42-day feeding trial was performed to assess the effects of dietary soybean meal on growth performance, apparent digestibility, intestinal digestive enzyme activity, and muscle growth–related gene expression of *Litopenaeus vannamei*. A total of 600 shrimp were randomly distributed into 20 tanks with 30 shrimp per tank and four tanks per group. The soybean meal was added to the diets at the rate of 20% (T20), 28% (T28), 35% (T35), 42% (T42), and 50% (T50), respectively. Shrimp were fed with apparent satiation three times daily. Results indicated that the final body weight, weight gain rate, specific growth rate, feed intake, intestinesomatic index, dressed weight percentage, and the apparent digestibility coefficients of dry matter, crude protein, crude lipid, and ash were linearly decreased ($p < 0.05$), but feed coefficient was linearly increased ($p < 0.05$) as dietary soybean meal increased from 20% to 50%. The intestinal trypsin and amylase activities were decreased ($p < 0.05$) as dietary soybean meal increased from 20% to 50%, and reached significance at the level of 35%, 42%, and 50%. Shrimp fed with T20 had higher ($p < 0.05$) intestinal lipase activity than those fed with other diets. The mRNA relative expression of growth hormone, myogenic regulatory factor 5, and target of rapamycin was downregulated ($p < 0.05$) as dietary soybean meal

increased from 20% to 50%. To conclude, dietary soybean meal exceeded 28% significantly inhibited growth performance of *L. vannamei*, mainly due to the negative impact of soybean meal on digestion and feed utilization and also the inhibition on the muscle growth and related gene expressions.

KEYWORDS

fishmeal, soybean meal, growth, digestion, muscle growth, *Litopenaeus vannamei*

Introduction

Fishmeal is one of the most important feed protein sources in aquatic feed. It has been reported that more than two-thirds of fishmeal is used as aquatic feed in the world and about 60% in China (Mai et al., 2021). As the global population increase, the demand of people for aquatic products consumption is unabated. However, the production of fishmeal is maintained at around 5 million tons in recent years, which could no longer continue to increase in the further. Combined with the increasing need of fishmeal in the development of aquaculture, professionals have spared no effort to find alternatives to fishmeal and solutions to improve the utilization of non-fishmeal protein. Over the last decade, the plant protein such as soybean meal has been widely used to replace fishmeal in aquafeed and exhibits as a promising alternative in various species (Jiang et al., 2015a; Chakraborty et al., 2019; Shukla et al., 2019; Bae et al., 2020; Bruce et al., 2022; Wu et al., 2021; Zhao et al., 2021; Ding et al., 2022). Even so, soybean meal is considered to have deficiencies including the presence of anti-nutritional factors (e.g., soybean agglutinin, glycinin, and β-conglycinin), imbalance of essential amino acids, and absence of certain fishmeal components (e.g., taurine, hydroxyproline, and vitamin D_3) (Mai et al., 2021), which may result in poor palatability and digestibility of diets and lead to the reduced growth performance of animals.

Litopenaeus vannamei belongs to crustacean species that is being widely cultured and welcomed in China. In 2020, China contributed 1.19 million tons of the farmed *L. vannamei*, which account for 70% of shrimp production (Fishery Administration of Ministry of Agriculture and Rural Affairs, 2021). Although replacing fishmeal with soybean meal in *L. vannamei* diet has been well reported, the suitable substitution ratio is inconsistent among studies. For comprehensive assessment from growth performance and nutrient utilization of *L. vannamei*, Amaya et al. (2007) indicated that fishmeal could be totally replaced by soybean meal (dietary concentration at 39.5%); Lim and Dominy (1990) suggested that the suitable substitution ratio of fishmeal (dietary concentration at 11.3%) by soybean meal is 60%; and the suitable substitution ratio were proposed to be 28% (dietary fishmeal at 38.2%) (Xu et al., 2021) and 20% (dietary fishmeal at 30.0%) (Yang et al., 2015). On the other hand, high dose of dietary soybean meal caused inhibition of growth and digestion of *L. vannamei*, whereas the reason and related mechanism are still not fully understood. Previous studies documented that the inhibition effect of dietary soybean meal on growth of aquatic animals is mainly attributed to endogenous anti-nutritional factors (Elumalai et al., 2019; Zhao, 2021; Zhou, 2021). A recent study screened the growth and development related functional genes (e.g., myogenic regulatory factors *Myf5* and *MyoG*) in the muscle of *L. vannamei* by transcriptome sequencing, but the information regarding the effects of dietary soybean meal on the muscle growth–related gene expressions is rare. Therefore, this study is conducted to evaluate the effects of dietary soybean meal on growth performance, apparent digestibility, intestinal digestive enzyme activity, and muscle growth–related gene expression of *L. vannamei*.

Materials and methods

Diet preparation

The ingredients and proximate compositions of experimental diets were shown in Table 1. The soybean meal (crude protein 46%) was added to the diets at the rate of 20% (T20), 28% (T28), 35% (T35), 42% (T42), and 50% (T50), to replace fishmeal (crude protein 66%) at the rate of 0%, 20%, 40%, 60%, and 80%, respectively. All ingredients were ground (AHZC1265 Hammer Mill, Buhler Machinery Co., Ltd., Guanghzou, China) to pass through a 320-μm sieve, mixed (AHML2000 Mixer, Buhler Machinery Co., Ltd., Changzhou, China) thoroughly and then extruded (SLX-80 Twin-screw Extruder, South China University of Technology Machinery Factory, Guangzhou, China) into 2-mm pellets, dried (HMO-205 Oven Dryer, Haiming Electronic Technology Co., Ltd., Dongguan, China) at 55°C for 12 h, and stored at −20°C until use.

10.3389/fmars.2022.945417

TABLE 1 Ingredients and proximate compositions (g/kg DM) of experimental diets.

Items	Diets				
	T20	T28	T35	T42	T50
Ingredients					
Fishmeal (Peru, crude protein, 68%)	250	200	150	100	50
Soybean meal (crude protein, 46%)	200	280	350	420	500
Peanut bran	120	120	120	120	120
Chicken meal	100	100	100	100	100
Wheat flour	220	200	180	160	130
Fish oil	20	20	20	20	20
Soy lecithin	20	24	27	30	34
Monocalcium phosphate	15	15	15	15	15
Vitamin premix	2	2	2	2	2
Mineral premix	5	5	5	5	5
Lysine	0	0	0.5	1	1.5
Methionine	2	3	3.5	4.5	5.5
Choline chloride	2	2	2	2	2
Salt	3	3	3	3	3
Sodium alginate	12	12	12	12	12
Cellulose	29	14	10	5.5	0
Total	1,000	1,000	1,000	1,000	1,000
Proximate composition					
Moisture	72	71	72	74	73
Crude protein	414	420	418	420	416
Crude lipid	84	84	83	82	82
Ash	75	77	74	75	77
Lysine	23	23	23	23	23
Methionine	8.8	9.0	8.7	8.8	9.0

T20–T50, diets containing 20%–50% of soybean meal.
One kilogram of diet provided: VA, 3,230 IU; VD, 1,600 IU; VE, 160 mg; VK_3, 4 mg; VB_1, 4 mg; VB_2, 8 mg; VB_6, 4.8 mg; VB_{12}, 0.016 mg; nicotinic acid, 28 mg; pantothenic acid calcium, 16 mg; biotin, 0.064 mg; folic acid, 1.285 mg; inositol, 40 mg; Ca, 1,150 mg; K, 180 mg; Mg, 45 mg; Fe, 50 mg; Zn, 40 mg; Mn, 9.5 mg; Cu, 7.5 mg; Co, 1.25 mg; I, 0.16 mg; Se, 0.25 mg.

Experimental design and feeding management

The protocol (No. GAAS20210501) and all procedures performed in this study were approved by the Institutional Animal Care and Use Committee of Institute of Animal Science, Guangdong Academy of Agricultural Sciences (Guangdong, China). A 42-day feeding trial was conducted by randomly distributing 600 *L. vannamei* (initial body weight, 5.8 ± 0.15 g) into 20 tanks (30 shrimp per tank) with four tanks being assigned to each diet. Shrimp were hand-fed with apparent satiation (approximately 4% of body weight per day) three times daily (08:00, 14:00, and 20:00). Uneaten feed was collected in 1 h after each meal, analyzed for dry matter, and subtracted from feed offered (dry matter basis) to calculate feed intake (FI) as described by Wang et al. (2019). During the feeding trial, water was continuously aerated and filtered through sand filter system at a rate of 1.6 L/min. Water temperature ranged from 25°C to 27°C, dissolved oxygen was above 5.0 mg/L, pH ranged from 7.6 to 8.0, salinity was 5‰–6‰, ammonia nitrogen and nitrite were below 0.01 mg/L, and the photoperiod regime was 12-h light and 12-h dark.

Sampling

Feces were continuously collected from each tank daily at the last 14 days of the feeding trial according to the siphon method described by Gumus (2011). The intact feces were picked out with a tweezers and stored at −20°C. Fecal samples collected from each tank were pooled and dried at 105°C to a constant weight to determine the apparent digestibility coefficients (ADCs) of dry matter, crude protein, crude lipid, and ash. Indigestible acid insoluble ash (AIA) in diet ingested and in feces samples was used as marker to estimate nutrient digestibility according to Peng et al. (2021).

At the end of the feeding trial, all shrimp were fasted and anesthetized with MS-222 (40 mg/L; Sigma, USA) prior to sampling. Shrimp per tank were counted and weighted to analyze for survival rate (SR), final body weight (FBW), weight gain rate (WGR), specific growth rate (SGR), and feed coefficient (FC). Six shrimp per tank were randomly selected, determined individual body and muscle weight, and slaughtered to analyze the hepatosomatic index (HSI), intestinesomatic index (ISI), and dressed weight percentage (DWP).

Intestines of three shrimp in each tank were sampled to determine trypsin (Ultraviolet colorimetry), lipase (colorimetry), and amylase (colorimetry) activities using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the corresponding instructions of the manufacturer.

The dorsal muscle of three shrimp in each tank was collected and immediately stored at −80°C for subsequent RNA extraction and real-time qPCR analysis of muscle growth–related gene expression.

Laboratory analyses

The nutrient compositions of experimental diets including moisture, crude protein, crude lipid, and ash were measured following the AOAC methods (AOAC, 1999). The contents of lysine and methionine were analyzed by chromatography using an automated amino acid analyzer (L8900, Hitachi, Japan) with a lithium high-performance column.

Total RNA of muscle samples was isolated using the RNAiso Plus kit (TaKaRa Biotechnology Co., Ltd.). The real-time qPCR analysis method was the same as described by Xin (2016). The primer sequences of the growth hormone (*GH*), myogenic regulatory factors (*Myf5* and *MyoG*), and target of rapamycin

(*TOR*) were listed in Table 2. The $2^{-\Delta\Delta CT}$ method was used to analyze the gene expression levels (Livak and Schmittgen, 2001).

Data calculations

Data were summarized and averaged for each tank. Growth performance parameters were calculated as follows:

SR (%) = 100 × (final number of shrimp/initial number of shrimp)

WGR (%) = 100 × [final body weight (g) − initial body weight (g)]/initial body weight (g)

SGR (%/d) = 100 × [ln final body weight (g) − ln initial body weight (g)]/days

FI (g/shrimp) = feed intake (g)/[(final number of shrimp + initial number of shrimp)/2]

FC = total feed intake (g)/[final body weight (g) − initial body weight (g)]

HSI (%) = 100 × hepatopancreas weight (g)/body weight (g)

ISI (%) = 100 × intestinal weight (g)/body weight (g)

DWP (%) = 100 × muscle weight (g)/body weight (g)

ADCs (%) = 100 × [1 − (a × b)/(c × d)]

where a and c are the AIA concentration in diet ingested and feces, respectively; b and d are the nutrient concentration in feces and diets ingested, respectively. a and d were calculated as difference between the diet offered and orts.

TABLE 2 Primers used for real-time qPCR.

Target Gene	Primer Sequence	Genbank Accession No.
GH	F: 5′-GATGGTTTGGGATCTGAGG AACA-3′ R: 5′-GGAAACTTATGGCATTAACAGGGA-3′	EU492542
Myf5	F: 5′-GGAACAACTACAACTTTGAAG CACA-3′ R: 5′-TCCCATCGCAACTCCTGTATCT-3′	KP715152
MyoG	F: 5′-AACCACCAACGCTGACCG-3′ R: 5′-CTGGTTGGGGTTGTTGGAAG-3′	KP715154
TOR	F: 5′-GACGGCAGTGCTCTATGA-3′ R: 5′-TGTTTGTGAGGCTTGGTG-3′	MK116884
β-Actin	F: 5′-TTGTACGAGGATCGAGTGGA-3′ R: 5′-ATGCTTTCGCAGTAGGTCGT-3′	GK26736

GH, growth hormone; Myf5 and MyoG, myogenic regulatory factors; TOR, target of rapamycin.

Statistical analysis

All data were subjected to normality test and homogeneity of variance by using Shapiro–Wilk and Levene's test for equal variance, respectively. If the data conform to the normal distribution, one-way ANOVA by using SPSS 17.0 analysis software (SPSS(Statistical Packages for Social Sciences, 2007) for Windows followed by the Tukey test with tank as statistical unit and treatment as fixed effect. Polynomial contrasts were used to determine linear and/or quadratic responses to the dietary soybean meal levels. Differences were regarded as significance when $p < 0.05$.

Results

Growth performance

All shrimp had similar ($p > 0.05$) SR and HSI regardless of the treatments (Table 3). The FBW, WGR, SGR, FI, ISI, and

TABLE 3 Growth performance of *L. vannamei* fed with experimental diets.

Items	Diets					SEM	*p*-value		
	T20	T28	T35	T42	T50		*P*	*L*	*Q*
SR, %	86.67	88.33	86.67	85.83	85.83	4.91	0.996	0.786	0.890
FBW, g	15.89^{a}	14.89^{b}	14.62^{b}	14.29^{b}	13.62^{c}	0.44	0.010	0.007	0.286
WGR, %	174.02^{a}	156.81^{b}	152.04^{b}	146.38^{b}	134.40^{c}	7.65	0.010	0.007	0.286
SGR, %/day	2.52^{a}	2.36^{b}	2.31^{b}	2.25^{b}	2.13^{c}	0.08	0.013	0.008	0.314
FI, g/shrimp	19.93^{a}	19.44^{ab}	19.07^{ab}	18.77^{ab}	18.30^{b}	0.53	0.183	0.018	0.953
FC	1.93^{c}	2.19^{b}	2.17^{b}	2.22^{b}	2.28^{a}	0.12	0.049	0.026	0.376
HSI, %	5.52	5.67	5.59	5.50	5.47	0.18	0.258	0.949	0.079
ISI, %	0.50^{a}	0.45^{b}	0.42^{b}	0.43^{b}	0.31^{c}	0.03	0.017	0.032	0.146
DWP, %	51.02^{a}	50.72^{a}	50.53^{a}	50.02^{ab}	49.23^{b}	0.57	0.164	0.048	0.831

T20–T50, diets containing 20%–50% of soybean meal.
SR, survival rate; FBW, final body weight; WGR, weight gain rate; SGR, specific growth rate; FI, feed intake; FC, feed coefficient; HSI, hepatosomatic index; ISI, intestinesomatic index; DWP, dressed weight percentage; SEM, mean standard error; P, overall effect; L, linear effect; Q, quadratic effect.
Different letters within a row indicate significant differences ($p < 0.05$).

DWP were linearly decreased ($p < 0.05$), but FC was linearly increased ($p < 0.05$) as dietary soybean meal increased from 20% to 50%.

ADCs of nutrients

The ADC of dry matter, crude protein, crude lipid, and ash was linearly decreased ($p < 0.001$) as dietary soybean meal increased from 20% to 50% (Table 4).

Intestinal digestive enzyme activities

The intestinal trypsin and amylase activities were decreased ($p < 0.05$) as dietary soybean meal increased from 20% to 50% and reached significance at the level of 35%, 42%, and 50% (Figure 1). Shrimp fed with T20 had higher ($p < 0.05$) intestinal lipase activity than those fed with other diets.

Muscle growth–related gene expression

All shrimp has similar ($p > 0.05$) mRNA relative expression of *MyoG* among diets (Figure 2). The mRNA relative expression of *GH*, *Myf5*, and *TOR* was downregulated ($p < 0.05$) as dietary soybean meal increased from 20% to 50%. Compared with T20, T28, T35, T42, and T50 had lower ($p < 0.05$) mRNA relative expression of GH and TOR, and T35, T42, and T50 had lower ($p < 0.05$) mRNA relative expression of *Myf5*.

Discussion

Growth performance

The similar SR among diets in this study indicated that dietary soybean meal at the rate of 20% to 50% did not affect survival of *L. vannamei*. However, the decreased SGR and FI suggested that the increased dietary soybean meal depressed feed palatability and growth performance of shrimp. This is consistent with the report by Yun et al. (2017) that the replacement ratio of dietary fishmeal with soybean meal at 33% reduced growth of *L. vannamei*. Xu et al. (2021) also reported that dietary fishmeal replaced by soybean meal at 56% reduced FI of *L. vannamei*. Similar results were also observed in various species, such as European catfish (*Silurus glanis*) (Kumar et al., 2017), Tiger puffer (*Takifugu rubripes*) (Lim et al., 2011), sharpsnout seabream (*Diplodus puntazzo*) (Hernandez et al., 2007), red snapper (*Lutjanus campechanus*) (Davis et al., 2005), *Pseudobagrus ussuriensis* (Wang et al., 2016), stellate sturgeon (*Acipenser stellatus*) (Emdadi et al., 2013), Jian carp (*Cyprinus carpio* var. Jian) (Jiang et al., 2015b), rainbow trout (*Oncorhynchus mykiss*) (Harlioglu, 2011), *Channa argus* (Zhang et al., 2020), *Epinephelus Fuscoguttatus* (Liu et al., 2018), bullfrog (*Rana catesbeiana*) (Ding et al., 2019; Wang et al., 2020), and Chinese mitten carb (*Eriocheir sinensis*) (Liu et al., 2021). In this study, the increased FC with increasing dietary soybean meal indicated that supplementation of soybean meal decreased feed utilization; this may account for the depressed growth performance of shrimp. It has been reported that FI is generally regulated by both digestibility and palatability of diet (Peng et al., 2016). The decreased FI as dietary soybean meal increased may partly attribute to decreased apparent digestibility of nutrients as observed in this study. In addition, soybean meal contains several anti-nutritional factors, e.g., soybean agglutinin, glycinin, and β-conglycinin, which have been well reported to reduce the palatability of diet and thereby decrease FI and growth performance of aquatic animals (Feng, 2006; Elumalai et al., 2019; Duan, 2019; Zhao, 2021; Zhou, 2021). Moreover, the decreased ISI and DWP as dietary soybean meal increased suggested that the inclusion of soybean meal in diet inhibited growth and development of the intestine and muscle of *L. vannamei*. This inhibition effect on the intestine may be due to the injury directly caused by soybean meal or indirectly induced by anti-nutritional factors. Zhang et al. (2020) documented that replacing 30% to 60% fishmeal with soybean meal induced intestinal injury of *Channa argus*. Ding et al. (2019) indicated that replacing 50% to 100% fishmeal with soybean meal damaged intestinal villus structure and caused enteritis of bullfrog (*Rana catesbeiana*). Zhou (2021) reported that dietary glycinin and β-conglycinin induced intestinal

TABLE 4 Apparent digestibility coefficients (ADCs) of nutrients by *L. vannamei* fed with experimental diets.

Items	Diets					SEM	*p*-value		
	T20	T28	T35	T42	T50		*P*	*L*	*Q*
ADC of dry matter, %	73.15^{a}	69.98^{b}	68.95^{b}	65.83^{c}	64.25^{c}	0.80	<0.001	<0.001	0.667
ADC of crude protein, %	92.28^{a}	88.73^{b}	86.48^{b}	85.50^{b}	81.50^{c}	0.82	<0.001	<0.001	0.306
ADC of crude lipid, %	87.68^{a}	84.68^{ab}	83.38^{ab}	81.25^{b}	77.28^{c}	2.46	<0.001	<0.001	0.490
ADC of ash, %	79.07^{a}	75.25^{b}	75.15^{b}	73.25^{b}	70.75^{c}	1.11	<0.001	<0.001	0.781

T20–T50, diets containing 20%–50% of soybean meal.
SEM, mean standard error; P, overall effect; L, linear effect; Q, quadratic effect.
Different letters within a row indicate significant differences ($p < 0.05$).

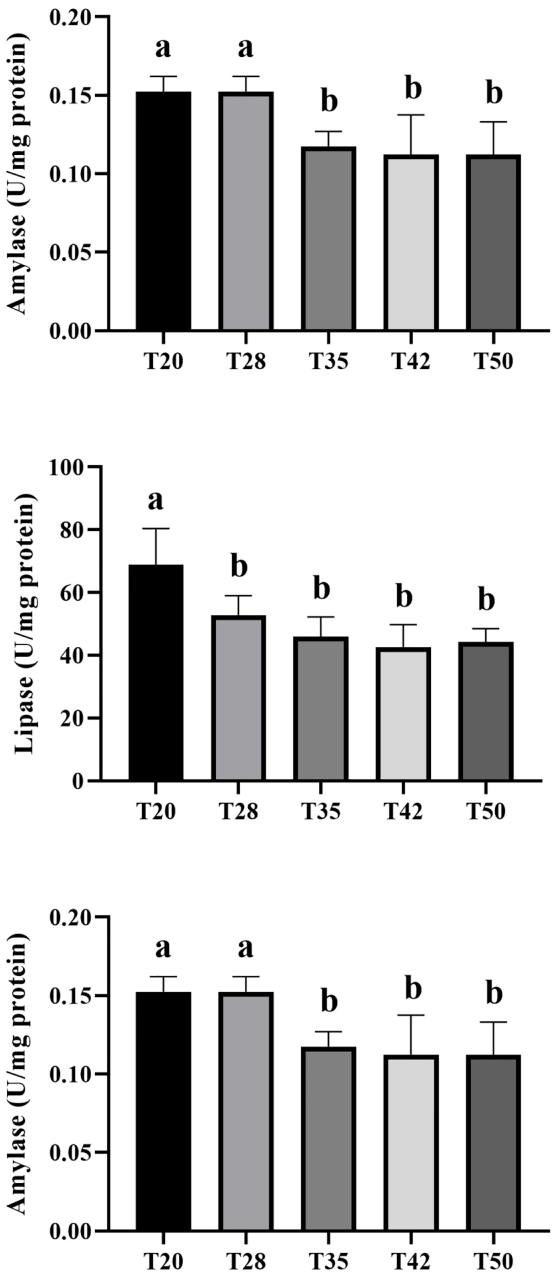


FIGURE 1
Intestinal digestive enzyme activities of *L. vannamei* fed with experimental diets. Different letters above the bars denote significant differences among diets ($p < 0.05$). T20–T50, diets containing 20%–50% of soybean meal.

damage and inflammation and thus inhibited growth and development of intestine in Songpu mirror carp by upregulating mRNA levels of apoptosis-related genes. Similar result was also reported by Duan (2019), in which the inclusion of β-conglycinin in grass carp diet caused enterocytes apoptosis and significantly reduced the ISI of fish. Similarly, Lu et al. (2018) also indicated that totally replacement of fishmeal by soybean meal in crayfish (*Cherax quadricarinatus*) diet significantly decreased crude protein content and depressed muscle growth. Xu et al. (2021) reported that replacing 28% to 70% fishmeal with soybean meal reduced the muscle nutrient compositions of *L. vannamei*.

Apparent digestibility and digestive enzyme activity

Digestibility of nutrients as reflected by the ADC values of crude protein, crude lipid, and ash usually indicates digestion and utilization of diets. These are closely related to the corresponding digestive enzyme activities in the intestine, because intestinal digestive enzymes, e.g., trypsin, lipase, and amylase, are commonly used as critical indicators to evaluate the changes in diets (Santigosa et al., 2008). In this study, the decreased ADCs of crude protein, crude lipid, and ash as dietary soybean meal increased suggest that the supplementation of soybean meal in shrimp diets decreased the digestion and utilization of diets. This is most likely due to the decreased intestinal digestive enzyme activities of trypsin, lipase, and amylase as observed in this study. The inhibition effect of intestinal digestive enzyme activities by replacing fishmeal with soybean meal in aquatic feed is a common phenomenon that has been well reported in previous studies. For instance, replacing 57% and 100% of fishmeal by soybean meal in Chinese mitten crab (*Eriocheir sinensis*) diets significantly reduced the intestinal digestive enzyme activities of trypsin, lipase, and amylase (Liu et al., 2021). Similar result was also reported by Zhang et al. (2015) that replacing 12% to 48% fishmeal with soybean meal significantly decreased the intestinal lipase and amylase activities of rice filed eel (*Monopterus albus*). Furthermore, Hernandez et al. (2007) documented that the inclusion of 60% soybean meal (approximately replacing 68% fishmeal) in sharpsnout seabream (*Diplodus puntazzo*) diet significantly reduced the ADCs of dry matter and crude protein. Yang et al. (2015) indicated that replacing 40% and 60% of fishmeal by soybean meal significantly decreased ADCs of dry matter and crude protein of *L. vannamei*. Tibaldi et al. (2006) also documented that substitution of 50% fishmeal by soybean meal significantly decreased ADCs of dry matter, crude protein, crude lipid, and ash. Although the reason why supplementation of soybean meal inhibited the intestinal digestive enzyme activities is not fully understood, this may be attributed to the increasing levels of the anti-nutritional factors (such as soybean agglutinin, glycinin, and β-conglycinin) with the increase of soybean meal in diets (Ding et al., 2019; Liu et al., 2021). Yue et al. (2012) also reported that anti-nutritional factors in soybean meal reduced the digestive enzyme activities in the gut of *L. vannamei*.

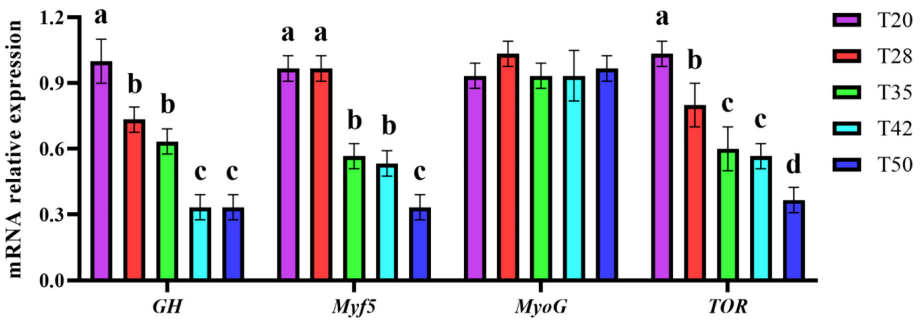


FIGURE 2
Muscle growth–related gene expression of *L. vannamei* fed with experimental diets. *GH*, growth hormone; *Myf5* and *MyoG*, myogenic regulatory factors; *TOR*, target of rapamycin. Different letters above the bars denote significant differences among diets ($p < 0.05$). T20–T50, diets containing 20%–50% of soybean meal.

Muscle growth–related gene expression

The growth and development of muscle that involves hyperplasia and hypertrophy is generally controlled by diverse genetic factors, such as growth hormone (*GH*), myogenic regulatory factors (*Myf5* and *MyoG*), and target of rapamycin (*TOR*) (Xin, 2016; Asaduzzaman et al., 2017). Therefore, the effects of formulated diets on muscle growth of aquatic animals were mainly investigated from the aspects of these muscle growth–related genes (Alami-Durante et al., 2018; Wei et al., 2020a; Wei et al., 2020b; Yang et al., 2021). *GH* stimulates the growth of muscle through inducing proliferation of myogenic cells and muscle hyperplasia and hypertrophy (Asaduzzaman et al., 2017). Myogenic regulatory factors, e.g., *Myf5* and *MyoG*, are the critical muscle development– and growth–related transcription factors (Funkenstein et al., 2007). *TOR*, an important regulatory factor in cellular central control system, plays a key role in cell growth and proliferation (Liang et al., 2020). Therefore, all of these genes act as a positive regulator of muscle growth that promote hyperplastic and hypertrophic muscular growth. In this study, the downregulated mRNA levels of *GH*, *Myf5*, and *TOR* as dietary soybean meal increased suggested that supplementation of soybean meal in shrimp diets decreased the growth and development of muscle. This may also account for the concomitantly decreased DWP as observed in this study. To our best knowledge, this study is the first to assess the effects of dietary soybean meal on these muscle-related gene expressions of *L. vannamei*. This is similar with the observation by Hu et al. (2018) that replacing 40% and 50% of fishmeal by fermented soybean meal in *Nibea albiflora* diets significantly decreased mRNA levels of muscle growth–related gene (insulin-like growth factor I). Ulloa et al. (2013) also reported that the dietary inclusion of plant proteins reduced the muscle growth–related gene expressions in the male zebrafish (*Danio rerio*). Despite the reason why the dietary inclusion of soybean meal downregulated the gene expression of muscle growth–related genes is not clear, this is most likely ascribed to the adverse effects of anti-nutritional factors exist in soybean meal on the proliferation of intestinal epithelial cells and thereby decreased digestion and absorption of nutrients in shrimp diet to provide necessary energy serving as basis contents for muscle growth. Numbers of studies documented that anti-nutritional factors extracted from soybean meal injured intestinal epithelial cells of animals (Feng, 2006; Guo, 2006; Xu, 2009; Duan, 2019; Peng, 2020; Zhou, 2021). These studies combined with the observation that supplementation of soybean meal inhibited nutrient digestibility of shrimp in this study may be the potential mechanisms to support this hypothesis. Further study is still needed to confirm this.

Conclusion

Dietary soybean meal that exceeded 28% significantly inhibited growth performance of *L. vannamei*. This growth inhibition effect may partly attribute to the negative impact of anti-nutritional factors that exist in soybean meal on the digestion and utilization of nutrients in shrimp diets and also the inhibition on the muscle growth and development as reflected by the decreased DWP and muscle growth–related gene expressions. This study provides a caution for the application of soybean meal in aquaculture, and further study is needed to clarify mechanisms regarding the inhibition effect of soybean meal on muscle growth of shrimp.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by the Animal Care and Use Committee of Guangdong Academy of Agricultural Sciences, Guangzhou, China.

Author contributions

KP and WH conceived and designed the experiments. KP, XC, HJL, JZ, YC, CL, and HL performed the experiments. KP analyzed the data and wrote the paper. All authors contributed to the article and approved the submitted version.

Funding

This study was funded by Innovation Fund project of Guangdong Academy of Agricultural Sciences (202137 and 202138), Special Fund for Scientific Innovation Strategy-Construction of High-Level Academy of Agriculture Science (R2021PY-QY001), and Independent Research and Development Projects of Maoming Laboratory (2021TDQD004 and 2021ZZ007).

Acknowledgments

We acknowledge those who donated the experimental animals and those who helped support this study at the Institute of Animal Science, Guangdong Academy of Agricultural Sciences.

Conflict of interest

Author HL is employed by Guangdong Jinyang 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.

The reviewer (HZ) declared a shared affiliation with the author (CL) to the handling editor at the time of review.

Publisher's note

References

Alami-Durante, H., Bazin, D., Cluzeaud, M., Fontagne-Dicharry, S., Kaushik, S., and Geurden, I. (2018). Effect of dietary methionine level on muscle growth mechanisms in juvenile rainbow trout (Oncorhynchus mykiss). *Aquaculture* 483, 273–285. doi: 10.1016/j.aquaculture.2017.10.030

Amaya, E., Davis, D. A., and Rouse, D. B. (2007). Replacement of fish meal in practical diets for the pacific white shrimp (Litopenaeus vannamei) reared under pond conditions. *Aquaculture* 262, 393–401. doi: 10.1016/j.aquaculture.2006.11.015

AOAC (1999). *Official methods of analysis of the association of official agricultural chemists, 16th ed* (Gaithersburg: AOAC International).

Asaduzzaman, M., Ikeda, D., Abol-Munafi, A. B., Bulbul, M., Ali, M. E., Kinoshita, S., et al. (2017). Dietary supplementation of inosine monophosphate promotes cellular growth of muscle and upregulates growth-related gene expression in Nile tilapia oreochromis niloticus. *Aquaculture* 468, 297–306. doi: 10.1016/j.aquaculture.2016.10.033

Bae, J., Hamidoghli, A., Djaballah, M. S., Maamri, S., Hamdi, A., Souffi, I., et al. (2020). Effects of three different dietary plant protein sources as fishmeal replacers in juvenile whiteleg shrimp, litopenaeus vannamei. *Fish. Aquat. Sci.* 23 (1), 2. doi: 10.1186/s41240-020-0148-x

Bruce, T. J., Oliver, L. P., Ma, J., Small, B. C., Hardy, R. W., Brown, M. L., et al. (2021). An initial evaluation of fishmeal replacement with soy protein sources on growth and immune responses of burbot (Lota lota maculosa). *Aquaculture* 545, 737157. doi: 10.1016/j.aquaculture.2021.737157

Chakraborty, P., Mallik, A., Sarang, N., and Lingam, S. S. (2019). A review on alternative plant protein sources available for future sustainable aqua feed production. *Int. J. Chem. Stud.* 7 (3), 1399–1404.

Davis, D. A., Miller, C. L., and Phelps, R. P. (2005). Replacement of fish meal with soybean meal in the production diets of juvenile red snapper, lutjanus campechanus. *J. Word. Aquacult. Soc* 36 (1), 114–119. doi: 10.1111/j.1749-7345.2005.tb00137.x

Ding, L., Wang, L., Lu, K., Song, K., and Zhang, C. (2019). Effects of replacement of fish meal with soybean meal on growth performance, digestive enzyme activity and intestinal health of rana catesbeiana. *Fresh. Fish.* 49 (2), 69–75. doi: 10.13721/j.cnki.dsyy.2019.04.011

Ding, Z., Zhou, D., Zheng, J., Chen, X., Kong, Y., Qi, C., et al. (2022). Replacing fishmeal with soybean meal affects survival, antioxidant capacity, intestinal microbiota, and mRNA expression of TOR and S6K1 in macrobrachium rosenbergii. *Chin. J. Oceanol. Limn.* 40, 805–817. doi: 10.1007/s00343-021-0494-2

Duan, X. (2019). *Effects of β-conglycinin on apoptosis in grass carp enterocytes and the involved mechanism* (Chengdu, China: Sichuan Agricultural University).

Elumalai, P., Rubeena, A. S., Arockiaraj, J., Wongpanya, R., Cammarata, M., Ringo, E., et al. (2019). The role of lectins in finfish: a review. *Rev. Fish. Sci. Aquacult.* 27 (2), 152–169. doi: 10.1080/23308249.2018.1520191

Emdadi, B., Sajadi, M. M., Yazdani, M. A., and Shakoorian, M. (2013). Influence of replacing fish meal with soybean meal on growth rate, feed conversion ratio and chemical composition of carcass, fillet and liver in juvenile stellate sturgeon (Acipenser stellatus). *Iran. Sci. Fish. J.* 22 (2), 23–34. doi: 10.22092/ISFJ.2017.110116

Feng, L. (2006). *Effects of soybean agglutinin on the proliferation, differentiation and functions of carp intestinal epithelial cells in primary culture* (Chengdu, China: Sichuan Agricultural University).

Fishery Administration of Ministry of Agriculture and Rural Affairs (2021). *China Fishery statistical yearbook* (Beijing, China: China Agriculture Press).

Funkenstein, B., Skopal, T., Rapoport, B., Rebhan, Y., Du, S. J., and Radaelli, G. (2007). Characterization and functional analysis of the 5′-flanking region of myosin light chain-2 gene expressed in fast skeletal muscle of the gilthead sea bream (Sparus aurata). *Comp. Biochem. Phys. D.* 2 (3), 187–199. doi: 10.1016/j.cbd.2007.04.001

Gumus, E. (2011). Effect of replacement of fishmeal with sand smelt (Atherina boyeri) meal on growth, feed utilization and body composition of mirror carp fry (Cyprinus carpio). *Kafkas. Univ. Vet. Fak. Derg.* 17 (3), 363–369. doi: 10.1038/laban0111-9a

Guo, L. (2006). *Effects of soybean β-conglycinin extract on the proliferation and function of carp intestinal-epithelial cells in primary culture* (Chengdu, China: Sichuan Agricultural University).

Harlioglu, A. G. (2011). The influence of replacing fish meal partially in diet with soybean meal and full-fat soya on growth and body composition of rainbow trout (Oncorhynchus mykiss). *Pakistan J. Zool.* 43 (1), 175–182. doi: 10.1515/MAMM.2010.076

Hernandez, M. D., Martinez, F. J., Jover, M., and Garcia, B. G. (2007). Effects of partial replacement of fish meal by soybean meal in sharpsnout seabream (Diplodus puntazzo) diet. *Aquaculture* 263, 159–167. doi: 10.1016/j.aquaculture.2006.07.040

Hu, S., Wang, L., Lou, B., Zeng, W., Tan, P., Zhan, W., et al. (2018). Effects of replacing fish meal with fermented soybean meal on amino acids in muscle and expression of IGF-I in liver and muscle of juvenile nibea albiflora. *J. Zhejiang. Ocean. Univ.* 37 (3), 196–202.

Jiang, S., Yang, Q., Huang, J., Lin, H., Li, X., and Jiang, S. (2015a). Impacts of diets with different protein levels on the growth and mRNA expression of growth-related genes in penaeus monodon families. *Mar. Fish.* 37 (3), 244–252. doi: 10.7668/hbnxb.2015.S1.020

Jiang, J., Zhou, X., Yin, L., Feng, L., Jiang, W., Liu, Y., et al. (2015b). The influence of replacing fish meal with soybean meal on growth, body composition and digestive ability of jian carp in practical extruded diet. *Acta Agric. Bor. Sin.* 30, 107–111. doi: 10.13233/j.cnki.mar.fish.2015.03.006

Kumar, S., Sandor Zs, J., Nagy, Z., Fazekas, G., Havasi, M., Sinha, A. K., et al. (2017). Potential of processed animal protein versus soybean meal to replace fish meal in practical diets for European catfish (Silurus glanis): growth response and liver gene expression. *Aquacult. Nutr.* 23, 1179–1189. doi: 10.1111/anu.12487

Liang, Q., Ou, M., Li, Z., Ren, Y., Wei, W., Qiao, X., et al. (2020). Functional analysis target of rapamycin (TOR) on the penaeus vannamei in response to acute low temperature stress. *Fish. Shell. Immunol.* 96, 53–61. doi: 10.1016/j.fsi.2019.11.070

Lim, C., and Dominy, W. (1990). Evaluation of soybean meal as a replacement for marine animal protein in diets for shrimp (Penaeus vannamei). *Aquaculture* 87, 53–63. doi: 10.1016/0044-8486(90)90210-E

Lim, S., Kim, S., Ko, G., Song, J., Oh, D., Kim, J., et al. (2011). Fish meal replacement by soybean meal in diets for tiger puffer, takifugu rubripes. *Aquaculture* 313, 165–170. doi: 10.1016/j.aquaculture.2011.01.007

Liu, Y., Meng, L., and Zhang, J. (2018). Effect of partial fish meal replacement by soybean meal on the growth performance, nutritional composition and blood biochemical indices of juvenile epinephelus fuscoguttatus. *China Feed.* 4, 60–64. doi: 10.15906/j.cnki.cn11-2975/s.20180412

Liu, Q., Wen, B., Man, D., Jiang, Y., Li, X., and Zuo, R. (2021). Substitution of fish meal with soybean meal for juvenile Chinese mitten crab (Eriocheir sinensis) reared at three salinities: Effects on survival, growth, antioxidant capacity and body composition. *Aquacult. Res.* 52, 3723–3735. doi: 10.1111/are.15217

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Method.* 25, 402–408. doi: 10.1006/meth.2001.1262

Lu, Y., Li, J., Zhang, X., Wang, A., Xian, J., and Wang, D. (2018). Effects of dietary replacement of fish meal protein by soybean meal protein on growth, muscle composition and enzyme activities of juvenile crayfish cherax quadricarinatus. *Hebei. Fish.* 4, 1–18. doi: 10.3969/j.issn.1004-6755.2018.04.001

Mai, K. S., Lv, M. D., and He, G. (2021). The issue of protein sources of aquafeed-a new approach to improve utilization efficiency of feed protein. *Feed. Ind.* 42 (1), 2–6. doi: 10.13302/j.cnki.fi.2021.01.001

Peng, C. (2020). *The mechanism of β-conglycinin and glycinin induced IPEC-J2 cell damage* (Hefei, China: Anhui Agricultural University).

Peng, K., Huang, W., Zhao, H., Sun, Y., and Chen, B. (2021). Dietary condensed tannins improved growth performance and antioxidant function but impaired intestinal morphology of litopenaeus vannamei. *Aquacult. Rep.* 21, 100853. doi: 10.1016/j.aqrep.2021.100853

Peng, K., Shirley, D. C., Xu, Z., Huang, Q., McAllister, T. A., Chaves, A. V., et al. (2016). Effect of purple prairie clover (Dalea purpurea vent.) hay and its condensed tannins on growth performance, wool growth, nutrient digestibility, blood metabolites and ruminal fermentation in lambs fed total mixed rations. *Anim. Feed. Sci. Technol.* 222, 100–110. doi: 10.1016/j.anifeedsci.2016.10.012

Santigosa, E., Sanchez, J., Medale, F., Kaushik, S., Perez-Sanchez, J., and Gallardo, M. A. (2008). Modifications of digestive enzymes in trout (Oncorhynchus mykiss) and sea bream (Sparus aurata) in response to dietary fish meal replacement by plant protein sources. *Aquaculture* 282, 68–74. doi: 10.1016/j.aquaculture.2008.06.007

Shukla, A., Kumar, M., Gupta, G., Pathak, N., and Mishra, V. (2019). A review on replacing fish meal in aqua feeds using plant and animal protein sources. *Int. J. Chem. Stud.* 7 (3), 4732–4739. doi: 10.13140/RG.2.2.13085.13285

SPSS (Statistical Packages for Social Sciences) (2007). *Statistical packages for social sciences (version 17.0)* (Chicago, IL, USA: SPSS Inc.).

Tibaldi, E., Hakim, Y., Uni, Z., Tulli, F., Francesco, M., Luzzana, U., et al. (2006). Effects of the partial substitution of dietary fish meal by differently processed soybean meals on growth performance, nutrient digestibility and activity of intestinal brush border enzymes in the European sea bass (Dicentrarchus labrax). *Aquaculture* 261, 182–193. doi: 10.1016/j.aquaculture.2006.06.026

Ulloa, P. E., Pena, A. A., Lizama, C. D., Araneda, C., Iturra, P., Neira, R., et al. (2013). Growth response and expression of muscle growth-related candidate genes in adult zebrafish fed plant and fishmeal protein-based diets. *Zebrafish* 10 (1), 99–109. doi: 10.1089/zeb.2012.0823

Wang, G., Peng, K., Hu, J., Yi, C., Chen, X., Wu, H., et al. (2019). Evaluation of defatted black soldier fly (Hermetia illucens l.) larvae meal as an alternative protein ingredient for juvenile Japanese seabass (Lateolabrax japonicus) diets. *Aquaculture* 507, 144–154. doi: 10.1016/j.aquaculture.2019.04.023

Wang, L., Wang, J., Lu, K., Song, K., Mai, K., Zhang, C., et al. (2020). Total replacement of fish meal with soybean meal in diets for bullfrog (Lithobates catesbeianus): Effects on growth performance and gut microbial composition. *Aquaculture* 524, 735236. doi: 10.1016/j.aquaculture.2020.735236

Wang, Y., Yu, S., Wang, Y., Che, J., Zhao, L., Bu, X., et al. (2016). Effect of replacing fish meal with soybean meal on growth, feed utilization and nitrogen and phosphorus excretion of juvenile pseudobagrus ussuriensis. *Aquacult. Res.* 47, 3145–3155. doi: 10.1111/are.12765

Wei, Y., Li, B., Xu, H., and Liang, M. (2020a). Fish protein hydrolysate in diets of turbot affects muscle fibre morphometry, and the expression of muscle growth-related genes. *Aquacult. Nutr.* 26, 1780–1791. doi: 10.1111/anu.13129

Wei, Y., Xu, H., and Liang, M. (2020b). Amino acid absorption and protein synthesis responses of turbot scophthalmus maximus to lysine and leucine in free, dipeptide and tripeptide forms. *Aquacult. Nutr.* 26, 358–367. doi: 10.1111/anu.12998

Wu, Y., Wang, Y., Ren, X., Huang, D., Si, G., and Chen, J. (2021). Replacement of fish meal with gamma-ray irradiated soybean meal in the diets of largemouth bass micropterus salmoides. *Aquacult. Nutr.* 27, 977–985. doi: 10.1111/anu.13239

Xin, F. (2016). *Gene cloning and functional study on the important genes of mTOR signalingin litopenaeus vannamei* (Beijing, China: University of Chinese Academy of Sciences).

Xu, J. (2009). *The influence of the extracted soybean glycinin and β-conglycinin on the function and molecular mechanism of mice intestinal-epithelial cells in primary cultivation* (Chengdu, China: Sichuan Agricultural University).

Xu, T., Xu, D., Liu, X., and Zhao, W. (2021). Effect of dehulled soybean meal instead of fish meal on growth and body composition of litopenaeus vannamei. *Feed. Res.* 13, 72–76. doi: 10.13557/j.cnki.issn1002-2813.2021.13.018

Yang, C., Chen, H., Peng, M., Li, Q., Zeng, D., Liu, Q., et al. (2021). Transcriptome sequencing and screening of genes related to muscle growth and development in litopenaeus vannamei. *J. Sou. Agric.* 52 (9), 2319–2328. doi: 10.3969/j.issn.2095-1191.2021.09.001

Yang, Q., Yan, B., Dong, X., Chi, S., and Liu, H. (2015). Effect of replacing fish meal with extruded soybean meal on growth, feed utilization and apparent nutrient digestibility of juvenile white shrimp (Litopenaeus vannamei). *J. Ocean. Univ. China* 14 (5), 865–872. doi: 10.1007/s11802-015-2572-6

Yue, Y. R., Liu, Y. J., Tian, L. X., Gan, L., Yang, H. J., and Liang, G. Y. (2012). Effects of replacing fishmeal with soybean meal and peanut meal on growth, feed utilization and haemolymph indexes for juvenile white shrimp litopenaeus vannamei. Boone. *Aquacult. Res.* 43, 1687–1696. doi: 10.1111/j.1365-2109.2011.02976.x

Yun, H., Shahkar, E., Hamidoghli, A., Lee, S., Won, S., and Bai, S. C. (2017). Evaluation of dietary soybean meal as fish meal replacer for juvenile whiteleg shrimp, litopenaeus vannamei reared in biofloc system. *Int. Aquat. Res.* 9, 11–24. doi: 10.1007/s40071-017-0152-7

Zhang, X., Han, P., Hu, J., Liu, L., Chen, Y., Xu, W., et al. (2020). Effects of replacement of fish meal by soybean meal on growth performance, protein utilization and intestinal tissue morphology of channa argus. *Chin. J. Anim. Nutr.* 32 (6), 2799–2807. doi: 10.3969/j.issn.1006-267x.2020.06.040

Zhang, J., Lv, F., Huang, Z., Liu, Z., Hu, Y., Wang, P., et al. (2015). Effects of fish meal replacement by different proportions of extruded soybean meal on growth performance, body composition, intestinal digestive enzyme activities and serum biochemical indices of rice filed eel (Monopterus albus). *Chin. J. Anim. Nutr.* 27 (11), 3567–3576. doi: 10.3969 /j.issn.1006-267x.2015.11.030

Zhao, X. (2021). *Effects of dietary glycinin or β-conglycinin addition on the growth performance and intestinal health of grouper (Epinephelus coioides)* (Xiamen, China: Jimei University).

Zhao, X., Wang, Y., Wang, X., and Ye, J. (2021). Growth performance, plasma components, and intestinal barrier in grouper (Epinephelus coioides) are altered by dietary fish meal replacement with extruded soybean meal. *Aquacult. Rep.* 21, 100863. doi: 10.1016/j.aqrep.2021.100863

Zhou, Z. (2021). *Effects of glycinin and β-conglycinin on growth performance, health of intestine and metabolic pathway of songpu mirror carp* (Shanghai, China: Shanghai Ocean University).

frontiers | Frontiers in Marine Science

TYPE Original Research
PUBLISHED 08 September 2022
DOI 10.3389/fmars.2022.987363

OPEN ACCESS

EDITED BY
Helena Peres,
University of Porto, Portugal

REVIEWED BY
Ida Kinasih,
Universitas Islam Negeri Sunan Gunung Djati Bandung, Indonesia
Fernanda Cornejo,
National Autonomous University of Mexico, Mexico
Jia-Song Zhang,
South China Sea Fisheries Research Institute (CAFS), China
Chengbo Sun,
Guangdong Ocean University, China

*CORRESPONDENCE
Yusheng Jiang
jys@dlou.edu.cn

SPECIALTY SECTION
This article was submitted to Aquatic Physiology, a section of the journal Frontiers in Marine Science

RECEIVED 07 July 2022
ACCEPTED 16 August 2022
PUBLISHED 08 September 2022

CITATION
He Y, Zhang N, Wang A, Wang S, Che Y, Huang S, Yi Q, Ma Y and Jiang Y (2022) Positive effects of replacing commercial feeds by fresh black soldier fly (*Hermetia illucens*) larvae in the diets of Pacific white shrimp (*Litopenaeus vannamei*): Immune enzyme, water quality, and intestinal microbiota.
Front. Mar. Sci. 9:987363.
doi: 10.3389/fmars.2022.987363

Positive effects of replacing commercial feeds by fresh black soldier fly (*Hermetia illucens*) larvae in the diets of Pacific white shrimp (*Litopenaeus vannamei*): Immune enzyme, water quality, and intestinal microbiota

Yuhang He[1,2], Naida Zhang[1], Aolin Wang[1], Sizhe Wang[1], Yanjin Che[1], Shu Huang[1], Qilin Yi[1], Yuexin Ma[1] and Yusheng Jiang[1]*

[1]Innovation Team for Crustacean Aquaculture and Restocking, Dalian Ocean University, Dalian, China, [2]Sanya Tropical Fisheries Research Institute, Sanya, China

This study investigated the effects of replacing commercial feeds with fresh black soldier fly larvae (BSFL) on the immune enzyme activities, water quality in the culturing environment, and intestinal microbiota of Pacific white shrimp (*Litopenaeus vannamei*). Five different feeding regimes were designed, in which 0% (control group), 25%, 50%, 75%, and 100% commercial feeds were replaced with equal wet weight of fresh BSFL, respectively. The experiment lasted for 45 days, and the results showed that the serum T-NOS (total nitric oxide synthase), AKP (alkaline phosphatase), and ACP (acid phosphatase) activity were significantly increased in the BSFL25% group. However, the immune enzymes of the hepatopancreas decreased significantly in all the BSFL-containing groups compared to the control group. Total ammonia nitrogen concentrations throughout the experimental phase and nitrate concentrations in the middle and later phases of the experiment were negatively correlated with the replacement rate of fresh BSFL in the diet, whereas pH was positively correlated with the replacement rate of fresh BSFL in the diet. Proteobacteria and Bacteroidetes were dominant phyla in the intestines of shrimps, but the relative abundances of Proteobacteria and Firmicutes decreased, with those of Bacteroidetes and Planctomycetes increased in the BSFL-containing groups. Among them, higher relative abundances of potential probiotics such as *Motilimonas*, *Shimia*, *Pseudoalteromonas*, and *Shewanella* and lower relative abundance of genus *Vibrio* were observed in the fresh BSFL-containing groups. Furthermore, shrimps fed with BSFL-containing diets had higher bacterial richness and diversity in the intestines. In conclusion, a proper replacement of commercial

feed with fresh BSFL had a positive effects on the immune-related enzyme activities, water quality, and intestinal health in the *L. vannamei*.

KEYWORDS

Pacific white shrimp *Litopenaeus vannamei*, immune enzyme activity, water quality, intestinal microbiota, black soldier fly *Hermetia illucens*

Introduction

Global aquaculture production of shrimp has increased dramatically within the past two decades. The Pacific white shrimp, *Litopenaeus vannamei*, is the most important farmed crustacean species, contributing 53% of the total farmed crustacean production (Yuan et al., 2021). With the upgrading of consumption, *L. vannamei* has become an attractive and popular species for aquaculture in the United States, Ecuador, Thailand, and China (Saoud et al., 2003; Mcgraw et al., 2010). Meanwhile, this has also brought challenges to the feed industry, because over 90% of farmed shrimp rely on high-protein diets containing a high percentage of marine fish meal (FM), which is a major ingredient in commercial feeds due to its high digestible protein content, balanced amino acid composition, and high palatability (Cruz-Suárez et al., 2007; Lemos et al., 2009; Council, 2011). However, FM also has a lot of disadvantages: the first of which is that the resource of FM cannot meet the continuously increasing demand of the aquaculture industry, and the contradiction is further aggravated by the natural resource decline and global competition for the FM from livestock and poultry industry (Adelizi et al., 1998; Samira and Mehdi, 2015) . In addition, the processing of FM produces a large number of pollutants containing nitrogen and sulfur compounds, which could be deleterious to human healthe and the environment. (Oyarzun et al., 2019). Finally, FM contains a large amount of phosphorus and nitrogen. It can cause the deterioration of farming water quality and the occurrence of diseases in aquatic animals when the unabsorbed nitrogen and phosphorus are discharged into the farming environment with residual bait and manure (Lai et al., 2009; Marinho-Soriano et al., 2009). Therefore, it has become an important topic for aquaculture industry to find a new protein source. The Food and Agricultural Organization (FAO) has highlighted the potential utility of insects as food and feed sources, which usually possess an outstanding capacity to upgrade low-quality organic material, require minimal water and cultivable land, and emit little greenhouse gases to produce large quantities of high-quality animal protein (Van Huis, 2013). Among them, *Locusta migratoria*, *Tenebrio molitor*, and *Hermetia illucens* are candidates to replace FM in commercial feed (EMEHINAIYE, 2012; Gasco et al., 2016; Bruni et al., 2018).

The black soldier fly (*Hermetia illucens*) is one of the most promising insect species for commercial exploitation in China, and it could live on animal manure or plant material by converting low-value organic waste into a protein-rich biomass (Diener et al., 2009). This makes it a suitable source of food for aquatic animals (Newton et al., 1977; Bondari and Sheppard, 1981). Recently, black soldier fly larvae (BSFL) has been used as a substitute for FM in several aquatic animal species including *Oncorhynchus mykiss* (Bruni et al., 2018), *Pelteobagrus fulvidraco* (Xiao et al., 2018), and *Cherax cainii* (Foysal et al., 2019). Interestingly, some studies have indicated that BSFL have the effect of enhancing the immunity of aquatic animals, such as *C. cainii* (Foysal et al., 2019) and *Cyprinus carpio* var. Jian (Li et al., 2017). In addition, BSFL have also been demonstrated to promote growth and nutrition absorptive capacity in aquatic animals (Li et al., 2017; Xiao et al., 2018).

Although there have been a lot of reports on feeding *L. vannamei* by BSFL (Cummins et al., 2017), these studies have more emphasis on the evaluation of the effects of replacing FM in commercial feed with BSFL in powder form on aquatic animals. Processing insects into powder form as the raw protein source is an industrial necessity and easy to preserve. However, fresh insects also have their advantages, such as simple feeding, low nutrient losses, and easier nutrition enhancement. A recent study by our team showed no significant negative effects on shrimp growth when up to 50% of the commercial feed was replaced by fresh BSFL (He et al., 2022). Aquaculture outcome is not defined solely by growth performance, but issues concerning aquatic animals' welfare also need to be taken into accounts, such as the water quality of the aquaculture environment and the immune capacity of different tissues. Commercial feeds have been found to negatively affect water quality parameters including pH, nitrite nitrogen (NO_2-N), and total ammonia nitrogen (TAN), which may in turn affect the growth of aquatic animals (Pullin and Lowe-McConnell, 1982). Fresh BSFL instead of commercial feed may be a potential method to reduce water

quality degradation in aquaculture environments. This capacity can maintain the health of aquatic animals and control disease outbreaks, which may improve farming profits and reduce the use of antibiotics in aquaculture. In addition, we would like to increase this replacement rate through more comprehensive studies. To the best of our knowledge, no information is available about the effects of feeding BSFL on the intestinal microbiota of *L. vannamei*. Intestinal microbiota helps the host to digest indigestible feed components such as cellulose, and it also participates in the maturation of intestinal mucosa, immune response, and disease resistance (Maslowski and Mackay, 2011; Llewellyn et al., 2014). Fresh insects contain bioactive compounds that appear to modulate intestinal microbiota and have a positive impact on animal health (Gasco et al., 2018). Therefore, it is necessary to evaluate whether the intestinal microbial changes of shrimp are the factors limiting the replacement rate and finding ways to improve the replacement rate.

In this experiment, the effects of fresh BSFL on hepatopancreas and serum immune enzyme activity, the water quality of the aquaculture environment, and the intestinal microbiota of *L. vannamei* were assessed to provide more information for farming *L. vannamei* using fresh BSFL as a protein source and to contribute for sustainable development of aquaculture industry.

Materials and method

Feeding regime

In this experiment, five feeding regimes were designed by replacing different levels (0%, 25%, 50%, 75%, and 100%, respectively) of commercial feeds with fresh BSFL. The commercial feeds (crude protein: 42%; crude lipid: 6%) were used as a control feeding regime. The BSFL were hatched in our laboratory and were fed the bran regularly every day (the bran and water mix by a volume ratio of 3:1). Then, the BSFL were collected and stored at −20°C before they turn into mature insects. The proximate composition of commercial feeds and fresh BSFL was shown in Table 1. Feeding regimes and theoretical proximate composition of ingested diets for each group were listed in Table 2.

Feeding experiment

L. vannamei postlarvae (body length is about 0.4 cm) were purchased from a commercial hatchery (Hainan Province, China) and were reared by our team on a basic non-insect diet. The larval shrimp were temporarily reared for 7 days before the experiment to acclimate to the rearing environment and experimental diets. Then, the experimental shrimp were fasted for 12 h and weighed. A total of 750 shrimp (body length: 3.27 ± 0.23 cm) were randomly distributed into 15 glass fiber tanks filled with 300 L of seawater, at a density of 50 shrimp per tank; the five experimental diets were randomly assigned to triplicate tanks. The shrimp used in the experiment were equivalent in size and weight. Shrimp were fed four times a day (6:00, 12:00, 18:00, and 24:00) during the 45-day feeding. The feeding weight of each group was based on the feeding amount from hand-feeding to visual satisfaction and on the weight of shrimp, whereas the replacement rate of fresh BSFL was controlled by adjusting the feeding times of fresh BSFL. The remaining feeds and feces were removed daily and about 20% of water was exchanged every day. The water temperature, salinity, and dissolved oxygen (DO) were maintained at 28°C ± 3°C, 27 ± 2, and 7.02 ± 0.69 mg/L, respectively.

Sample collection of shrimp

Shrimps were starved for 12 h before sampling. Then, the whole intestines of three shrimps from each tank were aseptically dissected, the intestinal contents were collected and immediately put in liquid nitrogen, and samples were preserved with dry ice and sent to Biomarker Technology Co. Ltd (Beijing, China) for DNA extraction and analyses by high-throughput sequencing. The blood of another five shrimps in each tank was collected and mixed with anticoagulant solution (1:1), and then the separated serum was removed by centrifuging (3000 rpm, 10 min) after keeping for 6 h at 4°C, which were immediately frozen in lipid

TABLE 1 Proximate composition of commercial feed and fresh BSFL (% weight).

Proximate composition	Commercial feed	Fresh BSFL
Crude protein	42.0	17.8
Crude ash	16.0	2.2
Crude lipid	4.0	7.4
phosphorus	1.2	0.3
moisture and other volatile	11.0	69.3

The black soldier fly larvae were hatched in our laboratory and were fed the bran regularly every day (the bran and water mix by a volume ratio of 3:1). Commercial feeds were bought from a local retailor and its protein sources were mainly fish meal and soybean meal.

TABLE 2 Feeding times and proximate composition of the experimental diets.

	Experiment diets				
	BSFL0%	**BSFL25%**	**BSFL50%**	**BSFL75%**	**BSFL100%**
Feeding times (times/day)					
Commercial feed	4	3	2	1	0
fresh BSFL	0	1	2	3	4
Proximate composition (% weight)					
Crude protein	42.00	35.95	29.90	23.85	17.80
Crude ash	16.00	12.55	9.10	5.65	2.20
Crude fat	4.00	4.85	5.70	6.55	7.40
phosphorus	1.20	0.98	0.75	0.53	0.30
moisture and other volatile	11.00	25.58	40.15	54.73	69.30

BSFL0% is the control group. The replacement rate was controlled by adjusting the feeding times of fresh BSFL.

nitrogen and stored at −80°C for serum immune enzyme activity analysis. In addition, the hepatopancreas of three shrimps from each tank was quickly removed, frozen in liquid nitrogen, then stored at −80°C until the determination of immune enzyme activities.

Assay of immune enzyme activities of serum and hepatopancreas

The hepatopancreas samples were homogenized in 10 volumes (w/v) of ice-cold physiological saline solution and centrifuged at 4000 rpm at 4°C for 20 min, and the supernatant was conserved at 4°C. The activity of alkaline phosphatase (AKP), acid phosphatase (ACP), and total nitric oxide synthase (T-NOS) in the hepatopancreas and serum was analyzed according to the instructions of commercially available kits (Nanjing Jiancheng Biotech Co., Nanjing, China). The protein content of hepatopancreas samples was determined as described by Bradford (1976).

Measurement of chemical water quality parameters

The DO (mg/L), temperature (T, °C), pH, and salinity (S) of the water in the shrimp tanks were measured daily using a portable water quality monitor-P1 (Zhongyi IOT Ltd. China) before feeding the shrimp. For other water quality parameters, 100 mL of water samples was gathered from tanks every 4 or 5 days and was used for further analysis. Subsequently, the water sample was inspected for NO_2-N and TAN following Standard Methods (Rice et al., 2012), and the chemical oxygen demand (COD) was measured according to Standard Methods (Eaton, 2005).

DNA extraction, PCR amplification of 16*S* amplicon sequencing, and sequence data processing

The shrimp intestinal microbiota was analyzed by 16*S* high-throughput sequencing. Total DNA was extracted using MN NucleoSpin 96 Soil Kit. Subsequently, the full-length 16s rRNA gene was amplified using barcoded conserved primers 27F (5′- AGRGTTTGATYNTGGCTCAG-3′) and 1492R (5′-TASGGHTACCTTGTTASGACTT-3′). The PCR amplification was performed in a total volume of 50 μL containing 25 μL of KOD FX Neo Buf (2×), 10 μL of 2 mM dNTP, 1 μL of KOD FX Neo (TOYOBO), 2.5 μL of 10 μM each primer, and 60 ng of DNA template, and finally, ddH_2O was added to 50 μL. PCR amplification was performed on the PCR system under the following conditions: (a) 1*(3 min at 95°C), (b) 25*(30 s at 95°C; 30 s at 55°C; 1 min at 72°C), and (c) 10 min at 72°C, 10°C until halted by the user (Yuan et al., 2019).

The PCR products were purified, quantified, and homogenized to form a sequencing library (SMRT Bell), and then the quality inspection was carried out. The product that qualified the library was sequenced on a PacBio Sequel platform at Biomarker Technology Co. Ltd (Beijing, China). Bioinformatics analysis for this research was performed with the help of BMK Cloud (Biomarker Technologies Co., Ltd., Beijing, China). Filtering and demultiplexing of raw reads were generated by sequencing to obtain circular consensus sequencing (CCS) reads by SMRT Link software (version 8.0) (*minPasses* ≥ 5 and *minPredictedAccuracy* ≥ 0.9). The CCS sequences were subsequently assigned to corresponding samples according to the barcode by lima (version 1.7.0). The Cutadapt quality control program (version 2.7) was used for quality filtering by identifying forward and reverse primers, and then discarding CCS reads without primers and reads beyond

the length range (1,200–1,650 bp). Chimeric sequences were detected and removed using the UCHIME algorithm (V 8.1) to obtain the clean reads.

Measurement of bacterial communities

The resulting datasets were analyzed using USEARCH (V 10.0). The obtained high-quality sequences were clustered as operational taxonomic units (OTUs) based on 97% similarity. and filtered for OTUs with reabundace<0.005%. Taxonomy annotation of OTUs was performed using the SILVA database (release132) by RDF classifier (V 2.2) (confidence threshold was 80%). Selected OTUs were aligned against the core alignment template of the SILVA database using PyNAST (V 1.2.2) to facilitate downstream diversity analysis. The OTU abundance information was normalized by a standard sequence number corresponding to the sample with the fewest sequences, and further analysis of alpha and beta diversity was performed based on the normalized output data. All sequences in this study have been deposited in the Sequence Read Archive (SRA) of the NCBI database under the accession number PRJNA859541. The beta diversity was analyzed by non-metric multidimensional scaling (NMDS) based on the unweighted UniFrac distance matrix to show differences in microbial community structure between each group. The linear discriminant analysis (LDA) effect size (LEfSe) analysis was used to analyze the differences in microbial community abundance between the control group and fresh BSFL-containing groups; the threshold for the LDA score was set at 4. The alpha diversity (Chao1, Ace, Shannon, and Simpson) and beta diversity were calculated and displayed by BMKCloud (www.biocloud.net).

Calculations and statistical analysis

One-way analysis of variance (ANOVA) was used to test the effect of dietary manipulation. Firstly, all the data were tested for normal distribution and homogeneity of variance. Then, the group means could be further compared using the LSD test for multiple comparisons. All statistical analyses were performed using the SPSS 23 software. The level of significant difference was set at $P < 0.05$ and the results were presented as means ± S.E.M. (standard error of the mean).

Results

The immune enzyme activities after farming experiment in serum and hepatopancreas

The activities of T-NOS, AKP, and ACP in the serum increased first and then decreased with the increase of the replacement rate of fresh BSFL. The activity of T-NOS was higher in the group containing fresh BSFL than that of the control group, and the maximum value was found in the BSFL75% group, with significant differences compared to the control group ($P < 0.05$). The maximum value of AKP activity was found in the BSFL25%, and when the replacement level was equal to or above 50%, the activity of AKP was significantly decreased compared to the control group ($P < 0.05$). The maximum activity of ACP was found in the BSFL25%, with significant differences ($P < 0.05$) compared to the control group and non-significant differences ($P>0.05$) in the other BSFL-containing replacement groups (Table 3).

The activity of T-NOS, AKP, and ACP of shrimp hepatopancreas was negatively related to the proportion of fresh BSFL in the diet, with T-NOS activity that significantly reduced ($P < 0.05$) when the BSFL replacement level was equal to or above 50%. The hepatopancreas AKP and ACP activity in all BSFL-containing groups was significantly lower ($P < 0.05$) than that of the control group (Table 4).

Chemical water parameters of shrimp tanks

Chemical water parameters were monitored and recorded in Figure 1. The results of water chemical parameters showed considerable variations among the experimental groups throughout the 45 days of the experimental period. The TAN and NO_2-N trends of shrimp tanks were negatively related to the dietary fresh BSFL replacement level, whereas the value of NO_2-

TABLE 3 The serum immune enzyme activities of *L. vannamei* fed different experimental diets for 8 weeks.

Enzyme activity	Diets				
	BSFL0%	BSFL25%	BSFL50%	BSFL75%	BSFL100%
T-NOS (U/ml)	16.27 ± 2.57^{b}	25.12 ± 4.66^{ab}	26.55 ± 2.42^{ab}	32.54 ± 0.86^{a}	21.98 ± 5.95^{ab}
AKP (U/100 ml)	13.49 ± 1.68^{a}	16.68 ± 2.44^{a}	3.21 ± 1.40^{b}	2.09 ± 0.41^{b}	3.03 ± 0.63^{b}
ACP (U/100 ml)	10.13 ± 2.84^{b}	15.01 ± 4.33^{a}	8.35 ± 1.11^{b}	7.69 ± 2.20^{b}	7.87 ± 2.49^{b}

Values are presented as mean ± S.E.M of samples.
Values of each parameter in the same row with different superscripts are significantly different ($P<0.05$).

TABLE 4 The hepatopancreatic immune enzyme activities of *L. vannamei* fed different experimental diets for 8 weeks.

Enzyme activity	Diets				
	BSFL0%	BSFL25%	BSFL50%	BSFL75%	BSFL100%
T-NOS (U/ml)	0.40 ± 0.04^{a}	0.36 ± 0.05^{ab}	0.28 ± 0.03^{b}	0.27 ± 0.03^{b}	0.25 ± 0.07^{b}
AKP (U/g prot)	16.04 ± 1.28^{a}	10.21 ± 0.34^{b}	10.50 ± 1.86^{b}	9.88 ± 1.69^{b}	8.16 ± 0.10^{b}
ACP (U/g prot)	17.62 ± 2.11^{a}	12.42 ± 1.88^{b}	12.09 ± 2.79^{bc}	9.57 ± 1.41^{bc}	7.91 ± 1.21^{c}

Values are presented as mean ± S.E.M of samples.
Values of each parameter in the same row with different superscripts are significantly different (P<0.05).

N in the middle and late periods of culture was decreased significantly in BSFL-containing diet groups than that of the control group (P<0.05). The highest concentration of TAN and NO_2-N was observed in the BSFL0% group with 0.88 ± 0.30 mg/L and 1.918 ± 0.17 mg/L, respectively, whereas the lowest concentration of TAN and NO_2-N was observed in the BSFL100% group with 0.13 ± 0.04 mg/L and 0.02 ± 0.01 mg/L, respectively. The differences in the value of COD of shrimp tanks were insignificant among the treatments (P>0.05). In the middle and late phase of culture, the value of pH of shrimp tanks was positively related to the replacement rate of fresh BSFL in the diet, where the pH value was significantly higher (P<0.05) than that of the control group when the BSFL replacement level was equal to or above 75%.

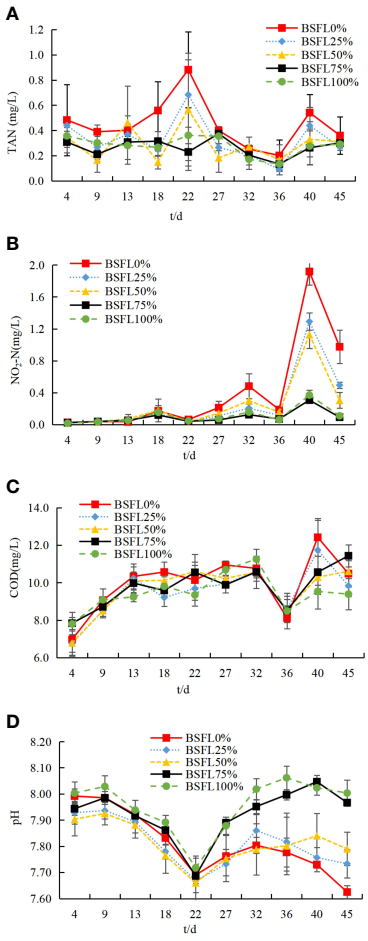


FIGURE 1
Trends of total ammonia nitrogen (TAN) **(A)**, NO_2-N **(B)** chemical oxygen demand (COD) **(C)** and pH **(D)** concentrations (mean±SD per sampling date) in different treatments throughout the experimental period.

PacBio sequencing and microbial complexity in shrimp intestine

A total of 177,569 CCS sequences were obtained after 15 samples were sequenced and identified by Barcode; each sample generated at least 6,081 CCS sequences with an average of 7,838 CCS sequences. The sequences with more than 97% similarity were clustered into OTUs to exploring the bacterial community diversity among all treatments. Both rarefaction analysis and Shannon index curves indicated that all sequenced samples reached the asymptote (Figure 2). The rarefaction analysis indicated that the rarefaction curves reached the asymptote and sufficient sampling depth was achieved for each sample when the number of random sampling sequences of each sample exceeds 5,000 (Figure 2). The bacterial richness and diversity indices were calculated from OTUs to estimate and compare the bacterial diversity in each diet (Table 5). The richness indices of Chao1 and Ace varied from 58 to 246.44 and from 67.42 to 242.65, respectively. The intestinal microbial richness of shrimp was positively correlated with dietary fresh BSFL levels according to the Chao1 and Ace indices, with a significant increase observed in the BSFL75% and BSFL100% groups ($P<0.05$). Bacterial diversity was estimated by the Shannon and Simpson indices, which varied from 1.15 to 3.61 and from 0.06 to 0.48, respectively. The Simpson and Shannon indices showed a trend of increasing and then decreasing intestinal microbial diversity of shrimp as the dietary fresh BSFL level increased; the maximum was found in the BSFL75% group, with a significant increase when the fresh BSFL level was equal to or above 75% ($P<0.05$). The Good's coverage value was > 0.99, indicating that a high degree of sequencing coverage, with all microbiota in each group, was represented by the number of OTUs identified.

Composition and difference of microbiota among five groups

In shrimp intestine, 87 OTUs (31.07%, 33.4%, 35.95%, 31.52%, and 29.39% in diet BSFL0%, BSFL25%, BSFL50%, BSFL75%, and BSFL100% groups, respectively) were shared among all samples (Figure 3). Significant differences were also observed among bacterial communities (beta diversity) in type (unweighted UniFrac) of taxa. The feeding effect on intestinal microbiota of shrimp was explained as displayed in unweighted NMDS (non-metric multi-dimensional scaling) (Figure 4). The bacterial communities of fresh BSFL-containing diet groups clustered together and separately from the control group were shown by the NMDS plot. It suggested that the composition of the shrimp intestinal community is significantly influenced by feeding on fresh BSFL. At the phylum level, the intestinal microbiota of shrimps was mainly dominated by Proteobacteria, Bacteroidetes, and Planctomycetes regardless of the diet. The relative abundance of Proteobacteria in all fresh BSFL-containing groups was lower than that of the control group, with a significant difference when the fresh BSFL replacement level was equal to or above 50%. The relative abundance of Planctomycetes was higher in all fresh BSFL-containing groups than that of the control group, with significant differences found in the BSFL100% group. The relative abundance of Bacteroidetes was higher in all groups containing fresh BSFL than that of the control group, with significant differences found in the BSFL25% and BSFL75% groups. Only Firmicutes were not the dominant phyla in the intestinal microbiota of shrimp whose diet contained fresh BSFL than that of the control group, whereas Verrucomicrobia and Actinobacteria were found to be dominant phyla in the higher fresh BSFL level groups (BSFL75% and BSFL100%) and control groups (Figure 5; Table 6). At the genus level, the intestinal microbiota of shrimp was mainly dominated by *Ruegeria* and *Vibrio* regardless of the diet. The relative abundance of *Ruegeria* was higher in all groups containing fresh BSFL than that of the control group, with significant differences found in the BSFL100% group. The trend in the relative abundance of *Vibrio* was the opposite compared to *Ruegeria*, with significant differences found in the BSFL100% group. Only *Fusibacter* and *Ralstonia* were not the dominant genera in the intestinal microbiota of the group containing fresh BSFL than that of the control group, while the intestinal microbiota of the groups

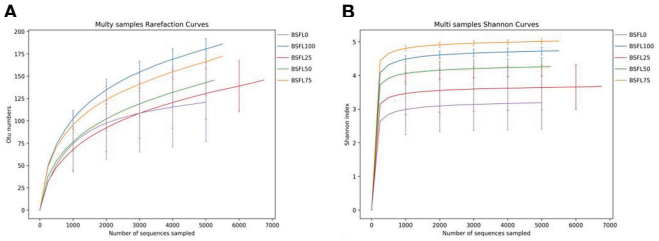


FIGURE 2
Rarefaction curves **(A)** and Shannon-Wiener curves **(B)** of the intestinal bacterial community of *L. vannamei* fed with diets having different levels of BSFL for 45 days.

10.3389/fmars.2022.987363

TABLE 5 Good's coverage value and alpha diversity indices values of intestinal microbiota of *L. vannamei* fed with diets having different levels of BSFL for 45 days.

Items	Diets				
	BSFL0%	BSFL25%	BSFL50%	BSFL75%	BSFL100%
Good's coverage (%)	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00
Observed OTUs	125 ± 64.25	150.33 ± 42.72	159.67 ± 18.63	177 ± 2.45	197.67 ± 20.86
Chao1	134.09 ± 58.34^{b}	181.34 ± 48.57^{ab}	190.36 ± 17.17^{ab}	224.52 ± 14.33^{a}	230.16 ± 14.12^{a}
Ace	136.7 ± 53.96^{b}	183.44 ± 41.56^{ab}	198.97 ± 17.60^{ab}	217.36 ± 9.09^{a}	229.22 ± 16.72^{a}
Simpson	0.28 ± 0.14^{a}	0.20 ± 0.06^{ab}	0.12 ± 0.03^{b}	0.07 ± 0.00^{b}	0.11 ± 0.01^{b}
Shannon	2.23 ± 0.78^{b}	2.55 ± 0.66^{ab}	2.97 ± 0.25^{ab}	3.49 ± 0.08^{a}	3.30 ± 0.12^{a}

Report data are expressed as mean ± S.E.M. The means were compared by ANOVA ($P<0.05$).
Different superscript letters on the same column indicate significant differences after post hoc Tukey's test.

containing fresh BSFL was characterized by a high relative abundance of *Motilimonas*, *Shimia*, *Pseudoalteromonas*, and *Donghicola*. *Shewanella* was the dominant genus found only in the BSFL50%, BSFL75%, and BSFL100% groups (Figure 6 and Table 7).

The statistical results were further supported by the LEfSe analysis, which showed significant differences in intestinal bacterial abundances between shrimps fed the diets containing fresh BSFL and those fed with commercial feeds (Figure 7). The results showed that the relative abundance of class Gammaproteobacteria and genus *Ralstonia* belonging to phylum Proteobacteria was significantly higher in the control group. Moreover, the significantly higher relative abundance of family Rubinisphaeraceae (Planctomycetes), order Planctomycetales (Planctomycetes), order Pirellulales (Planctomycetes), family Pirellulaceae (Planctomycetes), class Planctomycetacia (Planctomycetes), genus *Motilimonas* (Proteobacteria), family Psychromonadaceae (Proteobacteria), genus *Ruegeria* (Proteobacteria), order Flavobacteriales (Bacteroidetes), family Flavobacteriaceae (Bacteroidetes), class Bacteroidia (Bacteroidetes) was found in the fresh BSFL-containing groups.

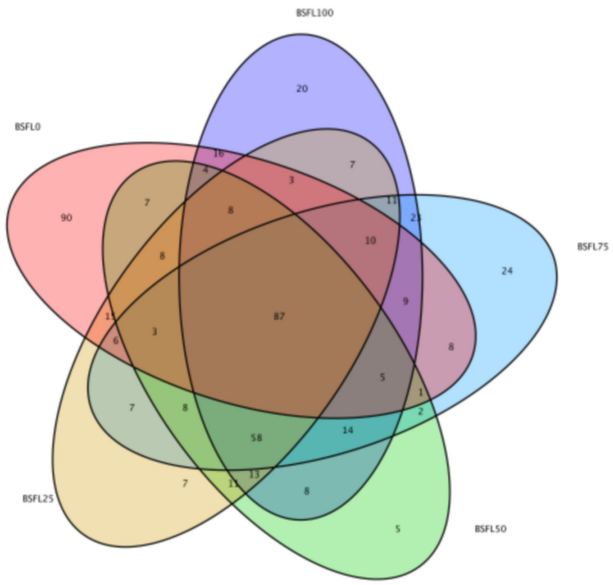


FIGURE 3
Venn diagram showing the distribution of all OTUs shared by *L. vannamei* in five groups. The figures in the Venn diagram indicate the number of the sequences that are associated with the OTUs in each group.

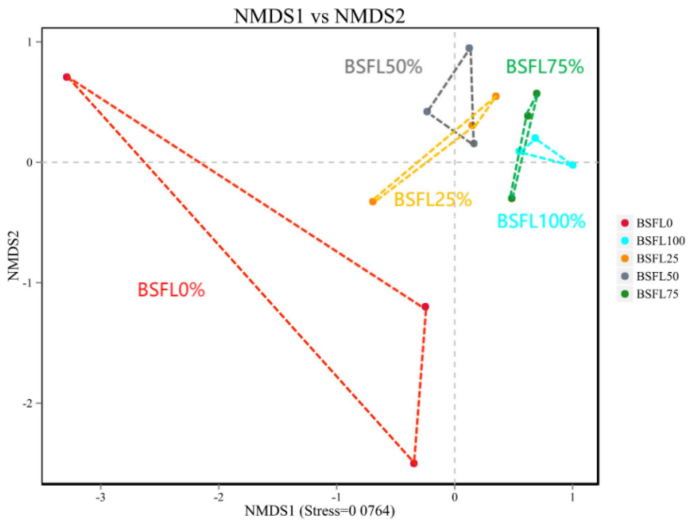


FIGURE 4
Non-MetricMulti-Dimensional Scaling (NMDS) of unweighted distances of intestinal microbiota associated to different diets.

Discussion

The effects on immune enzyme activity of shrimp

The proper level of fresh BSFL can increase the immune enzyme activity of shrimp serum but led to a decrease in hepatopancreas immune enzyme activity. Among the non-specific immune indicators of *L. vannamei*, the activity of AKP, ACP, and T-NOS is closely related to the health status and immune capacity of the organism. Inducible nitric oxide synthase (iNOS) produces large amounts of NO to kill pathogens after being stressed by immune stimulants or various environmental stressors (Chakravortty and Hensel, 2003). In addition, both ACP and AKP are marker enzymes for lysosomal enzymes, which are important for the immune system of crustaceans (Sgeirsson et al., 1995; Xiao et al., 2018). Higher activities of T-NOS, AKP, and ACP were detected in the shrimp serum when 25% commercial feed was replaced by fresh BSFL. This was following the previous study (Xiao et al., 2018), which lysozyme and phagocytic percentages in the serum of *P. fulvidraco* were increased by the appropriate level of BSFL meal supplemented in the diet. Furthermore, the defatted insect meal (*T. Molitor*) was added to the diet also increased the phenol oxidase activity of *L. vannamei* (Rios et al., 2019). We suggest that chitin in BSFL may be responsible for the improved immune enzyme activity of *L. vannamei*. Many studies have also shown that chitin and its derivatives could enhance the immune response of aquatic animals (Khoushab and Yamabhai, 2010; Ngo and Kim, 2014). However, fresh BSFL also has negative effects: elevated fat level and reduced protein level in shrimp diets with the increase of fresh BSFL replacement level in the diet. Some research indicated that feeding diets with excessive fat levels have a negative impact on the immune system of shrimp (Zhang et al., 2013). In addition, the protein level in the diet is also closely related to the immune capacity of aquatic animals (Council, 2011). Therefore, the replacement rate of fresh BSFL is limited by their nutrition composition.

Interestingly, we may break this limitation by changing the culture substrate of fresh BSFL. The fresh BSFL is like a capsule which nutrition composition is closely related to its culture substrate (Shumo et al., 2019). Meneguz et al. (2018) found that the protein content of BSFL was significantly increased by using agro-industrial by-products instead of organic waste. Fresh BSFL with a lower fat level and higher protein are obtained by adjusting the culture substrate. Furthermore, studies have shown that BSFL are deficient in essential amino acids and unbalanced EAA/NEAA (Cummins et al., 2017), and this disadvantage cannot be improved by changing the culture substrate of the BSFL. The amino acid content of larvae changed in a small range when BSFL were raised on different rearing substrates, especially when compared with the significant difference in amino acid composition of the culture substrates (Spranghers et al., 2017). This also suggests that if fresh BSFL are to be used in shrimp culture, it is better to supplement the diet with additives, such as the addition of free amino acids to improve the amino acid balance of the diet.

Water quality of shrimp tanks

Fresh BSFL can improve water quality and reduce water change, thus decreasing the chance of external disease invasion.

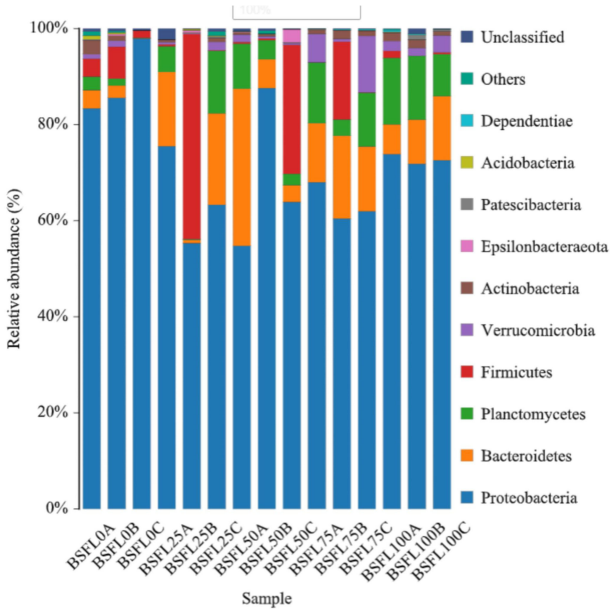


FIGURE 5
Relative abundances of bacterial phyla in the intestine of *L. vannamei* fed with diets having different levels of BSFL. The most dominant phyla (> 1% of the total sequences) are shown in the schematic individually, and the relative abundances of main phyla is indicated in Table 6.

Water quality has been observed to be influenced by feeding activities, which may affect the growth performance of aquatic animals. In this study, continuous monitoring of water quality in each group showed that fresh BSFL replacement level was negatively correlated with ammonia nitrogen and nitrate nitrogen and positively correlated with pH. This phenomenon may be explained by the deterioration of water quality due to feed losses (Cha et al., 2008). Previous studies indicated that only 20%–30% of feed nutrients are retained in the shrimp culture and the remaining 70%–80% are accumulated in the aquatic system, which can lead to eutrophication and water quality deterioration (Paez-Osuna, 2001). Furthermore, the residues of fresh BSFL are mainly chitin exoskeleton, which is more difficult to dissolve and easier to be removed by siphonage than the commercial feeds. Excessive pollutants in the culture environments (such as ammonia, nitrite, and COD derived from soluble excretions and organic decomposition) can lead to anoxic conditions which are harmful to benthic biota owing to the oxygen-consuming decomposition (Li et al., 2011; Wang et al., 2016). Stress in shrimp can be caused by the overload of toxic nitrogen metabolites, which can also reduce immune capacity leading to susceptibility to disease (Kautsky et al., 2000). In addition, the ammoniacal nitrogen was converted to microbial biomass by heterotrophic bacteria, along with the production of CO_2 (Ebeling et al., 2006), resulting in a decrease in the pH of aquaculture water environments. Previous studies have shown that the decrease in pH induces an increase of the oxygen consumption rate of shrimp (Chen and Lin, 1995). Large water exchange strategies are often adopted to maintain water quality in intensive and semi-intensive shrimp

TABLE 6 Percentage abundances (%) of bacterial phyla in the intestine of *L. vannamei*.

Phylum	BSFL0%	BSFL25%	BSFL50%	BSFL75%	BSFL100%
Proteobacteria	88.95 ± 6.40^{a}	64.71 ± 8.30^{ab}	68.75 ± 13.84^{b}	63.47 ± 3.26^{b}	72.75 ± 0.83b
Bacteroidetes	3.19 ± 0.62^{b}	17.25 ± 1.79^{a}	4.74 ± 1.24^{ab}	14.34 ± 2.10^{a}	9.58 ± 2.94^{ab}
Planctomycetes	2.11 ± 0.69	9.18 ± 3.83^{ab}	5.25 ± 3.00^{ab}	9.06 ± 4.05^{ab}	11.94 ± 2.28^{a}
Firmicutes	3.94 ± 1.45	—	—	—	—
Verrucomicrobia	1.1 ± 0.17^{b}	—	—	8.85 ± 2.9^{a}	2.42 ± 0.81^{b}
Actinobacteria	1.98 ± 0.95	—	—	1.23 ± 0.41	1.43 ± 0.43
Others	1.87 ± 0.87	1.86 ± 0.35	1.54 ± 0.42	0.81 ± 0.27	1.24 ± 0.4

Values are presented as the mean ± S.E.M of three replicates (n = 3, if there is a mutation value, removed). Values in the same row with different superscripts are significantly different ($P < 0.05$).

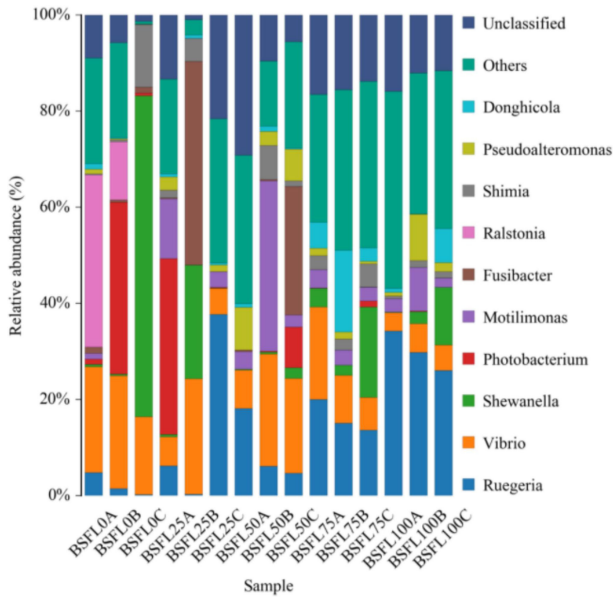


FIGURE 6
Relative abundances of bacterial genera in the intestine of *L. vannamei* fed with diets having different levels of BSFL. The most dominant genera (> 1% of the total sequences) are shown in the schematic individually, and the relative abundances of main genera is indicated in Table 7.

farming when water quality deteriorates, which increases the risk of pathogen invasion and transmission, energy consumption, and labor cost. Therefore, fresh BSFL are suitable for practical application in high-density shrimp culture.

Microbiota analysis of shrimp intestine

Generally, the high intestinal microbial richness and diversity are considered a positive phenomenon because they usually represent the better health status of the host (Sekirov et al., 2010; Apper et al., 2016). In this experiment, the richness and diversity of intestinal microbiota of *L. vannamei* can be enhanced when commercial feeds were replaced by fresh BSFL. This was in agreement with the findings of most studies, which indicate that the shrimp community is plastic and can be manipulated by the addition of insects to the diets. Specifically, Foysal et al. (2019) found that the diversity and richness of intestinal microbiota in marron (*C. cainii*) was positively correlated with dietary BSFL meal level. In addition, the

TABLE 7 Relative abundances (%) of bacterial genus in the intestine of *L. vannamei*.

Genus	BSFL0%	BSFL25%	BSFL50%	BSFL75%	BSFL100%
Ruegeria	2.17 ± 1.92^{b}	21.97 ± 15.74^{ab}	9.65 ± 6.02^{b}	16.23 ± 2.75^{ab}	30.07 ± 3.35^{a}
Vibrio	20.55 ± 3.14^{a}	11.83 ± 8.61^{ab}	16.99 ± 6.54^{ab}	12.00 ± 5.26^{ab}	5.00 ± 0.90^{b}
Shewanella	—	—	—	3.03 ± 0.84	7.26 ± 4.74
Motilimonas	—	7.83 ± 4.61	3.04 ± 0.52	3.25 ± 0.4	4.63 ± 3.21
Fusibacter	1.01 ± 0.41	—	—	—	—
Ralstonia	23.98 ± 11.85	—	—	—	—
Shimia	—	3.22 ± 1.58	4.13 ± 2.98	3.34 ± 1.06	1.06 ± 0.37
Pseudoalteromonas	—	2.1 ± 0.66	6.12 ± 2.46	1.20 ± 0.46	4.11 ± 3.94
Donghicola	—	—	—	8.38 ± 6.17	—
Others	16.56 ± 9.99	18.63 ± 11.07	23.54 ± 7.26	32.05 ± 6.81	34.42 ± 5.86

Only the top 10 genera in relative abundances are shown, and the other genera are combined into others for display in the figure and table. Values are presented as the mean ± S.E.M of three replicates (n = 3, if there is a mutation value, removed). Values in the same row with different superscripts are significantly different ($P < 0.05$).

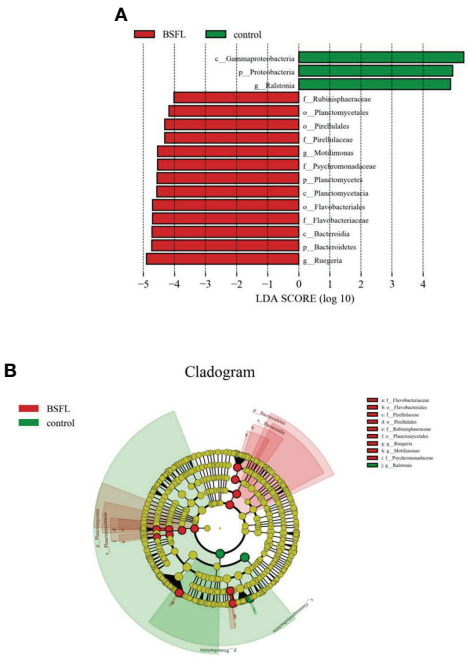


FIGURE 7
The LDA effects size (LEfSe) analysis of bacterial taxa between the control and fresh BSFL-containing groups. **(A)** Bar chart displays LDA scores of the control and fresh BSFL-containing groups. The LDA scores represented the differencein relative abundance with exponent fold change of 10 between two treatments. Significant differences are defined as $P < 0.05$ and LDA score >0.04. **(B)** Cladogram displays significantly enriched bacterial taxa (from the phylum to the genus level).

richness and diversity of intestinal microbiota in rainbow trout were significantly improved after FM was partially replaced by BSFL meal in the diets (Terova et al., 2019). In addition, the composition of the dominant intestinal microbiota of *L. vannamei* was also significantly affected by the diet containing fresh BSFL. Previous studies showed that the intestinal microbiota of *L. vannamei* was dominated by Proteobacteria and Bacteroidetes, which constitute the core flora, regardless of the diet administered (Yuan et al., 2019). However, chronic intestinal microecological disorders or intestinal inflammation was often caused by the excessive relative abundance of Proteobacteria (Mukhopadhya et al., 2012; Shin et al., 2015). In addition, intestinal inflammation is usually accompanied by the increase of Proteobacteria and by the decrease of Bacteroidetes. We suggest that chitin in BSFL may be a key factor in improving the intestinal health of shrimp. It was known that the insects contain a large amount of chitin which is a mucopolysaccharide polymer hardly digested by many aquatic animal species. However, many studies found chitin added to the diet of aquatic animals can improve the diversity of intestinal microbiota and inhibit the growth of pathogens in the intestine (Zhou et al., 2013; Karlsen et al., 2017; Udayangani et al., 2017). In addition, fresh BSFL are also rich in lauric acid, a medium-chain fatty acid that inhibits Gram-positive bacteria and plays a role in regulating the composition of intestinal bacteria (Skřivanová et al., 2005; Skřivanová et al., 2006).

However, feeding fresh BSFL inhibited the relative abundance of the Firmicutes. In this experiment, only the control group was characterized by a high abundance of Firmicutes. This was inconsistent with previous findings in rainbow trout (*O. mykiss*), a higher relative abundance of Firmicutes was found in the insect-containing groups (Terova et al., 2019). Bruni et al. (2018) found that Firmicutes were only detected in the fecal samples of *O. mykiss* fed with BSFL. Firmicutes include different lactic acid bacteria, which are generally considered to be beneficial microbes. Firmicutes play an important role in breaking down indigestible carbohydrates such as starches and fibers, thereby contributing to making more efficient use of energy in diets (Smriga et al., 2010; Catalán et al., 2018). Thus, feeding fresh BSFL has a negative effect on the digestibility of shrimp. It was analyzed that the relative abundance of Firmicutes in the intestine of shrimp may be limited by two factors. Firstly, the plant component of the diet was typically associated with higher Firmicutes found in several studies (Heikkinen et al., 2006; Desai et al., 2012). The decrease in the relative abundance of Firmicutes may be caused by a reduction in the plant component of the fresh BSFL feeding regime. Secondly, the composition of the intestinal microbiota of

aquatic animals was also modulated by the insect-rearing substrates. The highest inhibitory activities against Gram-positive bacteria were found in larvae reared on chitin, cellulose, bacteria, and plant oil (Vogel et al., 2018). The wheat bran was used as a rearing substrate for BSFL in this study, which may also explain the lower relative abundance of Firmicutes as Gram-positive bacteria in the intestine of shrimp. These results also remind us that additional probiotics belonging to Firmicutes should be added to the diet to improve the digestibility of shrimp when commercial feeds are replaced by fresh BSFL, such as *Streptococcus*, *Lactobacillus*, and *Carnobacterium*.

At the level of genus, fresh BSFL can promote the relative abundance of probiotic bacteria while suppressing the relative abundance of *Vibrio*. Some unique probiotics were only found in the fresh BSFL-containing groups, such as *Motilimonas*, *Shimia*, *Pseudoalteromonas*, and *Shewanella*. These bacteria designated as probiotics had been reported to be positively correlated with diets containing insects in general (Terova et al., 2019). *Pseudoalteromonas* can produce a variety of extracellular active substances with antimicrobial, bacteriolytic, galactose decomposition, cellulose degradation, and other functions, so it has high utilization potential and important ecological implications (Holmström and Kjelleberg, 1999). *Shewanella* as the dominant bacteria was found in the high fresh BSFL replacement groups, and some studies have shown that *Shewanella* contributes to the nutrition absorption of rainbow trout (Koziska and Pêkala, 2004). In addition, the prolific growth of *Vibrio* can lead to the whole microecological balance being destroyed. This ultimately results in the growth and immune capacity being inhibited and even death burst in aquatic animals (Sung et al., 2001). In the present experiment, the fresh BSFL-containing groups had better water quality, which may have suppressed the relative abundance of *Vibrio*. Beyond that, the proliferation of beneficial bacteria can be stimulated by chitin, which can effectively compete with *Vibrio*.

In addition, the relative abundances of microbial taxa across the control group and fresh BSFL-containing groups in this trial were contrasted using the LEfSe analysis, which revealed that the significantly enriched probiotic bacteria again validated the positive effect of feeding fresh BSFL on the shrimp intestinal microbiota. Interestingly, the emergence of Planctomycetes as the dominant bacteria in the intestine of shrimp has not been reported in previous studies, but our results showed a significant increase in the relative abundance of Planctomycetes in the fresh BSFL-containing groups. Planctomycetes are widely found in natural systems such as seawater, freshwater, soil, and other artificial systems such as wastewater. They play an important role in farming wastewater nitrogen removal systems by generating nitrogen (N_2) from total ammonia nitrogen (NH_4^+) and nitrite (NO_2–N). Thus, Planctomycetes are considered eco-friendly bacteria (Kuypers et al., 2003; Van Teeseling et al., 2015). We suggested that the elevated relative abundance of the Planctomycetes in the shrimp intestine may be related to the change in the aquatic environment. Some studies have shown that the intestinal microbiota of aquatic animals interacts with the microflora in the water environment. In addition, the positive effect of fresh insects on water quality might benefit the enrichment of the Planctomycetes in the shrimp intestine. However, this hypothesis requires further research.

Conclusions

This study demonstrated that replacing commercial feeds by fresh black soldier fly larvae in the diets at a proper rate had positive effects on immune and digestive system in Pacific white shrimp *L. vannamei* with an improvement on water quality during culturing experiment. The decreased immune enzyme activities in hepatopancreas was found in groups with BSFL replacement rate above 50%, suggesting an optimal proportion of BSFL replacement should be under 50%. In the coming work, the replacement level of commercial feeds with fresh BSFL could be improved by changing the culture substrates for the insects, as well as a dietary supplementation with nutritional ingredients or probiotics, which can contribute to sustainable shrimp farming by reducing FM consumption.

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/-PRJNA859541.

Author contributions

YH designed and performed the experiments with other teammates with the help of YJ. YH drafted the manuscript. QY and AW took samples. SW and NZ measured the activity of immune enzymes. YC and AW performed the statistical analysis of the original data. SH and YM revised the manuscript. All authors contributed to the article and approved the submitted version.

Acknowledgments

We are sincerely grateful to thank Prof. Jianguang Qin, Prof. Xiaodong Li and Associate Prof. Rantao Zuo for their pieces of advice and comments on this manuscript. This work was financially supported by the National Key R&D Program of China (Grant No. 2020YFD0900202 and 2018YFD0900400), Project of Selecting Best Candidates to Lead Key Researches in

Liaoning 2021JH1/10400040 and Grant for marine economic development from Liaoning provincial department of natural resources (202233).

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

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Adelizi, P. D., Rosati, R. R., Warner, K., Wu, Y. V., and Muench, T. R. (1998). Evaluation of fish-meal free diets for rainbow trout, Oncorhynchus mykiss. *Aquac. Nutr.* 4, 255–262. doi: 10.1046/j.1365-2095.1998.00077.x

Apper, E., Weissman, D., Respondek, F., Guyonvarch, A., Baron, F., Boisot, P., et al. (2016a). Hydrolysed wheat gluten as part of a diet based on animal and plant proteins supports good growth performance of Asian seabass (*Lates calcarifer*), without impairing intestinal morphology or microbiota. *Aquaculture* 453, 40–48. doi: 10.1016/j.aquaculture.2015.11.018

Bondari, K., and Sheppard, D. C. (1981). Soldier fly larvae as feed in commercial fish production. *Aquaculture* 24, 103–109. doi: 10.1016/0044-8486(81)90047-8

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochem.* 72, 248–254. doi: 10.1016/0003-2697(76)90527-3

Bruni, L., Pastorelli, R., Viti, C., Gasco, L., and Parisi, G. (2018). Characterisation of the intestinal microbial communities of rainbow trout (*Oncorhynchus mykiss*) fed with *Hermetia illucens* (black soldier fly) partially defatted larva meal as partial dietary protein source. *Aquaculture* 487, 56–63. doi: 10.1016/j.aquaculture.2018.01.006

Catalán, N., Villasante, A., Wacyk, J., Ramírez, C., and Romero, J. (2018). Fermented soybean meal increases lactic acid bacteria in gut microbiota of atlantic salmon (*Salmo salar*). *Probiotics Antimicrobial Proteins* 10, 1–11. doi: 10.1007/s12602-017-9366-7

Chakravortty, D., and Hensel, M. (2003). Inducible nitric oxide synthase and control of intracellular bacterial pathogens. *Microbes Infection* 5, 621–627. doi: 10.1016/S1286-4579(03)00096-0

Cha, S. H., Lee, J. S., Song, C. B., Lee, K. J., and Jeon, Y. J. (2008). Effects of chitosan-coated diet on improving water quality and innate immunity in the olive flounder, *Paralichthys olivaceus*. *Aquaculture* 278, 110–118. doi: 10.1016/j.aquaculture.2008.01.025

Chen, J. C., and Lin, C. Y. (1995). Responses of oxygen consumption, ammonia-n excretion and urea-n excretion of *Penaeus chinensis* exposed to ambient ammonia at different salinity and pH levels. *Aquaculture* 136, 243–255. doi: 10.1016/0044-8486(95)01060-2

Cruz-Suárez, L. E., Nieto-López, M., Guajardo-Barbosa, C., Tapia-Salazar, M., Scholz, U., Ricque-Marie, D, et al. (2007). Replacement of fish meal with poultry by-product meal in practical diets for *Litopenaeus vannamei*, and digestibility of the tested ingredients and diets. *Aquaculture* 272, 466–476. doi: 10.1016/j.aquaculture.2007.04.084

Council, N. R. (2011). *Nutrient requirements of fish and shrimp*. (National Academies Press), 57–125.

Cummins, V. C., Rawles, S. D., Thompson, K. R., Velasquez, A., Kobayashi, Y., and Hager, J, et al. (2017). Evaluation of black soldier fly (*Hermetia illucens*) larvae meal as partial or total replacement of marine fish meal in practical diets for pacific white shrimp (*Litopenaeus vannamei*). *Aquaculture* 473, 337–344. doi: 10.1016/j.aquaculture.2017.02.022

Desai, A. R., Links, M. G., Collins, S. A., Mansfield, G. S., and Hill, J. E. (2012). Effects of plant-based diets on the distal gut microbiome of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 350-353, 134–142. doi: 10.1016/j.aquaculture.2012.04.005

Diener, S., Zurbruegg, C., and Tockner, K. (2009). Conversion of organic material by black soldier fly larvae: Establishing optimal feeding rates. *Waste Manag. Res.* 27, 603–610. doi: 10.1177/0734242X09103838

Eaton, A. D., Clesceri, L. S., Rice, E. W., Greenberg, A. E., and Franson, M. (2005). *Standard methods for the examination of water and waste water*. Centennial Edition., APHA, AWWA, WEF, Washington, DC

Ebeling, J. M., Timmons, M. B., and Bisogni, J. J. (2006). Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic removal of ammonia-nitrogen in aquaculture systems. *Aquaculture* 257, 346–358. doi: 10.1016/j.aquaculture.2006.03.019

Emehinaiye, P. A. (2012). *Growth performance of oreochromis niloticus fingerlings fed with varing levels of migratory locust (locustamigratoria) meal*. Bachelor of Aquaculture and Fisheries Management, Federal University of Agriculture, Abeokuta.

Foysal, M. J., Fotedar, R., Tay, C. Y., and Gupta, S. K. (2019). Dietary supplementation of black soldier fly (*Hermetica illucens*) meal modulates gut microbiota, innate immune response and health status of marron (*Cherax cainii*, Austin 2002) fed poultry-by-product and fishmeal based diets. *PeerJ* 7, e6891. doi: 10.7717/peerj.6891

Gasco, L., Finke, M., and Huis, A. V. (2018). Can diets containing insects promote animal health? *J. Insects as Food Feed* 4, 1–4. doi: 10.3920/JIFF2018.x001

Gasco, L., Henry, M., Piccolo, G., Marono, S., Gai, F., Renna, M., et al. (2016). Tenebrio molitor meal in diets for European sea bass (*Dicentrarchus labrax* l.) juveniles: Growth performance, whole body composition and *in vivo* apparent digestibility. *Anim. Feed Sci. Technol.* 220, 34–45. doi: 10.1016/j.anifeedsci.2016.07.003

Heikkinen, J., Vielma, J., Kemil Inen, O., Tiirola, M., Eskelinen, P., Kiuru, T., et al. (2006). Effects of soybean meal based diet on growth performance, gut histopathology and intestinal microbiota of juvenile rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 261 (1), 259–268. doi: 10.1016/j.aquaculture.2006.07.012

He, Y., Liu, X., Zhang, N., Wang, S., Wang, A., Zuo, R., et al. (2022). Replacement of commercial feed with fresh black soldier fly (*Hermetia illucens*) larvae in pacific white shrimp (*Litopenaeus vannamei*). *Aquacult. Nutr.* 2022, 9130400. doi: 10.1155/2022/9130400

Holmström, C., and Kjelleberg, S. (1999). Marine pseudoalteromonas species are associated with higher organisms and produce biologically active extracellular agents. *FEMS Microbiol. Ecol.* 30, 285–293. doi: 10.1111/j.1574-6941.1999.tb00656.x

Karlsen, Ø., Amlund, H., Berg, A., and Olsen, R. E. (2017). The effect of dietary chitin on growth and nutrient digestibility in farmed Atlantic cod, Atlantic salmon and Atlantic halibut. *Aquacult. Res.* 48, 123–133. doi: 10.1111/are.12867

Kautsky, N., Rönnbäck,P., Tedengren, M., and Troell, M. (2000). Ecosystem perspectives on management of disease in shrimp pond farming. *Aquaculture* 191, 145–161. doi: 10.1016/S0044-8486(00)00424-5

Khoushab, F., and Yamabhai, M. (2010). Chitin research revisited. *Mar. Drugs* 8, 1988–2012. doi: 10.3390/md8071988

Koziska, A., and Pękala, A. (2004). First isolation of shewanella putrefaciens from freshwater fish - a potential new pathogen of fish. *Bulletin- Eur. Assoc. Fish. Pathol.* 24 (4), 189–193.

Kuypers, M. M. M., Sliekers, A. O., Lavik, G., Schmid, M., Jørgensen, B. B., Kuenen, J. G., et al. (2003). Anaerobic ammonium oxidation by anammox bacteria in the black Sea. *Nature* 422 (6932), 608–611. doi: 10.1038/nature01472

Lai, H., Hou, J., Su, C., and Chen, C. (2009). Effects of chloramphenicol, florfenicol, and thiamphenicol on growth of algae chlorella pyrenoidosa, isochrysis galbana, and tetraselmis chui. *Ecotoxicol. Environ. Saf.* 72, 329–334. doi: 10.1016/j.ecoenv.2008.03.005

Lemos, D., Lawrence, A. L., and Iii, A. (2009). Prediction of apparent protein digestibility of ingredients and diets by *in vitro* pH-stat degree of protein hydrolysis with species-specific enzymes for juvenile pacific white shrimp *Litopenaeus vannamei*. *Aquaculture* 295, 89–98. doi: 10.1016/j.aquaculture.2009.06.011

Li, S., Ji, H., Zhang, B., Zhou, J., and Yu, H. (2017). Defatted black soldier fly (Hermetia illucens) larvae meal in diets for juvenile Jian carp (Cyprinus carpio var. Jian): Growth performance, antioxidant enzyme activities, digestive enzyme activities, intestine and hepatopancreas histological structure. *Aquaculture* 477, 62–70. doi: 10.1016/j.aquaculture.2017.04.0157

Li, X., Li, J., Wang, Y., Fu, L., Fu, Y., Li, B., et al. (2011). Aquaculture industry in china: Current state, challenges, and outlook. *Rev. Fish. Sci.* 19 (3), 187–200. doi: 10.1080/10641262.2011.573597

Llewellyn, M. S., Boutin, S., Hoseinifar, S. H., and Derome, N. (2014). Teleost microbiomes: The state of the art in their characterization, manipulation and importance in aquaculture and fisheries. *Front. Microbiol.* 5, 207. doi: 10.3389/fmicb.2014.00207

Marinho-Soriano, E., Nunes, S. O., Carneiro, M., and Pereira, D. C. (2009). Nutrients' removal from aquaculture wastewater using the macroalgae gracilaria birdiae. *Biomass Bioenergy* 33, 327–331. doi: 10.1016/j.biombioe.2008.07.002

Maslowski, K. M., and Mackay, C. R. (2011). Diet, gut microbiota and immune responses. *Nat. Immunol.* 12, 5–9. doi: 10.1038/ni0111-5

Mcgraw, W. J., Davis, D. A., Oddington, D. T., and Rouse, D. B. (2010). Acclimation of *litopenaeus vannamei* postlarvae to low salinity: Influence of age, salinity endpoint, and rate of salinity reduction. *J. World Aquacult. Soc.* 33, 78–84. doi: 10.1111/j.1749-7345.2002.tb00481.x

Meneguz, M., Schiavone, A., Gai, F., Dama, A., Lussiana, C., Renna, M., et al. (2018). Effect of rearing substrate on growth performance, waste reduction efficiency and chemical composition of black soldier fly (*Hermetia illucens*) larvae. *J. Sci. Food Agric.* 98, 5776–5784. doi: 10.1002/jsfa.9127

Mukhopadhya, I., Hansen, R., El-Omar, E. M., and Hold, G. L. (2012). IBD–what role do proteobacteria play? *Nat. Rev. Gastroenterol. Hepatol.* 9, 219–230. doi: 10.1038/nrgastro.2012.14

Newton, G. L., Booram, C. V., Barker, R. W., and Hale, O. M. (1977). Dried hermetia illucens larvae meal as a supplement for swine. *J. Anim. Sci.* 44, 395–400. doi: 10.2527/jas1977.443395x

Ngo, D. H., and Kim, S. K. (2014). Antioxidant effects of chitin, chitosan, and their derivatives. *Adv. Food Nutr. Res.* 73, 15–31. doi: 10.1016/B978-0-12-800268-1.00002-0

Oyarzun, P., Alarcon, L., Calabriano, G., Bejarano, J., Nunez, D., et al. (2019). Trickling filter technology for biotreatment of nitrogenous compounds emitted in exhaust gases from fishmeal plants. *J. Environ. Manage.* 232, 165–170. doi: 10.1046/j.1365-2095.1998.00077.x

Paez-Osuna, F. (2001). The environmental impact of shrimp aquaculture: A global perspective. *Environ. pollut.* 112, 229–231. doi: 10.1016/S0269-7491(00)00111-1

Pullin, R. S., and Lowe-McConnell, R. H. (1982). *The biology and culture of tilapias: Proceedings of the international conference on the biology and culture of tilapias, 2-5 September 1980 at the study and conference center of the Rockefeller foundation* (Bellagio, Italy: WorldFish).

Rios, A., Lefebvre, T., Hong, D., Henry, M., and Motte, C. (2019). Replacing fish meal with defatted insect meal (Yellow mealworm tenebrio molitor) improves the growth and immunity of pacific white shrimp (*Litopenaeus vannamei*). *Anim. Open Access J. From MDPI* 9 (5), 258. doi: 10.3390/ani9050258.

Rice, E. W., Baird, R. B., Eaton, A. D., and Clesceri, L. S. (2012). *Standard methods for the examination of water and wastewater*. Washington, DC: American Public Health Association.

Samira, H., and Mehdi, S. M. (2015). The effect of replacing fish meal in the diet with Enzyme-Treated soybean meal (HP310) on growth and body composition of rainbow trout fry. *Molecules* 20(12). doi: 10.3390/molecules201219751.

Saoud, I. P., Davis, D. A., and Rouse, D. B. (2003). Suitability studies of inland well waters for *Litopenaeus vannamei* culture. *Aquaculture* 217, 373–383. doi: 10.1016/S0044-8486(02)00418-0

Sekirov, I., Russell, S. L., Antunes, L. C. M., and Finlay, B. B. (2010). Gut microbiota in health and disease. *Physiol. Rev* 90 (3), 859–904. doi: 10.1152/physrev.00045.2009

Sgeirsson, B., Hartemink, R., and Chlebowski, J. F. (1995). Alkaline phosphatase from Atlantic cod (*Gadus morhua*). kinetic and structural properties which indicate adaptation to low temperatures. *Comp. Biochem. Physiol. Part B Biochem. Mol. Biol.* 110, 315–329. doi: 10.1016/0305-0491(94)00171-P

Shin, N. R., Whon, T. W., and Bae, J. W. (2015). Proteobacteria: Microbial signature of dysbiosis in gut microbiota. *Trends Biotechnol.* 33 (9), 496–503. doi: 10.1016/j.tibtech.2015.06.011

Shumo, M., Osuga, I. M., Khamis, F. M., Tanga, C. M., Fiaboe, K., Subramanian, S., et al. (2019). The nutritive value of black soldier fly larvae reared on common organic waste streams in Kenya. *Sci. Rep.* 9 (1), 10110. doi: 10.1038/s41598-019-46603-z

Skřivanová, E., Marounek, M., Benda, V., and Březina, P. (2006). Susceptibility of escherichia coli, salmonella sp. and clostridium perfringens to organic acids and monolaurin. *Veterinární Medicína* 51, 81–88.

Skřivanová, E., Marounek, M., Dlouha, G., and Kaňka, J. (2005). Susceptibility of clostridium perfringens to C2–C18 fatty acids. *Lett. Appl. Microbiol.* 41, 77–81. doi: 10.1111/j.1472-765X.2005.01709.x

Smriga, S., Sandin, S. A., and Azam, F. (2010). Abundance, diversity, and activity of microbial assemblages associated with coral reef fish guts and feces. *FEMS Microbiol. Ecol.* 73, 31–42. doi: 10.1111/j.1574-6941.2010.00879.x

Spranghers, T., Ottoboni, M., Klootwijk, C., Ovyn, A., and Smet, S. D. (2017). Nutritional composition of black soldier fly (*Hermetia illucens*) prepupae reared on different organic waste substrates. *J. Sci. Food Agric.* 97, 2594–2600. doi: 10.1002/jsfa.8081

Sung, H. H., Hsu, S. F., Chen, C. K., Ting, Y. Y., and Chao, W. L. (2001). Relationships between disease outbreak in cultured tiger shrimp (*Penaeus monodon*) and the composition of vibrio communities in pond water and shrimp hepatopancreas during cultivation. *Aquaculture* 192, 101–110. doi: 10.1016/S0044-8486(00)00458-0

Terova, G., Rimoldi, S., Ascione, C., Gini, E., Ceccotti, C., Gasco, L., et al. (2019). Rainbow trout (*Oncorhynchus mykiss*) gut microbiota is modulated by insect meal from hermetia illucens prepupae in the diet. *Rev. Fish. Biol. Fish.* doi: 10.1007/s11160-019-09558-y

Udayangani, R. M. C., Dananjaya, S. H. S., Nikapitiya, C., Heo, G. J., and Zoysa, M. D. (2017). Metagenomics analysis of gut microbiota and immune modulation in zebrafish (*Danio rerio*) fed chitosan silver nanocomposites. *Fish. Shellfish Immunol.* 66, 173–184. doi: 10.1016/j.fsi.2017.05.018

Van Huis, A. (2013). Potential of insects as food and feed in assuring food security. *Annu. Rev. Entomology* 58, 563–583. doi: 10.1146/annurev-ento-120811-153704

Van Teeseling, M. C., Mesman, R. J., Kuru, E., Espaillat, A., Cava, F., Brun, Y. V., et al. (2015). Anammox planctomycetes have a peptidoglycan cell wall. *Nat. Commun.* 6 (1), 1–6. doi: 10.1038/ncomms7878

Vogel, H., Müller, A., Heckel, D., Gutzeit, H., and Vilcinskas, A. (2018). Nutritional immunology: Diversification and diet-dependent expression of antimicrobial peptides in the black soldier fly *Hermetia illucens*. *Dev. Comp. Immunol* 78, 141–148. doi: 10.1016/j.dci.2017.09.008

Wang, Q., Li, Z., Lian, Y., Du, X., Zhang, S., Yuan, J., et al. (2016). Farming system transformation yields significant reduction in nutrient loading: Case study of hongze lake, Yangtze river basin, China. *Aquaculture* 457, 109–117. doi: 10.1016/j.aquaculture.2016.02.025

Xiao, X., Jin, P., Zheng, L., Cai, M., Yu, Z., Yu, J., et al. (2018). Effects of black soldier fly (*Hermetia illucens*) larvae meal protein as a fishmeal replacement on the growth and immune index of yellow catfish (*Pelteobagrus fulvidraco*). *Aquacult. Res.* 49 (4), 1569–1577. doi: 10.1111/are.13611

Yuan, Y., Jin, M., Luo, J., Xiong, J., Ward, T. L., Ji, F., et al. (2019). Effects of different dietary copper sources on the growth and intestinal microbial communities of pacific white shrimp (*Litopenaeus vannamei*). *Aquacult. Nutr.* 25 (4), 828–840. doi: 10.1111/anu.12901

Yuan, J., Zhang, X., Li, F., and Xiang, J. (2021). Genome sequencing and assembly strategies and a comparative analysis of the genomic characteristics in penaeid shrimp species. *Front. Genet.* 12, 658619. doi: 10.3389/fgene.2021.658619

Zhang, S. P., Li, J. F., Wu, X. C., Zhong, W. J., Xian, J. A., et al. (2013). Effects of different dietary lipid level on the growth, survival and immune-relating genes expression in pacific white shrimp, *Litopenaeus vannamei*. *Fish. Shellfish Immunol.* 34 (5), 1131–1138. doi: 10.1016/j.fsi.2013.01.016

Zhou, Z., Karlsen, Ø., He, S., Olsen, R. E., Yao, B., Ringø, E., et al. (2013). The effect of dietary chitin on the autochthonous gut bacteria of a tlantic cod (*Gadus morhua* l.). *Aquacult. Res.* 44 (12), 1889–1900. doi: 10.1111/j.1365-2109.2012.03194.x

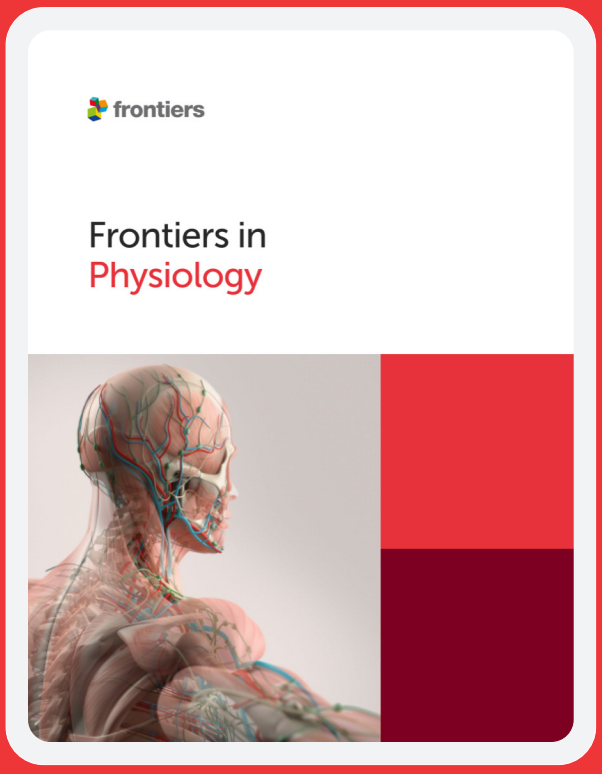
Frontiers in Physiology

Understanding how an organism's components work together to maintain a healthy state

The second most-cited physiology journal, promoting a multidisciplinary approach to the physiology of living systems - from the subcellular and molecular domains to the intact organism and its interaction with the environment.

Discover the latest Research Topics

See more →



Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact