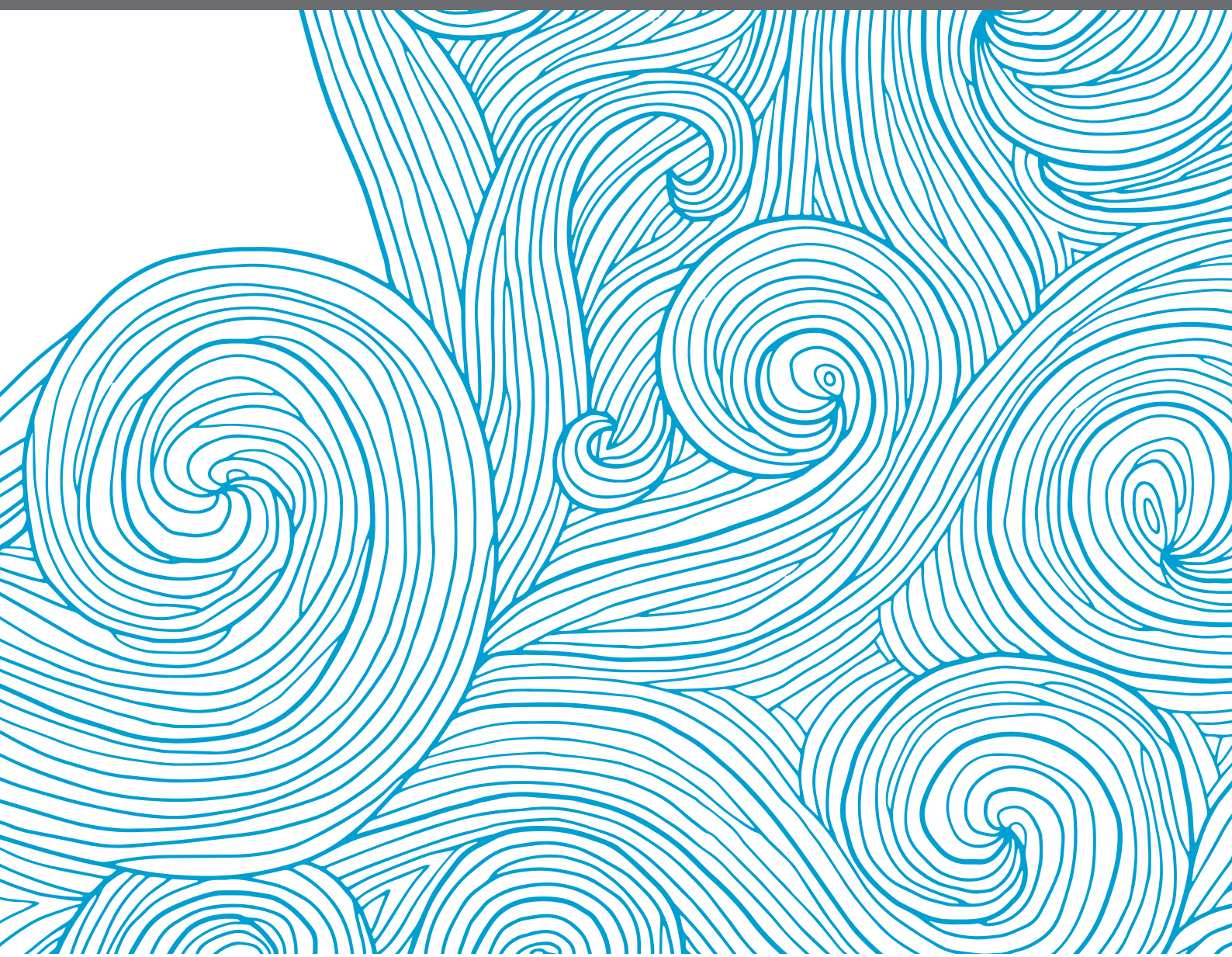
OMICS AND FISH NUTRITION

EDITED BY: Kang-le Lu, Samad Rahimnejad and Songlin Li
PUBLISHED IN: Frontiers in Marine Science



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-83250-352-2
DOI 10.3389/978-2-83250-352-2

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

OMICS AND FISH NUTRITION

Topic Editors:
Kang-le Lu, Jimei University, China
Samad Rahimnejad, University of South Bohemia in České Budějovice, Czechia
Songlin Li, Shanghai Ocean University, China

Citation: Lu, K.-l., Rahimnejad, S., Li, S., eds. (2022). Omics and Fish Nutrition. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83250-352-2

Table of Contents

frontiers | Frontiers in Marine Science

EDITORIAL
published: 25 May 2022
doi: 10.3389/fmars.2022.912884

Editorial: Omics and Fish Nutrition

Kang-le Lu[1], *Samad Rahimnejad*[2] *and Song-lin Li*[3]*

[1] Key Laboratory of Healthy Mariculture for the East China Sea, Ministry of Agriculture and Rural Affairs, P.R.China, Fisheries College, Jimei University, Xiamen, China, [2] South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, České Budějovice, Czechia, [3] College of Fisheries and Life Science, Shanghai Ocean University, Shanghai, China

Keywords: aquafeed, metabolomics, transcriptomics, nutrients, proteomics

Editorial on the Research Topic

Omics and Fish Nutrition

OPEN ACCESS

Edited and reviewed by:
Yngvar Olsen,
Norwegian University of Science and Technology, Norway

***Correspondence:**
Song-lin Li
slli@shou.edu.cn

Specialty section:
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

Received: *05 April 2022*
Accepted: *11 May 2022*
Published: *25 May 2022*

Citation:
Lu K-l, Rahimnejad S and Li S-l (2022) Editorial: Omics and Fish Nutrition. Front. Mar. Sci. 9:912884. doi: 10.3389/fmars.2022.912884

According to a new report published by Food and Agriculture Organization (FAO, 2020), global fish production reached around 179 million tons and aquaculture production accounted for 63.7% of the total production. The rapid expansion of aquaculture industry has been associated with numerous challenges such as shortage of resources for sustainable production of aquafeeds (Zhou et al., 2019; Bruni et al., 2021). For a long time, the focus of traditional fish nutrition studies has been primarily on nutrients requirement and fish metabolism, but nowadays the interaction of nutrients and physiological responses has drawn attention of fish nutritionists. Omics technologies including transcriptomics, proteomics, and metabolomics have made a large contribution to better understanding of fish response to nutrients and dietary manipulations. The omics approaches have been successfully implemented for exploring the molecular basis of complex traits such as feed efficiency, muscle myopathies, immunity, and disease tolerance (Vallejos-Vidal et al., Vallejos-Vidal et al., 2022).

TRANSCRIPTOMICS IN UNDERSTANDING FISH PERFORMANCE

Transcriptomics is often used in identifying the expression of genes in mRNA transcripts in response to different factors. Nutrients can modify gene transcription and translation thereby regulating the animals' growth and reproduction performances. For example, the liver transcriptomic analysis revealed that n-3 LC-PUFA can promote the ovary development through upregulating the expression of vitellogenesis and zonagenesis related genes (Wang et al., 2021). It was earlier assumed that protein requirement of fish cultured under optimal and high temperatures differs, and interestingly the transcriptomic analysis in a recent study indicated that such variations in dietary protein requirement are associated with the metabolic alterations of three major nutrients in the liver (Cai et al., 2020).

PROTEOMICS IN EVALUATING PROTEIN METABOLISM

Proteomic analysis has been employed as an efficient and useful tool in assessment of the metabolism of proteins. For example, it suggested that different forms of lysine and leucine affect

the absorption, synthesis and degradation of protein and ultimately the fish growth (Wei et al.). Moreover, the proteomics suggested that the impairment of growth performance in fish fed terrestrial proteins corresponds with the inhibition of cellular protein biosynthesis (Cao et al.). In the future, proteomics could be implemented as a key tool for understanding the role of specific amino acids in satisfying the physiological needs of fish.

METABOLOMICS PROMOTES NEW INSIGHTS IN FISH NUTRITION

Metabolomics can provide new insight into the alterations of nutrients metabolism by identifying small molecule metabolites. The results obtained from metabolomics can indicate the holistic vision of fish metabolism and health monitoring (Roques et al., 2019). Fish fed plant-based diets showed metabolic disturbances illustrated by lower muscle PUFA concentration. Moreover, the metabolomics data revealed the increased muscle malate, fumarate and glycine concentrations which are associated with energy metabolism (Wei et al., 2017). Also, metabolomic analysis suggested that taurine modulates carbohydrate, amino acids, and lipids metabolic pathways in Nile tilapia (Shen et al., 2018). Furthermore, metabolomics results in a rainbow trout study indicated that fish fed the diets in which fish meal and fish oil were replaced with alternative sources exhibited higher energy requirements (Cao et al.).

Overall, omics provides a global insight into molecular response and metabolism of fish in response to nutritional factors. In the future, it could provide novel methods and specific markers for evaluating the nutritional and health status of fish. It will also contribute to elucidating the hidden effects of specific nutrients or feedstuffs in fish.

AUTHOR CONTRIBUTIONS

K-LL and SR were responsible for the idea of this Research Topic and wrote and reviewed this editorial. S-LL contributed to the review of the editorial. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by National Natural Science Foundation of China (32072984), Natural Science Foundation of Fujian Province (2020J01664) and Shanghai Talent Development Fund (2019097).

REFERENCES

Bruni, L., Secci, G., Husein, Y., Faccenda, F., Lira De Medeiros, A. C., and Parisi, G. (2021). Is It Possible to Cut Down Fishmeal and Soybean Meal Use in Aquafeed Limiting the Negative Effects on Rainbow Trout (*Oncorhynchus Mykiss*) Fillet Quality and Consumer Acceptance? *Aquaculture* 543, 736996. doi: 10.1016/j.aquaculture.2021.736996

Cai, L., Wang, L., Song, K., Lu, K., Zhang, C., and Rahimnejad, S. (2020). Evaluation of Protein Requirement of Spotted Seabass (*Lateolabrax Maculatus*) Under Two Temperatures, and the Liver Transcriptome Response to Thermal Stress. *Aquaculture* 516, 734615. doi: 10.1016/j.aquaculture.2019.734615

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

Roques, S., Deborde, C., Richard, N., Skiba Cassy, S., Moing, A., and Fauconneau, B. (2019). Metabolomics and Fish Nutrition: A Review in the Context of Sustainable Feed Development. *Rev. Aquacult.* 12 (1), 261–282. doi: 10.1111/raq.12316

Shen, G., Huang, Y., Dong, J., Wang, X., Cheng, K. K., Feng, J., et al. (2018). Metabolic Effect of Dietary Taurine Supplementation on Nile Tilapia (*Oreochromis Nilotictus*) Evaluated by NMR-Based Metabolomics. *J. Agr. Food Chem.* 66, 368–377. doi: 10.1021/acs.jafc.7b03182

Vallejos-Vidal, E., Reyes-Cerpa, S., Tort, L., Polo, J., Reyes-López, F. E., and Gisbert, E. (2022). Spray-Dried Porcine Plasma Promotes the Association Between Metabolic and Immunological Processes at Transcriptional Level in Gilthead Sea Bream (*Sparus Aurata*) Gut. *Front. Marine. Sci.* 9. doi: 10.3389/fmars.2022.814233

Wang, T., Jiang, D., Shi, H., Mustapha, U. F., Deng, S., Liu, Z., et al. (2021). Liver Transcriptomic Analysis of the Effects of Dietary Fish Oil Revealed a Regulated Expression Pattern of Genes in Adult Female Spotted Scat (*Scatophagus Argus*). *Front. Marine. Sci.* 8. doi: 10.3389/fmars.2021.784845

Wei, Y., Liang, M., Mai, K., Zheng, K., and Xu, H. (2017). ^{1}h NMR–based Metabolomics Studies on the Effect of Size-Fractionated Fish Protein Hydrolysate, Fish Meal and Plant Protein in Diet for Juvenile Turbot (*Scophthalmus Maximus L.*). *Aquac. Nutr.* 23, 523–536. doi: 10.1111/anu.12420

Zhou, W., Rahimnejad, S., Lu, K., Wang, L., and Liu, W. (2019). Effects of Berberine on Growth, Liver Histology, and Expression of Lipid-Related Genes in Blunt Snout Bream (*Megalobrama Amblycephala*) Fed High-Fat Diets. *Fish. Physiol. Biochem.* 45, 83–91. doi: 10.1007/s10695-018-0536-7

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 in Marine Science

ORIGINAL RESEARCH
published: 13 April 2021
doi: 10.3389/fmars.2021.649747

Influence of Non-protein Diets on Hepatic Metabolism and Endocrine in Barramundi (*Lates calcarifer*)

Zhengyi Fu[1,2,3†], Shengjie Zhou[1,2,3†], Gang Yu[1,2,3], Rui Yang[1,2,3], Mingyang Han[1,2,3] and Zhenhua Ma[1,2,3]*

[1] Tropical Aquaculture Research and Development Center, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Sanya, China, [2] Key Laboratory of South China Sea Fishery Resources Exploitation and Utilization, Ministry of Agriculture and Rural Affairs, Guangzhou, China, [3] Sanya Tropical Fisheries Research Institute, Sanya, China

This study evaluated the effects of different dietary non-protein energy sources on hepatic metabolism and endocrine of barramundi. Fish were fed iso-energy diets (18 MJ kg^{-1}) with two types of non-protein energy source in the experimental group and a regular diet was used as the control. The feeding trial lasted 56 days. In the present study, CPT1 and SCD expression in fish fed high lipid diet were upregulated and liver lipid metabolism was more active than the control group. Serum biochemical parameters including serum glucose, serum triacylglycerol and serum cholesterol were significantly increased. The IGF-I and IGF-II expressions were significantly upregulated, and growth performances were improved in fish fed the high lipid diet. When barramundi were fed with the high carbohydrate diet, GK expression was downregulated and cPEPCK expression was upregulated, indicating that glycogen might accumulate in liver. The fluctuation of serum biochemical parameters and the growth performance were not significantly different compared to the control group. In conclusion, high lipid diet can shorten the raising period, but it causes the change of metabolic level and the increase of useless adipose tissue; The high carbohydrate diet did not significantly improve the growth performance, and no significant metabolic abnormalities were observed, indicating that carbohydrate has the potential to be the feed energy supply source for juvenile barramundi. The results provide insights for further understanding the availability of non-protein energy sources in the diet of juvenile barramundi.

Keywords: ***Lates calcarifer*, non-protein energy sources, growth performance, serum biochemical parameters, metabolism, endocrine**

OPEN ACCESS

Edited by:
Kang-le Lu,
Jimei University, China

Reviewed by:
Shan He,
Huazhong Agricultural University, China
Fotini Kokou,
Wageningen University and Research, Netherlands

***Correspondence:**
Zhenhua Ma
zhenhua.ma@hotmail.com

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

Received: *05 January 2021*
Accepted: *23 March 2021*
Published: *13 April 2021*

Citation:
Fu Z, Zhou S, Yu G, Yang R, Han M and Ma Z (2021) Influence of Non-protein Diets on Hepatic Metabolism and Endocrine in Barramundi (Lates calcarifer). Front. Mar. Sci. 8:649747. doi: 10.3389/fmars.2021.649747

INTRODUCTION

In intensive aquaculture, feed supply is the main component of production costs, and protein is the most expensive ingredient in most fish diets because of the non-renewability of fish meal and the presence of anti-nutrient factors in plant proteins (Muzinic et al., 2006; Cashion et al., 2017). Energy density of feed is generally increased by increasing the protein content, but high protein content is not only increases the cost of raising but also has negative impacts of environment due to potential

nitrogenous losses such as residues and feces (Dosdat, 2001; Lazzari and Baldisserotto, 2008). An increase in the proportion of lipids and carbohydrates added to the feed effectively alleviates this problem, with some additional benefits. Lipids are known to be the major non-protein energy with high energy density in fish feed. Dietary lipids are considerable sources of essential fatty acids for fish, and some researchers have indicated that appropriate dietary lipid content can improve the quality of the end product (NRC, 2011). Carbohydrates are more attractive and potential included in diet because of their abundant availability and cheap (Wilson, 1994). The addition of this nutrient also has a beneficial effect to the pelleting quality of feed (Thomas et al., 1998).

Animals have developed metabolic system accurate and complex enough to accommodate to diversified nutritional states in their natural environment (Buettner et al., 2007; Soengas, 2014; Gyamfi et al., 2019). The changes in the metabolism are a tool that reflect the adaptability to different dietary conditions (Moraes and Bidinotto, 2004; Bibiano Melo et al., 2006). To elucidate the reasons for the variation in metabolism, the understanding of the change in the key enzymes involved in each metabolic pathway is essential. Previous studies have shown that feed nutrition would cause changes in hepatic function by regulating lipid metabolism, thereby changing cell function (Li et al., 2016; Meng et al., 2018). High dietary carbohydrate: lipid ratios (>3.41) by increases *de novo* lipogenesis and reduces FA β-oxidation and lipoprotein clearance in the liver, thereby accelerating the liver lipid deposition of juvenile tilapia, *Oreochromis niloticus* (Xie et al., 2017). To maintain the optimum health condition in carnivorous fish, it is necessary to understand the adaptative strategy of energy metabolism and metabolic feedback in the feeding practice.

As an aquatic species widely cultivated in Australia and Asian countries, barramundi (*Lates calcarifer*) has the characteristics of fast growth, easy propagation and domestication (Liu et al., 2018; Ma et al., 2018, 2019). Barramundi also has a long history of breeding and research, as well as extensive studies on its lipid and carbohydrate requirements. There are two kinds of common feed formulation for barramundi in Australia that are implemented by adjusting the protein and lipid levels, one is low energy density (15 MJ/k digestible energy, ∼ 46% digestible protein, 12% lipid) and the other is medium energy density (18 MJ/k digestible energy, ∼ 40% digestible protein, 20% lipid) (Glencross, 2006, 2008). Studies have tried to raise carbohydrate levels to save feed costs for barramundi. A recommended protein level is 42.5%, lipid level is 6–12%, and the carbohydrate level is raised to 20% of the feed, and this will not significantly affect the growth of barramundi (Catacutan and Coloso, 1997). However, little investigation has been paid to the metabolism of this species, and the adaptive strategies to non-protein energy sources intake have not been well studied. In the present study, fish oil and α-starch were used as the potential energy source to evaluate the endocrine status and hepatic metabolism of barramundi to dietary lipids and carbohydrate. Results from the present study could pave the way to further understand on energy metabolism of barramundi and provide a basis for optimizing feed formula of barramundi with a cheaper dietary source.

MATERIALS AND METHODS

Experimental Diets

Three iso-energy diets (**Table 1**) referenced from previous study (Fu et al., 2019), the control group (C) used the diet formula according to crude protein 475 g kg^{-1}, crude lipid 100 g kg^{-1}, and nitrogen-free extract 180 g kg^{-1} (Glencross et al., 2017). In the experimental group, fish oil and α-starch were used as the source of lipid and carbohydrate, respectively (NRC, 2011). The crude lipid in the high lipid group (HL) increased to 160 g kg^{-1}, and the nitrogen-free extract in the high carbohydrates group (HC) increased to 220 g kg^{-1}. The preparation and preservation methods of the feed were also referred to in previous study (Fu et al., 2019).

Experiment Design and System

The same batch of Juvenile barramundi (33.69 ± 3.63 g, 12.08 ± 2.13 cm) were obtained from Tropical Aquaculture Research and Development Center, Sanya, China, and grown in an indoor seawater recirculating system being fed a commercial feed (Santong Bio-engineering Co., Ltd., Weifang, China). Fish were grouped and weaned according to previous study (Fu et al., 2019). A total of 117 fish were randomly divided to 9

TABLE 1 | Feed composition (g kg^{-1}), proximate composition of the diets (air-dry basis g kg^{-1}) and gross energy content (MJ kg^{-1}) in three experimental diets (Fu et al., 2019).

Ingredients	C	HL	HC
Fish meal	560	560	560
Peeled soybean meal	236	176	186
α-starch	100	100	150
Fish oil	60	120	60
Choline chloride	2	2	2
Vitamin premix[a]	10	10	10
Mineral premix[b]	10	10	10
Yttrium oxide	2	2	2
Calcium dihydrogen phosphate	10	10	10
Ethoxy quin	10	10	10
Total	1,000	1,000	1,000
Proximate composition			
Dry matter	929.9	914.1	932
Crude protein	474.9	445.7	451.4
Lipid	99.9	153.1	94
Ash	173.6	167	167.2
Nitrogen-free extract	179.99	145.04	218.10
Gross energy(MJ kg^{-1})	18.26	19.08	18.13

[a]*Vitamin premix (mg kg^{-1} diet or specified): vitamin A 900 0000 (IU kg^{-1} diet),vitamin D 250 0000 (IU kg^{-1} diet),vitamin K3 600 (IU kg^{-1} diet),vitamin E 500 (IU kg^{-1} diet),vitamin B1 3200,vitamin B2 1 0900,vitamin B5 2 0000,vitamin B6 5000,vitamin B12 1160,vitamin C 5 0000,niacin 400,folic acid 50,calcium pantothenate 200,phaseomannite 1500,biotin 2.*

[b]*Mineral premix(mg kg^{-1} diet or specified): MgSO4·7H2O 300; KCl 70; KI 1.5; ZnSO4·7H2O 14; MnSO4·4H2O 3; CuCl2 5; CoCl2·6H2O 0.5; FeSO4·7H2O 15; KH2 PO4·H2O 4.5 (g kg^{-1} diet); CaCl2 2.8 (g kg^{-1} diet). The dietary energy was calculated as protein: 23. 64 MJ·kg^{-1},lipid: 39. 54 MJ·kg^{-1}·carbohydrate: 17. 15 MJ·kg^{-1}. C, Control; HL, High lipid; HC, High carbohydrate.*

experimental tanks (fiber glass, 800 L) for a 12 day acclimation. Each experimental tank contained 13 fish and experimental feed was randomly assigned to the experimental tank. The experiment lasted 8 weeks. The experiment was conducted in outdoor seawater tanks with filtered natural seawater. Fish were fed *ad libitum* twice a day at 08:00 and 16:00 h until apparent satiation on the basis of visual observation, and the feed consumed per tank were recorded. One hour after fed, the residual feeds and feces were sucked away. During the experimental period, the water quality parameters were measured daily and maintained at dissolved oxygen > 7.0 mg L^{-1}, ammonia nitrogen < 0.1 mg L^{-1}, nitrite nitrogen < 0.02 mg L^{-1}, salinity $33 \pm 1‰$ and pH 7.8. The fish were reared and fed the diets under natural daylight cycle.

Sampling

At the time of the experiment ending, all fish were anaesthetized in 7 mg L^{-1} eugenol (Shangchi Dental Material Co., Ltd., Changshu, China), weighed to estimate growth performance and three fish were sampled from each tank (i.e., nine fish per treatment) for somatic parameters, biochemical and gene expression analyses. Blood was withdrawn from the caudal vein by a sterile injector (1 mL), allowed to stand at room temperature for 4 h, centrifuged at 3,500 × g for 10 min at 4°C, and the supernatant was collected and then stored at −80°C for further analysis. Thereafter, whole body, viscera, liver and intraperitoneal fat were weighed for determination of viscera ratio (VR), hepatosomatic index (HSI), intraperitoneal fat ratio (IPF). Liver tissues were snap-frozen in liquid nitrogen, then preserved at −80°C until use.

Proximate Composition Analysis

The proximal compositions of the diet, fish body and muscle samples were determined based on previous study (Glencross et al., 2017; Ma et al., 2019). Moisture was determined by oven dry to a constant weight at 103°C in an air-blower-driven drying closet (Nocchi Instrument Co., Ltd., China). Crude protein content (N × 6.25) was determined using a rapid N exceed (Elementar Co., Ltd., Germany). Ash (% dry weight) was quantified by combustion at 550°C in a muffle furnace (Laboratory Instrument Co., Ltd., China). Crude lipid (% dry weight) was determined by ether extraction using a Soxtec System (Zhejiang Tuopu Instrument Co., Ltd., China).

Serum Biochemical Parameters Analysis

The serum biochemical parameters were determined according to the manufacturer's instructions using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) i.e., glucose(GLU): Glucose Oxsidase—Peroxidase Aminoantypirin method; triglycerides(TG): Phosphate oxidase—Peroxidase Aminoantypirin method; total cholesterol(TC): Cholesterol Oxidase—Peroxsidase Aminoantypirin method; high-density lipoprotein cholesterol(HDL-C) and low-density lipoprotein cholesterol (LDL-C): direct method. All serum biochemical parameters analyses were performed in triplicate.

Gene Expression Analysis

Methods for RNA extraction and cDNA synthesis experiments were refined based on the methods published at Cold Spring Harbor Laboratory (Green and Sambrook, 2012). Total RNA was extracted using TRIzol (Invitrogen, United States). RNA integrity and concentration were verified by agarose gel electrophoresis and spectrophotometry (Bioteke Corporation Co., Ltd., China). The RNA was immediately used for cDNA synthesis. Subsequently, reverse transcription was performed on 1 μg of total RNA using TransScript-Uni One-Step gDNA Removal and cDNA Synthesis SuperMix (Transgen Biotech Co., Ltd., China) following the manufacturer's instructions. The synthesized cDNA samples were stored at −20°C until further use.

The genes chosen for analysis by qPCR were selected from the *L. calcarifer* NCBI database[1]. The sequences of specific primer pairs and genes accession numbers are shown in **Table 2**. The specific primers of ELOVL fatty acid elongase 5 (ELOVL5), fatty acid desaturase 2 (FADS2), cytosolic phosphoenolpyruvate carboxykinase (cPEPCK), insulin like growth factor 1 (IGF-I), insulin like growth factor 2 (IGF-II) and actin beta (β-actin) were designed using the Primer Premier 5 program. The primers of stearoyl CoA deasaturase (SCD), carnitine palmitoyl transferase 1 (CPT1), ATP-citrate synthase (ACLY) and glucokinase (GK) were previously designed and validated

[1]https://www.ncbi.nlm.nih.gov/

TABLE 2 | Primers of SCD, CPT1, ELOVL5, FADS2, ACLY, GCK, cPEPCK, IGF-I, IGF-II, and β-actin genes in barramundi used in qPCR.

Gene abbreviation	Primer sequence (5′–3′)	Amplicon size (bp)	Accession number
Metabolism genes			
SCD	F: CCTGGTACTTCTGGGGTGAA	217	XM_018696772.1
	R: AAGGGGAATGTGTGGTGGTA		
CPT1	F: TGATGGTTATGGGGTGTCCT	186	XM_018678316.1
	R: CGGCTCTCTTCAACTTTGCT		
ELOVL5	F: ATCCAGTTCTTCTTAACCGT	277	GQ214180.1
	R: GGTTTCTCAAATGTCAATCCAC		
FADS2	F: TCATACTACCTTCGCTACTTCTC	104	GU047383.2
	R: ACAAACCAGTGACTCTCCAG		
ACLY	F: CAACACCATTGTCTGTGCTC	271	XM_018703332.1
	R: GAAATGCTGCTTAACAAAGTCC		
GK	F: CTGGTGTGATCAACCGAATG	179	XM_018673057.1
	R: ACCACTCCCCTCCTCTGACT		
cPEPCK	F: TCCGCCATCGGCTACCT	119	XM_018678244.1
	R: TCCTCCACCTCCCTTTGC		
Growth hormone genes			
IGF-I	F: TGTGGACGAGTGCTGCTT	144	EU136176.1
	R: TGCCCTGCGGTACTAACCT		
IGF-II	F: AGTATTCCAAATACGAGGTGTG	131	XM_018664155.1
	R: GAAGATAACCTGCTCCTGTG		
Control gene			
β-actin	F: AACCAAACGCCCAACAACT	112	XM_018667666.1
	R: ATAACTGAAGCCATGCCAATG		

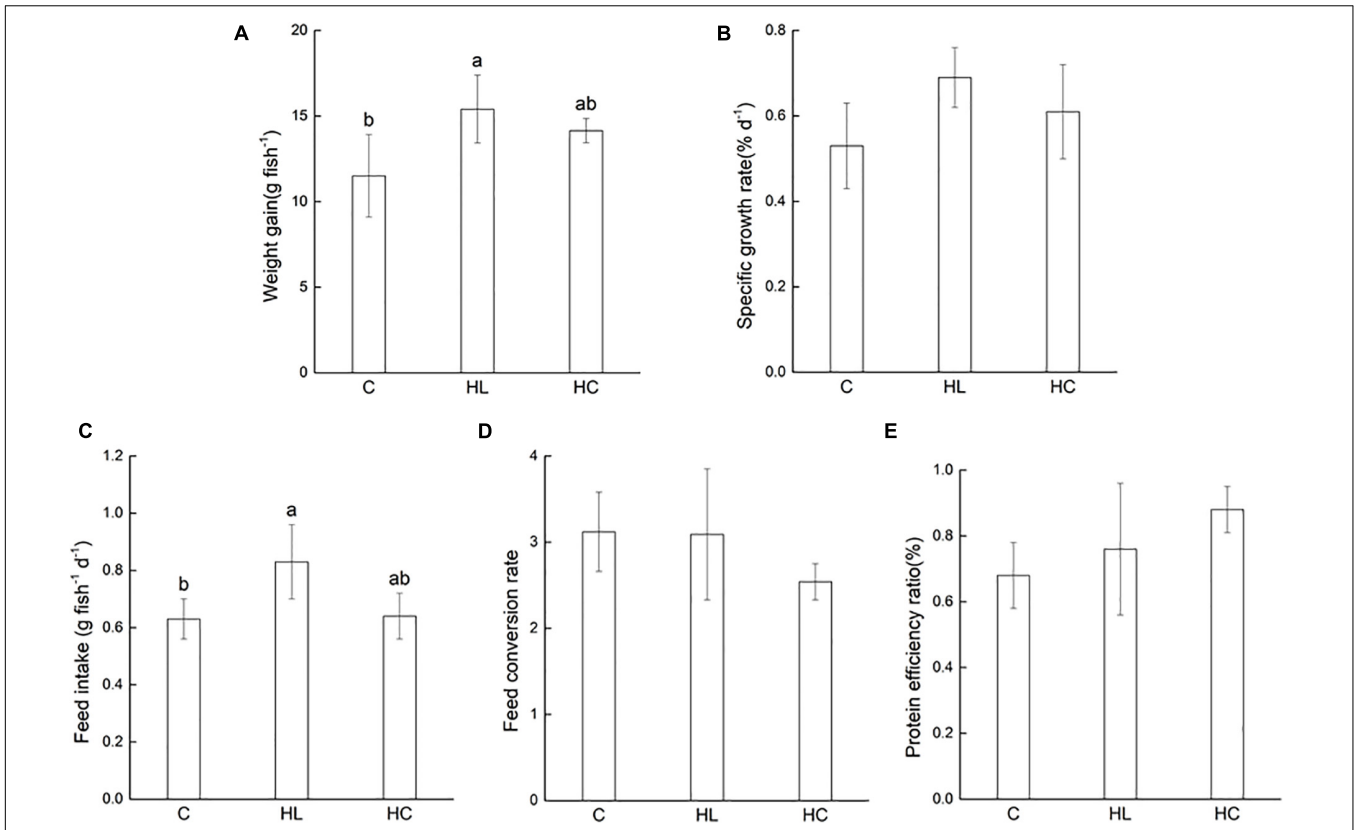


FIGURE 1 | Effects of three experimental diets on growth performance (**(A)** Weight gain, **(B)** Specific grown rate, **(C)** Feed intake, **(D)** Feed conversion rate, **(E)** Protein efficiency ratio) of barramundi fed different experimental diets (C-Control, HL-High lipid, HC-High carbohydrate). Different superscript letters indicate significant differences among treatments ($P < 0.05$). Error bars represent standard error.

by Wade et al. (2014). The qPCR was amplified in a Real-time qPCR machine (Hangzhou Longgene Scientific Instrument Co., Ltd., China) using SYBR Green (Tiangen Biotech Co., Ltd., China) following the manufacturer's recommendations. Before the Real-time qPCR experiments, the specificity and efficiency of the primes above were detected. Each primer product was validated by melt-curve analysis to ensure only specific products were obtained with no formation of primer dimers. Constructing standard curves using serial dilution of cDNA. After verification of PCR efficiency to be around 100%, and Pearson's coefficients of determination (R^2) > 0.98. The 20 μl of reaction including 10 μl 2 × RealUniversal PreMix, 0.6 μl of each primer (10 μM) and 2 μl of diluted cDNA was initially denatured at 95°C for 15 min and then amplified for 40 cycles (95°C, 10 s, 58°C, 20 s, and 72°C, 30 s). Each assay was repeated three times in this study. No template control (NTC) was included with each assay to verify that PCR master mixes were free of contamination. The $^{\Delta\Delta}C_t$ method was used to calculate the relative expression with β-actin as a reference gene, and normalized as a control (Green and Sambrook, 2012).

Calculations and Statistical Analysis

Specific growth rate (SGR), Feed conversion ratio (FCR), Feed intake (FI), Protein efficiency ratio (PER), Condition factor (CF), Viscera ratio (VR), Hepatosomatic index (HIS) and Intraperitoneal fat ratio (IPF) were calculated by referring to the method of Fu and Ma (Fu et al., 2019; Ma et al., 2019).

The data were presented as the mean ± standard deviation (SD). Statistical analyses were carried out by PASW Statistics (version 18). Assumptions of homogeneity of variances were checked using Levene's equal variance test. The dissimilarities between different groups were conducted by one-way ANOVA and LSD test, and the level of significance was set at $P < 0.05$. All percentage data were transformed using square root to satisfy the assumptions of ANOVA.

RESULTS

Growth Performance

The fish growth performance was presented in **Figure 1**. The SGR, FCR and PER were not significantly different among the three groups of *Lates calcarifer*. Only WG and FI showed significant difference ($P < 0.05$) and the trend was similar, among which the HL and C treatments showed significant difference ($P < 0.05$), but HC showed no significant difference with the other two groups ($P > 0.05$).

Proximate Composition and Somatic Parameters

In order to better explain the experimental results in conjunction with other data, data on Proximate composition and somatic parameters were referenced from previous study (Fu et al., 2019). There was no significant difference in moisture content among the three groups ($P > 0.05$, **Table 3**). The crude protein in the whole body and muscle of the HL group was significantly lower than that of the other two groups ($P < 0.05$), and the crude fat was significantly higher than that of the other two groups ($P < 0.05$). Nevertheless, the consistency between the whole body and the muscle was not reflected in the crude ash content, and the crude ash content in the HL group was significantly lower than that in the other two groups ($P < 0.05$). In the muscle, the coarse ash content was C > HL > HC, and there was a significant difference between the C and HL groups ($P < 0.05$). There was no significant difference in the condition factor (CF) and the viscera ratio (VR) in all groups ($P > 0.05$, **Table 3**). The HL was the highest group in the hepatosomatic index (HSI) and intraperitoneal fat ratio (IPF). Meanwhile, the HSI was significantly different between the HL and HC groups, and the IPF in the HL group was significantly different from that in the other two groups ($P < 0.05$).

Serum Biochemical Parameters Analysis

GLU, TG, TC, HDL-C, and LDL-C were significantly affected by experimental diets ($P < 0.05$, **Table 4**), and the highest value was found in the HL group.

TABLE 3 | Effects of three experimental diets on body composition and somatic parameters in barramundi [Partly referred to Fu et al. (2019)].

Items	Experimental diets		
	C	HL	HC
Whole body composition			
Moisture contect%	70.41 ± 0.75	67.94 ± 1.03	68.81 ± 2.06
Crude protein(N% × 6.25)	63.19 ± 0.30^{a}	54.27 ± 1.25^{c}	61.30 ± 0.19^{b}
Ash content% DW	18.17 ± 0.24^{a}	16.92 ± 0.21^{b}	18.31 ± 0.13^{a}
Crude lipid% DW	18.13 ± 0.44^{c}	26.89 ± 0.39^{a}	20.11 ± 0.53^{b}
Muscle composition			
Moisture contect%	78.54 ± 1.02	78.08 ± 2.98	77.80 ± 0.39
Crude protein(N% × 6.25)	90.07 ± 1.03^{a}	88.04 ± 0.75^{b}	90.34 ± 0.73^{a}
Ash content% DW	5.84 ± 0.08^{a}	5.77 ± 0.08^{ab}	5.66 ± 0.06^{b}
Crude lipid% DW	2.19 ± 0.11^{b}	3.81 ± 0.19^{a}	2.03 ± 0.10^{b}
Somatic parameters			
CF (g cm^{-3})	3.42 ± 0.05	3.62 ± 0.04	3.39 ± 0.47
VR (%)	8.28 ± 0.47	9.46 ± 0.66	8.04 ± 2.22
HIS (%)	1.47 ± 0.27^{ab}	1.96 ± 0.27^{a}	1.36 ± 0.25^{b}
IPF (%)	1.91 ± 0.24^{b}	3.84 ± 0.59^{a}	1.48 ± 0.32^{b}

Data are given as the mean ± SD. In the same row, values with same small letter superscripts or no letter superscripts mean no significant difference ($P > 0.05$); different small letter superscripts mean significant difference ($P < 0.05$).
CF, Condition factor; VR, Viscera ratio; HSI, Hepatosomatic index; IPF, Intraperitoneal fat ratio.

Gene Expression of Liver

In fatty acid metabolism genes, the SCD, CPT1, fasd2, and ACLY expressions in the HC group were significantly upregulated ($P < 0.05$), while the ELOVL5 expression showed no significant difference among treatments ($P > 0.05$, **Figure 2A**). In glucose metabolism genes, the acyl and cPEPCK expressions in the HC group were significantly upregulated ($P < 0.05$), and the GK expression in the HL and HC groups were significantly downregulated ($P < 0.05$, **Figure 2B**). In growth hormone genes, the IGF-I and IGF-II expression levels were similar in each treatment. The expressions of IGF-I and IGF-II were significantly upregulated in the HL group, while those were significantly downregulated in the HC group ($P < 0.05$, **Figure 2C**).

DISCUSSION

In the present study, the adaptive mechanisms of barramundi to high lipid (160 g kg^{-1}) or high carbohydrate (220 g kg^{-1}) diets were evaluated to gain a better understanding on the metabolic strategy of non-protein energy sources in barramundi. There was found that different non-protein energy sources had effects on growth performance, food intake, somatic proximate composition and serum biochemical parameters. According to the hepatic expression level of metabolic genes, barramundi had different strategies for different non-protein energy sources.

The Response of Barramundi to High Lipid Diet

In fish, dietary lipids are an important source of essential fatty acids (FA) for regular growth, body functions, health and reproduction (Turchini et al., 2009). In this study, the whole body lipid content increased in the HL group. Consistently, the concentration of serum triglyceride and glucose also increased, indicating an active endogenous lipid transport (Chatzifotis et al., 2010), and the oxidative decomposition of lipid consumes a large amount of the intermediate product of glycolysis (a-glycerophosphate), which blocks glycolysis and makes the serum glucose increase (Tzur et al., 1964). In addition, the increase in

TABLE 4 | Effects of three experimental diets on Serum biochemical parameters in barramundi.

Items	Experimental diets		
	C	HL	HC
GLU (mmol/L)	11.20 ± 1.74^{b}	14.41 ± 0.6^{a}	9.88 ± 0.53^{b}
TG (mmol/L)	1.44 ± 0.01^{b}	1.72 ± 0.08^{a}	1.33 ± 0.03^{c}
TC (mmol/L)	5.99 ± 0.43^{ab}	6.43 ± 0.53^{a}	5.14 ± 0.40^{b}
HDL-C (mmol/L)	2.68 ± 0.18^{ab}	2.98 ± 0.21^{a}	2.51 ± 0.80^{b}
LDL-C (mmol/L)	2.16 ± 0.23^{ab}	3.03 ± 0.31^{a}	1.85 ± 0.06^{b}

Data are given as the mean ± SD. In the same row, values with same small letter superscripts or no letter superscripts mean no significant differences ($P > 0.05$); different small letter superscripts mean significant differences ($P < 0.05$).
C, Control; HL, High lipid; HC, High carbohydrate. GLU, glucose; TG, triacylglycerol; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

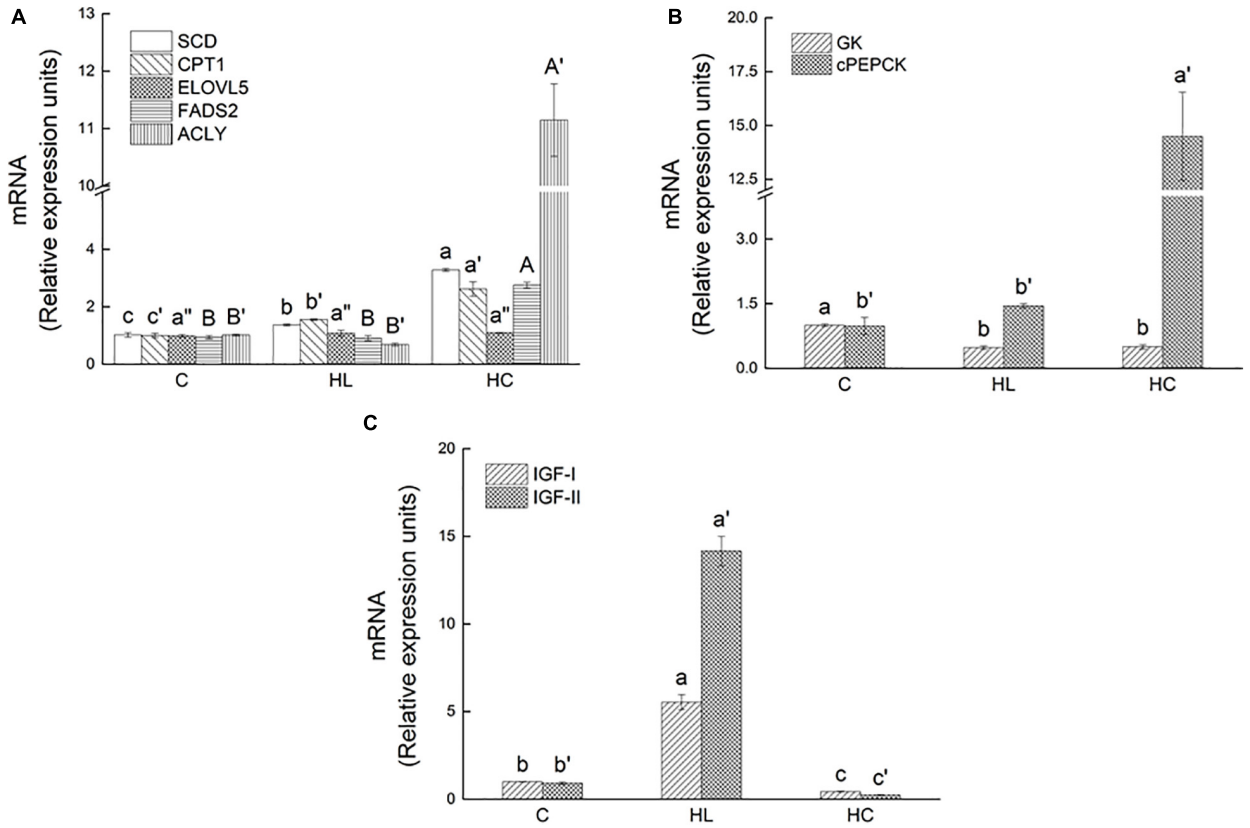


FIGURE 2 | Relative expression of lipid metabolism genes (**(A)** SCD, CPT1, ELOVL5, FADS2), carbohydrate metabolism genes (**(B)** ACLY, GK, cPEPCK) and Growth genes (**(C)** IGF-I, and IGF-II) in barramundi fed different experimental diets (C-Control, HL-High lipid, HC-High carbohydrate). Different superscript letters indicate significant differences among treatments ($P < 0.05$). Error bars represent standard error. (put the figures in a single column of three).

dietary lipid content leads to an increase in dietary energy density, which will induce fish to control energy intake by reducing food intake (Nankervis et al., 2000), but the result is just the opposite in the present study. We found that the enthusiasm of barramundi fed the high lipid diet was much higher than that of the other two groups. It can be speculated that the increase in food intake of the high lipid group may be due to the attraction of fish oil. It could also be that the high lipid diet was softer and palatable. The increase in feed intake directly leads to the increase in weight. Excessive dietary lipid intake induces to excessive fat deposition in muscle, liver and visceral cavity in fish (Martino et al., 2002; Alves Martins et al., 2007). In this study, the increase in weight includes two parts: effective weight gain and ineffective weight gain. Ineffective weight gain mainly refers to the accumulation of abdominal adipose tissue. It is calculated that the effective weight gain accounts for about a quarter of the total weight gain, which still leaves most of the energy devoted to the effective weight gain. Although the results of high lipid group showed metabolic abnormalities, they still gave us some enlightenment that adding appropriate amount of lipids in the diet could improve the feeding enthusiasm and growth performance of barramundi.

The result of certain important hepatic lipometabolic gene expressions showed that ELOVL5, FADS2, and ACLY had no significant difference compared with the control group, and CPT1 and SCD were significantly upregulated in the present study. CPT1 participates in the key step of mitochondrial fatty acid oxidation and can catalyze the transformation of fatty acyl- CoAs into fatty acid carnitines into the mitochondrial matrix (Kerner and Hoppel, 2000; Laura et al., 2018). SCD plays a significant part in the synthesis of unsaturated fatty acids (Flowers and Ntambi, 2008; Ardiyanti et al., 2012). The synthetic product contains oleate, it indispensable to the biosynthesis of triglycerides and other lipids (Hsieh et al., 2004; Gonzalez-Baró et al., 2007). Similar trends in the expression of the related genes of lipid metabolism were found in both grass carp (*Ctenopharyngodon idellus*) and tilapia (*Oreochromis niloticus*) (Li et al., 2016; Xie et al., 2017). In the present study, GK expression was significantly downregulated. The glucose phosphorylation reaction catalyzed by liver GK can provide the substrate for glycolysis, glycogenesis and the pentose phosphate pathway. It is very important for maintaining blood glucose homeostasis (Engelking, 2010). In mammals, fish oil in the diet would reduce the expression of GK at both the enzyme and mRNA levels. In fact, the principle is that long-chain polyunsaturated fatty acids (PUFA) in fish oil can inhibit hepatic GK gene expression (Jump et al., 1994; Jump and Clarke, 1999). Whether the cause of GK

expression reduction in barramundi is similar to that in mammals remains to further investigation. The intrahepatic adaptations of barramundi in response to high lipid intake were to increase SCD, induce CPT1 and simultaneously reduce GK to cope with excessive lipid intake.

Insulin like growth factors (IGFs) are mitogenic peptides, and they are generally believed that their expression are regulated by Endogenous (nutritional state and humoral factors) and exogenous (temperature and photoperiod) factors (Duan, 1998; Tatar et al., 2003). The process of nutrition regulation of IGFs is firstly caused by the hypothalamus integrated nutritional status clues, which leads to the release of stimulant (growth hormone-releasing hormone) or inhibitor (somatostatins) signals to the anterior pituitary gland to produce somatotrophs, which then enters the blood circulation and binds to hepatic GH receptors to stimulate the compound of IGFs (Picha et al., 2008). Several studies have suggested that IGFs have a significant role in nutrient metabolism, growth and development of fish and can be judged by the liver mRNA expression (Rolland et al., 2015). In previous studies, expression of the IGF-I and IGF-II genes were proved to be positively correlated with growth performance in channel catfish (*Ictalurus punctatus*) (Peterson et al., 2005), clownfish (*Amphiprion ocellaris*) (Avella et al., 2009), and golden pompano (*Trachinotus ovatus*) (Tan et al., 2017). In this study, IGF-I and IGF-II in the HL group were significantly upregulated, and the gene expression was consistent with the growth performance (WG and SGR). The increase in growth performance seems to be related to the IPF. This association can also be explained by the upregulation of IGF-I, which also promotes the proliferation and differentiation of adipose tissue (Chang et al., 2016). Therefore, dietary lipids promote IGFs secretion, and thus have a positive impact on growth performance. Similar results were found in rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (*Oncorhynchus niloticus*) (Vera Cruz et al., 2006; Meng et al., 2019).

The Response of Barramundi to High Carbohydrate Intake

The effect of high carbohydrate diet on fat accumulation in barramundi was not as significant as that of high-fat diet, suggesting that energy may be stored in other forms, such as glycogen (Hemre et al., 2002). There was no significant change in blood glucose, indicating that barramundi could adapt to the high-carbohydrate diet used in the study, and the serum TG, TC, LDL-C, and HDL-C were all reduced in various degrees, indicating that the intake of high-carbohydrate diet inhibited the utilization and transport of lipids.

The mRNA expression of metabolic key enzyme was significantly regulated by the high carbohydrate diet, and the expression of key enzyme genes for lipid synthesis (Fasd2, ACLY, and SCD) and decomposition (CPT1) was upregulated. Particularly, SCD and CPT1 were also higher than those in the HL group. Dietary carbohydrate administration also promote lipogenesis (Kamalam et al., 2012). There are two routes to excess glucose after carbohydrate feeding, one is amassed in the form of glycogen, or converted to triglycerides through lipogenesis (Skiba-Cassy et al., 2009; Polakof et al., 2012). The increased transcripts of liver lipid metabolism gene induced by dietary carbohydrate were found in gilthead sea bream *Sparus aurata* and rainbow trout *Oncorhynchus mykiss* (Castro et al., 2016; Song et al., 2018). Although there is no direct link between lipogenesis and glucose metabolism, this pathway possibly play an important part in glucose homeostasis because excessive glucose is converted to fatty acids. GK expression was downregulated and PEPCK expression was upregulated. This indicates that the extra carbohydrate can be converted into glycogen in barramundi fed the high carbohydrate diet. In some species such as carp *Cyprinus carpio*, rainbow trout *Oncorhynchus mykiss* and perch *Perca fluviatilis* and, the hepatic GK activity rises with dietary carbohydrate (Borrebaek et al., 2003; Capilla et al., 2003; Encarnación et al., 2004), but our findings do not support these findings. As a carnivorous fish, barramundi are insensitive to dietary carbohydrates, and the regulation of GK expression level remains to be further studied. Thus, the intrahepatic adaptation of barramundi in response to high carbohydrate intake were induces glycogen accumulation (GK and cPEPCK), enhance lipid synthesis (fasd2, ACLY, and SCD) and induce mitochondrial FA oxidative (CPT1) to activate lipid metabolism.

In the expression of growth hormone gene, IGF-I and IGF-II were downregulated, but there was no significant difference in the growth performance compared with the control group. The possible explanation is that the downregulation is not strong enough to affect growth or other factors like insulin, steroids and thyroid hormones, which allow the fine control of growth and adaptation to endogenous and external changes (Mommsen and Moon, 2001). The other hypothesis is that the increase of carbohydrate through α-starch in high carbohydrate diets leads to a lower FI due to its poor palatability. This hypothesis, however, is in contrast to the attractivity of high lipid diets to fish oil which lead to a direct response of endocrine and growth changes (Picha et al., 2008). Therefore, further research on feed intake as well as the role of other hormones associated with growth regulation is required in future.

CONCLUSION

This study demonstrates that when barramundi were fed with a high lipid diet, liver lipid metabolism was more active than in the control group. Serum biochemical parameters were significantly increased, and muscle and intraperitoneal fat were significantly accumulated, leading to improvement of growth performance. When barramundi were fed with a high carbohydrate diet, glycogen in the liver has the tendency of accumulation. The fluctuation of serum biochemical parameters was not obvious, and the accumulation of muscle and intraperitoneal fat were also not clearly evidenced. The growth performance in the HC and HL treatments was not significantly different from that of the control group. The intake of high lipid and high carbohydrate diets caused significant changes in metabolism, revealing the

mechanism of the response to non-protein energy sources of juvenile barramundi.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

ZM and GY: conceptualization. SZ: experimental operation. ZF, SZ and MH: field sampling. ZF and RY: sample determination. ZF: writing—original draft preparation. ZM: writing—review and editing. All authors read and approved the final manuscript.

FUNDING

This study was supported by the Central Public-interest Scientific Institution Basal Research Fund (CAFS No. 2020TD55), Guangxi Innovation Driven Development Special Fund Project (Grant No. Guike AA18242031), Central Public-interest Scientific Institution Basal Research Fund, South China Sea Fisheries Research Institute (Grant No. CAFS-2018ZD01).

REFERENCES

Alves Martins, D., Afonso, L. O. B., Hosoya, S., Lewis-McCrea, L. M., Valente, L. M. P., and Lall, S. P. (2007). Effects of moderately oxidized dietary lipid and the role of vitamin E on the stress response in *Atlantic halibut* (*Hippoglossus hippoglossus* L.). *Aquaculture* 272, 573–580. doi: 10.1016/j.aquaculture.2007.08.044

Ardiyanti, A., Abe, T., Tameoka, N., Kobayashi, E., Shoji, N., Ohtani, Y., et al. (2012). Effects of growth hormone gene polymorphism on lipogenic gene expression levels in diaphragm tissues of japanese black heifers. *Asian Australas J. Anim. Sci.* 25, 1055–1062. doi: 10.5713/ajas.2012.12029

Avella, M. A., Olivotto, I., Silvi, S., Place, A. R., and Carnevali, O. (2009). Effect of dietary probiotics on clownfish: a molecular approach to define how lactic acid bacteria modulate development in a marine fish. *Am. J. Physiol.Regulatory Integr. Compar. Physiol.* 298, R359–R371.

Bibiano Melo, J. F., Lundstedt, L. M., Metón, I., Baanante, I. V., and Moraes, G. (2006). Effects of dietary levels of protein on nitrogenous metabolism of Rhamdia quelen (*Teleostei*: *Pimelodidae*). *Compar. Biochem. Physiol. A Mol. Integr. Physiol.* 145, 181–187. doi: 10.1016/j.cbpa.2006.06.007

Borrebaek, B., Christophersen, B., and Sundby, A. (2003). Metabolic function of hepatic hexokinase in perch, *Perca fluviatilis*. *Aquac. Res.* 34, 235–239. doi: 10.1046/j.1365-2109.2003.00809.x

Buettner, R., Schölmerich, J., and Bollheimer, L. C. (2007). High-fat diets: modeling the metabolic disorders of human obesity in rodents. *Obesity* 15, 798–808. doi: 10.1038/oby.2007.608

Capilla, E., Médale, F., Navarro, I., Panserat, S., Vachot, C., Kaushik, S., et al. (2003). Muscle insulin binding and plasma levels in relation to liver glucokinase activity, glucose metabolism and dietary carbohydrates in rainbow trout. *Regulatory Peptides* 110, 123–132. doi: 10.1016/s0167-0115(02)00212-4

Cashion, T., Le Manach, F., Zeller, D., and Pauly, D. (2017). Most fish destined for fishmeal production are food-grade fish. *Fish Fish.* 18, 837–844. doi: 10.1111/faf.12209

Castro, C., Corraze, G., Firmino-Diógenes, A., Larroquet, L., Panserat, S., and Oliva-Teles, A. (2016). Regulation of glucose and lipid metabolism by dietary carbohydrate levels and lipid sources in gilthead sea bream juveniles. *Brit. J. Nutr.* 116, 19–34. doi: 10.1017/s000711451600163x

Catacutan, M. R., and Coloso, R. M. (1997). Growth of juvenile Asian seabass, *Lates calcarifer*, fed varying carbohydrate and lipid levels. *Aquaculture* 149, 137–144. doi: 10.1016/s0044-8486(96)01432-9

Chang, H. R., Kim, H. J., Xu, X., and Ferrante, A. W. (2016). Macrophage and adipocyte IGF1 maintain adipose tissue homeostasis during metabolic stresses. *Obesity* 24, 172–183. doi: 10.1002/oby.21354

Chatzifotis, S., Panagiotidou, M., Papaioannou, N., Pavlidis, M., Nengas, I., and Mylonas, C. C. (2010). Effect of dietary lipid levels on growth, feed utilization, body composition and serum metabolites of meagre (*Argyrosomus regius*) juveniles. *Aquaculture* 307, 65–70. doi: 10.1016/j.aquaculture.2010.07.002

Dosdat, A. (2001). Environmental impact of aquaculture in the Mediterranean: nutritional and feeding aspects. *Cah Options Mediterr.* 55, 23–36.

Duan, C. (1998). Nutritional and developmental regulation of Insulin-like growth factors in fish. *J. Nutr.* 128, 306S–314S.

Encarnación, C., Françoise, M., Stephane, P., Christiane, V., Paulo, R., Emidio, G., et al. (2004). Response of hexokinase enzymes and the insulin system to dietary carbohydrates in the common carp, *Cyprinus carpio*. *Reprod. Nutr. Dev.* 44, 233–242. doi: 10.1051/rnd:2004027

Engelking, L. (2010). *Overview of Carbohydrate Metabolism*. Amsterdam: Academic Press.

Flowers, M. T., and Ntambi, J. M. (2008). Role of stearoyl-coenzyme a desaturase in regulating lipid metabolism. *Curr. Opin. Lipidol.* 19, 248–256. doi: 10.1097/mol.0b013e3282f9b54d

Fu, Z., Yang, R., Chen, X., Qin, J. G., and Ma, Z. (2019). Dietary non-protein energy source regulates antioxidant status and immune response of barramundi (*Lates calcarifer*). *Fish Shellfish Immun.* 95, 697–704. doi: 10.1016/j.fsi.2019.11.018

Glencross, B. (2006). The nutritional management of barramundi, *Lates calcarifer*–a review. *Aquacult. Nutr.* 12, 291–309. doi: 10.1111/j.1365-2095.2006.00410.x

Glencross, B. D. (2008). A factorial growth and feed utilization model for barramundi, *Lates calcarifer* based on Australian production conditions. *Aquacult. Nutr.* 14, 360–373. doi: 10.1111/j.1365-2095.2007.00543.x

Glencross, B. D., Blyth, D., Bourne, N., Cheers, S., Irvin, S., and Wade, N. M. (2017). An analysis of partial efficiencies of energy utilisation of different macronutrients by barramundi (*Lates calcarifer*) shows that starch restricts protein utilisation in carnivorous fish. *Brit. J. Nutr.* 117, 500–510. doi: 10.1017/s0007114517000307

Gonzalez-Baró, M. R., Lewin, T. M., and Coleman, R. A. (2007). Regulation of triglyceride metabolism. II. function of mitochondrial GPAT1 in the regulation of triacylglycerol biosynthesis and insulin action. *Am. J. Physiol. Gastrointestinal Liver Physiol.* 292, G1195–G1199.

Green, M. R., and Sambrook, J. (2012). *Molecular Cloning: a Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.

Gyamfi, D., Ofori Awuah, E., and Owusu, S. (2019). *Lipid Metabolism: an Overview*. Cambridge, MA: Academic Press.

Hemre, G., Mommsen, T. P., and Krogdahl, Å (2002). Carbohydrates in fish nutrition: effects on growth, glucose metabolism and hepatic enzymes. *Aquacult. Nutr.* 8, 175–194. doi: 10.1046/j.1365-2095.2002.00200.x

Hsieh, S. L., Chang, H. T., Wu, C. H., and Kuo, C. M. (2004). Cloning, tissue distribution and hormonal regulation of stearoyl-CoA desaturase in tilapia, *Oreochromis mossambicus*. *Aquaculture* 230, 527–546. doi: 10.1016/s0044-8486(03)00408-3

Jump, D. B., and Clarke, S. D. (1999). Regulation of gene expression by dietary fat. *Annu. Rev. Nutr.* 19, 63–90.

Jump, D. B., Clarke, S. D., Thelen, A., and Liimatta, M. (1994). Coordinate regulation of glycolytic and lipogenic gene expression by polyunsaturated fatty acids. *J. Lipid Res.* 35, 1076–1084. doi: 10.1016/s0022-2275(20)40103-8

Kamalam, B. S., Medale, F., Kaushik, S., Polakof, S., Skiba-Cassy, S., and Panserat, S. (2012). Regulation of metabolism by dietary carbohydrates in two lines of rainbow trout divergently selected for muscle fat content. *J. Exp. Biol.* 215, 2567–2578. doi: 10.1242/jeb.070581

Kerner, J., and Hoppel, C. (2000). Fatty acid import into mitochondria. *Biochimica et Biophysica Acta* 1486, 1–17. doi: 10.1016/s1388-1981(00)00044-5

Laura, M., Bianca, X. G., Hong, C., and Yuan-Xiang, P. (2018). Epigenetic regulation of carnitine palmitoyltransferase 1 (Cpt1a) by high fat diet. *Biochim. Biophys. Acta. Gene Regulatory Mech.* 1862, 141–152. doi: 10.1016/j.bbagrm.2018.12.009

Lazzari, R., and Baldisserotto, B. (2008). Nitrogen and phosphorus waste in fish farming. *Bol. Inst. Pesca.* 34, 591–600.

Li, A., Yuan, X., Liang, X., Liu, L., Li, J., Li, B., et al. (2016). Adaptations of lipid metabolism and food intake in response to low and high fat diets in juvenile grass carp (*Ctenopharyngodon idellus*). *Aquaculture* 457, 43–49. doi: 10.1016/j.aquaculture.2016.01.014

Liu, Y., Hu, J., Zhou, S., Yang, R., Qin, J., Ma, Z., et al. (2018). Effect of acute ammonia stress on antioxidant enzymes and digestive enzymes in barramundi *Lates calcarifer* Larvae. *Israeli J. Aquac. Bamidgeh.* 70, 1508–1519.

Ma, Z., Hassan, M. M., Allais, L., He, T., Leterme, S., Ellis, A. V., et al. (2018). Replacement of fishmeal with commercial soybean meal and EnzoMeal in juvenile barramundi *Lates calcarifer*. *Aquac. Res.* 49, 3258–3269. doi: 10.1111/are.13790

Ma, Z., Hassan, M. M., Allais, L., He, T., Leterme, S., Ellis, A., et al. (2019). Comparison of partial replacement of fishmeal with soybean meal and EnzoMeal on growth performance of Asian seabass *Lates calcarifer*. *Compar. Biochem. Physiol. C Toxicol. Pharmacol.* 216, 29–37. doi: 10.1016/j.cbpc.2018.10.006

Martino, R. C., Cyrino, J. E. P., Portz, L., and Trugo, L. C. (2002). Effect of dietary lipid level on nutritional performance of the surubim, *Pseudoplatystoma coruscans*. *Aquaculture* 209, 209–218. doi: 10.1016/s0044-8486(01)00738-4

Meng, X., Li, S., Qin, C., Zhu, Z., Hu, W., Yang, L., et al. (2018). Intestinal microbiota and lipid metabolism responses in the common carp (*Cyprinus carpio* L.) following copper exposure. *Ecotox. Environ. Saf.* 160, 257–264. doi: 10.1016/j.ecoenv.2018.05.050

Meng, Y., Qian, K., Ma, R., Liu, X., Han, B., Wu, J., et al. (2019). Effects of dietary lipid levels on sub-adult triploid rainbow trout (*Oncorhynchus mykiss*): growth performance, digestive ability, health status and expression of growth-related genes. *Aquaculture* 513:734394. doi: 10.1016/j.aquaculture.2019.734394

Mommsen, T. P., and Moon, T. W. (2001). *Hormonal Regulation of Muscle Growth*. Cambridge, MA: Academic Press, 251–308.

Moraes, G., and Bidinotto, P. M. (2004). Digestive proteases of pacu, *Piaractus mesopotamicus*, fed on distinct protein-starch diets. *J. Appl. Aquac.* 15, 197–207. doi: 10.1300/j028v15n03_17

Muzinic, L. A., Thompson, K. R., Metts, L. S., Dasgupta, S., and Webster, C. D. (2006). Use of turkey meal as partial and total replacement of fish meal in practical diets for sunshine bass (*Morone chrysops*×*Morone saxatilis*) grown in tanks. *Aquacult. Nutr.* 12, 71–81. doi: 10.1111/j.1365-2095.2006.00387.x

Nankervis, L., Matthews, S. J., and Appleford, P. (2000). Effect of dietary non-protein energy source on growth, nutrient retention and circulating insulin-like growth factor I and triiodothyronine levels in juvenile barramundi, *Lates calcarifer*. *Aquaculture* 191, 323–335. doi: 10.1016/s0044-8486(00)00436-1

NRC (2011). *Nutrient Requirements of Fish and Shrimp*. Washington DC: The National Academies Press.

Peterson, B. C., Waldbieser, G. C., and Bilodeau, L. (2005). Effects of recombinant bovine somatotropin on growth and abundance of mRNA for IGF-I and IGF-II in channel catfish (*Ictalurus punctatus*). *J. Anim. Sci.* 83, 816–824. doi: 10.2527/2005.834816x

Picha, M. E., Turano, M. J., Beckman, B. R., and Borski, R. J. (2008). Endocrine biomarkers of growth and applications to aquaculture: a minireview of growth hormone, insulin-like growth factor (IGF)-I, and IGF-binding proteins as potential growth indicators in fish. *N. Am. J. Aquacult.* 70, 196–211. doi: 10.1577/a07-038.1

Polakof, S., Panserat, S., Soengas, J. L., and Moon, T. W. (2012). Glucose metabolism in fish: a review. *J. Compar. Physiol. B.* 182, 1015–1045. doi: 10.1007/s00360-012-0658-7

Rolland, M., Dalsgaard, J., Holm, J., Gómez-Requeni, P., and Skov, P. V. (2015). Dietary methionine level affects growth performance and hepatic gene expression of GH–IGF system and protein turnover regulators in rainbow trout (*Oncorhynchus mykiss*) fed plant protein-based diets. *Compar. Biochem. Physiol. B Biochem. Mol. Biol.* 181, 33–41. doi: 10.1016/j.cbpb.2014.11.009

Skiba-Cassy, S., Lansard, M., Panserat, S., and Médale, F. (2009). Rainbow trout genetically selected for greater muscle fat content display increased activation of liver TOR signaling and lipogenic gene expression. *Am. J. Physiol. Regulatory Integr. Compar. Physiol.* 297, R1421–R1429. doi: 10.1152/physiolgenomics.00114.2006

Soengas, J. L. (2014). Contribution of glucose- and fatty acid sensing systems to the regulation of food intake in fish. a review. *Gen. Comp. Endocr.* 205, 36–48. doi: 10.1016/j.ygcen.2014.01.015

Song, X., Marandel, L., Dupont-Nivet, M., Quillet, E., Geurden, I., and Panserat, S. (2018). Hepatic glucose metabolic responses to digestible dietary carbohydrates in two isogenic lines of rainbow trout. *Biology Open.* 7:32896.

Tan, X., Sun, Z., Huang, Z., Zhou, C., Lin, H., Tan, L., et al. (2017). Effects of dietary hawthorn extract on growth performance, immune responses, growth- and immune-related genes expression of juvenile golden pompano (*Trachinotus ovatus*) and its susceptibility to Vibrio harveyi infection. *Fish Shellfish Immun.* 70, 656–664. doi: 10.1016/j.fsi.2017.09.041

Tatar, M., Bartke, A., and Antebi, A. (2003). The endocrine regulation of aging by insulin-like signals. *Science* 299, 1346–1351. doi: 10.1126/science.1081447

Thomas, M., van Vliet, T., and van der Poel, A. F. B. (1998). Physical quality of pelleted animal feed 3. Contribution of feedstuff components. *Anim. Feed Sci. Technol.* 70, 59–78. doi: 10.1016/s0377-8401(97)00072-2

Turchini, G. M., Torstensen, B. E., and Ng, W. (2009). Fish oil replacement in finfish nutrition. *Rev. Aquacult.* 1, 10–57. doi: 10.1111/j.1753-5131.2008.01001.x

Tzur, R., Tal, E., and Shapiro, B. (1964). α-Glycerophosphate as regulatory factor in fatty acid esterification. *Biochim. Biophys. Acta* 84, 18–23. doi: 10.1016/0926-6542(64)90096-4

Vera Cruz, E. M., Brown, C. L., Luckenbach, J. A., Picha, M. E., Bolivar, R. B., and Borski, R. J. (2006). Insulin-like growth factor-I cDNA cloning, gene expression and potential use as a growth rate indicator in Nile tilapia, *Oreochromis niloticus*. *Aquaculture* 251, 585–595. doi: 10.1016/j.aquaculture.2005.06.039

Wade, N. M., Skiba-Cassy, S., Dias, K., and Glencross, B. D. (2014). Postprandial molecular responses in the liver of the barramundi, *Lates calcarifer*. *Fish Physiol. Biochem.* 40, 427–443. doi: 10.1007/s10695-013-9854-y

Wilson, R. P. (1994). Utilization of dietary carbohydrate by fish. *Aquaculture* 124, 67–80. doi: 10.1016/0044-8486(94)90363-8

Xie, D., Yang, L., Yu, R., Chen, F., Lu, R., Qin, C., et al. (2017). Effects of dietary carbohydrate and lipid levels on growth and hepatic lipid deposition of juvenile tilapia, *Oreochromis niloticus*. *Aquaculture* 479, 696–703. doi: 10.1016/j.aquaculture.2017.07.013

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.

frontiers in Marine Science

ORIGINAL RESEARCH
published: 23 June 2021
doi: 10.3389/fmars.2021.691404

Liver Metabolome and Proteome Response of Turbot (*Scophthalmus maximus*) to Lysine and Leucine in Free and Dipeptide Forms

Yuliang Wei[1,2], *Benxiang Li*[1], *Houguo Xu*[1,2] *and Mengqing Liang*[1,2]*

[1] *Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, China,* [2] *Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China*

Omics approaches provide more metabolic information to explain the relationship between dietary nutrition and fish growth. This study aimed to explore the metabolome and proteome response of turbot (*Scophthalmus maximus*) fed diets containing lysine and leucine in free and dipeptide forms by the approaches of integrated liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based metabolomics and isobaric tags for relative and absolute quantification (iTRAQ)-based proteomics. Plant protein-based diets were formulated to contain the equivalent of lysine and leucine in free amino acid [crystalline amino acid (CAA)] and synthetic Lys-Leu (Lys-Leu) forms. The metabolome and proteome profiles of the liver were screened in fish fed either the CAA diet or the Lys-Leu diet after an 8-week feeding trial. Fish fed the Lys-Leu diet showed a significantly higher final body weight and a specific growth rate compared with fish fed the CAA diet. Protein- and amino acid-related metabolic processes in the liver were identified between the Lys-Leu and CAA groups based on differential metabolites and proteins. The proteolytic enzymes and amino acid transporters from differential proteins of the liver showed that the process of protein digestion and absorption may be affected by the different forms of lysine and leucine in the feed. A mechanistic target of rapamycin complex 1 and ubiquitin proteasome pathways were identified by differential proteins, which were involved in the processes of protein synthesis and degradation in the liver. Lysine degradation, tryptophan metabolism, alanine, aspartate, and glutamate metabolism, arginine biosynthesis, arginine and proline metabolism, and glycine, serine, and threonine metabolism were identified based on differential metabolites and proteins, which showed that the metabolism of various amino acids, including lysine, had been affected by both the CAA and Lys-Leu groups. In conclusion, the data of integrated metabonomics and proteomics suggested that different forms of lysine and leucine in the feed may affect liver metabolic processes including protein digestion and absorption, protein synthesis and degradation, and amino acid metabolism. In addition, a good correlation between differential metabolites and proteins was observed in amino acid metabolism by using the approaches of integrated LC-MS/MS-based metabolomics and iTRAQ-based proteomics.

Keywords: dipeptide, free amino acid, liver, metabolomics, proteomics, turbot

OPEN ACCESS

Edited by:
Songlin Li,
Shanghai Ocean University, China

Reviewed by:
Fei Song,
South China Normal University, China
Mansour Torfi Mozanzadeh,
South Iran Aquaculture Research Center, Iran
Dizhi Xie,
South China Agricultural University, China

***Correspondence:**
Mengqing Liang
liangmq@ysfri.ac.cn

Specialty section:
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

Received: *06 April 2021*
Accepted: *26 May 2021*
Published: *23 June 2021*

Citation:
Wei Y, Li B, Xu H and Liang M (2021) Liver Metabolome and Proteome Response of Turbot (Scophthalmus maximus) to Lysine and Leucine in Free and Dipeptide Forms. Front. Mar. Sci. 8:691404. doi: 10.3389/fmars.2021.691404

INTRODUCTION

With the rising demand and price of fish meal, expanding the amount of plant protein in aquafeeds was an effective strategy to replace fish meal (Daniel, 2017). When more plant ingredients were formulated in diets, it was also necessary to supplement essential amino acids based on the requirement of fish (Furuya et al., 2004; El-Husseiny et al., 2017). Nutritionists proposed that essential amino acids in feed could be supplemented with synthetic peptides or crystalline forms (Dabrowski et al., 2003; Ostaszewska et al., 2013). Those studies in fish species mainly focused on a comparison of dietary amino acid sources in peptides or free amino acids forms using classical nutritional methods, such as the evaluation of growth and feed utilization and the analysis of digestive enzyme activity and amino acid composition of fish whole body (Mamauag et al., 2012; Kim and Lee, 2013; Kamaszewski et al., 2019, 2020). Regarding turbot (*Scophthalmus maximus*), our previous studies showed that the utilization efficiency of dietary Lys-Leu dipeptide was not only higher than that of dietary crystalline lysine and leucine but also higher than that of dietary Lys-Leu-Lys/Leu-Lys-Leu, Lys-Gly, or Gly-Leu (Wei et al., 2020a,b). However, the effect of dietary amino acid in free and peptide forms on fish growth was still controversial in different fish species, such as carp (*Cyprinus carpio*) (Kamaszewski et al., 2014), red sea bream (*Pagrus major*) (Mamauag et al., 2012), and olive flounder (*Paralichthys olivaceus*) (Rahimnejad and Lee, 2014). It implied that the application of classical nutritional methods may not comprehensively understand how the forms of dietary amino acids regulated the metabolic process of fish, which, in turn, affected their growth. Hence, in order to address this issue, omics approaches, including metabolomics and proteomics, should be introduced in fish nutrition (Sales et al., 2014).

Metabolomics and proteomics are the functional continuation of transcriptomics and can be used to further elucidate the role of nutritional changes in diets on fish growth by understanding the metabolic pathways (de Vareilles et al., 2012; Rodrigues et al., 2012; Ceciliani et al., 2018). Metabolomics can help to gain insight into the alterations of metabolism by identifying small molecule metabolites (Deborde et al., 2021). Proteomics focuses on diverse processes, such as cytoskeletal dynamics, energy and carbohydrate metabolism, lipoprotein metabolism, amino acid metabolism, and protein metabolism, by identifying proteins in the cell and tissue (de Vareilles et al., 2012). However, to date, only a few studies had explored the response of fish to dietary amino acid imbalance or protein source changes by metabolomics or proteomics (Gómez-Requeni et al., 2011; de Vareilles et al., 2012; Wei et al., 2017a,b; Deborde et al., 2021). For instance, Gómez-Requeni et al. (2011) and de Vareilles et al. (2012) reported that a proteomics approach was used to explore the effects of dietary lysine imbalances on the protein expression of whole body or muscle in zebrafish (*Danio rerio*). Our previous studies evaluated the metabolome profile of liver and muscle in turbot fed diets containing fish meal and fish protein hydrolysate (low molecular weight peptides and free amino acids) (Wei et al., 2017a,b). Omics approaches in those studies were often used separately–proteomics and metabolomics–and an integrated analysis using these approaches was rarely conducted in fish nutrition. Thus, in order to provide more metabolic information to explain the relationship between dietary nutrition and fish growth, it was necessary to combine metabolomics with proteomics in nutritional studies with fish (Guo et al., 2019; Min et al., 2020; Yu et al., 2020).

The aim of this study was to explore the liver metabolome and proteome response of turbot fed diets containing lysine and leucine in free and dipeptide forms. The growth and the feed utilization of turbot are investigated after an 8-week feeding trial (Wei et al., 2020a), and the metabolome and proteome profiles of the liver were screened by the approaches of integrated liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based metabolomics and isobaric tags for relative and absolute quantification (iTRAQ)-based proteomics.

MATERIALS AND METHODS

Experimental Diets and Feeding Trail

Our previous study provided evidence that the inclusion of dietary dipeptide formed by lysine and leucine was effective for growth and feed utilization in turbot (Wei et al., 2020a). Thus, the equivalent of lysine and leucine, but in different forms (free or dipeptide), was used in plant protein-based diets (**Table 1**). Briefly, two isoproteic (480 g kg^{-1} crude protein) diets were formulated to contain 150 g kg^{-1} fish meal and 380 g kg^{-1} peanut meal. Then, one of them was supplemented with crystalline lysine (21.1 g kg^{-1}) and leucine (18.9 g kg^{-1}) [the Crystalline amino acid (CAA) diet]; the other was supplemented with synthetic Lys-Leu (40.0 g kg^{-1}) (the Lys-Leu diet). CAA pre-mix without lysine and leucine was added to the diets according to Peres and Oliva-Teles (2005). The analysis of amino acid composition in diets revealed that the contents of total lysine and leucine were similar in the two diets, while the contents of free lysine and leucine in the CAA diet were higher than those in the Lys-Leu diet (**Table 2**).

Turbot was purchased and cultured at Yantai Tianyuan Aquatic Product Co., Ltd. (Shandong, China). The fish were transported from a cemented tank (5.0 m × 5.0 m × 1.5 m) to a large cylindrical fiberglass tank (water volume, 300 L) and fed a commercial feed for 2 weeks to acclimate to new culture conditions. Then, 150 fish with initial weight (11.98 ± 0.03 g) were stocked in six tanks (water volume, 120 L; 25 fish per tank). Each diet was assigned to three replicate tanks. The fish were hand-fed to apparent satiation twice daily (06:30 and 16:30) in a flow-through system with continuous aeration and water flow rate of 3 L min^{-1}. The uneaten feed was siphoned from the tank bottom and weighed to accurately estimate the feed consumed. The feeding trail lasted for 8 weeks from August to October. During this period, natural photoperiod was used, and water temperature, pH, salinity, and dissolved oxygen were 15 ± 1°C, 7.7 ± 0.2, 32 ppt, and 7 mg L^{-1}, respectively. At the termination of the feeding trail, the fish were starved for 24 h, and then the number and weight of the fish from each tank were recorded. Three fish from each tank were collected at 2 h after feeding and then anesthetized with eugenol (1:10,000). After blood sampling, the liver sample of each fish was flash-frozen

in liquid nitrogen and then stored at −80°C before use. The experimental protocols followed the guidelines approved by the Institutional Animal Care and Use Committee of the Yellow Sea Fisheries Research Institute.

Non-targeted Metabolomic and Analysis

Extraction of Metabolites

Metabolites of the liver in turbot were extracted using a modification of the organic protein precipitation method (Chen et al., 2018; Dong et al., 2020). The liver sample (25 mg) was transferred into a tube with 800 μl frozen solution of methanol/acetonitrile/water (2:2:1) and two small steel balls.

The sample was homogenized using a bead mill homogenizer (TissueLyser LT; Qiagen Hilden, Germany) at 60 Hz for 4 min, the steel balls were taken out, and then the sample was sonicated (80 Hz) in an ice bath for 10 min. The mixture was placed at −20°C for 120 min and then centrifuged at 25,000 × *g* for 15 min at 4°C. The supernatant was collected and transferred to a new tube. Subsequently, the centrifugation was repeated once, and 600 μl of supernatant was collected and freeze-dried. Finally, the dried supernatant sample was dissolved in 50% methanol solution, sonicated (80 Hz) in an ice bath for 10 min, and then centrifuged at 25,000 × *g* for 15 min at 4°C. The supernatant with metabolites was collected again for a subsequent analysis. In addition, 50 μl from each supernatant sample was pooled as the quality control (QC) sample.

LC-MS/MS Analysis

The supernatant with metabolites were determined by a liquid chromatography (LC)-mass spectrometer (MS) system. The injection volume for each supernatant sample was 5 μl. An ultra-performance liquid chromatography (UPLC) (Waters Corp., United Kingdom) was performed using an ACQUITY UPLC HSS T3 column (internal diameter, 2.1 mm; column length, 100 mm; and particle size, 1.8 μm; Waters Corp., United Kingdom). After the metabolites from the supernatant sample were eluted, a high-resolution tandem mass spectrometer (Xevo G2 XS Q-TOF, Waters Corp., United Kingdom) was used in both the positive and the negative ion modes. Mass spectrometry data were achieved by Centroid MS^E mode (where *E* represents the collision energy). The centroid mode range was 50–1,200 Da, and the scan time was 0.2 s. During MS/MS acquisition, all precursors were fragmented with 20–40 eV, and the scan time was 0.2 s. In the process of data acquisition, mass accuracy was calibrated by

TABLE 1 | Formulation and chemical composition of experimental diets (g kg^{-1} of dry matter).

Ingredients	Crystalline amino acid	Lys-Leu
Fish meal	150	150
Peanut meal	380	380
Wheat meal	164.4	164.4
Amino acid pre-mix[a]	112.6	112.6
L-Lysine[b]	21.1	
L-Leucine[b]	18.9	
Lys-Leu[c]		40
Fish oil	45	45
Soybean oil	35	35
Soybean lecithin	15	15
Mineral pre-mix[d]	15	15
Vitamin pre-mix[e]	8	8
$Ca(H_2PO_4)_2$	15	15
DMPT	5	5
Choline chloride	10	10
Vitamin C	5	5
Proximate composition		
Dry matter	932.9	929.3
Ash	90.2	91.7
Crude protein	482.6	486.9
Crude lipid	112.1	110.1

[a]*Amino acid mixture composition: (g kg^{-1} diet; all are L-form amino acids unless otherwise indicated): arginine, 11.7; histidine; 3.0; isoleucine, 9.4; d/l-methionine, 14.5; cystine, 4.2; phenylalanine, 3.1; threonine, 12.1; tryptophan, 2.0; valine, 9.8; taurine, 10.0; hydroxyproline, 6.0; aspartic acid, 4.0; glutamic acid, 4.0; serine, 4.0; glycine, 14.8; and alanine, 4.0.*

[b]*L-lysine and L-leucine were purchased from Hebei Huayang Amino Acids Group Company Limited.*

[c]*Lys-Leu (lysine–leucine) was purchased from GL Biochem (Shanghai) Ltd.*

[d]*Mineral pre-mix (mg or g kg^{-1} diet): $MgSO_4{\cdot}7H_2O$, 1,200 mg; $CuSO_4{\cdot}5H_2O$, 10 mg; $ZnSO_4{\cdot}H_2O$, 50 mg; $FeSO_4{\cdot}H_2O$, 80 mg; $MnSO_4{\cdot}H_2O$, 45 mg; $CoCl_2{\cdot}6H_2O$ (1%), 50 mg; $NaSeSO_3{\cdot}5H_2O$ (1%), 20 mg; $Ca(IO_3)_2{\cdot}6H_2O$ (1%), 60 mg; and zoelite, 13.49 g.*

[e]*Vitamin pre-mix (mg or g kg^{-1} diet): thiamin, 25 mg; riboflavin, 45 mg; pyridoxine HCl, 20 mg; vitamin B12, 0.1 mg; vitamin K3, 10 mg; inositol, 800 mg; pantothenic acid, 60 mg; niacin, 200 mg; folic acid, 20 mg; biotin, 1.2 mg; retinol acetate, 32 mg; cholecalciferol, 5 mg; alpha-tocopherol, 120 mg; and wheat middling, 6.66 g.*

DMPT, dimethyl-β-propiothetin.

TABLE 2 | Total and free amino acid composition of experimental diets (g kg^{-1} dry matter).

	Crystalline amino acid		Lys-Leu	
	AA	FAA	AA	FAA
	EAA			
Threonine	17.6	9.5	18.3	9.2
Valine	24.3	8.0	25.1	7.7
Methionine	17.2	11.1	17.5	10.8
Isoleucine	17.5	6.5	18.3	6.2
Leucine	35.8	14.7	38.9	0.7
Phenylalanine	17.8	3.2	18.9	3.6
Lysine	25.8	13.4	27.3	0.8
Histidine	7.7	3.2	8.2	3.1
Arginine	50.6	10.2	53.0	10.7
	NEAA			
Taurine	9.8	9.6	10.2	9.9
Aspartic acid	31.2	3.5	33.0	3.4
Serine	15.1	3.5	15.4	3.4
Glutamic acid	62.8	4.4	66.5	4.4
Glycine	22.5	8.3	24.0	8.2
Alanine	28.0	3.8	29.8	3.8
Cystine	23.4	2.3	24.3	2.4
Tyrosine	10.2	0.2	10.6	0.2
Proline	11.3	3.8	11.5	3.8

AA, amino acid; FAA, free AA; EAA, essential AA; NEAA, non-essential AA.

acquiring the leucine–encephalin signal every 3 s. Furthermore, in order to evaluate the stability of the instrument system, a QC sample was collected for every 10 samples.

Data Processing and Statistical Data Analysis

The raw data from the mass spectrometer were imported into Progenesis QI (version 2.2) for a data matrix to obtain metabolite-related mass-to-charge ratio (m/z), retention time, and ion intensity. The normalized peak data were pre-processed using metaX software, including imputing the remaining peaks with missing values using the k-nearest neighbor algorithm, removing low-quality ions (50% of QC samples or 80% of biological samples), and then filtering out ions with a relative standard deviation >30% in QC samples (Wen et al., 2017). Differential metabolites in the liver of different treatments were identified using univariate analysis and multivariate statistics. Univariate analysis was performed by the methods of *t*-test and fold change (FC) analysis ($P < 0.05$; FC $\geq$ 1.2 or $\leq$0.8333). Multivariate statistics were performed by partial least squares-discriminate analysis (PLS-DA) with variable importance for the projection (VIP) values ($\geq$1.0). Heat map was used to visualize differential metabolites, and Kyoto Encyclopedia of Genes and Genomes (KEGG)[1] was used to help in understanding the functions of metabolites.

Proteomics Analysis

Protein Extraction and Digestion

Proteins of the liver in turbot were extracted using the method reported by Wu et al. (2019). The liver tissue from each fish was homogenized in lysis buffer 3 [8 mol/L urea, 40 mmol/L Tris–HCl, or tetraethylammonium bicarbonate (TEAB) with 1 mmol/L phenylmethanesulfonyl fluoride, 2 mmol/L ethylenediaminetetraacetic acid, and 10 mmol/L dithiothreitol (DTT), pH 8.5] by TissueLyser (Qiagen, Hilden, Germany). After centrifugation at 25,000 × *g* for 20 min at 4°C, 10 mmol/L DTT was added to the supernatant at 56°C for 1 h, and then the mixture was incubated with 55 mmol/L iodoacetamide in the dark at room temperature for 45 min. The precipitated protein was washed three times with acetone. The quality and the quantity of the protein sample were measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis and Bradford method, respectively.

The protein solutions (100 μg) were digested with 2.5 μg Trypsin gold (Promega, Madison, WI, United States) at 37°C for 4 h. Trypsin gold was added once more with the same ratio, and trypsin digestion was continued at 37°C for 8 h. After trypsin digestion, the peptides were desalted with a Strata X C18 column (Phenomenex, Torrance, CA, United States) and vacuum-dried.

iTRAQ Labeling and Peptide Fractionation

The vacuum-dried peptides were dissolved in 30 μl of 0.5 mol/L TEAB with vortexing. After the iTRAQ labeling reagents were recovered to ambient temperature, peptide labeling was performed by iTRAQ Reagent 8-plex Kit (Applied Biosystems, Foster City, CA, United States). The labeled samples were desalted with a Strata X C18 column and then vacuum-dried.

The peptides were separated using a Shimadzu LC-20AB HPLC Pump system (Kyoto, Japan) equipped with Gemini C18 column (internal diameter, 4.6 mm; column length, 250 mm; and particle size, 5 μm; Phenomenex, Torrance, CA, United States). The peptides were dissolved with buffer A [5% acetonitrile (ACN), 95% H_2O, pH 9.8] to 2 ml. Elution is monitored by measuring absorbance at 214 nm, and fractions are collected every 1 min. The eluted peptides are pooled as 20 fractions and vacuum-dried.

LC-MS/MS Analysis

Each fraction was reconstituted in buffer A (2% ACN and 0.1% formic acid) and centrifuged at 20,000 × *g* for 10 min. The supernatant was loaded on an Ultimate 3000 UHPLC system (Thermo Scientific, Waltham, MA, United States), equipped with a trap and an analytical column. The samples were desalted in a trap column and then eluted into a nanocapillary C18 column (inner diameter, 75 μm; column diameter, 3 μm; and column length, 25 μm). The peptides separated from nano-high-performance liquid chromatography were subjected to tandem mass spectrometry Q Exactive HF X (Thermo Fisher Scientific, San Jose, CA, United States) for data-dependent acquisition detection by nano-electrospray ionization.

Protein Identification and Functional Annotation

The raw data was firstly transformed into mascot generic format (MGF) for bioinformatics analysis. Then, the exported MGF files were searched using the Mascot search engine (version 2.3.02; Matrix Science, London, United Kingdom). For protein quantitation, an automated software (IQuant software) was used to analyze the labeled peptides with isobaric tags (Wen et al., 2014). The peptide-spectrum matches were pre-filtered at a false discovery rate (FDR) of 1% to assess the confidence of the

TABLE 3 | Growth performance and feed utilization of juvenile turbot fed different diets.

	Crystalline amino acid	Lys-Leu	*P*-value
Final body weight (g)	36.43 ± 1.20[a]	39.75 ± 0.71[b]	0.022
Survival rate (%)[a]	98.67 ± 1.33	98.67 ± 1.33	1.000
Specific growth rate (%/day)[b]	1.98 ± 0.06[a]	2.14 ± 0.03[b]	0.027
Feed intake (%/day)[c]	1.65 ± 0.03	1.52 ± 0.03	0.150
Feed efficiency ratio[d]	1.09 ± 0.05	1.26 ± 0.01	0.085
Protein efficiency ratio[e]	2.31 ± 0.10	2.59 ± 0.02	0.129
Protein productive value (%)[f]	35.26 ± 1.63	35.93 ± 0.78	0.803

Data are presented as mean ± SE of three replicate tanks (n = 3).
Values in the same row followed by different superscript letters are significantly different (P < 0.05).
[a]*Survival rate = 100 × (final fish number/initial fish number).*
[b]*Specific growth rate = 100 × [ln (final weight)–ln (initial weight)]/feeding days.*
[c]*Feed intake = 100 × total feed intake/[feeding days × (final weight + initial weight)/2].*
[d]*Feed efficiency ratio = body weight gain/dry feed intake.*
[e]*Protein efficiency ratio = (final weight–initial weight)/protein intake.*
[f]*Protein productive value = 100 × (final protein content–initial protein content)/protein intake.*

[1]http://www.kegg.jp/

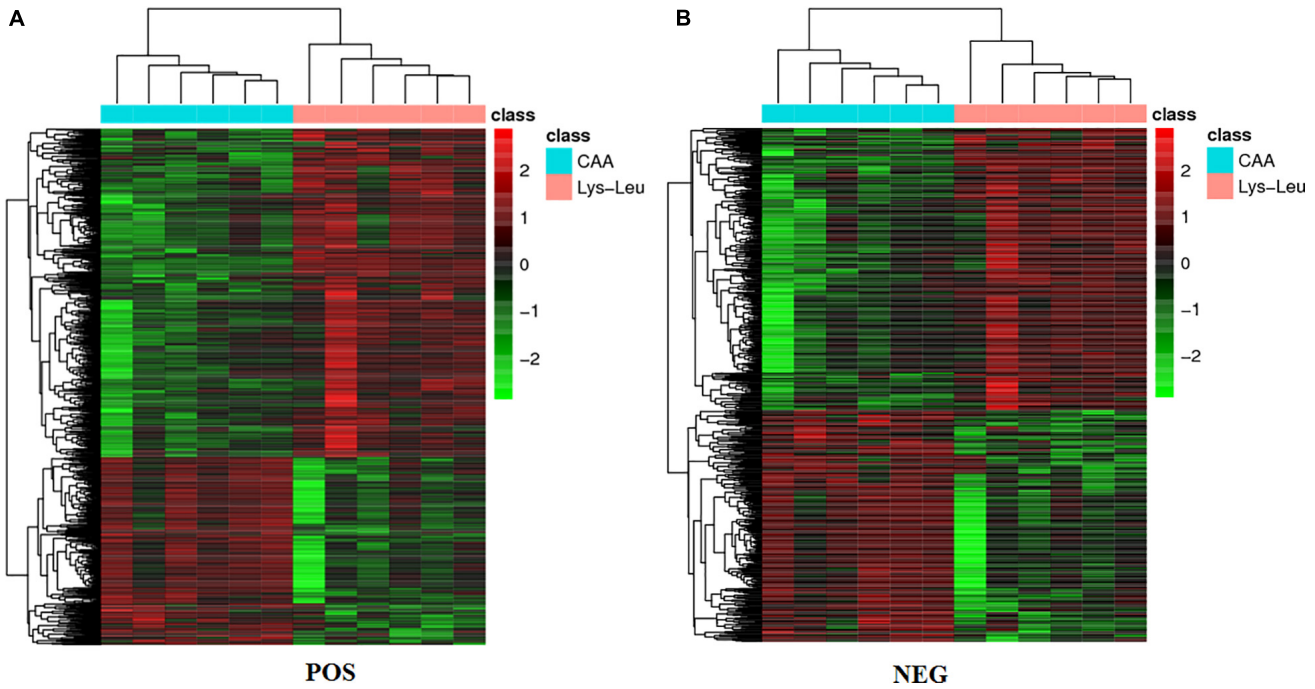


FIGURE 1 | Heat maps of differential metabolites between the CAA and Lys-Leu treatments. **(A)** The positive ion mode. **(B)** The negative ion mode. Red color represents the value that is higher than the mean value. Green color represents the value that is lower than the mean value. Black color represents the value that is close to the mean value.

peptides. Then, identified peptide sequences were assembled into proteins. In order to reduce the rate of false-positive at the protein level, the proteins were identified at a FDR of 1% (Savitski et al., 2015). Proteins with 1.2-fold change (mean value) and *P*-value (*t*-test) less than 0.05 were defined as differential proteins. Cluster analysis was used to visualize differential proteins. For the pathway analysis, KEGG was used to predict the main metabolic pathways of the differential proteins.

RESULTS

Growth Performance

The analysis of *t*-test revealed that the final body weight ($P = 0.022$) and the specific growth rate ($P = 0.027$) offish fed the Lys-Leu diet were significantly higher than those of fish fed the CAA diet (**Table 3**). However, no significant differences among survival rate, feed intake, feed efficiency ratio, protein efficiency ratio, and protein productive value were observed between dietary treatments ($P > 0.05$).

Differential Metabolites of the Liver Between Dietary Treatments

Differential metabolites were screened from all identified metabolites based on $P < 0.05$, FC ≥ 1.2, or FC ≤ 0.8333 in the *t*-test and VIP ≥ 1 in the PLS-DA model (**Figure 1**). A total of 1,140 and 661 identified metabolites were obtained between the CAA and Lys-Leu treatments in the POS and the NEG modes, respectively (**Supplementary Tables 1**, **2** and the annotation information of metabolites is provided in **Supplementary Table 3** based on compound.ID). Then, the KEGG database was used to annotate and enrich the pathway analysis of these differential metabolites from the POS and the NEG modes.

Key metabolic pathways of protein- and amino acid-related metabolic processes were selected from 148 metabolic pathways in positive ion mode and 89 metabolic pathways in negative ion mode (**Table 4**). Lysine biosynthesis and degradation as well as valine, leucine, and isoleucine metabolism in the liver were affected when lysine and leucine were added to diets in the form of dipeptide (Lys-Leu) or free amino acids. In addition to lysine and leucine metabolism, differential metabolites between the Lys-Leu and CAA groups were involved in other amino acid metabolisms, such as tryptophan metabolism, phenylalanine metabolism, alanine, aspartate, and glutamate metabolism, arginine biosynthesis, arginine and proline metabolism, and glycine, serine, and threonine metabolism.

Differential Proteins of the Liver Between Dietary Treatments

According to iTRAQ quantification, 935,928 spectrums were generated and 32,261 peptides and 5,708 proteins were identified with 1% FDR. A total of 618 differential proteins were identified between the Lys-Leu and CAA treatments. Among them, 431 (**Supplementary Table 4**) significantly downregulated and 187 (**Supplementary Table 5**) significantly upregulated differential proteins were screened using IQuant software based on FC > 1.2

TABLE 4 | Key differential metabolites and their pathways annotated by KEGG.

Metabolic pathways	Count	Metabolites	
		Downregulation[a]	Upregulation[b]
		Amino acid metabolism	
Lysine biosynthesis	4	N-Acetyl-LL-2,6-diaminoheptanedioate; N-succinyl-L-2,6-diaminopimelate, LL-2,6-diaminopimelate, meso-2,6-diaminopimelate	
Lysine degradation	8	Lysopine, N6-acetyl-N6-hydroxylysine, N2-citryl-N6-acetyl-N6-hydroxylysine, N6-acetyl-L-lysine, N6,N6,N6-trimethyl-L-lysine, (3S)-3-hydroxy-N6,N6,N6-trimethyl-L-lysine	5-Aminopentanal, 5-aminovaleric acid
Branched chain amino acid metabolism	3	L-Valine, L-isoleucine	L-Leucine
Tryptophan metabolism	25	L-Tryptophan, N-formylkynurenine, 8-methoxykynurenate, indole, N-acetylindoxyl, 3-indoleglycolaldehyde, oxitriptan, 5-hydroxyindolepyruvate, serotonin, 5-hydroxyindoleacetaldehyde, 5-hydroxyindoleacetate, 5-methoxyindoleacetate, indolepyruvate, (R)-(indol-3-yl)lactate, indole-3-acetaldehyde, tryptophol, N-hydroxyl-tryptamine, desulfoglucobrassicin, indoleacetic acid, 3-methyldioxyindole, indole	5-Methoxytryptamine, N-methylserotonin, 2-aminomuconate semialdehyde, picolinic acid
Phenylalanine metabolism	12	L-Phenylalanine, D-phenylalanine; N-acetyl-D-phenylalanine, *trans*-cinnamate, pyruvophenone, cathinone, N-acetyl-L-phenylalanine, phenylacetic acid, alpha-phenylacetamide, phenylethyl alcohol	Pseudoephedrine; ephedrine
Alanine, aspartate, and glutamate metabolism	3	L-Glutamine, L-arginosuccinic acid	Beta-citrylglutamate
Arginine biosynthesis	3	L-Glutamine, N-acetylornithine, L-arginosuccinic acid	
Arginine and proline metabolism	9	N2-succinyl-L-arginine, N2-succinyl-L-ornithine, feruloylputrescine, gamma-glutamyl-gamma-aminobutyrate, 4-oxoproline, L-1-pyrroline-3-hydroxy-5-carboxylate, linatine; 1-pyrroline-4-hydroxy-2-carboxylate	5-Aminovaleric acid
Glycine, serine, and threonine metabolism	4	L-Tryptophan, N4-acetyl-L-2,4-diaminobutyrate, N-alpha-acetyl-L-2,4-diaminobutyrate	Betaine

[a] *Downregulation indicates that the metabolites of the Lys-Leu group are less abundant compared to the CAA group.*
[b] *Upregulation indicates that the metabolites of the Lys-Leu group are more abundant compared to the CAA group.*

and $P < 0.05$ (**Figure 2**). Then, the database of KEGG was used for the functional annotation of differential proteins.

Key metabolic pathways of protein- and amino acid-related metabolic processes were selected from 305 metabolic pathways using the KEGG database (**Table 5**). For protein digestion and absorption, digestive enzymes including trypsin, pancreatic elastase II, carboxypeptidase A1, carboxypeptidase A2, and carboxypeptidase B as well as amino acid transporter including CD98 heavy chain (SLC3A2) were downregulated in the Lys-Leu group compared with the CAA group. For protein synthesis and degradation, the differential proteins between the Lys-Leu and CAA groups were enriched in mammalian target of rapamycin (mTOR) signaling pathway and ubiquitin-mediated proteolysis. For amino acid metabolism, the differential proteins between the Lys-Leu and CAA groups were involved in lysine degradation as well as other amino acid metabolisms (tryptophan metabolism, alanine, aspartate, and glutamate metabolism, arginine biosynthesis, arginine and proline metabolism, and glycine, serine, and threonine metabolism).

DISCUSSION

Studies of the comparison of dietary amino acid sources in dipeptides or free amino acid forms showed that olive flounder (*P. olivaceus*) could utilize lysine or leucine of dipeptide form more efficiently than that of free amino acid form (Kim and Lee, 2013; Rahimnejad and Lee, 2014). The data of growth performance in this study had been provided in a separate study (Wei et al., 2020a), which suggested that similar results to those of olive flounder were observed in turbot regarding the utilization of lysine and leucine in dipeptide and free amino acid forms. However, in addition to the analysis of growth performance, our previous study only investigated the effects of the different forms of dietary amino acids on the concentrations of serum-free amino acids and the expressions of intestinal amino acids and peptide transporters (Wei et al., 2020a). This study attempted to use the approaches of integrated metabolomics and proteomics to comprehensively elucidate the effects of dietary lysine and leucine in the forms of dipeptides and free amino acids on protein- and amino acid-related metabolic processes.

Protein Digestion and Absorption

Trypsin, pancreatic elastase II, carboxypeptidase A1, carboxypeptidase A2, and carboxypeptidase B are proteolytic enzymes, and their main physiological function is to digest the protein (Cohen et al., 1981; Neurath, 1984; Zambonino Infante and Cahu, 2007). In this study, the expressions of trypsin, pancreatic elastase II, carboxypeptidase A1, carboxypeptidase A2, and carboxypeptidase B were downregulated in fish fed the Lys-Leu diet compared with fish fed the CAA diet (**Table 5**), which showed that dietary synthetic Lys-Leu may decrease the activities of proteolytic enzymes compared to the dietary free form of lysine and leucine. However, the growth performance in the Lys-Leu group was higher than that in the CAA group. Obviously, the activities of proteolytic enzymes and the growth

were contradictory between the two treatments. A possible reason was that the proteolytic enzyme activities were related to the sampling time. At the sampling time (2 h after feeding) of this study, it happened that proteolytic enzyme activities decreased in the Lys-Leu group compared to the CAA group, but regardless of whether proteolytic enzyme activities increased or decreased between the two treatments, different forms of lysine and leucine in the feed may firstly cause changes in the activities of proteolytic enzymes and then affect the digestion of dietary protein. For amino acid absorption, the proteome profile of the liver also showed a lower expression of the heavy chain of the 4F2 antigen (4F2hc) in the Lys-Leu treatment. The 4F2hc, named CD98 heavy chain, is a type II membrane N-glycoprotein that forms heterodimers with associated transporters. These glycoprotein-associated amino acid transporters include L-type amino acid transporters (4F2hc/LAT1 and 4F2hc/LAT2), y^+ L-type amino acid transporters (4F2hc/y^+LAT1 and 4F2hc/y^+LAT2), the alanine, serine, and cysteine transporter 1 (4F2hc/Asc-1), and the cystine and glutamate exchange transporter (4F2hc/xCT), which play a key role in the transport of a variety of amino acids including lysine and leucine (Fotiadis et al., 2013). Thus, the proteome profile of the liver in the process of protein digestion and absorption showed that the different forms of dietary lysine and leucine not only affect the digestion of dietary protein but also affect the absorption of a variety of amino acids.

Protein Synthesis and Degradation

Protein synthesis can be promoted by activating the mechanistic target of rapamycin complex 1 (mTORC1) (Laplante and Sabatini, 2012). As a member of the branched-chain amino acid family, leucine is considered as the potent activator of mTORC1 (Kim and Guan, 2011). Therefore, we hypothesized that free or dipeptide leucine in this study may impact protein synthesis by regulating the mTORC1 signaling pathway. Obviously, differential proteins in the liver supported this hypothesis (**Table 5**). Based on the proteome profile of the liver, the effects of dietary lysine and leucine in free and dipeptide forms on the mTORC1 signaling pathway were achieved by two upstream signals. One of the upstream signals may be phosphatidylinositol 3-kinase (PI3K)-protein kinase B (Akt) signals. Compared with fish fed the CAA diet, the mTORC1 signaling pathway was affected by downregulating the expression of insulin and protein kinase B (Akt2) and upregulating the expression of tuberous sclerosis 2 in fish fed the Lys-Leu diet (Ma and Blenis, 2009). The other upstream signal may be the cellular entry of leucine because 4F2hc/LAT1 and 4F2hc/LAT2 are considered as the primary routes (Kim and Guan, 2011). In the present study, the downregulated expression of 4F2hc in fish fed the Lys-Leu indicated that it may directly affect the mTORC1 signaling pathway through the transport of leucine.

Ubiquitin-mediated proteolysis is the primary pathway for the degradation of intracellular proteins in all tissues (Lecker et al., 2006). At the transcription level, our previous studies found that dietary dipeptides, tripeptides, and free forms of lysine and leucine may affect protein degradation by regulating the expression of muscle RING finger 1 (ubiquitin protein ligase-related gene) (Wei et al., 2020b). Similarly, in this study,

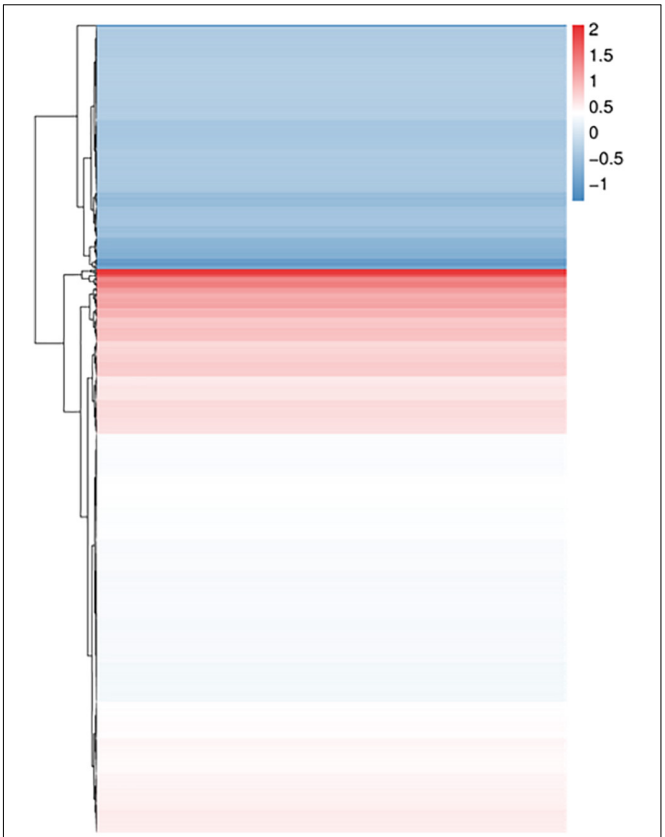


FIGURE 2 | Cluster analysis of differential proteins between the CAA and Lys-Leu treatments. Blue color means downregulation of differential proteins, and red color means upregulation of differential proteins.

differential proteins related to ubiquitin-mediated proteolysis were identified between the Lys-Leu and CAA groups (**Table 5**). In the ubiquitin proteasome pathway (UPP), there are distinct enzymatic components, among which E1 (ubiquitin-activating enzyme) is responsible for activating the C-terminal glycine of ubiquitin, E2s (ubiquitin carrier or conjugating proteins) are responsible for transporting the activated ubiquitin, and E3s (ubiquitin protein ligase) is responsible for catalyzing the covalent binding of ubiquitin to the substrate (Ciechanover et al., 2000; Lecker et al., 2006). Based on the differential proteins in the liver, different forms of lysine and leucine in the feed may regulate protein degradation *via* the E2s and E3s of the UPP.

Amino Acid Metabolism

Since this study focused on the different forms of lysine and leucine in feed, the metabolism of lysine and leucine in the liver was analyzed firstly. Lysine biosynthesis and degradation as well as branched-chain amino acid (valine, leucine, and isoleucine) metabolism were annotated using differential metabolites between dietary treatments (**Table 4**). The *de novo* biosynthesis of lysine contains two main pathways: the diaminopimelate and the α-aminoadipate pathways (Hall and da Costa, 2018). The differential metabolites showed that different forms of dietary lysine and leucine may affect the diaminopimelate pathway,

which belonged to the aspartate-derived biosynthetic family. For lysine degradation, the pipecolate and saccharopine pathways were considered as the main pathways in the liver (Pena et al., 2016). However, by combining the data of metabolomics and proteomics, it was shown that dietary lysine in dipeptide and free amino acid forms may not mainly affect the two degradation pathways of lysine but also affected the synthesis of carnitine using lysine as a precursor (Vaz and Wanders, 2002). For branched-chain amino acid metabolism, since these three amino acids (leucine, isoleucine, and valine) may share common transport systems and the same enzymes of degradation (Harper et al., 1984; Peganova and Eder, 2003), antagonistic effects had been found in some fish species (Yamamoto et al., 2004). In this study, a higher level of leucine and lower levels of valine and isoleucine in liver metabolites were observed in fish fed the Lys-Leu diet compared to fish fed the CAA diet, which indicated that different forms of dietary lysine and leucine may affect the antagonism between branched-chain amino acids.

In addition to the metabolism related to lysine and leucine, the data of metabolomics and proteomics showed that tryptophan metabolism, alanine, aspartate, and glutamate metabolism, arginine biosynthesis, arginine and proline metabolism, and glycine, serine, and threonine metabolism were identified between dietary treatments (**Tables 4**, **5**). For tryptophan metabolism, tryptophan, and leucine share 4F2hc/LAT1, which may lead to the concentrations of these two amino acids showing opposite trends in the liver (**Table 4**). A lower level of tryptophan in the Lys-Leu group may further affect tryptophan degradation in the downstream pathways of serotonin, indoleacetic acid, and kynuric acid (Hoseini et al., 2019). For alanine, aspartate, and glutamate metabolism, arginine biosynthesis, and arginine and proline metabolism, different forms of dietary lysine and leucine may mainly affect glutamine and glutaminase, which may be a central mediator metabolite and enzyme. Glutamine may not only be used as the carbon for arginine and proline synthesis but may also be converted into glutamate by glutaminase (Curthoys and Watford, 1995; Morris, 2016). In glycine, serine, and threonine metabolism, the choline–glycine pathway may be responsible for the methyl donor (Wang et al., 2013). According to metabolomics and proteomics data, dietary Lys-Leu upregulated the level of betaine and downregulated the enzyme activity of betaine-homocysteine S-methyltransferase. Thus, it implied that different forms of amino acids in the feed may affect the supply of methyl *via* the choline–glycine pathway.

CONCLUSION

In this study, a total of 1,140 (POS mode) and 661 (NEG mode) differential metabolites as well as a total of 618

TABLE 5 | Key differential proteins and their pathways annotated by KEGG.

Metabolic pathways	Count	Proteins	
		Downregulation[a]	Upregulation[b]
		Protein digestion and absorption	
Protein digestion and absorption	6	Trypsin, pancreatic elastase II, carboxypeptidase A1, carboxypeptidase A2, carboxypeptidase B, 4F2 antigen (4F2hc, SLC3A2)	
		Protein synthesis	
Mammalian target of rapamycin (mTOR) signaling pathway	4	Insulin, protein kinase B (Akt2), 4F2hc, Ras homolog gene family, member A	Tuberous sclerosis 2
		Protein degradation	
Ubiquitin-mediated proteolysis	6	Ubiquitin-conjugating enzyme E2 G1, E3 ubiquitin-protein ligase HERC1, F-box and WD-40 domain protein CDC4, cullin 4	Ubiquitin-conjugating enzyme E2 R, elongin-C
		Amino acid metabolism	
Lysine degradation	3	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1, collagen beta-1,O-galactosyltransferase	[Histone H3]-lysine4 N-trimethyltransferase ASH1L
Tryptophan metabolism	2		Indoleamine 2,3-dioxygenase, kynurenine–oxoglutarate transaminase
Alanine, aspartate and glutamate metabolism	3	Asparagine synthase (glutamine-hydrolyzing), glutaminase	Aspartoacylase
Arginine biosynthesis	2	Glutaminase	Nitric oxide synthase
Arginine and proline metabolism	4	Glycine amidinotransferase, guanidinoacetate N-methyltransferase, creatine kinase	Nitric oxide synthase
Glycine, serine, and threonine metabolism	7	Guanidinoacetate N-methyltransferase, glycine amidinotransferase, betaine-homocysteine S-methyltransferase, glyoxylate/hydroxypyruvate reductase, D-3-phosphoglycerate dehydrogenase, threonine 3-dehydrogenase	Glycine hydroxymethyltransferase

[a] *Downregulation indicates that the proteins of the Lys-Leu group are less abundant compared to the CAA group.*
[b] *Upregulation indicates that the proteins of the Lys-Leu group are more abundant compared to the CAA group.*

differential proteins were identified between the Lys-Leu and CAA treatments. Based on integrated data of differential metabolites and proteins, the protein- and amino acid-related metabolic processes of the liver included protein digestion and absorption, protein synthesis and degradation, and amino acid metabolism. In the process of protein metabolism, the differential proteins of the liver were related to proteolytic enzymes and amino acid transporters as well as mTORC1 and UPPs, respectively. In the process of amino acid metabolism, differential metabolites and proteins of the liver were related to lysine degradation, tryptophan metabolism, alanine, aspartate, and glutamate metabolism, arginine biosynthesis, arginine and proline metabolism, and glycine, serine, and threonine metabolism. Thus, the mechanism of the effects of dietary lysine and leucine on the growth of turbot can be understood from three aspects: protein digestion and absorption, protein synthesis and degradation, and amino acid metabolism. In addition, amino acid metabolism, in this study, revealed a good correlation between differential metabolites and protein using the approaches of integrated LC–MS/MS-based metabolomics and iTRAQ-based proteomics.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD025276.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the Yellow Sea Fisheries Research Institute.

AUTHOR CONTRIBUTIONS

ML, YW, and HX conceived and designed this experiment, drafted and reviewed the manuscript. BL accomplished turbot rearing, the feeding trial, and the sampling process. All authors reviewed and commented on the manuscript.

FUNDING

This research was supported by the National Natural Science Foundation of China (31902387, 31972803, and 31672663), the Central Public-Interest Scientific Institution Basal Research Fund, CAFS (2020TD48), and China Agriculture Research System of MOF and MARA (CARS-47).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.691404/full#supplementary-material

REFERENCES

Ceciliani, F., Lecchi, C., Urh, C., and Sauerwein, H. (2018). Proteomics and metabolomics characterizing the pathophysiology of adaptive reactions to the metabolic challenges during the transition from late pregnancy to early lactation in dairy cows. *J. Proteomics* 178, 92–106. doi: 10.1016/j.jprot.2017.10.010

Chen, X. Q., Elsheikha, H. M., Hu, R. S., Hu, G. X., Guo, S. L., Zhou, C. X., et al. (2018). Hepatic metabolomics investigation in acute and chronic murine toxoplasmosis. *Front. Cell. Infect. Microbiol.* 8:189. doi: 10.3389/fcimb.2018.00189

Ciechanover, A., Orian, A., and Schwartz, A. L. (2000). Ubiquitin-mediated proteolysis: biological regulation via destruction. *Bioessays* 22, 442–451. doi: 10.1002/(SICI)1521-1878(200005)22:5<442::AID-BIES6>3.0.CO;2-Q

Cohen, T., Gertler, A., and Birk, Y. (1981). Pancreatic proteolytic enzymes from carp (*Cyprinus carpio*)-II. Kinetic properties and inhibition studies of trypsin, chymotrypsin and elastase. *Comp. Biochem. Physiol. B* 69, 647–653. doi: 10.1016/0305-0491(81)90365-5

Curthoys, N. P., and Watford, M. (1995). Regulation of glutaminase activity and glutamine metabolism. *Annu. Rev. Nutr.* 15, 133–159. doi: 10.1146/annurev.nu.15.070195.001025

Dabrowski, K., Lee, K. J., and Rinchard, J. (2003). The smallest vertebrate, teleost fish, can utilize synthetic dipeptide-based diets. *J. Nutr.* 133, 4225–4229. doi: 10.1093/jn/133.12.4225

Daniel, N. (2017). Status of aquaculture with respect to nutrition and feed. *Int. J. Fish. Aquat. Stud.* 5, 333–345.

de Vareilles, M., Conceição, L. E., Gómez-Requeni, P., Kousoulaki, K., Richard, N., Rodrigues, P. M., et al. (2012). Dietary lysine imbalance affects muscle proteome in zebrafish (*Danio rerio*): a comparative 2D-DIGE study. *Mar. Biotechnol.* 14, 643–654. doi: 10.1007/s10126-012-9462-3

Deborde, C., Hounoum, B. M., Moing, A., Maucourt, M., Jacob, D., Corraze, G., et al. (2021). Putative imbalanced amino acid metabolism in rainbow trout long term fed a plant-based diet as revealed by 1H-NMR metabolomics. *J. Nutr. Sci.* 10:e13. doi: 10.1017/jns.2021.3

Dong, X., Liu, Q., Kan, D., Zhao, W., Guo, H., and Lv, L. (2020). Effects of ammonia-N exposure on the growth, metabolizing enzymes, and metabolome of *Macrobrachium rosenbergii*. *Ecotoxicol. Environ. Safe.* 189:110046. doi: 10.1016/j.ecoenv.2019.110046

El-Husseiny, O., El-Haroun, E. R., Goda, A., Hassan, I., Woodward, B., and Suloma, A. (2017). Effects of lysine and tryptophan supplementations in plant protein-based diets on the performance of Nile tilapia (*Oreochromis niloticus*). *J. Appl. Aquac.* 29, 266–276. doi: 10.1080/10454438.2017.1364684

Fotiadis, D., Kanai, Y., and Palacín, M. (2013). The SLC3 and SLC7 families of amino acid transporters. *Mol. Aspects Med.* 34, 139–158. doi: 10.1016/j.mam.2012.10.007

Furuya, W. M., Pezzato, L. E., Barros, M. M., Pezzato, A. C., Furuya, V. R., and Miranda, E. C. (2004). Use of ideal protein concept for precision formulation of amino acid levels in fish-meal-free diets for juvenile Nile tilapia (*Oreochromis niloticus* L.). *Aquac. Res.* 35, 1110–1116. doi: 10.1111/j.1365-2109.2004.01133.x

Gómez-Requeni, P., de Vareilles, M., Kousoulaki, K., Jordal, A. E. O., Conceição, L. E., and Rønnestad, I. (2011). Whole body proteome response to a dietary lysine imbalance in zebrafish Danio rerio. *Comp. Biochem. Physiol. Part D Genomics Proteomics* 6, 178–186. doi: 10.1016/j.cbd.2011.02.002

Guo, C., Sun, D., Wang, X., and Mao, S. (2019). A combined metabolomic and proteomic study revealed the difference in metabolite and protein expression profiles in ruminal tissue from goats fed hay or high-grain diets. *Front. Physiol.* 10:66. doi: 10.3389/fphys.2019.00066

Hall, C. J., and da Costa, T. P. S. (2018). Lysine: biosynthesis, catabolism and roles. *Wiki J. Sci.* 1:4. doi: 10.15347/wjs/004

Harper, A. E., Miller, R., and Block, K. P. (1984). Branched-chain amino acid metabolism. *Ann. Rev. Nutr.* 4, 409–454. doi: 10.1146/annurev.nu.04.070184.002205

Hoseini, S. M., Pérez-Jiménez, A., Costas, B., Azeredo, R., and Gesto, M. (2019). Physiological roles of tryptophan in teleosts: current knowledge and perspectives for future studies. *Rev. Aquacult.* 11, 3–24. doi: 10.1111/raq.12223

Kamaszewski, M., Ostaszewska, T., Napora-Rutkowski, Ł, Wójcik, M., and Dabrowski, K. (2019). The role of dipeptide on fish growth and digestive enzyme activity modulation in common carp (*Cyprinus carpio* L.). *Anim. Sci. Pap. Rep.* 37, 75–86.

Kamaszewski, M., Prasek, M., Ostaszewska, T., and Dabrowski, K. (2014). The influence of feeding diets containing wheat gluten supplemented with dipeptides or free amino acids on structure and development of the skeletal muscle of carp (*Cyprinus carpio*). *Aquac. Int.* 22, 259–271. doi: 10.1007/s10499-013-9683-0

Kamaszewski, M., Wójcik, M., Krawczyńska, A., and Ostaszewska, T. (2020). The influence of diet containing wheat gluten supplemented with dipeptides or amino acids on the morphology of white muscle of yellow perch (*Perca flavescens*). *Animals* 10:388. doi: 10.3390/ani10030388

Kim, J., and Guan, K. L. (2011). Amino acid signaling in TOR activation. *Annu. Rev. Biochem.* 80, 1001–1032. doi: 10.1146/annurev-biochem-062209-094414

Kim, S. S., and Lee, K. J. (2013). Comparison of leucine requirement in olive flounder (*Paralichthys olivaceus*) by free or synthetic dipeptide forms of leucine. *Anim. Feed Sci. Tech.* 183, 195–201. doi: 10.1016/j.anifeedsci.2013.05.008

Laplante, M., and Sabatini, D. M. (2012). mTOR signaling in growth control and disease. *Cell* 149, 274–293. doi: 10.1016/j.cell.2012.03.017

Lecker, S. H., Goldberg, A. L., and Mitch, W. E. (2006). Protein degradation by the ubiquitin–proteasome pathway in normal and disease states. *J. Am. Soc. Nephrol.* 17, 1807–1819. doi: 10.1681/ASN.2006010083

Ma, X. M., and Blenis, J. (2009). Molecular mechanisms of mTOR-mediated translational control. *Nat. Rev. Mol. Cell Bio.* 10, 307–318. doi: 10.1038/nrm2672

Mamauag, R. E. P., Gao, J., Thanh Nguyen, B., Ragaza, J. A., Koshio, S., Ishikawa, M., et al. (2012). Supplementations of dl-methionine and methionine dipeptide in diets are effective for the development and growth of larvae and juvenile red sea bream, Pagrus major. *J. World Aquac. Soc.* 43, 362–374. doi: 10.1111/j.1749-7345.2012.00563.x

Min, C. W., Hyeon, H., Gupta, R., Park, J., Cheon, Y. E., Lee, G. H., et al. (2020). Integrated proteomics and metabolomics analysis highlights correlative metabolite-protein networks in soybean seeds subjected to warm-water soaking. *J. Agric. Food Chem.* 68, 8057–8067. doi: 10.1021/acs.jafc.0c00986

Morris, S. M. Jr. (2016). Arginine metabolism revisited. *J. Nutr.* 146, 2579S–2586S. doi: 10.3945/jn.115.226621

Neurath, H. (1984). Evolution of proteolytic enzymes. *Science* 224, 350–357. doi: 10.1126/science.6369538

Ostaszewska, T., Dabrowski, K., Kamaszewski, M., Kwasek, K., Grodzik, M., and Bierla, J. (2013). The effect of dipeptide, Lys-Gly, supplemented diets on digestive tract histology in juvenile yellow perch (*Perca flavescens*). *Aquac. Nutr.* 19, 100–109. doi: 10.1111/j.1365-2095.2012.00948.x

Peganova, S., and Eder, K. (2003). Interactions of various supplies of isoleucine, valine, leucine and tryptophan on the performance of laying hens. *Poultry Sci.* 82, 100–105. doi: 10.1093/ps/82.1.100

Pena, I. A., Marques, L. A., Laranjeira, A. B., Yunes, J. A., Eberlin, M. N., and Arruda, P. (2016). Simultaneous detection of lysine metabolites by a single LC–MS/MS method: monitoring lysine degradation in mouse plasma. *Springerplus* 5:172. doi: 10.1186/s40064-016-1809-1

Peres, H., and Oliva-Teles, A. (2005). The effect of dietary protein replacement by crystalline amino acid on growth and nitrogen utilization of turbot *Scophthalmus maximus* juveniles. *Aquaculture* 250, 755–764. doi: 10.1016/j.aquaculture.2005.04.046

Rahimnejad, S., and Lee, K. J. (2014). Comparison of free and dipeptide lysine utilization in diets for juvenile olive flounder *Paralichthys olivaceus*. *Fish. Aquat. Sci.* 17, 433–439. doi: 10.5657/FAS.2014.0433

Rodrigues, P. M., Silva, T. S., Dias, J., and Jessen, F. (2012). Proteomics in aquaculture: applications and trends. *J. Proteomics* 75, 4325–4345. doi: 10.1016/j.jprot.2012.03.042

Sales, N. M. R., Pelegrini, P. B., and Goersch, M. C. (2014). Nutrigenomics: definitions and advances of this new science. *J. Nutr. Metabol.* 2014:202759. doi: 10.1155/2014/202759

Savitski, M. M., Wilhelm, M., Hahne, H., Kuster, B., and Bantscheff, M. (2015). A scalable approach for protein false discovery rate estimation in large proteomic data sets. *Mol. Cell. Proteomics* 14, 2394–2404. doi: 10.1074/mcp.M114.046995

Vaz, F. M., and Wanders, R. J. (2002). Carnitine biosynthesis in mammals. *Biochem. J.* 361, 417–429. doi: 10.1042/bj3610417

Wang, W., Wu, Z., Dai, Z., Yang, Y., Wang, J., and Wu, G. (2013). Glycine metabolism in animals and humans: implications for nutrition and health. *Amino Acids* 45, 463–477. doi: 10.1007/s00726-013-1493-1

Wei, Y., Li, B., Xu, H., and Liang, M. (2020a). Effects of lysine and leucine in free and different dipeptide forms on the growth, amino acid profile and transcription of intestinal peptide, and amino acid transporters in turbot (*Scophthalmus maximus*). *Fish Physiol. Biochem.* 46, 1795–1807. doi: 10.1007/s10695-020-00828-2

Wei, Y., Liang, M., Mai, K., Zheng, K., and Xu, H. (2017a). 1H NMR—based metabolomics studies on the effect of size-fractionated fish protein hydrolysate, fish meal and plant protein in diet for juvenile turbot (*Scophthalmus maximus* L.). *Aquac. Nutr.* 23, 523–536. doi: 10.1111/anu.12420

Wei, Y., Liang, M., Mai, K., Zheng, K., and Xu, H. (2017b). The effect of ultrafiltered fish protein hydrolysate levels on the liver and muscle metabolic profile of juvenile turbot (*Scophthalmus maximus* L.) by 1H NMR-based metabolomics studies. *Aquac. Res.* 48, 3515–3527. doi: 10.1111/are.13178

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. *Aquac. Nutr.* 26, 358–367. doi: 10.1111/anu.12998

Wen, B., Mei, Z., Zeng, C., and Liu, S. (2017). metaX: a flexible and comprehensive software for processing metabolomics data. *BMC Bioinformatics* 18:183. doi: 10.1186/s12859-017-1579-y

Wen, B. O., Zhou, R., Feng, Q., Wang, Q., Wang, J., and Liu, S. (2014). IQuant: an automated pipeline for quantitative proteomics based upon isobaric tags. *Proteomics* 14, 2280–2285. doi: 10.1002/pmic.201300361

Wu, X., Pan, X., Cao, S., Xu, F., Lan, L., Zhang, Y., et al. (2019). iTRAQ-based quantitative proteomic analysis provides insights into strong broodiness in Muscovy duck (*Cairina moschata*) combined with metabolomics analysis. *J. Proteomics* 204, 103401. doi: 10.1016/j.jprot.2019.103401

Yamamoto, T., Shima, T., and Furuita, H. (2004). Antagonistic effects of branched-chain amino acids induced by excess protein-bound leucine in diets for rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 232, 539–550. doi: 10.1016/S0044-8486(03)00543-X

Yu, K., Matzapetakis, M., Horvatić, A., Terré, M., Bach, A., Kuleš, J., et al. (2020). Metabolome and proteome changes in skeletal muscle and blood of pre-weaning calves fed leucine and threonine supplemented diets. *J. Proteomics* 216, 103677. doi: 10.1016/j.jprot.2020.103677

Zambonino Infante, J. L., and Cahu, C. L. (2007). Dietary modulation of some digestive enzymes and metabolic processes in developing marine fish: applications to diet formulation. *Aquaculture* 268, 98–105. doi: 10.1016/j.aquaculture.2007.04.032

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.

frontiers in Marine Science

ORIGINAL RESEARCH
published: 10 August 2021
doi: 10.3389/fmars.2021.706463

Evaluation of the Dietary Black Soldier Fly Larvae Meal (*Hermetia illucens*) on Growth Performance, Intestinal Health, and Disease Resistance to *Vibrio parahaemolyticus* of the Pacific White Shrimp (*Litopenaeus vannamei*)

Yongkang Chen[1], *Shuyan Chi*[1,2,3], *Shuang Zhang*[1,2,3], *Xiaohui Dong*[1,2,3], *Qihui Yang*[1,2,3], *Hongyu Liu*[1,2,3], *Beiping Tan*[1,2,3]* *and Shiwei Xie*[1,2,3]*

[1] *Laboratory of Aquatic Nutrition and Feed, College of Fisheries, Guangdong Ocean University, Zhanjiang, China,* [2] *Aquatic Animals Precision Nutrition and High-Efficiency Feed Engineering Research Centre of Guangdong, Zhanjiang, China,* [3] *Key Laboratory of Aquatic, Livestock and Poultry Feed Science and Technology in South China, Ministry of Agriculture, Zhanjiang, China*

The present study evaluated the effects of the dietary black soldier fly larvae meal (BSF) on growth performance, intestinal health, and susceptibility to *Vibrio parahaemolyticus* in the Pacific white shrimp *Litopenaeus vannamei*. The basal diet was formulated to contain 25% fish meal (FM), and then the FM was replaced with BSF for 10, 20, and 30% of the FM protein in the experimental diets, which are referred to as FM, BSF10, BSF20, and BSF30, respectively. Four hundred and eighty healthy and uniform-sized shrimp (∼0.88 g) were distributed among four groups of three replicates, each with 40 shrimp in a 300-L tank and they were fed four times daily for 7 weeks. The results showed that the growth performance did not change significantly in shrimp fed with BSF10 and BSF20 diets, but significantly decreased in those fed with BSF30 diet compared to the ones fed with FM diet. After feeding trial and sampling, a *V. parahaemolyticus* infection challenge trial was conducted on shrimp. The results showed that the survival rate of shrimp fed with BSF10 was significantly higher than those fed with FM. The results of the midgut histology showed that the width and height of intestinal mucosal folds decreased significantly in shrimp fed with BSF20 and BSF30, and the early signs of apoptosis in the intestinal cells were found in shrimp fed with BSF30. The mRNA levels of non-specific immune-related genes *dorsal* and *relish* were downregulated in shrimp fed with BSF20 and BSF30 diets. The mRNA levels of antimicrobial peptides–related genes *alf* (anti-lipopolysaccharide factor) were upregulated in shrimp fed with BSF10 but downregulated in shrimp fed with BSF30. The mRNA level *pen3* (penaeidins 3) was upregulated in shrimp fed with BSF10 and BSF20 diets. The intestinal bacterial communities on operational

OPEN ACCESS

Edited by:
Kang-le Lu,
Jimei University, China

Reviewed by:
Ana Teresa Gonçalves,
GreenCoLAB, Portugal
Wuying Chu,
Changsha University, China

***Correspondence:**
Shiwei Xie
xswzsdx@163.com
Beiping Tan
bptan@126.com

Specialty section:
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

Received: *07 May 2021*
Accepted: *15 July 2021*
Published: *10 August 2021*

Citation:
Chen Y, Chi S, Zhang S, Dong X, Yang Q, Liu H, Tan B and Xie S (2021) Evaluation of the Dietary Black Soldier Fly Larvae Meal (Hermetia illucens) on Growth Performance, Intestinal Health, and Disease Resistance to Vibrio parahaemolyticus of the Pacific White Shrimp (Litopenaeus vannamei). Front. Mar. Sci. 8:706463. doi: 10.3389/fmars.2021.706463

taxonomic unit levels among groups were not significantly differentiated according to the beta diversity analysis. At the genus levels, a decrease in *Vibrio*, *Photobacterium*, and *Candidatus_Bacilloplasma*, as well as the increase in *Bacillus* and *Pseudoalteromonas* abundance, indicated the improvement of intestinal microbiota in shrimp fed with dietary BSF. Therefore, the use of BSF in shrimp diet should be controlled at a dosage of 20% of the FM, which can improve the intestinal microbiota without causing any negative effects.

Keywords: *Litopenaeus vannamei*, black soldier fly larvae meal, intestinal histology, non-specific immune, intestinal microbiota

INTRODUCTION

Pacific white shrimp *Litopenaeus vannamei* has become an important species in global aquaculture due to the advantages of its rapid growth and strong stress resistance (FAO, 2020). However, some gastrointestinal diseases, such as white feces syndrome (WFS) (Hou et al., 2018) and early mortality syndrome (EMS) (Zorriehzahra and Banaederakhshan, 2015) have caused significant losses to shrimp farming in recent years. As a vital organ for nutrient uptake and immune defense, the intestinal structure and biochemical barrier are closely related to the health status of the shrimp (Suo et al., 2017).

The intestinal structure and the biochemical barrier refer to the integrity of the histological structure of the intestine. The midgut of shrimp consists of the outer epidermis, muscular layers, protruding mucosal folds, and the inner epidermis, and an intact intestinal structure forms the basis for the functioning of the intestine (Xie et al., 2020). In addition, the immune function of the gut should be considered, as pathogens in the external environment can pass through the intestine (Pilotto et al., 2020). The antimicrobial peptides such as *alf* (anti-lipopolysaccharide factor), *cru* (crustins), and *pen3* (penaeidins 3) are regulated by secreted cytokines and immune signaling pathways, such as the Toll and IMD (immune deficiency). When Toll and IMD pathways are activated in early response to pathogens, the expression of downstream factors *dorsal* and *relish* can regulate pathways activities (Qiu et al., 2014). Intestinal microbiota are an important part of the intestinal biochemical barrier (Qi et al., 2017). The intestinal microbiota have been extensively studied and shown to benefit aquatic animals with its functions in immune response, nutrient uptake, and homeostasis maintenance (Li et al., 2018), which is mainly influenced by environmental and dietary factors (Egerton et al., 2018). Although close contact with the aquatic environment may influence the early gut colonization (Roeselers et al., 2011), feed causes a continuous effect on the intestinal microbiota composition over a longer culture period (Ringø et al., 2016).

In shrimp diets, fish meal (FM) is the most commonly used protein source, usually accounting for 25–35% of the formulation (Xie et al., 2016). The depletion and shortage of fishery resources and the high cost of FM have encouraged researchers to look for alternatives (Cai et al., 2020). In the last decade, experimental studies on feeding of insect meal–based diets have been carried out in *L. vannamei*, which has been conducted on mealworm (*Tenebrio molitor*), silkworm pupae (*Bombyx mori* L.), as well as fly maggots (Cao et al., 2012; Choi et al., 2018; Rahimnejad et al., 2019). Besides, the black soldier fly is a saprophytic insect (*Hermetia illucens*) commonly used for the sustainable recycling of animal waste and other organic wastes. Its larvae are rich in proteins and lipids, and also it is considered as a potential protein source for aquatic animals (Barragan-Fonseca et al., 2017). Several studies have been conducted to evaluate the effectiveness of BSF as an FM replacement in the diets of *L. vannamei*. In general, most growth responses could be obtained if the amount of BSF replacement FM is limited to 25% of the ratio without changing the nutrition levels of the diet (Cummins et al., 2017). Another study showed that BSF substitution of <30% FM in diets did not negatively affect the growth performance of *L. vannamei* (Hu et al., 2019). Also, dietary BSF has been shown to affect the intestinal health status; however, it causes different effects on different animals. In previous studies, a total replacement of the FM with BSF did not impair the intestinal health of Atlantic salmon (*Salmo salar* L.) (Li et al., 2020). However, replacement of 75% FM with defatted BSF led to significant pathological changes in the intestinal wall of Jian carp (*Cyprinus carpio* var. Jian) (Li et al., 2017). It is still unclear how dietary BSF affects the intestinal health of *L. vannamei* and our experiments were conducted to study the effect of BSF on the growth performance, intestinal structure, and immunity as well as the intestinal microbiota communities of *L. vannamei*.

MATERIALS AND METHODS

Ethics Statement

This study was carried out following the recommendations of Care and Use of Laboratory Animals in China, Animal Ethical and Welfare Committee of China Experimental Animal Society. The protocol was approved by the Animal Ethical and Welfare Committee of Guangdong Ocean University, Guangdong, China.

Diet Preparation

The black soldier fly larvae meal used in this experiment was provided by (Guangzhou Fishtech Biotechnology Co., Ltd, Guangdong, China) and contained 35.17% crude protein and 32.60% crude lipid. The basal diet was formulated to contain 25% FM and then the FM was replaced with BSF for 10, 20, and 30% of the FM protein, which we referred to as FM, BSF10, BSF20, and BSF30, respectively. With the increase of BSF addition, the level of fish oil, soybean oil, and soybean lecithin was gradually reduced to balance the crude lipid levels among the four diets.

Several essential amino acids were supplemented to the BSF diets to obtain similar amino acid profiles as the FM diet. Diets were processed as follows: all the ingredients were smashed and sifted out with an 80-mesh sieve. Then, all the ingredients were weighed, combined, and then mixed to homogeneity (M-256, South China University of Technology, Guangdong, China). It is important to note that BSF and soybean meal were mixed (1:1) and then crushed and sieved before being mixed with other ingredients. The 1.0- and 1.5-mm diameter pellets were extruded using a pelletizer (Institute of Chemical Engineering, South China University of Technology, Guangdong, China) and then ripened in an electric oven at 60°C for 30 min, and stored frozen (−20°C) before use. Formulation and proximate composition of the diets are given in **Table 1**.

Shrimp and Experimental Conditions

Juvenile *L. vannamei* were obtained from the nursery base of Guangdong Yuehai Seed Co., Ltd (Zhanjiang, China). The shrimp were fed with commercial feed (Guangdong HAID Group, crude protein 48.0%, crude fat 8.0%) and acclimatized to the experimental conditions for a week. After starvation treatment for 24 h, healthy and uniform-sized juvenile *L. vannamei* (∼0.88 g) were distributed among four groups with triplicate fiberglass tanks (300 L) per group and 40 shrimp in each tank. Shrimp were fed to apparent satiation four times daily at 7:20, 11:20, 16:00, and 21:00 h for 7 weeks. In the first 4 weeks, shrimp were fed 1.0 mm diameter feed, while they were fed 1.5 mm diameter feed in the last 3 weeks. During the experiment, 60% of the water was exchanged each day by seawater disinfected with chlorine dioxide to maintain water quality. Water temperature and salinity were measured every day in a range of 27–31°C and 25–30%, respectively.

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

Ingredients	Diets			
	FM	BSF10	BSF20	BSF30
Brown fish meal[a]	25.0	22.5	20.0	17.5
Soybean meal	25	25	25	25
Peanut meal	10	10	10	10
Wheat flour	24.05	22.93	21.82	20.70
Beer yeast	3	3	3	3
Shrimp shell meal	5	5	5	5
Black soldier fly larvae meal[b]	0	4.75	9.5	14.25
Fish oil	2.00	1.45	0.90	0.35
Soybean oil	2.00	1.45	0.90	0.35
Soybean lecithin	1.00	0.85	0.70	0.55
Choline chloride	0.3	0.3	0.3	0.3
Vitamin and Mineral Premix[c]	1	1	1	1
Calcium monophosphate	1.5	1.5	1.5	1.5
Vitamin C	0.1	0.1	0.1	0.1
Methionine[d]	0	0.04	0.07	0.11
Lysine[d]	0	0.08	0.16	0.24
Threonine[d]	0	0	0	0.01
Ethoxyquin	0.05	0.05	0.05	0.05
Proximate composition				
Dry matter (%)	92.01	92.47	92.71	92.62
Crude protein (% DM)	41.42	41.95	42.51	42.31
Crude lipid (% DM)	7.79	7.11	7.04	7.29
Ash (% DM)	10.73	11.01	11.10	11.67

[a] *Brown fishmeal: 68.21% crude protein, 9.00% crude lipid, bought from Zhanjiang HaiBao Feed Co. Ltd., Zhanjiang, Guangdong, China.*

[b] *Black soldier fly larvae meal: 35.17% crude protein, 32.60% crude lipid, provided by Guangzhou Fishtech Biotechnology Co., Ltd., Guangzhou, China.*

[c] *Vitamin and Mineral Premix (per kg of diet): thiamine, 5 mg; riboflavin, 10 mg; vitamin A, 5,000 IU; vitamin E, 40 mg; vitamin D3, 1,000 IU; menadione, 10 mg; pyridoxine, 10 mg; biotin, 0.1 mg; cyanocobalamin, 0.02 mg; calcium pantothenate, 20 mg; folic acid, 1 mg; niacin, 40 mg; vitamin C, 150 mg; iron, 100 mg; iodine, 0.8 mg; cupper, 3 mg; zinc, 50 mg; manganese, 12 mg; selenium, 0.3 mg; cobalt, 0.2 mg.*

[d] *All the crystalline amino acids were bought from Shanghai Sanjie Biotechnology Co., Ltd, China.*

Sample Collection and Analysis

Diets of each group were collected for moisture, crude protein, crude lipid, ash, amino acid analysis, and fatty acid profiles. Crude protein was determined by the Dumas Nitrogen method with a Primacs100 analyzer (Skalar, Dutch); crude lipid was determined by the ether extraction method with an XT15 extractor (Ankom, United States); moisture was determined by oven drying at 105°C. Amino acid composition was determined according to the standard of GB/T 18246-2019 in the national standard of P. R. China (**Table 2**). Samples were hydrolyzed in 6 N HCl at 110°C for 22 h, then separated by the ion exchange column and reaction with ninhydrin solution, and the concentrations of amino acids were obtained through spectrophotometry. Fatty acids composition of diets and ingredients were determined by GB/T 5009.168-2016 in the

TABLE 2 | Amino acid compositions of experimental diets (% dry matter).

Amino acids	Diets			
	FM	BSF10	BSF20	BSF30
Aspartic acid	4.12	4.23	4.08	3.89
Threonine	1.68	1.74	1.66	1.55
Serine	1.90	1.99	1.90	1.79
Glutamic acid	7.49	7.72	7.41	7.00
Glycine	2.09	2.13	2.06	2.02
Alanine	2.15	2.22	2.15	2.12
Cystine	0.50	0.50	0.51	0.46
Valine	2.03	2.14	1.98	1.98
Methionine	0.70	0.71	0.68	0.65
Isoleucine	1.72	1.77	1.69	1.62
Leucine	3.12	3.22	3.06	2.95
Tyrosine	1.12	1.22	1.37	1.24
Phenylalanine	1.93	2.00	1.87	1.82
Lysine	2.63	2.64	2.64	2.60
Histidine	1.10	1.06	1.02	1.04
Arginine	2.81	2.87	2.92	2.69
Proline	1.90	2.05	1.95	1.98
Total	38.99	40.22	38.96	37.39

national standard of the P. R. China with the assistance of Willtest Technology Co. Ltd., Sichuan, China (**Table 3**).

After the feeding trial, the shrimp in each tank were counted and weighed. Ten shrimp from each tank were randomly selected and the intestines were removed on ice, immediately placed in liquid nitrogen, and transferred to −80°C for RNA extraction and intestinal microbiota analysis.

Challenge Trial

Prior to the pathogen challenge trial, the shrimp fed with commercial diets (mentioned above, crude protein 48.0%, crude fat 8.0%) and raised under the same conditions were divided into four subgroups ($n = 10$), injected with graded concentrations of *Vibrio parahaemolyticus* (10^6, 10^7, 10^8, and 10^9 CFU/ml for 40 μl) and continually monitored for 1 week to determine the 50% lethal concentration. After the feeding trial, 10 shrimp from each tank were injected with 40 μl *V. parahaemolyticus* (5×10^8 CFU/ml) for the challenge trial. The challenge trial continued for 12 days after injection to finalize the survival rate.

Intestinal Histology Analysis

In each tank, midguts from three shrimp were fixed in Bouin's fixative solution for 24 h and then transferred to 70% ethanol. Then gut samples were dehydrated in a graded series of ethyl alcohol and embedded in paraffin. Sections were stained with H&E and observed under a microscope (Nikon Ni-U, Japan). Another three midguts were collected for transmission electron microscopy (TEM) examination. The method was described by Xie et al. (2020), briefly, samples were fixed in 2.5% glutaraldehyde solution (4°C) for 24 h, post-fixed in 1% osmium tetroxide (OsO_4) for 1 h, dehydrated in a graded series of ethyl alcohol, and finally embedded with resin. Ultrathin sections (90 nm) were placed on copper grids and were stained with saturated uranyl acetate solution for 30 min, rinsed with distilled water, and post stained with lead citrate for 30 min. Ultrathin sections were screened and observed with a TEM (Hitachi HT7700 TEM, Japan). The statistical study of intestinal histology was carried out by measuring the height of microvilli, height and width of mucosal folds, as well as the thickness of muscle layer by ImageJ software. Each index was randomly measured six times in one section, and one-way ANOVA followed by Tukey's multiple-range test was used to determine significant differences among groups. The probability value of $P < 0.05$ was deemed to be statistically significant.

Quantitative Real-Time PCR Analysis

Total RNA of intestine was extracted from four shrimp as a pooled sample in each tank using the TransZol Up Plus RNA kit (TransGen Biotech Co., Ltd, China). Agarose gel electrophoresis and spectrophotometric analysis (Nanodrop 2000, Thermo, USA) were used to assess the RNA quality and concentration. cDNA was synthesized using a PrimeScript RT reagent kit with gDNA Eraser (Takara, Japan), according to the instructions of the manufacturer. Briefly, oligo dT primers and random 6-mers were used to reverse transcribe 1,000 ng RNA in the presence

TABLE 3 | Fatty acid compositions of ingredients and diets (% of total fatty acids).

Fatty acids	Ingredients		Diets			
	Fishmeal	Black soldier fly larvae meal	FM	BSF10	BSF20	BSF30
12:0	0.10	19.50	0.06	3.59	6.99	10.20
14:0	7.52	4.53	2.19	2.86	3.57	3.87
15:0	0.64	0.20	0.24	0.25	0.25	0.25
16:0	22.90	21.00	16.60	17.90	19.20	20.60
18:0	5.20	3.95	5.01	4.77	4.58	4.43
20:0	0.21	0.17	0.44	0.39	0.35	0.32
ΣSFA	36.56	49.34	24.54	29.76	34.95	39.68
16:1n-7	8.38	2.54	2.98	2.98	3.03	2.98
18:1n-9	7.65	24.00	14.70	16.40	17.90	19.60
22:1n-9	0.14	0.01	0.11	0.10	0.06	0.04
ΣMUFA	16.17	26.55	17.79	19.48	20.99	22.62
18:2n-6	3.06	19.60	29.20	27.20	25.40	24.00
20:4n-6	1.32	0.32	0.78	0.70	0.59	0.50
ΣPUFA n-6	4.38	19.92	29.98	27.90	25.99	24.50
18:3n-3	1.10	1.79	3.94	3.52	3.02	2.68
20:5n-3	0.23	0.46	11.30	8.94	6.60	4.20
22:6n-3	19.50	0.07	8.95	7.23	5.52	3.73
ΣPUFA n-3	20.83	2.32	24.19	19.69	15.14	10.61
n-3/n-6	4.76	0.12	0.81	0.71	0.58	0.43

ΣSFA, ΣMUFA, ΣPUFA, ΣPUFA n-3, and ΣPUFA n-6 are the sum of saturated, monounsaturated, polyunsaturated, polyunsaturated n3, and polyunsaturated n6, respectively.

of PrimeScript RT enzyme Mix I, 5×PrimeScript buffer, and RNase-free H_2O at 37°C for 15 min, following inactivation at 85°C for 5 s.

Real-time PCR for the target genes was performed using a SYBR Green Premix Pro Taq HS qPCR Kit II (Accurate Biotechnology Hunan Co., Ltd, China) and quantified on the LightCycler 480 (Roche Applied Science, Switzerland) using the following program: 0.5 μM of forward- and reverse-specific primers, 5 μl of 2×SYBR Green Pro Taq HS Premix II, 10 ng of cDNA template and nuclease-free water to a final volume of 10 μl, denaturation step at 95°C for the 30 s, followed by 40 amplification cycles of 5 s denaturation at 95°C, 30 s annealing at 60°C, followed by a melt-curve analysis, and cooling to 4°C. *β-actin* was used as the reference gene according to Xie et al. (2019). Primers of *relish* and *dorsal* were referred to a previous study (Qiu et al., 2014), while other primers were designed by Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (**Table 4**).

Intestinal Microbiota Analysis

Microbial DNA of the intestine from six shrimp as two pooled samples in each tank was extracted using E.Z.N.A. stool DNA Kit (Omega Bio-tek, Inc., United States) following the protocols of the manufacturer. To analyze the microbial population, the V3–V4 variable region of the 16S ribosomal RNA gene was amplified by the primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 806R (5′-GGACTACHVGGGTATCTAAT-3′) where the barcode is an eight-base sequence unique to each sample. The reactions were carried out in triplicate 50 μl mixture containing 100 ng of template DNA, 5 μl of 10 × KOD Buffer, 5 μl of 2.5 mM dNTPs, 1.5 μl of each primer (5 μM), and 1 μl of KOD polymerase. The PCR was run as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 98°C for 10 s, 62°C for 30 s, and 68°C for 30 s, followed by a final extension at 68°C for 5 min.

High-throughput sequencing of the purified PCR products was carried out using Illumina NovaSeq 6000 sequencing system. Amplicons were extracted from 2% agarose gels and purified using the AMPure XP Beads (Beckman Agencourt, United States) following the protocols of the manufacturer and quantified using ABI StepOnePlus Real-Time PCR System (Life Technologies, Foster City, United States). Purified amplicons were pooled in equimolar and paired-end sequenced (PE250) in the Illumina platform according to the standard protocols. After sequencing, the raw reads containing more than 10% of unknown nucleotides or containing less than 50% of bases with quality (Q-value) > 20 were removed, and the paired-end clean reads were merged as raw tags using FLASH (version 1.2.11) (Magoč and Salzberg, 2011). Raw tags were filtered following Bokulich et al.'s (2013) condition to obtain high-quality clean tags. The clean tags were clustered into operational taxonomic units (OTUs) of ≥97% similarity using UPARSE (version 9.2.64) pipeline (Edgar, 2013). All chimeric tags were removed using UCHIME algorithm and finally obtained effective tags for further analysis. The tag sequence with the highest abundance was selected as a representative sequence within each cluster. The representative OTU sequences were classified into organisms using RDP classifier based on SILVA database (version 132), with the confidence threshold value of 0.8. Among groups, Venn analysis was performed in R project Venn Diagram package (version 1.6.16) to identify unique and common species of OTUs. The stacked bar plot of the community composition on phylum level was visualized in the R project ggplot2 package (version 2.2.1). Heatmap of genus abundance was plotted using pheatmap package (version 1.0.12) in R project. Alpha diversity analysis, including ACE, Chao1, Simpson, and Shannon were calculated in QIIME (version 1.9.1). The alpha index comparison among groups was computed by Tukey's HSD test in SPSS 21.0. The principal coordinates analysis (PCoA) of weighted-UniFrac distances of multivariate statistical techniques and analysis of similarities (Anosim) test of Bray–Curtis distances performed on OTU level were calculated by the R project Vegan package (version 2.5.3) (Oksanen et al., 2013) and plotted in the R project ggplot2 package.

TABLE 4 | Primers used for quantitative real-time PCR.

Gene name	Sequence of primer (5′-3′)	GenBank no.
relish	CTACATTCTGCCCTTGACTCTGG	EF432734
	GGCTGGCAAGTCGTTCTCG	
dorsal	TGGGGAAGGAAGGATGC	FJ998202.1
	CGTAACTTGAGGGCATCTTC	
alf	CGCTTCACCGTCAAACCTTAC	GQ227486.1
	GCCACCGCTTAGCATCTTGTT	
cru	GGTGTTGGTGGTGGTTTCCC	AY486426.1
	CAGTCGCTTGTGCCAGTTCC	
pen3	ATACCCAGGCCACCACCCTT	DQ206403.1
	TGACAGCAACGCCCTAACC	
β-actin	GAGCAACACGGAGTTCGTTGT	AF300705.2
	CATCACCAACTGGGACGACATGGA	

alf, anti-lipopolysaccharide factor; cru, crustins; pen3, penaeidins 3.

Calculations and Statistical Analysis

The growth performance was calculated as follows:

Weight gain (WG, %)

= 100 × (final body weight - initial body weight)/initial body weight

Specific growth rate (SGR, % per day)

= 100 × (Ln final body weight - Ln initial body weight)/t

Survival (%)

= 100 × (final number of shrimp)/ (initial number of shrimp)

Feed conversion rate (FCR)

= feed consumed (g)/ (final body weight - initial body weight)

where t is the experimental duration in days.

The results were presented as the means with SEM. All data were subjected to ANOVA followed by Tukey's HSD test to determine significant differences among treatments using SPSS

TABLE 5 | The effect of black soldier fly larvae meal (BSF) on growth performance of *Litopenaeus vannamei*.

Parameter	Group			
	FM	BSF10	BSF20	BSF30
IBW	0.88 ± 0.001	0.88 ± 0.002	0.88 ± 0.001	0.88 ± 0.002
FBW	7.76 ± 0.09^{b}	7.66 ± 0.19^{ab}	7.55 ± 0.13^{ab}	7.06 ± 0.16^{a}
WG	776.38 ± 10.3^{b}	767.58 ± 25^{ab}	758.11 ± 13.51^{ab}	698.35 ± 18.95^{a}
SGR	4.34 ± 0.02^{b}	4.32 ± 0.05^{ab}	4.29 ± 0.03^{ab}	4.15 ± 0.04^{a}
Survival	93.75 ± 1.25	96.66 ± 3.11	94.16 ± 8.24	100 ± 0
FCR	1.62 ± 0.04^{ab}	1.6 ± 0.03^{ab}	1.55 ± 0^{a}	1.7 ± 0.04^{b}

Data represent mean ± SEM of three replicates (n = 3). Values in the same row with different letters are significantly different (P < 0.05) based on Tukey's HSD test. The lack of superscript letter indicates no significant differences among groups.
Where: IBW, initial body weight (g); FBW, final body weight (g); WG, weight gain (%); SGR, specific growth rate (% day^{-1}); FCR, feed conversion rate.

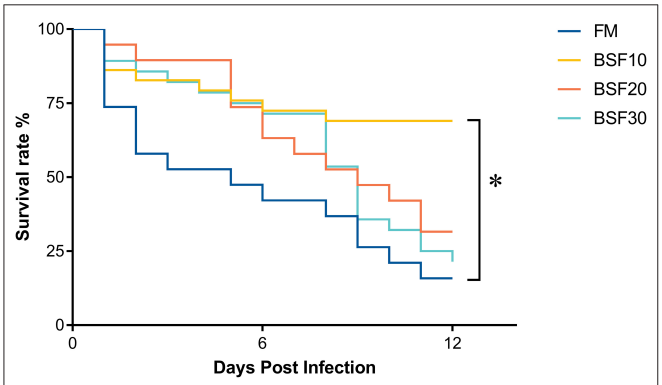


FIGURE 1 | Effect of black soldier fly larvae meal (BSF) on the survival rate of *Litopenaeus vannamei* after infection of *Vibrio parahaemolyticus*. Asterisk (*) indicates significantly different ($P < 0.05$) among groups based on Tukey's HSD test.

21.0 (SPSS, Chicago, IL, United States). A probability value of $P < 0.05$ was deemed to be statistically significant.

RESULTS

Growth Performance and Cumulative Mortality After Challenge

As shown in **Table 5**, the final body weight (FBW), weight gain (WG), and the specific growth rate (SGR) decreased with the increasing BSF addition, and FBW, WG, and SGR of shrimp fed with BSF30 were significantly lower than those fed with FM ($P < 0.05$). Feed conversion rate (FCR) first decreased and then increased with the increasing dietary BSF levels, and FCR of shrimp fed with BSF20 diet was significantly lower than those fed with BSF30 ($P < 0.05$). The results of the challenge trial are shown in **Figure 1**. The survival rate of shrimp fed with BSF10 significantly increased compared with those fed with FM after infection for 12 d ($P < 0.05$).

TABLE 6 | The effect of black soldier fly larvae meal (BSF) on intestinal histology of *Litopenaeus vannamei*.

Parameter	Diets			
	FM	BSF10	BSF20	BSF30
Microvilli height (μm)	1.72 ± 0.12^{b}	2.00 ± 0.20^{c}	1.24 ± 0.12^{a}	1.62 ± 0.14^{b}
Mucosal folds height (μm)	58.85 ± 5.87^{d}	48.40 ± 4.08^{c}	39.82 ± 2.03^{b}	26.50 ± 3.30^{a}
Mucosal folds width (μm)	51.73 ± 7.70^{b}	51.60 ± 8.36^{b}	37.96 ± 9.17^{a}	28.35 ± 4.80^{a}
Muscle layer thickness (μm)	41.47 ± 6.25^{a}	59.36 ± 4.60^{b}	42.68 ± 4.69^{a}	39.23 ± 5.30^{a}

Data represent mean ± SEM of three replicates (n = 3). Values in the same row with different letters are significantly different (P < 0.05) based on Tukey's HSD test. The lack of superscript letter indicates no significant differences among groups.

Intestinal Histology

The statistical results of intestinal histology are shown in **Table 6**. Results showed that the height of the microvilli significantly increased in shrimp fed with BSF10 diet, but decreased in those fed with BSF20 diet ($P < 0.05$). The height of the mucosal folds significantly decreased with the increasing BSF addition ($P < 0.05$). The width of the mucosal folds of shrimp fed with FM and BSF10 were significantly more than those fed with BSF20 and BSF30 ($P < 0.05$). The thickness of the muscle layer of shrimp fed with BSF10 was significantly higher than the other three groups ($P < 0.05$). As shown in **Figure 2**, the results of TEM revealed that shrimp fed with BSF20 and BSF30 showed obvious swelling of the nuclear membrane, and in particular, the chromatin of shrimp fed with BSF30 was abnormally agglutinated and distributed along the nuclear membrane. Mitochondrial cristae of shrimp fed with BSF20 were irregular and the mitochondrial structure of shrimp fed with BSF30 was disrupted. The cystic lumen of the endoplasmic reticulum of shrimp fed with BSF30 swelled and a large number of vesicles appeared in the cytoplasm.

Expression of Non-specific Immune-related Genes in the Intestine

As shown in **Figure 3**, mRNA expression of *dorsal* and *relish* significantly decreased in shrimp fed with BSF20 and BSF30, while no significant change was found in shrimp fed with BSF10 compared to those fed with FM ($P < 0.05$). The mRNA expression of *alf* (anti-lipopolysaccharide factor) significantly increased in shrimp fed BSF10 but decreased in shrimp fed BSF30 compared to those fed FM ($P < 0.05$). The mRNA expression of *cru* (crustins) showed no significant difference between the shrimp fed FM and BSF diets ($P > 0.05$). The mRNA expression of *pen3* (penaeidins 3) significantly increased in shrimp fed with BSF10 and BSF20 ($P < 0.05$), but no significant changes were observed in shrimp fed with BSF10 compared to those fed with FM ($P > 0.05$).

Intestinal Microbiota Analysis

A total of 998,755 raw tags were obtained from the gut samples, with an average of 110,972.8 raw tags per sample (ranging from

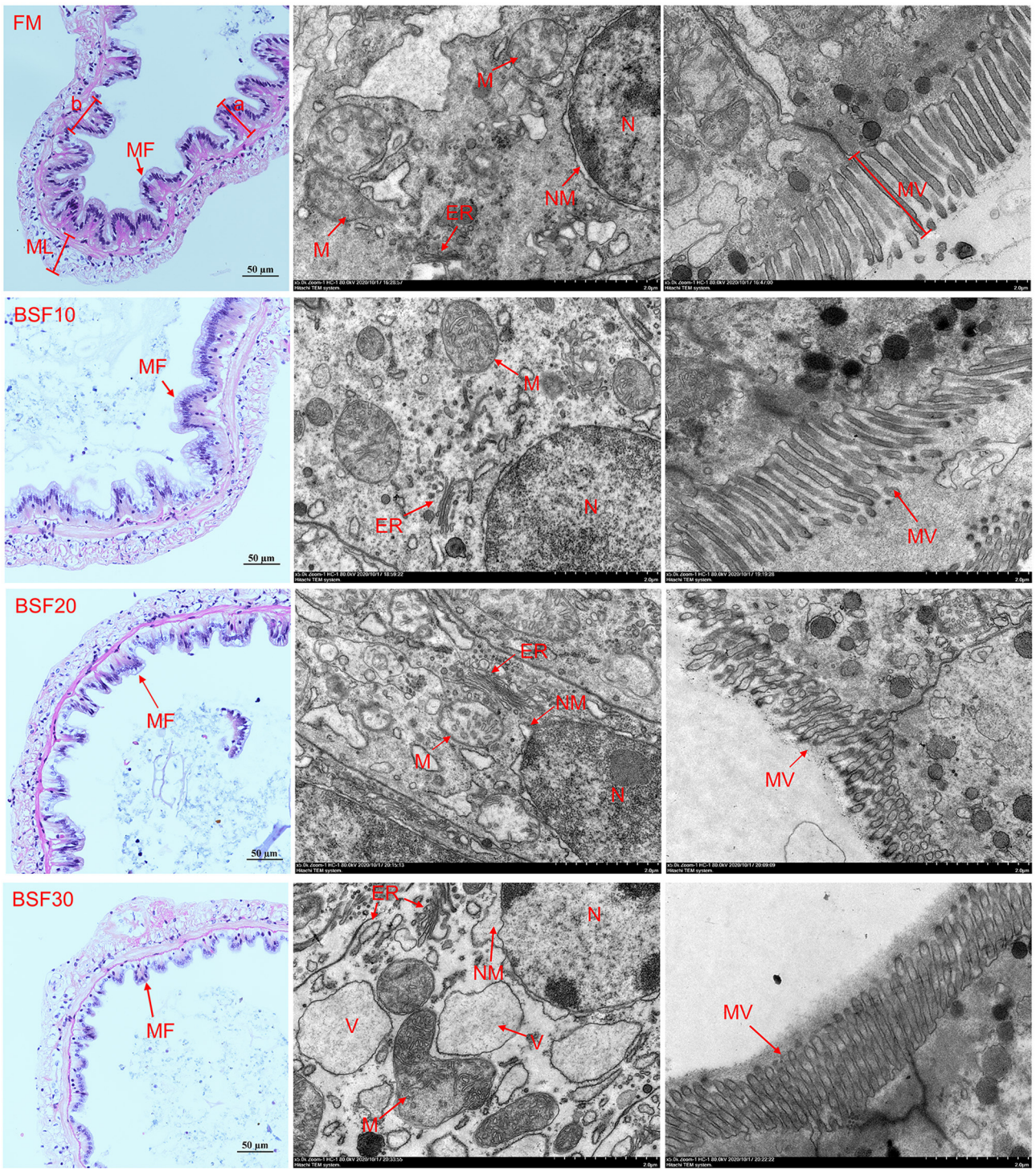


FIGURE 2 | Effect of BSF on midgut histology of *L. vannamei*. Where: ER, endoplasmic reticulum; N, cell nucleus; NM, nucleus membrane; MF, mucosal folds; ML, muscle layer; M, mitochondria; MV, microvilli; V, Vesicles. a shows height of mucosal folds; and b shows width of mucosal folds. Pictures in the same row are considered as the same group.

102,809 to 116,331 sequences). After sequence analysis and filtering, we finally got a total of 942,176 effective tags with an average of 104,686.2 effective tags per sample (ranging from 100,932 to 111,704 sequences). Sequences with more than 97% similarity were clustered into OTUs. As shown in **Figure 4A**, 224 OTUs were overlapped among the three groups. To be

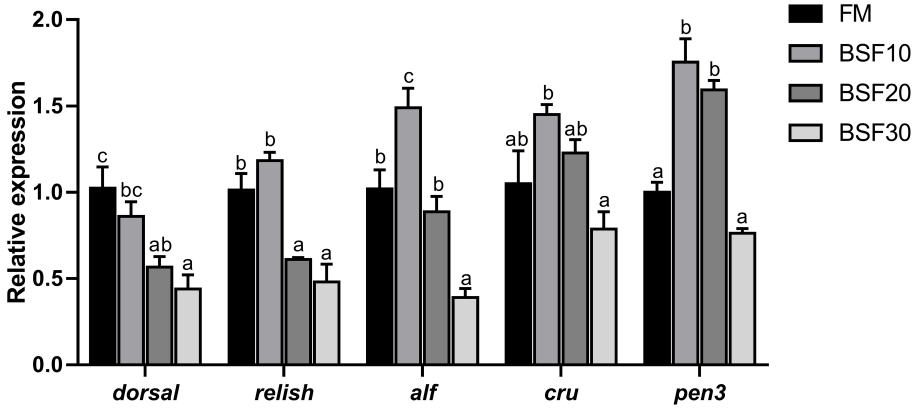


FIGURE 3 | Effect of BSF on mRNA levels of *dorsal*, *relish*, *alf* (anti-lipopolysaccharide factor), *cru* (crustins), and *pen3* (penaeidins 3) in the intestine of *L. vannamei*. Vertical bars represent the mean ± SEM ($n = 3$). Data marked with different letters differ significantly ($P < 0.05$) among groups based on Tukey's HSD test.

specific, the core OTU numbers in the shrimp fed with FM (104) were the highest, followed by the shrimp fed with BSF30 (104) and BSF20 (51). The PCoA based on weighted-UniFrac distances and analysis of similarities (Anosim) using the Bray–Curtis distance matrix on OUT levels were performed to assess the modification in microbiota composition after the feeding trial. As shown in **Figure 4B**, the composition of the intestine microbiota from the FM-fed group was clustered closer to BSF30-fed group rather than BSF20-fed group. However, the results of $R > 0$ and $P > 0.05$ in Anosim test indicated that the differences in microbiota composition among FM-, BSF20-, and BSF30-fed groups were not significant ($P > 0.05$) (**Supplementary Figure 1**). As for the alpha diversity indices, there were no significant differences in bacterial richness parameters (Chao1 and Ace) and bacterial diversity parameters (Shannon and Simpson) among the three groups ($P > 0.05$) (**Table 7**). After the feeding trial, the microbial composition of the intestinal microbiota in *L. vannamei* was altered by the BSF replacement. As shown in **Figure 5**, Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, Verrucomicrobia, Planctomycetes, Cyanobacteria, Tenericutes, Chloroflexi, and Gemmatimonadetes were the top 10 phyla in *L. vannamei*. The heatmap of the top 20 abundance intestinal microbiota is shown in **Figure 6**. The abundance of genus *Vibrio*, *Photobacterium*, *Haloferula*, *ZOR0006*, *Rubritalea*, *Candidatus_Bacilloplasma*, *Marivita*, and *Hoppeia* increased in shrimp fed with FM diet. The abundance of *Ruegeria*, *Halocynthiibacter*, *Pseudoalteromonas*, *Tenacibaculum*, *Tamlana*, *Pir4_lineage*, *Marivita*, *Ilumatobacter*, *Hoppeia*, and *Blastopirellula* increased in shrimp fed with BSF20, while the abundance of *Bacillus*, *Acinetobacter*, and *ZOR0006* increased in shrimp fed with BSF30.

DISCUSSION

Although differences in the metamorphic stage of insects (larvae and pupae) (Huyben et al., 2019), nutrient composition caused by different food sources (Barroso et al., 2017), and processing techniques (Larouche et al., 2019) may lead to distinguishing experimental results, many studies have demonstrated the potential of BSF for application in aquatic animal feeds (Li et al., 2017; Renna et al., 2017; Wang et al., 2019). Similar to the previous studies, the present study showed that better growth performance was obtained when 20% of FM was replaced with BSF, but replacing 30% of FM significantly reduced the final body weight, weight gain, and specific growth rate of the shrimp (Cummins et al., 2017; Hu et al., 2019). Due to the high lipid content (10–40%), BSF is often processed by oil separation methods into protein-rich components to minimize the risk of fat oxidation and compositional changes. The use of defatted BSF in shrimp feed has also been studied, and defatted BSF can replace 60% of FM without negatively affecting the growth performance and antioxidant and immune capacity of shrimp (Wang et al., 2021). This suggests that the inhibition of growth performance by the addition of high levels of BSF to shrimp diets may be due to the fatty acid composition in BSF. Under the present experimental conditions, the fatty acid composition of BSF was rich in lauric acids, but very low in EPA and DHA, compared to FM. Lipids are not only a major source of energy and carriers of fat-soluble vitamins but also serve as components of cell membranes and precursors for many important metabolites (Zhou et al., 2019; Xie et al., 2020). Replacement of FM with BSF in the diet reduced the highly unsaturated fatty acid contents in the diet, and the hepatocyte disruption was observed in *L. vannamei* (Wang et al., 2021) and *Cyprinus carpio var*. Jian (Li et al., 2017). In addition to BSF, other insect proteins have been also studied in shrimp diet, such as silkworms (*Bombyx mori* L.). Replacing 75% of FM with defatted silkworm meal did not affect the growth performance of *L. vannamei* but higher levels of silkworm meal would lead to hepatopancreas tubular damage (Rahimnejad et al., 2019). However, the effect of dietary BSF on the intestinal health remains unknown. Excessive replacement of FM may lead to impaired nutritional sensitivity and metabolism (Xu et al., 2016; Shao et al., 2020). Besides, intestinal histology is directly related to the digestion and absorption of nutrients by the shrimp, thus affecting growth.

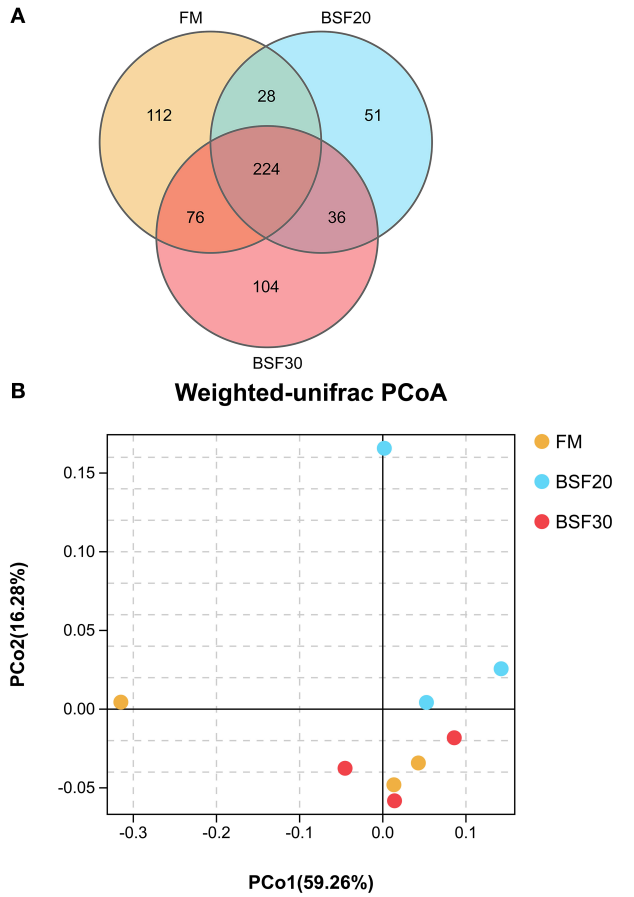


FIGURE 4 | Intestine microbiota responses of *L. vannamei* fed with diets containing different contents of BSF. **(A)** Venn diagrams of OTUs among groups; **(B)** Principal coordinates analysis (PCoA) based on weighted-UniFrac analysis of bacterial profiles.

TABLE 7 | The effect of black soldier fly larvae meal (BSF) on alpha diversity indices in the intestinal microbiota of *Litopenaeus vannamei*.

Diets	Richness estimators		Diversity estimators	
	Ace	Chao1	Simpson	Shannon
FM	458.95 ± 27.85	450.86 ± 43.36	0.8 ± 0.01	3.59 ± 0.11
BSF20	446.58 ± 40.31	419.91 ± 33.42	0.88 ± 0.03	4.11 ± 0.49
BSF30	464.43 ± 7.98	464.46 ± 11.18	0.82 ± 0.05	3.69 ± 0.55

Data represent mean ± SEM of three replicates (n = 3). Values in the same row with different letters are significantly different (P < 0.05). The lack of superscript letter indicates no significant differences among groups.

In addition to the growth status of shrimp, issues related to animal welfare need to be considered, which includes the effects of immune status, oxidative status, and diet on the intestinal structure, directly affecting the digestive function and immune response of shrimp (Bruni et al., 2018). The intestinal structural barrier separates the internal from the external environment and is the first line of defense against invasion by pathogens (Rahimnejad et al., 2018). In the results of HE-staining sections, it was clear that the width and height of intestinal mucosal folds decreased with increasing BSF replacement. The shrinkage of intestinal mucosal folds will reduce the contact area between the intestine and the food of shrimp, which is not conducive to food digestion and nutrient absorption. Besides, results of TEM showed that shrimp fed with BSF30 showed early signs of apoptosis: chromatin consolidation, separation and distribution along the nuclear membrane, swelling of the endoplasmic reticulum vesicles, and abnormal mitochondrial structures, demonstrating that BSF replacement for 30% FM can damage the intestinal epithelial cell structure of shrimp and may explain the reduction of growth performance (Xie et al., 2018). In a previous study, the reduction of FM in diets upregulated the expression of endoplasmic reticulum stress-related genes, resulting in severe endoplasmic reticulum stress in the intestine (Xie et al., 2020). The content of highly unsaturated fatty acids in the diet decreases with the proportion of FM and fish oil in the diet, which negatively affects the biophysical properties of cell membranes and may lead to apoptosis (O'shea et al., 2009).

The non-specific immune system of the shrimp plays an important role in the resistance to pathogen invasion (Gui et al., 2019). To evaluate the effect of BSF on resistance to *V. Parahaemolyticus* infection, a challenge trial was conducted to compare the disease resistance of shrimp fed diets containing different levels of BSF. Colonizing in the intestine, *V. parahaemolyticus* would result in EMS, also known as acute hepatopancreatic necrosis disease (Zorriehzahra and Banaederakhshan, 2015; Qi et al., 2017). In this study, the survival rate of shrimp fed with BSF10 was significantly higher than those fed with FM after infection, indicating that BSF replacement of 10% FM could improve the resistance of *L. vannamei* to *V. Parahaemolyticus*.

Furthermore, the effect of BSF on the non-specific immune gene expression in the intestine was investigated. *dorsal* and *relish* are Rel/NF-κB transcription factors, the former factor activating antifungal and antimicrobial responses in the Toll pathway through a cascade amplification effect, and the latter one activating antimicrobial peptide gene expression in the IMD pathway (Li and Xiang, 2013). The Toll and IMD pathways induce immune-related gene expression during pathogen invasion, thereby regulating the early stages of the immune response (Jin et al., 2018). The results showed that intestinal *dorsal* and *relish* mRNA levels were maintained at the control level in the shrimp fed with BSF10 but downregulated in those fed with BSF20 and BSF30. Furthermore, antimicrobial peptides are products of the immune response and are capable of directly killing or removing pathogens (Bachère, 2003; Wang et al., 2007). Anti-lipopolysaccharide factor has been reported in *L. vannamei* (De La Vega et al., 2008), *Fenneropenaeus chinensis* (Li et al., 2013), and *Penaeus monodon* (Supungul et al., 2004), which showed a broad-spectrum of antimicrobial activity against Gram-negative bacteria and endotoxins (Silveira et al., 2018). Crustins are antimicrobial peptides prevalent in crustaceans with antibacterial activity against Gram-positive bacteria, as well as inhibition of fungi and white spot syndrome virus (Shockey et al., 2009; Chen et al., 2019). Penaeidins 3 is an antimicrobial peptide isolated from *L. vannamei* (Destoumieux et al., 1997)

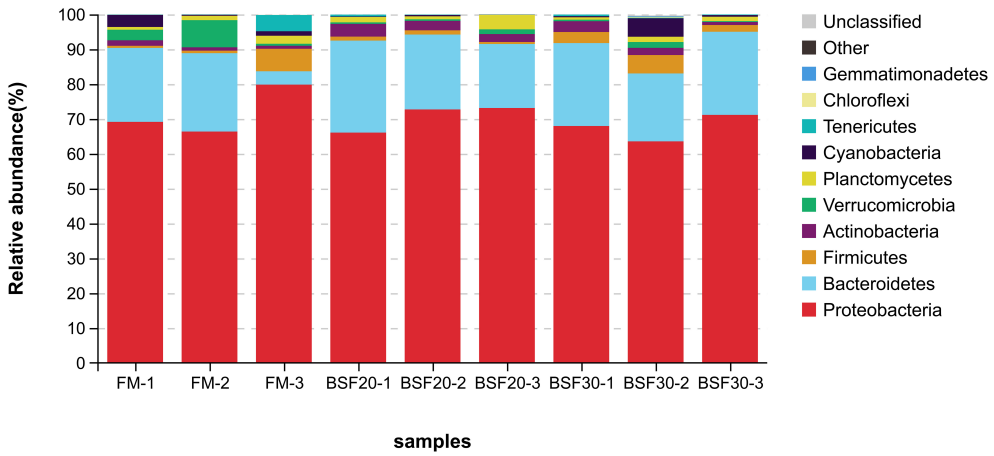


FIGURE 5 | Relative abundance comparison of intestinal microbiota in *L. vannamei* fed with different diets at the phylum level of the taxonomy ($n = 3$).

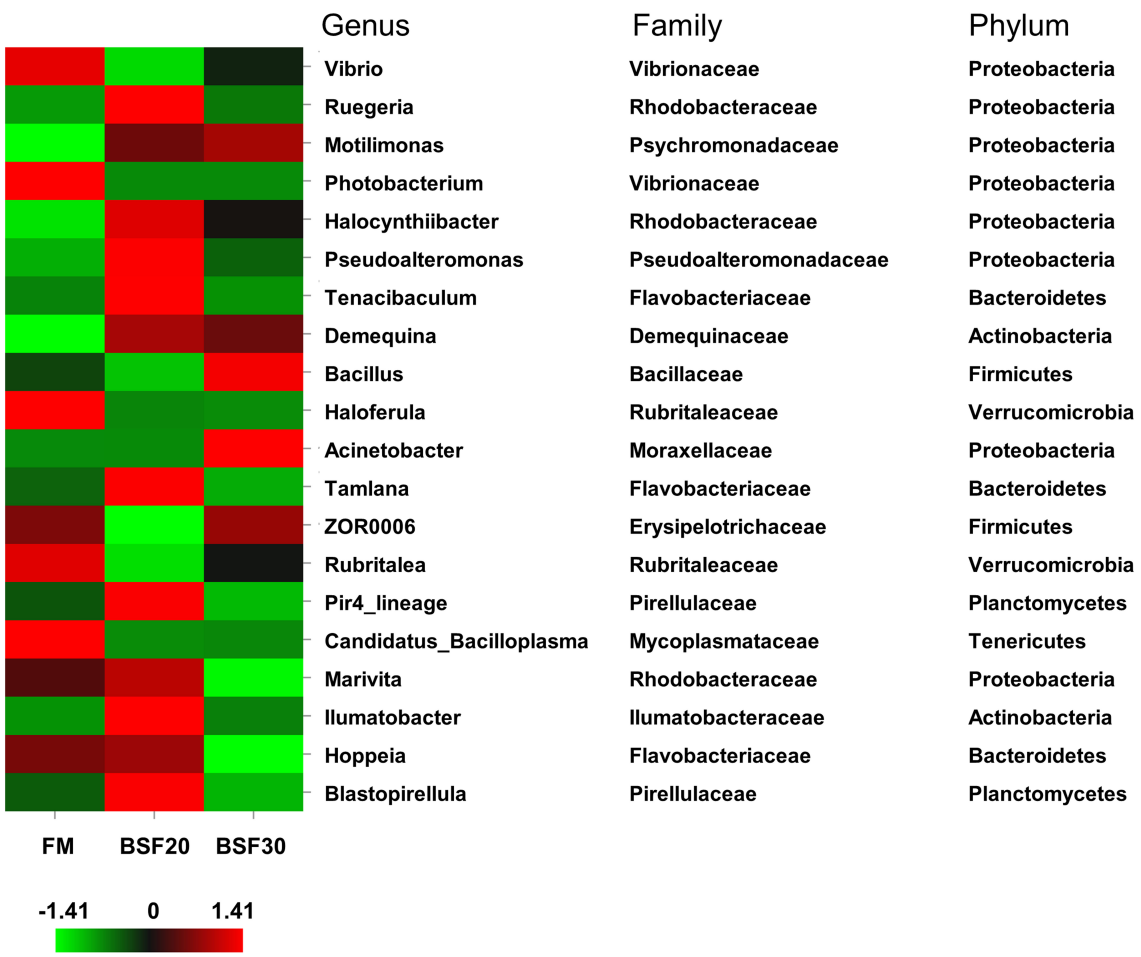


FIGURE 6 | Heatmap of top 20 abundance intestinal microbiota in *L. vannamei* fed with different diets at the genus level. The color bar of each genus is average of three samples in each group. The taxonomy of the genus (family and phylum) is depicted on the right.

with antibacterial activity against Gram-positive bacteria and fungi (Takuji, 2007). In the present study, mRNA level of *alf* upregulated in shrimp fed with BSF10, while downregulated in those fed with BSF30, which was consistent with the results of the challenge trial, indicating that dietary BSF replacing 10% FM can improve intestinal immunity in shrimp.

In addition to the immune defense of shrimp, the gut microbiota has been found to have a remarkable effect on animal health, growth, and survival and is of increasing interest to researchers and breeders (He et al., 2017; Li et al., 2018). To date, the effect of BSF on the intestinal microbiota has been studied on aquatic animals, such as rainbow trout (Terova et al., 2019), zebrafish (*Danio rerio*) (Osimani et al., 2019), rice field eel (*Monopterus albus*) (Hu et al., 2020), marron (*Cherax cainii*) (Foysal et al., 2019), and other animals. Since there was no significant difference in the growth performance of shrimp fed with BSF10 and BSF20, we explored the intestinal microbiota of shrimp fed with FM, BSF20, and BSF30 diets. Results showed that there were no significant differences in Anosim tests of beta diversity analysis, indicating that the intestinal microbiota community was not separated in shrimp fed with FM, BSF20, and BSF30 diets. At the phylum level, Proteobacteria was the most common bacteria in the shrimp gut, followed by Bacteroidetes, Firmicutes, and Actinobacteria, which was in line with the results of a previous study (Amoah et al., 2020). Proteobacteria do not change in abundance with environment or diet and are the most stable bacteria in *L. vannamei*, while the abundance of the other major bacterial phyla (Firmicutes, Bacteroidetes, Actinobacteria) may vary with developmental stage, diet, and environmental factors (Li et al., 2018). At the genus level, *Vibrio* and *Photobacterium* are known as opportunistic pathogens and have been reported in shrimp diseases (Avendaño-Herrera et al., 2006; Liu and Qiu, 2007; Wang et al., 2020). *Tenacibaculum* and *Acinetobacter* have also been reported as bacterial pathogens, while the former was identified in marine fish, which causes an ulcerative disease (Avendaño-Herrera et al., 2006) as well as the latter was identified as a potential pathogen of red leg disease in the Pacific white shrimp (Huang et al., 2020). Moreover, *Candidatus_Bacilloplasma* may contribute to WFS in *L. vannamei* (Hou et al., 2018). In the present study, the reduction of some harmful bacteria in shrimp fed with BSF20 may help improve intestinal health. On the other hand, some beneficial bacteria also differed among FM, BSF20, and BSF30. *Haloferula* has been reported in sea cucumbers (*Apostichopus japonicus*) and was reduced by the high levels of selenium and vitamin C supplementation, indicating that *Haloferula* is related to the antioxidant capacity of the gut (Zeng et al., 2020). Moreover, *Pseudoalteromonas* has been shown to produce extracellular antibacterial compounds, which could enhance the resistance of shrimp to *V. parahaemolyticus* (Wang et al., 2018). *Bacillus* is a non-pathogenic Gram-positive bacterium and is beneficial to the growth performance and immunity of the Pacific white shrimp (Wen et al., 2011; Zokaeifar et al., 2012; Tang et al., 2015). The increase of *Pseudoalteromonas* in BSF20-fed group and *Bacillus* in BSF30-fed group may enhance the intestinal health. According to the present study, the increase of beneficial bacteria and the reduction of harmful bacteria suggest that BSF20 diet may improve the intestinal bacterial communities of *L. vannamei*.

CONCLUSION

The present study evaluated the effects of BSF on growth, intestinal health, and susceptibility to *V. parahaemolyticus* of *L. vannamei*. Results showed that BSF replacement of 10% FM did not negatively affect the growth performance and intestinal histology of the shrimp *L. vannamei*, but improved the survival rate after infection of the bacteria *V. parahaemolyticus*. When the replacement of FM was increased to 20%, there was no negative effect on the intestinal integrity and promoted a positive modulation in the intestinal microbiota. However, FM replacement up to 30% promoted intestinal cell apoptosis and degeneration and was deleterious to the shrimp.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA729583.

ETHICS STATEMENT

This animal study was reviewed and approved by the Animal Ethical and Welfare Committee of Guangdong Ocean University.

AUTHOR CONTRIBUTIONS

YC and SX designed the experiments. YC carried out the experiments and drafted the manuscript. SC, SZ, XD, QY, and HL: accountable for some aspects (such as ingredients and sites) of the study in ensuring that the experiment can be carried out properly. SX and BT reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the fund of the National Key R&D Program of China (2019YFD0900200), the National Natural Science Foundation of China (32002402), and the Guangdong Basic and Applied Basic Research Foundation (2019A1515011970, 2021A1515010428).

ACKNOWLEDGMENTS

We are grateful to Guangzhou Genedenovo Biotechnology Co., Ltd (Guangdong, China) for assisting in sequencing and bioinformatics analysis (http://www.omicsmart.com).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.706463/full#supplementary-material

Supplementary Figure 1 | Analysis of similarities (Anosim) test of Bray–Curtis distances performed on OTU level.

REFERENCES

Amoah, K., Dong, X., Tan, B., Zhang, S., Chi, S., Yang, Q., et al. (2020). Administration of probiotic *Bacillus licheniformis* induces growth, immune and antioxidant enzyme activities, gut microbiota assembly and resistance to *Vibrio parahaemolyticus* in *Litopenaeus vannamei*. *Aquacult. Nutr.* 26, 1604–1622. doi: 10.1111/anu.13106

Avendaño-Herrera, R., Toranzo, A. E., and Magariños, B. (2006). Tenacibaculosis infection in marine fish caused by *Tenacibaculum maritimum*: a review. *Dis. Aquat. Org.* 71, 255–266. doi: 10.3354/dao071255

Bachère, E. (2003). Anti-infectious immune effectors in marine invertebrates: potential tools for disease control in larviculture. *Aquaculture* 227, 427–438. doi: 10.1016/S0044-8486(03)00521-0

Barragan-Fonseca, K. B., Dicke, M., and Van Loon, J. J. (2017). Nutritional value of the black soldier fly (*Hermetia illucens* L.) and its suitability as animal feed–a review. *J. Insects Food Feed* 3, 105–120. doi: 10.3920/JIFF2016.0055

Barroso, F. G., Sánchez-Muros, M.-J., Segura, M., Morote, E., Torres, A., Ramos, R., et al. (2017). Insects as food: enrichment of larvae of *Hermetia illucens* with omega 3 fatty acids by means of dietary modifications. *J. Food Composition Anal.* 62, 8–13. doi: 10.1016/j.jfca.2017.04.008

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., et al. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat. Methods* 10, 57–59. doi: 10.1038/nmeth.2276

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

Cai, L., Wang, L., Song, K., Lu, K., Zhang, C., and Rahimnejad, S. (2020). Evaluation of protein requirement of spotted seabass (*Lateolabrax maculatus*) under two temperatures, and the liver transcriptome response to thermal stress. *Aquaculture* 516:734615. doi: 10.1016/j.aquaculture.2019.734615

Cao, J., Yan, J., Wang, G., Huang, Y., Zhang, R., Zhou, T., et al. (2012). Effects of replacement of fish meal with housefly maggot meal on digestive enzymes, transaminases activities and hepatopancreas histological structure of *Litopenaeus vannamei*. *South China Fish. Sci.* 8, 72–79. doi: 10.3969/j.issn.2095-0780.2012.05.011

Chen, Y., Lian, Y., He, H., Yuan, K., Zhang, C., Yue, G., et al. (2019). Functional characterization of an ER-stress responding Crustin gene in *Litopenaeus vannamei*. *Fish Shellfish Immunol.* 84, 541–550. doi: 10.1016/j.fsi.2018.10.047

Choi, I.-H., Kim, J.-M., Kim, N.-J., Kim, J.-D., Park, C., Park, J.-H., et al. (2018). Replacing fish meal by mealworm (*Tenebrio molitor*) on the growth performance and immunologic responses of white shrimp (*Litopenaeus vannamei*). *Acta Scientiarum Anim. Sci.* 40:e39077. doi: 10.4025/actascianimsci.v40i1.39077

Cummins, V. C., Rawles, S. D., Thompson, K. R., Velasquez, A., Kobayashi, Y., 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

De La Vega, E., O'leary, N. A., Shockey, J. E., Robalino, J., Payne, C., Browdy, C. L., et al. (2008). Anti-lipopolysaccharide factor in *Litopenaeus vannamei* (LvALF): a broad spectrum antimicrobial peptide essential for shrimp immunity against bacterial and fungal infection. *Mol. Immunol.* 45, 1916–1925. doi: 10.1016/j.molimm.2007.10.039

Destoumieux, D., Bulet, P., Loew, D., Van Dorsselaer, A., Rodriguez, J., and Bachere, E. (1997). Penaeidins, a new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda). *J. Biol. Chem.* 272, 28398–28406. doi: 10.1074/jbc.272.45.28398

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Egerton, S., Culloty, S., Whooley, J., Stanton, C., and Ross, R. P. (2018). The gut microbiota of marine fish. *Front. Microbiol.* 9:873. doi: 10.3389/fmicb.2018.00873

FAO (2020). *FAO Yearbook. Fishery and Aquaculture Statistics 2018*. Rome.

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

Gui, L., Mai, H., Chi, S., Zhou, W., Li, Y., Tan, B., et al. (2019). Effects of yeast culture on growth performance, hematological parameters, immunity and disease resistance of *Litopenaeus vannamei*. *J. Guangdong Ocean Univ.* 39, 30–37. doi: 10.3969/j.issn.1673-9159.2019.03.005

He, Y., Chi, S., Tan, B., Zhang, H., Dong, X., Yang, Q., et al. (2017). Effect of yeast culture on intestinal microbiota of *Litopenaeus vannamei*. *J. Guangdong Ocean Univ.* 37, 21–27. doi: 10.3969/j.issn.1673-9159.2017.04.004

Hou, D., Huang, Z., Zeng, S., Liu, J., Wei, D., Deng, X., et al. (2018). Intestinal bacterial signatures of white feces syndrome in shrimp. *Appl. Microbiol. Biotechnol.* 102, 3701–3709. doi: 10.1007/s00253-018-8855-2

Hu, Y., Huang, Y., Tang, T., Zhong, L., Chu, W., Dai, Z., et al. (2020). Effect of partial black soldier fly (*Hermetia illucens* L.) larvae meal replacement of fish meal in practical diets on the growth, digestive enzyme and related gene expression for rice field eel (*Monopterus albus*). *Aquacult. Rep.* 17:100345. doi: 10.1016/j.aqrep.2020.100345

Hu, J., Wang, G., Huang, W., Zhao, H., Mo, W., and Huang, Y. (2019). Effects of fish meal replacement by black soldier fly (*Hermetia illucens*) larvae meal on growth performance, body composition, serum biochemical indexes and antioxidant ability of juvenile *Litopenaeus vannamei*. *Chin. J. Animal Nutr.* 31, 5292–5300. doi: 10.3969/j.issn.1006-267x.2019.11.046

Huang, X., Gu, Y., Zhou, H., Xu, L., Cao, H., and Gai, C. (2020). *Acinetobacter venetianus*, a potential pathogen of red leg disease in freshwater-cultured whiteleg shrimp *Penaeus vannamei*. *Aquacult. Rep.* 18:100543. doi: 10.1016/j.aqrep.2020.100543

Huyben, D., Vidaković, A., Hallgren, S. W., and Langeland, M. (2019). High-throughput sequencing of gut microbiota in rainbow trout (*Oncorhynchus mykiss*) fed larval and pre-pupae stages of black soldier fly (*Hermetia illucens*). *Aquaculture* 500, 485–491. doi: 10.1016/j.aquaculture.2018.10.034

Jin, M., Xiong, J., Zhou, Q., Yuan, Y., Wang, X., and Sun, P. (2018). Dietary yeast hydrolysate and brewer's yeast supplementation could enhance growth performance, innate immunity capacity and ammonia nitrogen stress resistance ability of Pacific white shrimp (*Litopenaeus vannamei*). *Fish Shellfish Immunol.* 82, 121–129. doi: 10.1016/j.fsi.2018.08.020

Larouche, J., Deschamps, M.-H., Saucier, L., Lebeuf, Y., Doyen, A., and Vandenberg, G. W. (2019). Effects of killing methods on lipid oxidation, colour and microbial load of black soldier fly (*Hermetia illucens*) larvae. *Animals* 9:182. doi: 10.3390/ani9040182

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. Aquacult.* 26, 381–399. doi: 10.1080/23308249.2018.1440530

Li, F., and Xiang, J. (2013). Recent advances in researches on the innate immunity of shrimp in China. *Dev. Compar. Immunol.* 39, 11–26. doi: 10.1016/j.dci.2012.03.016

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.2017.04.015

Li, S., Zhang, X., Sun, Z., Li, F., and Xiang, J. (2013). Transcriptome analysis on Chinese shrimp *Fenneropenaeus chinensis* during WSSV acute infection. *PLoS ONE* 8:e58627. doi: 10.1371/journal.pone.0058627

Li, Y., Kortner, T. M., Chikwati, E. M., Belghit, I., Lock, E.-J., and Krogdahl, Å. (2020). Total replacement of fish meal with black soldier fly (*Hermetia illucens*) larvae meal does not compromise the gut health of Atlantic salmon (*Salmo salar*). *Aquaculture* 520:734967. doi: 10.1016/j.aquaculture.2020.734967

Liu, W., and Qiu, D. (2007). Effect of *Vibrio alginolyticus* on nitric oxide and oxygen free radical in serum of *Penaeus vannamei* Boone. *J. Guangdong Ocean Univ.* 27, 60–63. doi: 10.3969/j.issn.1673-9159.2007.03.013

Magoč, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R., et al. (2013). Package 'vegan'. *Community ecology package, version* 2, 1–295.

O'shea, K. M., Khairallah, R. J., Sparagna, G. C., Xu, W., Hecker, P. A., Robillard-Frayne, I., et al. (2009). Dietary ω-3 fatty acids alter cardiac mitochondrial phospholipid composition and delay Ca^{2+}-induced permeability transition. *J. Mol. Cell. Cardiol.* 47, 819–827. doi: 10.1016/j.yjmcc.2009.08.014

Osimani, A., Milanović, V., Roncolini, A., Riolo, P., Ruschioni, S., Isidoro, N., et al. (2019). *Hermetia illucens* in diets for zebrafish (*Danio rerio*): a study of bacterial diversity by using PCR-DGGE and metagenomic sequencing. *PLoS ONE* 14:e0225956. doi: 10.1371/journal.pone.0225956

Pilotto, M. R., Argenta, N., Forte, J. M., Hostins, B., Menezes, F. G. R., Maggioni, R., et al. (2020). Environmental rearing conditions are key determinants of changes in immune gene expression patterns in shrimp midgut. *Dev. Compar. Immunol.* 106:103618. doi: 10.1016/j.dci.2020.103618

Qi, C., Wang, L., Liu, M., Jiang, K., Wang, M., Zhao, W., et al. (2017). Transcriptomic and morphological analyses of *Litopenaeus vannamei* intestinal barrier in response to *Vibrio paraheamolyticus* infection reveals immune response signatures and structural disruption. *Fish Shellfish Immunol.* 70, 437–450. doi: 10.1016/j.fsi.2017.09.004

Qiu, W., Zhang, S., Chen, Y., Wang, P., Xu, X., Li, C., et al. (2014). *Litopenaeus vannamei* NF-κB is required for WSSV replication. *Dev. Compar. Immunol.* 45, 156–162. doi: 10.1016/j.dci.2014.02.016

Rahimnejad, S., Hu, S., Song, K., Wang, L., Lu, K., Wu, R., et al. (2019). Replacement of fish meal with defatted silkworm (*Bombyx mori* L.) pupae meal in diets for Pacific white shrimp (*Litopenaeus vannamei*). *Aquaculture* 510, 150–159. doi: 10.1016/j.aquaculture.2019.05.054

Rahimnejad, S., Yuan, X., Wang, L., Lu, K., Song, K., and Zhang, C. (2018). Chitooligosaccharide supplementation in low-fish meal diets for Pacific white shrimp (*Litopenaeus vannamei*): effects on growth, innate immunity, gut histology, and immune-related genes expression. *Fish Shellfish Immunol.* 80, 405–415. doi: 10.1016/j.fsi.2018.06.025

Renna, M., Schiavone, A., Gai, F., Dabbou, S., Lussiana, C., Malfatto, V., et al. (2017). Evaluation of the suitability of a partially defatted black soldier fly (*Hermetia illucens* L.) larvae meal as ingredient for rainbow trout (*Oncorhynchus mykiss* Walbaum) diets. *J. Anim. Sci. Biotechnol.* 8, 1–13. doi: 10.1186/s40104-017-0191-3

Ringø, E., Zhou, Z., Vecino, J. 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, 219–282. doi: 10.1111/anu.12346

Roeselers, G., Mittge, E. K., Stephens, W. Z., Parichy, D. M., Cavanaugh, C. M., Guillemin, K., et al. (2011). Evidence for a core gut microbiota in the zebrafish. *ISME J.* 5, 1595–1608. doi: 10.1038/ismej.2011.38

Shao, J., Wang, L., Shao, X., and Liu, M. (2020). Dietary different replacement levels of fishmeal by fish silage could influence growth of *Litopenaeus vannamei* by regulating mTOR at transcriptional level. *Front. Physiol.* 11:359. doi: 10.3389/fphys.2020.00359

Shockey, J. E., O'leary, N. A., De La Vega, E., Browdy, C. L., Baatz, J. E., and Gross, P. S. (2009). The role of crustins in *Litopenaeus vannamei* in response to infection with shrimp pathogens: an *in vivo* approach. *Dev. Compar. Immunol.* 33, 668–673. doi: 10.1016/j.dci.2008.11.010

Silveira, A. S., Matos, G. M., Falchetti, M., Ribeiro, F. S., Bressan, A., Bachère, E., et al. (2018). An immune-related gene expression atlas of the shrimp digestive system in response to two major pathogens brings insights into the involvement of hemocytes in gut immunity. *Dev. Compar. Immunol.* 79, 44–50. doi: 10.1016/j.dci.2017.10.005

Suo, Y., Li, E., Li, T., Jia, Y., Qin, J. G., Gu, Z., et al. (2017). Response of gut health and microbiota to sulfide exposure in Pacific white shrimp *Litopenaeus vannamei*. *Fish Shellfish Immunol.* 63, 87–96. doi: 10.1016/j.fsi.2017.02.008

Supungul, P., Klinbunga, S., Pichyangkura, R., Hirono, I., Aoki, T., and Tassanakajon, A. (2004). Antimicrobial peptides discovered in the black tiger shrimp *Penaeus monodon* using the EST approach. *Dis. Aquat. Org.* 61, 123–135. doi: 10.3354/dao061123

Takuji, O. (2007). Effects of lipopolysaccharide on gene expression of antimicrobial peptides (penaeidins and crustin), serine proteinase and prophenoloxidase in haemocytes of the Pacific white shrimp, *Litopenaeus vannamei*. *Fish Shellfish Immunol.* 22, 68–76. doi: 10.1016/j.fsi.2006.03.013

Tang, J., Huang, Y., Cai, J., Lu, Y., Wu, Z., and Jian, J. (2015). Effects of a probiotics combined with chinese herbal medicine on growth performance, water quality and resistance to diseases for *Litopenaeus vannamei*. *J. Guangdong Ocean Univ.* 35, 47–52. doi: 10.3969/j.issn.1673-9159.2015.06.009

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

Wang, G., Peng, K., Hu, J., Mo, W., Wei, Z., and Huang, Y. (2021). Evaluation of defatted Hermetia illucens larvae meal for *Litopenaeus vannamei*: effects on growth performance, nutrition retention, antioxidant and immune response, digestive enzyme activity and hepatic morphology. *Aquacult. Nutr.* 27, 986–997. doi: 10.1111/anu.13240

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, H., Wang, C., Tang, Y., Sun, B., Huang, J., and Song, X. (2018). *Pseudoalteromonas* probiotics as potential biocontrol agents improve the survival of *Penaeus vannamei* challenged with acute hepatopancreatic necrosis disease (AHPND)-causing *Vibrio parahaemolyticus*. *Aquaculture* 494, 30–36. doi: 10.1016/j.aquaculture.2018.05.020

Wang, Y., Chang, P., and Chen, H. (2007). Tissue expressions of nine genes important to immune defence of the Pacific white shrimp *Litopenaeus vannamei*. *Fish Shellfish Immunol.* 23, 1161–1177. doi: 10.1016/j.fsi.2007.04.004

Wang, Z., Shi, C., Wang, H., Wan, X., Zhang, Q., Song, X., et al. (2020). A novel research on isolation and characterization of *Photobacterium damselae* subsp. *damselae* from Pacific white shrimp, *Penaeus vannamei*, displaying black gill disease cultured in China. *J. Fish Dis.* 43, 551–559. doi: 10.1111/jfd.13153

Wen, C., Xiu, M., Zhang, L., Li, X., and Wu, Y. (2011). Detection of *Bacillus* preparations used for aquaculture. *J. Guangdong Ocean Univ.* 31, 39–44. doi: 10.3969/j.issn.1673-9159.2011.01.008

Xie, S., Liu, Y., Tian, L., Niu, J., and Tan, B. (2020). Low dietary fish meal induced endoplasmic reticulum stress and impaired phospholipids metabolism in juvenile Pacific white shrimp, *Litopenaeus vannamei*. *Front. Physiol.* 11:1024. doi: 10.3389/fphys.2020.01024

Xie, S., Liu, Y., Zeng, S., Niu, J., and Tian, L. (2016). Partial replacement of fishmeal by soy protein concentrate and soybean meal based protein blend for juvenile Pacific white shrimp, *Litopenaeus vannamei*. *Aquaculture* 464, 296–302. doi: 10.1016/j.aquaculture.2016.07.002

Xie, S., Wei, D., Yin, P., Zheng, L., Guo, T., Liu, Y., et al. (2019). Dietary replacement of fish-meal impaired protein synthesis and immune response of juvenile Pacific white shrimp, *Litopenaeus vannamei* at low salinity. *Compar. Biochem. Physiol. Part B Biochem. Mol. Biol.* 228, 26–33. doi: 10.1016/j.cbpb.2018.11.002

Xie, S., Zheng, L., Wan, M., Niu, J., Liu, Y., and Tian, L. (2018). Effect of deoxynivalenol on growth performance, histological morphology, anti-oxidative ability and immune response of juvenile Pacific white shrimp, *Litopenaeus vannamei*. *Fish Shellfish Immunol.* 82, 442–452. doi: 10.1016/j.fsi.2018.08.053

Xu, D., He, G., Mai, K., Zhou, H., Xu, W., and Song, F. (2016). Postprandial nutrient-sensing and metabolic responses after partial dietary fishmeal replacement by soyabean meal in turbot (*Scophthalmus maximus* L.). *Br. J. Nutr.* 115, 379–388. doi: 10.1017/S0007114515004535

Zeng, F., Rabbi, M. H., Hu, Y., Li, Z., Ren, X., Han, Y., et al. (2020). Synergistic effects of dietary selenomethionine and vitamin C on the immunity, antioxidant status, and intestinal microbiota in sea cucumber (*Apostichopus japonicus*). *Biol. Trace Elem. Res.* 9, 1–13. doi: 10.1007/s12011-020-02483-3

Zhou, W., Rahimnejad, S., Lu, K., Wang, L., and Liu, W. (2019). Effects of berberine on growth, liver histology, and expression of lipid-related genes in blunt snout bream (*Megalobrama amblycephala*) fed high-fat diets. *Fish Physiol. Biochem.* 45, 83–91. doi: 10.1007/s10695-018-0536-7

Zokaeifar, H., Balcázar, J. L., Saad, C. R., Kamarudin, M. S., Sijam, K., Arshad, A., et al. (2012). Effects of *Bacillus subtilis* on the growth performance, digestive enzymes, immune gene expression and disease resistance of white shrimp, *Litopenaeus vannamei*. *Fish Shellfish Immunol.* 33, 683–689. doi: 10.1016/j.fsi.2012.05.027

Zorriehzahra, M., and Banaederakhshan, R. (2015). Early mortality syndrome (EMS) as new emerging threat in shrimp industry. *Adv. Anim. Vet. Sci.* 3, 64–72 doi: 10.14737/journal.aavs/2015/3.2s.64.72

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 in Marine Science

ORIGINAL RESEARCH
published: 22 September 2021
doi: 10.3389/fmars.2021.741780

Dietary Analysis Based on 18S rDNA, and Stable Carbon and Nitrogen Isotopes in Juvenile *Eriocheir sinensis* Crabs Reared Under Three Feeding Modes

Zhenzhen Lu[1,2,3†], *Yunfei Sun*[1,2,3†], *Changlun Xiao*[1,2,3] *and Yongxu Cheng*[1,2,3*]

[1] Key Laboratory of Freshwater Aquatic Genetic Resources, Ministry of Agriculture, Shanghai Ocean University, Shanghai, China, [2] Shanghai Engineering Research Center of Aquaculture, Shanghai Ocean University, Shanghai, China, [3] National Demonstration Centre for Experimental Fisheries Science Education, Shanghai Ocean University, Shanghai, China

OPEN ACCESS

Edited by:
Kang-Le Lu,
Jimei University, China

Reviewed by:
Song Yang,
Sichuan Agricultural University, China
Bin Xia,
Qingdao Agricultural University, China
Ce Shi,
Ningbo University, China

***Correspondence:**
Yongxu Cheng
yxcheng@shou.edu.cn

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Marine Fisheries, Aquaculture, and Living Resources, a section of the journal Frontiers in Marine Science

Received: 15 July 2021
Accepted: 30 August 2021
Published: 22 September 2021

Citation:
Lu Z, Sun Y, Xiao C and Cheng Y (2021) Dietary Analysis Based on 18S rDNA, and Stable Carbon and Nitrogen Isotopes in Juvenile Eriocheir sinensis Crabs Reared Under Three Feeding Modes. Front. Mar. Sci. 8:741780. doi: 10.3389/fmars.2021.741780

To investigate the growth and feeding conditions of the Chinese mitten crab *Eriocheir sinensis* under different feeding modes: traditional (mainly consisting of wheat, bran, and soybean meal), formulated, and mixed feeds (1:1 mixture of traditional and formulated feeds) were fed in different crab breeding ponds in this study. During the experiment, the stomach contents of juvenile crabs under the different feeding modes were collected. The main potential eukaryotic food components were studied using 18S ribosomal DNA sequencing, and the contribution of different feeding modes to the feeding source of juvenile crabs were analyzed using C and N stable isotopes. The terminal weight and weight gain rate of crabs under the formulated feeding mode were significantly higher than those in the traditional and mixed feeding modes ($P < 0.05$). No differences were observed in the diversity and abundance of the main potential eukaryotic feed components of male and female crabs under different feeding modes ($P > 0.05$). Thirty-four phyla, composed mainly of benthic organisms, were identified, with Arthropoda (mainly including Malacostraca, 30.25–51.48%), Phragmoplastophyta (mainly including Embryophyta and Trebouxiophyceae, 5.08–24.74%), and Diatomea (3.13–8.43%) being the most abundant. The $\delta^{13}C$ and $\delta^{15}N$ values of the feeding sources and muscle of crabs ranged from −34.45 to −22.21‰, and from 0.27 to 5.66‰, respectively, varying greatly among the three feeding modes and $\delta^{15}N$ value of muscle under formulated feeding mode was significantly higher than that in traditional feeding mode ($P < 0.05$). The proportion of particulate organic matter (11.92–17.50%) is similar to *Alternanthera philoxeroides* (11.24–16.03%) in three feeding modes. There was no significant difference in feeding habits between male and female crabs under the same and different feeding modes. Juvenile crabs feed on both plant- and animal-based feeds in an aquaculture pond, but they are not complete predators and selectively feed on animal or plant feeds as supplements of that which is deficient, in addition to their main feed.

Keywords: *Eriocheir sinensis*, juvenile crab, feeding mode, 18S rDNA, stable isotope analysis

INTRODUCTION

The Chinese mitten crab, *Eriocheir sinensis*, which belongs to the phylum Arthropoda, subphylum Crustacea, order Decapoda, and family Graspidae, is one of the major economically important farmed crab species in China (Huang et al., 2019). In 2020, its production from aquaculture reached 775 887 tons (MOAC, 2020). It is mainly produced using the pond culture method (Wang and Wang, 2013), and its breeding cycle usually takes 2 years, with the first year involving juvenile crab breeding from the big-eyed larvae to juvenile crabs weighing 5–10 g (Cheng et al., 2008) and the second year involving adult crab breeding until gonad maturation (Cheng et al., 2008). Que et al. (2012) found that the survival rate, weight gain rate, and first molting time of crabs in the second year could be improved if they were fed with high-quality bait at the latter stage of juvenile culture. Therefore, it is necessary to understand the nutrient requirements of crabs in the juvenile culture stage. At present, three feeding modes for juvenile crab culture are prevalent in China: whole-course formulated feeds, traditional feeds, and mixed feeds (1:1 mixture of traditional and formulated feeds) (Fu et al., 2014). The traditional feeding mode, which plays a leading role in the culture of juvenile *E. sinensis* in China, mainly contains soybean meal, bran, rapeseed meal and wheat (Wang and Wang, 2013; He et al., 2014). This feeding mode is cheaper, but will lead to problems such as early maturity of crab species, poor quality of adult crab and deterioration of water quality, which seriously restricts the green and sustainable development of the culture industry of *E. sinensis* (Pan et al., 2016). Consequently, nutritionally balanced artificial formulated feed is increasingly being adopted to facilitate sustainable development of industry (Han et al., 2021). However, the food composition of *E. sinensis* under these different feeding modes and the contribution of the different feed sources to its growth have not been reported yet.

Chinese mitten crab is an omnivorous animal (Lafontaine and Veilleux, 2002). Using the stomach content analysis to study the feeding habits of different life stages of wild *E. sinensis*, Rudnick and Resh (2005) showed that the zoea and megalopa stages mainly feed on phytoplankton and zooplankton; the juvenile crab feeds mainly on aquatic plants (Rudnick et al., 2000); and the second instar adult crab often feeds on plants, shrimp, shellfish, and aquatic insects, being more inclined to a carnivorous diet (Chen et al., 1989). There are related reports on the feeding habits of *E. sinensis*, but owing to technical limitations, there is a lack of accurate quantitative data on its feeding habits. In recent years, with the development of molecular techniques, DNA barcoding has been widely used in the analysis of aquatic animal feeding habits. At present, there are three methods of DNA barcoding for stomach content analysis, including the mitochondrial cytochrome-oxidase I (*COI*) gene (Bade et al., 2014), internal transcribed spacer (*ITS*) (Bachy et al., 2013), and 18S ribosomal DNA (rDNA) (Riemann et al., 2010). However, the amplified fragments of *COI* and *ITS* are longer than those of 18S rDNA, and the variation is higher. Therefore, the amplified fragments of *COI* and *ITS* are suitable for species with a single feeding level. The 18S rDNA can be applied to the analysis of the feeding habits of aquatic animals (Wang X. F. et al., 2017), including the main feed source of organisms (Zhou et al., 2020), change in feeding habits during the transformation of growth (Zeng, 2010), environment (Wang X. F. et al., 2017), and even the discovery of feed sources that have not been found in previous studies (O'Rorke et al., 2012). For example, Arthropoda was the most dominant phylum in the gut of *Carcinus maenas*, followed by Ochrophyta and Mollusca (Cordone et al., 2020). The feeding habits of *Ostrea gigas* Thunberg did not change significantly before and after the *Enteromorpha* transit, and the dominant species belonged to the genus *Streptophyta* (Wang X. F. et al., 2017).

After the determination of feed sources, the contribution of different feed sources to the growth of aquatic animals should be quantitatively studied, and stable isotopes have been developed as a relatively advanced technique (Lin, 2013). The simultaneous use of ^{13}C and ^{15}N stable isotopes can determine the contribution ratio of different feed sources (Sun, 2012) under natural conditions in the wild (Lan et al., 2020) as well as during artificial breeding (Li et al., 2018). For example, under natural conditions, shellfish is the main feed for *Oratosquilla oratoria*, with an average contribution rate of 38.6%, which is significantly higher than the average contribution rate of fish (only 8.9%) (Ning et al., 2016). Paddy-farmed *Procampus clarkii* were mainly fed with feed (18.86–44.17%), followed by particulate organic matter POM (16.77–23.2%). Therefore, the combination of 18S rDNA and stable isotope analysis can be used for improved study of the feeding habits of crabs.

It is necessary to understand the changes in the intestinal content and food composition under different feeding modes to develop a feed formula more conducive to the growth of juvenile crab. To date, the effect of these three feeding modes on the diet of crabs has not been evaluated. In this study, 18S rDNA sequencing and stable isotope analysis were used to determine the effects of three different feeding modes on the growth of juvenile crabs, the composition and contribution of the main potential feed sources in the stomach of juvenile crabs, and to compared the feeding habits of male and female crabs. Understanding pond aquaculture nutrition ecology in the process of juvenile crab breeding is of great significance and can provide a theoretical basis for feeding ecology for the research and development of high-efficiency formulated feed for *E. sinensis* in artificial culture.

MATERIALS AND METHODS

Breeding Management

The experiment was carried out in nine mud aquaculture ponds with length, width, and depth of 70, 30, and 1.5 m, respectively, in Dongtan Aquaculture Base at Shanghai Daohong Aquaculture Technology Co., Ltd (31.62° N, 121.40° E) from July to November 2018. To begin the experiment, 6,000 juvenile crabs weighing between 1 and 2g were placed in each aquaculture pond. *Alternanthera philoxeroides* was planted in the center of the pond, and the surrounding area was a "large rectangle embedded with a small rectangle." To provide shelter and clean water, *A. philoxeroides* was transplanted evenly in the

circular groove of the pond and accounted for 75% of the experimental pond, and the area of hollow algal species in each pond was strictly controlled, with these species being removed when exceeding this area or supplemented in case of being insufficient. The outside of the pond was surrounded by a 40 cm-high fence to prevent crabs from escaping. The crabs were fed under three different feeding modes: traditional (consisting of wheat, bran, and soybean meal), formulated (Zhejiang Aohua Feed Co., Ltd., Jiaxing, China), and mixed (1:1 mixture of traditional and formulated feeds) feeds. The conventional biochemical composition of the different feeding modes is shown in **Table 1**. Each feeding mode was applied in three replicates. During the breeding period, the feeding ratio of each feeding mode was consistent, and the amount fed was approximately 1–3% of the total body weight of the crabs, at approximately 16:00 h every day.

Sample Collection and Data Determination

A Hach HQd portable water quality analyser (HQd, Hach Water Analysis Instrument Co., Ltd., Shanghai, China) was used to measure the temperature, dissolved oxygen, and pH of the water at 9–10 am in each pond. Water quality changes in the breeding process are shown in **Figure 1**. During the experiment, 1–2 g of formulated feed was collected, freeze-dried, crushed (particle size < 0.1 mm), and then cryopreserved at −40°C for subsequent analysis. The water sample collected using a water collector was passed through a 100 μm sieve, and the water samples removed from zooplankton were filtered using a glass fiber membrane (GF/F Whatman, Little Chalfont, United Kingdom; 47 mm diameter, 0.7 μm aperture) to obtain POM, which was wrapped in aluminum foil and frozen for subsequent analysis. At the end of the experiment, a fresh *A. philoxeroides* sample was collected from the crab breeding pond and washed slowly and repeatedly with double distilled water to remove soil on its surface and then dried, freeze-dried, and crushed (particle size < 0.1 mm) before storing it at −20°C for subsequent stable isotope analysis. In October, three male and three female crabs with sound appendages and good vitality in each pond were collected and selected for the experiment, and three samples were combined into one sample according to gender. The collected crabs were anesthetized on ice, and the surface was disinfected with 70% ethanol. The specimens were then dissected, and their stomach contents were extracted and transferred to 2.0 mL labeled cryopreservation tubes. The tubes were immersed into a liquid nitrogen tank and then stored in a refrigerator at −80°C for subsequent 18S rDNA analysis. The three *E. sinensis* specimens collected in each pond in November were anesthetized on ice, and the surface was disinfected with 70% ethanol. The shell was separated from the body along the side of the shell, and the hepatopancreas, head breastplate, appendages, and gills were removed to get body. Then, the muscle of the crab was freeze-dried, crushed (particle size < 0.1 mm), and cryopreserved at −40°C for subsequent stable isotope analysis.

Twenty male and twenty female crabs were randomly taken from each pond in the middle of each month to understand their growth during the experiment. The crabs were dried with a dry towel and accurately weighed on an electronic balance (accurate to 0.01g). The weight gain rate (WGR) of each juvenile *E. sinensis* was calculated as follows:

$$WGR\ (\%) = \frac{W_t - W_n}{W_t} \times 100\%$$

where, Wt and Wn are the average body weight (g) of crabs in months t and n, respectively.

TABLE 1 | Conventional biochemical composition of different baits (% dry weight).

Modes	Moisture	Crude protein	Crude lipid	Ash
Traditional feeding mode	12.12 ± 0.34^{c}	39.09 ± 2.83	5.48 ± 0.15^{a}	6.20 ± 0.48^{a}
Formulated feeding mode	6.99 ± 0.09^{a}	38.70 ± 0.47	8.56 ± 0.09^{c}	10.39 ± 0.17^{c}
Mixture feeding mode	9.11 ± 0.27^{b}	39.08 ± 1.80	7.39 ± 0.11^{b}	8.66 ± 0.46^{b}

Values in the same line with different superscripts are significantly different (P < 0.05).

Genomic DNA Extraction, Polymerase Chain Reaction Amplification, Construction of Illumina PE Library, and Computer Sequencing

Genomic DNA from the stomach contents of *E. sinensis* was extracted using the E.Z.N.A. SOIL DNA Kit (Omega

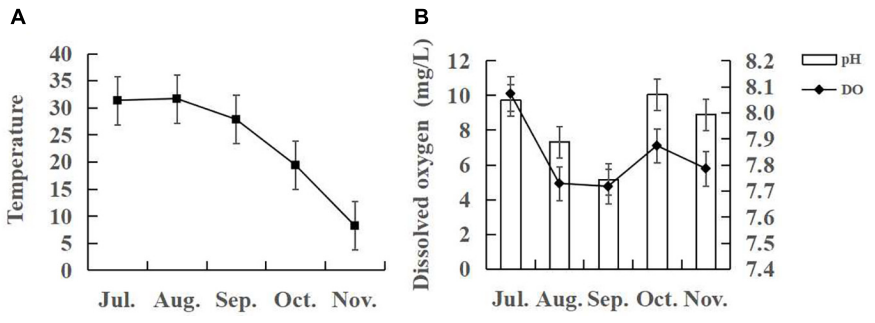


FIGURE 1 | Trends in **(A)** temperature and **(B)** dissolved oxygen and pH in pond water used to culture juvenile E. sinensis from July to November, 2018.

Bio-Tek, Norcross, GA, United States). The diluted genomic DNA was used as a template to amplify the target gene with specific primers. The primer sequences were TAREF (5′-CCAGCASCYGCGGTAATTCC-3′) and TAREF (5′-AC TTTCGTTCTTGATYRA-3′). The ABI Geneamp ® 9700 PCR (Applied Biosystems, Foster City, CA, United States) instrument was used for PCR using TransGen AP221-02: TransStart FastPFU DNA polymerase. Using high-efficiency and high-fidelity enzymes for PCR can ensure amplification efficiency and accuracy (Bokulich and Mills, 2013). The PCR amplification was carried out in a total volume of 20 μL containing 4 μL of 5× FastPFU Buffer, 0.8 μL of each primer (5 μmol/L), 0.4 μL of FastPFU Polymerase, 10 ng of template DNA, and 2 μL of 2.5 mmol/L dNTPs. The following cycling program was used: initial denaturation at 95°C for 2 min; 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 5 min. The PCR products were detected using 2% agarose gel electrophoresis and quantified using the Quantifluor^{TM}-ST Blue Fluorescence Quantitative System (Promega, Madison, WI, United States) according to the preliminary quantitative results of electrophoresis. The PCR products were mixed and purified using 2% agarose gel electrophoresis in 1× TAE buffer, followed by gel extraction. PCR products with the main band size between 300 and 400 bp were selected, recovered by gel-elution using the AxyPrep DNA Gel Recovery Kit (Axygen Biosciences, Union City, CA, United States), and eluted with Tris-HCl.

The purified PCR products were quantified on a Qubit 3.0 fluorometer (Life Technologies, Invitrogen, Carlsbad, CA, United States), and all 24 barcoded amplicons were evenly mixed and used to construct an Illumina antithesis library, according to the preparation procedure of the Illumina genomic DNA library. Sequencing was performed on the Illumina MiSeq platform (Shanghai Biozeron Co., Ltd., Shanghai, China) (2 × 250).

Stable Isotope Determination

The crab samples were small and comprised a mix of three individual samples. After freeze-drying, the processed samples were ground, and screened using an 80-mesh sieve. The stable isotopes of the samples were determined at the Laboratory of Ingestion Ecology, Shanghai Ocean University. The samples were covered with aluminum foil, and the muscle of *E. sinensis*, POM, *A. philoxeroides*, and feed samples weighed 1.00, 5.00, 2.00, and 1.50 mg, respectively. A stable isotope mass spectrometer (ISOPRIME100, Isoprime Corporation, Cheadle, United Kingdom) was used to measure the stable isotope ($\delta^{13}C$ and $\delta^{15}N$) values of crab samples, international standard material (PBD), and purified atmospheric N_2 as reference standards; $\delta^{13}C$ and $\delta^{15}N$ were calculated using the following formula:

$$\delta X\,(‰) = [(R_{sample}/R_{standard} - 1) \times 1000]$$

where, X is ^{13}C or ^{15}N; R_{sample} is the sample's isotope ratio of $^{13}C/^{12}C$ or $^{15}N/^{14}N$; and $R_{standard}$ is the isotope ratio of the standard (Caut et al., 2009). To ensure the accuracy of the experimental results and the stability of the instrument, one standard sample was inserted every ten samples for calibration. The analysis accuracies of the $\delta^{13}C$ and $\delta^{15}N$ values of the samples were 0.05 and 0.06‰, respectively.

Data Analysis

Sequences were classified as operational taxonomic units (OTUs) at 97% sequence similarity level using UPASE (version 7.1), and UCHime was used to identify and delete chimeric sequences (Edgar, 2010) for OTU clustering and species classification analysis based on the valid data, so as to obtain the corresponding species information and abundance distribution. In addition, species composition and alpha diversity were analyzed to obtain the composition and richness information of eukaryotes in river crabs (Bokulich and Mills, 2013; Mueller et al., 2014).

The software program SPSS 26.0 (SPSS, Chicago, IL, United States) was used to analyze the obtained data. One-way analysis of variance was used to compare the differences in $\delta^{13}C$ or $\delta^{15}N$ in feeding sources under different feeding modes. To determine homogeneity of variance, inverse sine or square root processing was performed on the percentage data. One-way analysis of variance was then applied, using the Tukey's-b (K) method for multiple comparisons. If homogeneity of variance was not satisfied after data conversion, then multiple comparisons were performed with Games-Howell Parametric tests. $P < 0.05$ was considered significant. To assess the contribution of different feed sources to the isotopic characteristics of *E. sinensis*, the stable isotope mixing model "Stable Isotope Analysis in R" (SIAR; Parnell and Jackson, 2013) package (version 4.2) was used in R 3.3.2 (R Development Core Team, 2016). The SIAR model used the $\delta^{13}C$ and $\delta^{15}N$ values of consumers and their feed sources to estimate the potential contribution of each feed source that consumers prey on. The model was run in R, allowing variability to be included in the stable isotope ratios of predators and potential feed sources (Parnell et al., 2010). The SIAR model was used to identify the stomach contents and obtain stable isotopic values according to the study of Caut et al. (2009), who found that the $\delta^{15}N$ fractionation factor of *E. sinensis*, i.e., the Δ value, was 2.75 ± 0.01 ‰, and the fractionation factor of $\delta^{13}C$ was 0.75 ± 0.11 ‰.

RESULTS

Growth Performance of Juvenile *Eriocheir sinensis* Under Three Feeding Modes

Changes in the average weight and WGR of juvenile crabs during culture under three feeding modes are shown in **Figure 2**. As shown in **Figure 2A**, the average weight of juvenile *E. sinensis* in all three feeding modes gradually increased as the crabs matured to the breeding stage. At the end of the breeding stage (November), there was no significant difference in the average weight between the traditional feeding mode (8.43 ± 2.13) and the mixed feeding mode (8.36 ± 2.39) ($P > 0.05$), which was

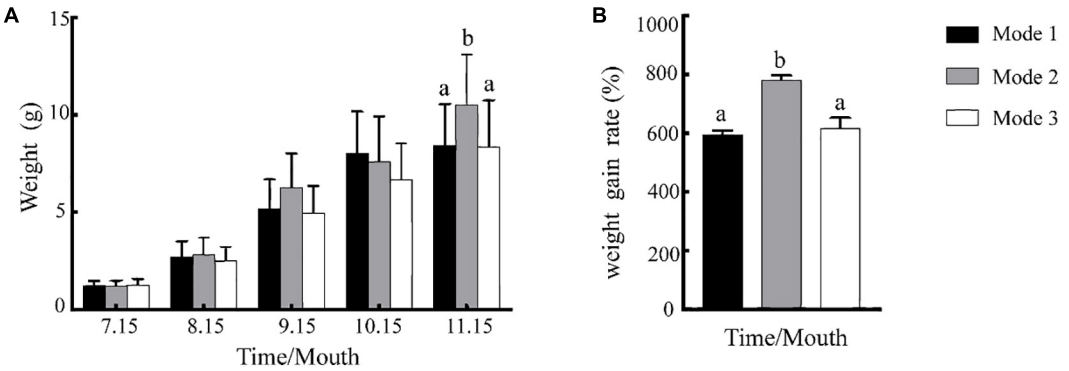


FIGURE 2 | Changes of body weight **(A)** and comparison of juvenile crab weight gain rates **(B)** during juvenile crab breeding under three feeding modes. The values on the bar graph represent the average; the error bars represent the standard deviation (n = 40). Letters above the bars of the same series indicate significant differences between treatments ($P < 0.05$). Mode 1, 2, and 3 represent traditional, formulated, and mixture feeding modes, respectively.

significantly lower than the average weight of the juvenile crabs in the formulated feeding mode (10.52 ± 2.60) ($P < 0.05$). As shown in **Figure 2B**, there is no significant difference in the WGR between the traditional feeding mode (593.23 ± 15.29) and the mixed feeding mode (615.91 ± 38.09) ($P > 0.05$), and was significantly lower than that in the formulated feeding mode (593.23 ± 15.29) ($P < 0.05$).

Diversity Index and Abundance Index of the Stomach Contents of *Eriocheir sinensis* Under Three Feeding Modes

The alpha diversity of the stomach contents of male and female crabs under the three feeding modes is shown in **Figure 3**. The dilution (**Figure 3A**) and Shannon–Wiener diversity index curves (**Figure 3B**), which were based on the number of OTUs, tend to saturation.

The Simpson's diversity indices of the stomach contents of female crabs under traditional, formulated, and mixed feeding modes were 0.27 ± 0.07, 0.32 ± 0.11, and 0.27 ± 0.03, respectively, indicating no differences between any two groups (**Figure 3C**; $P > 0.05$), and the corresponding Simpson's diversity indices of the stomach contents of male crabs were 0.25 ± 0.14, 0.24 ± 0.09, and 0.57 ± 0.30, respectively, also indicating no differences between any two groups (**Figure 3C**; $P > 0.05$). There were no differences in Simpson's diversity indices between male and female crabs under the same feeding mode ($P > 0.05$).

The Chao's species richness indices of the stomach contents of female crabs under traditional, formulated, and mixed feeding modes were 61.67 ± 26.77, 24.33 ± 7.42, and 65.33 ± 20.50, respectively, indicating no differences between any two groups (**Figure 3D**; $P > 0.05$). Similarly, the Chao's species richness indices of the stomach contents of male crabs under traditional, formulated, and mixed feeding modes are 37.00 ± 4.04, 53.00 ± 22.50, and 22.33 ± 8.41, respectively, indicating no differences between any two groups (**Figure 3D**; $P > 0.05$). There were no differences in the Chao's species richness indices between male and female crabs under the same feeding mode ($P > 0.05$). The abundance of the main potential eukaryotes in the stomach of male and female crabs was not different under the different feeding modes, but the compositions of their stomach contents were different.

Analysis of the Main Potential Eukaryotic Components in the Stomach of *Eriocheir sinensis* Under Three Feeding Modes

The species with the highest OTU abundance under the three feeding modes are shown in **Table 2**. A total of 34 phyla were identified in the stomach contents of crabs. The number of phyla identified in the stomach contents of crabs reared under traditional, formulated, and mixed feeding modes were 24, 24, and 29, respectively.

No differences ($P > 0.05$) in the abundance of stomach phyla were observed between crabs reared under the three feeding modes, with Arthropoda showing the highest overall phylum abundance in the stomach contents of crabs reared under the three feeding modes. The content of Arthropoda in the stomach of male (45.68 ± 0.14%) and female (51.48 ± 0.12) crabs was the highest under the traditional feeding mode, but the opposite was true under the formulated feeding mode. The results showed that the contents of Phragmoplastophyta in the stomach of male (23.20 ± 0.15%) and female (18.99 ± 0.16%) crabs was the highest under the formulated feeding mode, and the contents of Diatom in the stomach of male (8.43 ± 0.02%) and female (6.96 ± 0.02%) crabs was the highest under the mixed feeding mode, while the contents of Phragmoplastophyta (in male crabs abundance accounted for 6.80 ± 0.01%; in female crabs abundance accounted for 16.90 ± 0.13%) and Diatom (male crabs abundance accounted for 4.35 ± 0.02%; in female crabs abundance accounted for 3.72 ± 0.03%) in the stomach of crabs were the lowest under the traditional feeding mode.

The class level of abundance of the main potential feed sources of crabs under the different feeding modes is shown in **Figure 4**, in which Malacostraca has the highest overall abundance in the stomach contents of crabs under the three feeding modes, with the lowest being 71.02% (formulated feeding mode), the highest

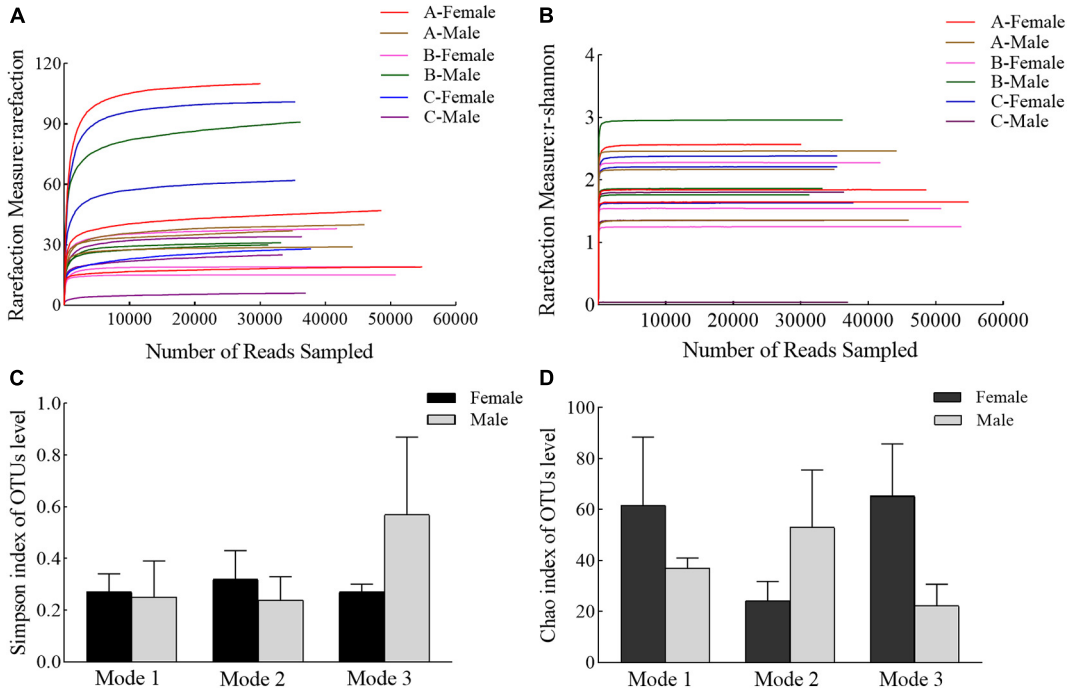


FIGURE 3 | Alpha diversity comparisons of the stomach contents of juvenile *Eriocheir sinensis* reared under different feeding modes. **(A)** Rarefaction curve, **(B)** Shannon-Wiener curve using Shannon's index as the metric for crab fed with various diets, **(C)** Simpson's diversity indices in different feeding modes, and **(D)** Chao's species richness indices in different feeding modes. Values on the bar graph represent the average; error bars represent the standard deviation (n = 9). Mode 1, 2, and 3 represent traditional, formulated, and mixture feeding modes, respectively.

TABLE 2 | Operational taxonomic units (OTUs) of stomach content abundance exceeding 5% in crab samples reared under three feeding modes (%).

Phylum	Gender	Traditional feed mode	Formulated feed mode	Mixture feed mode
Arthropoda	Female	51.48 ± 0.12	50.87 ± 0.11	40.93 ± 0.11
	Male	45.68 ± 0.14	30.25 ± 0.08	36.76 ± 0.27
Phragmoplastophyta	Female	16.90 ± 0.13	23.20 ± 0.15	24.74 ± 0.11
	Male	6.80 ± 0.01	18.99 ± 0.16	5.08 ± 0.04
Diatomea	Female	3.72 ± 0.03	8.2 ± 0.04	8.43 ± 0.02
	Male	4.35 ± 0.02	3.13 ± 0.03	6.96 ± 0.02

61.84% (mixed feeding mode), and an average of 67.44%. The proportions of Malacostraca, Embryophyta, Trebouxiophyceae, Bivalvia, Chlorophyceae, Insecta, and Intramacronucleata decreased successively to 69.45, 9.01, 8.34, 4.34, 3.30, 3.18, and 2.39%, respectively, under the traditional feeding mode. The proportions of Malacostraca, Trebouxiophyceae, Embryophyta, Chlorophyceae, Intramacronucleata, Insecta, and Bivalvia decreased successively to 71.02, 13.39, 8.95, 6.15, 0.48, 0.1, and 0.08%, respectively, under the formulated feeding mode. The proportions of Malacostraca, Embryophyta, Trebouxiophyceae, Chlorophyceae, Intramacronucleata, Insecta, and Bivalvia decreased successively to 61.84, 12.91, 5.45, 3.5, 0.29, 0.1, and 0.09%, respectively, under the mixed feeding mode. The taxa with different feeding patterns at different class levels are the same but have different proportions (**Figure 4**). Among the three feeding modes, the top three taxa were Malacostraca, Embryophyta, and Trebouxiophyceae.

$\delta^{13}C$ and $\delta^{15}N$ Stable Isotopic Characteristics of *Eriocheir sinensis* Under the Three Feeding Modes

The $\delta^{13}C$ and $\delta^{15}N$ values of *E. sinensis* juvenile crab muscle under three different feeding modes are shown in **Table 3**. The muscle $\delta^{13}C$ values (‰) of juvenile crabs under the formulated, mixed, and traditional feeding modes were −24.02 ± 0.72, −24.45 ± 1.22, and −23.31 ± 1.19, respectively, indicating no difference between the three feeding modes ($P > 0.05$). The muscle $\delta^{15}N$ values (‰) of juvenile crabs in the formulated, mixed, and traditional feeding modes were 3.37 ± 0.64, 3.08 ± 0.37, and 2.13 ± 0.57, respectively, and that of the formulated feeding mode was significantly higher than that of the traditional feeding mode ($P < 0.05$).

$\delta^{13}C$ and $\delta^{15}N$ Stable Isotopic Characteristics of Potential Feed Sources of *Eriocheir sinensis*

The overall distribution of $\delta^{13}C$ and $\delta^{15}N$ stable isotopes of the potential feeding sources of crabs under the three feeding modes is shown in **Figure 5**. The $\delta^{13}C$ values ranged from −33.90 ± 0.54‰ to −23.19 ± 0.11‰, and the $\delta^{15}N$ values ranged from 0.49 ± 0.02‰ to 5.63 ± 0.01‰. Among them,

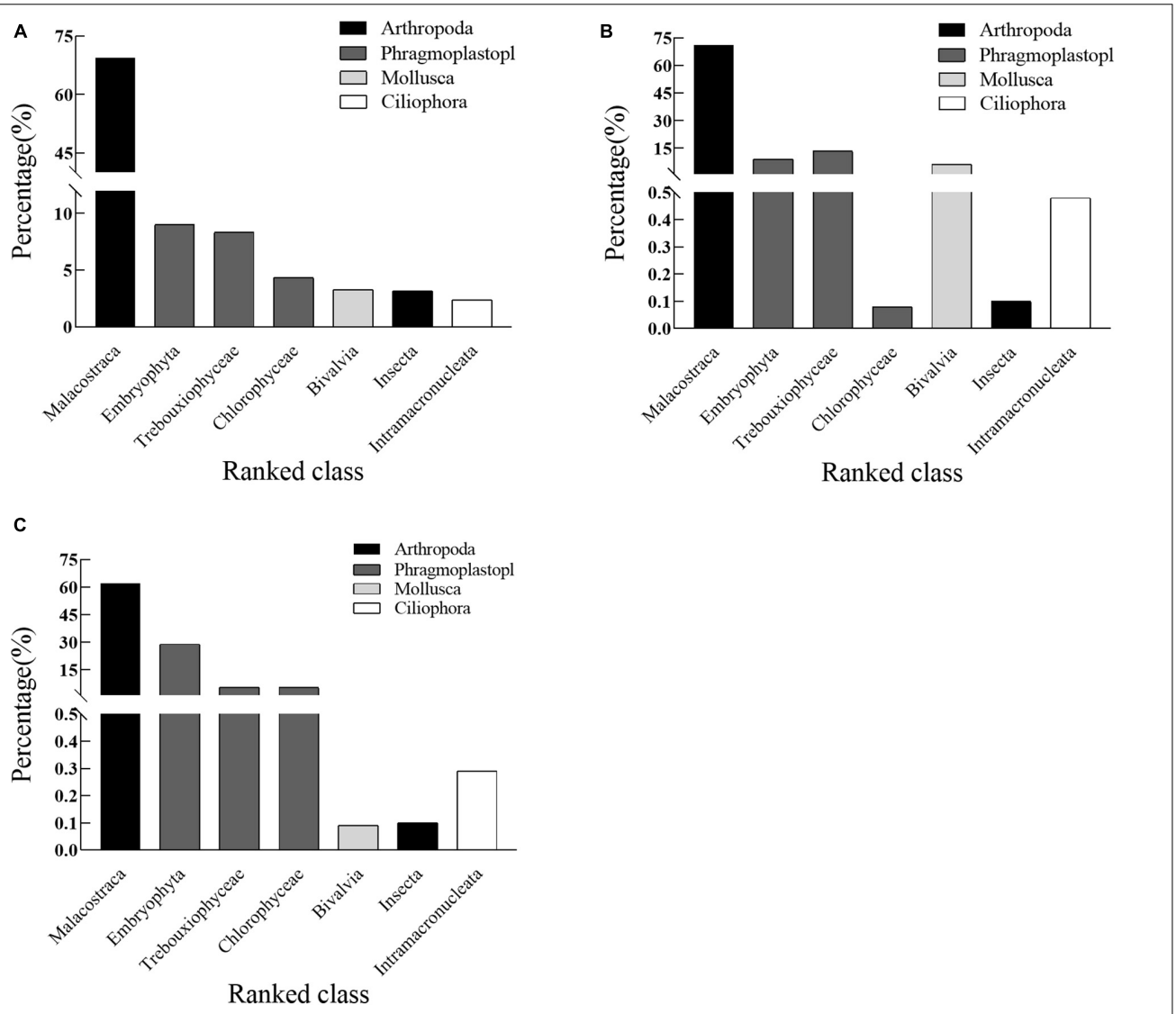


FIGURE 4 | Rank abundance of the main potential prey reads of juvenile *Eriocheir sinensis* crabs at the class level under **(A)** traditional, **(B)** formulated, and **(C)** mixture feeding modes based on 18S rDNA. Values on the bar graph represent the average.

TABLE 3 | Analysis of variance of ^{13}C and ^{15}N isotope contents in muscle of Chinese mitten crab in different feeding modes (‰).

Stable isotopes	Traditional feed mode	Formulated feed mode	Mixture feed mode
C	−23.31 ± 1.19	−24.02 ± 0.72	−24.45 ± 1.22
N	2.13 ± 0.57^{a}	3.37 ± 0.64^{b}	3.08 ± 0.37^{ab}

Values with different small letters mean significant differences in the same row ($P < 0.05$). The data in the table are expressed as mean ± standard deviation (−X ± SD), n = 3.

the $\delta^{13}C$ variation ranges of the potential feed sources of crabs under the traditional, formulated, and mixed feeding modes were −33.43 ± 0.71‰ to −26.04 ± 0.19‰, −32.98 ± 0.93‰ to −23.19 ± 0.12‰, and −33.90 ± 0.54‰ to −24.47 ± 0.05‰, which changed substantially, like the corresponding $\delta^{15}N$ variation under the three feeding modes (0.49 ± 0.02–5.63 ± 0.01‰, 2.01 ± 0.07–5.63 ± 0.01‰, and 1.09 ± 0.21–5.63 ± 0.01‰, respectively).

Potential Feed Source Composition of *Eriocheir sinensis* Under Three Feeding Modes Based on Stable Isotope Analysis

The average feed contribution rate in *E. sinensis* was analyzed using SIAR (**Figure 6**). Under the traditional feeding mode, the main diet of *E. sinensis* was a traditional feed, with an average contribution of 71.89%, followed successively by POM and *A. philoxeroides*, with average contributions of 16.66 and 11.46%, respectively. Under the formulated feeding mode, the main diet of crabs was formulated feed, with an average contribution of 66.48%, followed successively by

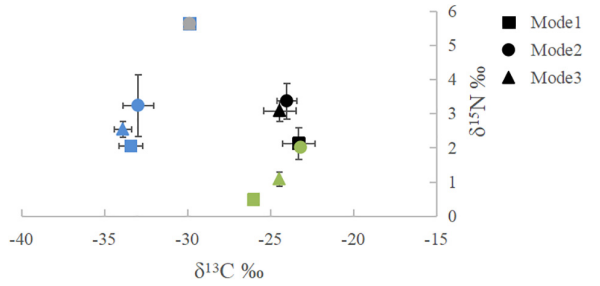


FIGURE 5 | Isotopic compositions of ^{13}C and ^{15}N stability of muscles and possible feed sources under the traditional, formulated, and mixture feeding modes. Error bars are ± SD. Mode1, 2, and 3 represent traditional, formulated, and mixted feeding modes, respectively. Color of symbol denotes different sources: black are muscle samples; gray are *Alternanthera philoxeroides* samples; bule are particulate organic matter (POM) samples; green are feed samples.

A. philoxeroides and POM, with average contributions of 17.50% and 16.03%, respectively. Under the mixed feeding mode, the main diet of crabs was a mixed feed, with an average contribution of 76.84%, followed by POM (11.92%) and *A. philoxeroides* (11.24%), whose average contribution rates were not significantly different.

DISCUSSION

Differences in the Growth and Dietary Composition of *Eriocheir sinensis* Under the Three Feeding Modes

In this study, 18S rDNA was used to analyze the stomach feed composition of male and female juvenile crabs under traditional, formulated, and mixed feeding modes. The flat curve of Rarefaction index and Shannon–Wiener curve indicated that the sequencing data covering the feeding source species of juvenile crabs and were sufficient to reflect the feed source diversity of crabs under different feeding modes. The Simpson and Chao indexes represented the diversity and abundance of food composition in the stomach of the crabs, respectively. The higher the Simpson index, the lower the diversity index, while the Chao index of abundance showed a contrary trend. The results showed that there was no significant difference among the three feeding modes ($P > 0.05$), but the terminal

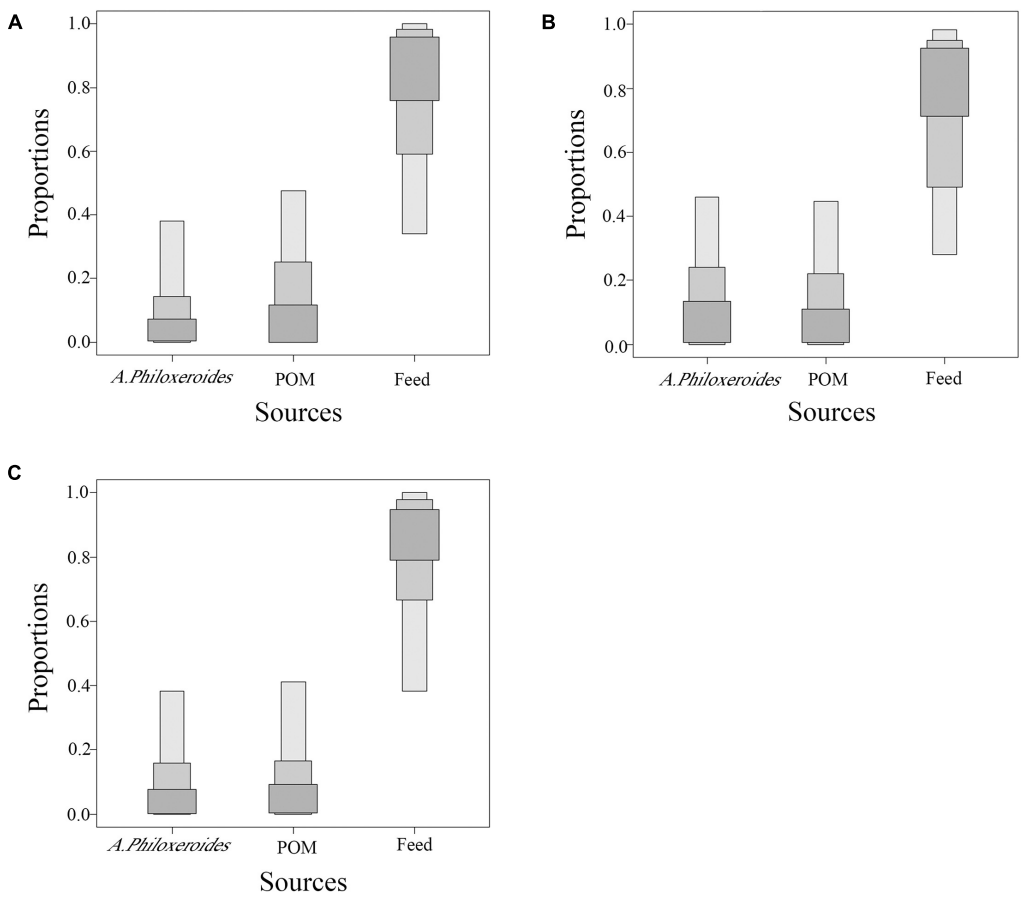


FIGURE 6 | Feed contribution rate of juvenile *Eriocheir sinensis* based on the stable isotope analysis in R package (SIAR) analysis. **(A)** Traditional feeding mode, **(B)** Formulated feeding mode, and **(C)** Mixture feeding mode.

body weight and WGR under the formulated feeding mode were significantly higher than those under traditional feeding mode and mixed feeding mode. This result may be due to the traditional, formulated, and mixed feeds made up the majority of the crabs' diet, so the diversity and abundance of other feed items were different but not significantly so. But the growth of *E. sinensis* is affected by factors such as feed and culture environment (Shao et al., 2013, 2014), which can reflect the quality of feed (Yang et al., 2014). The formulated feed containing high protein and fat (Yang et al., 2011), which is conducive to the growth of juvenile crabs. However, the traditional feeding mode had fewer nutrients, so the above indexes of juvenile crabs fed with formulated feed alone were significantly higher than those of the other two feeding modes.

The most representative phyla reported for the sampling site (Arthropoda, Phragmoplastophyta, and Diatomea) also showed the highest occurrence in the dietary samples analyzed. The phylum Arthropoda, with the highest proportion, may be composed of arthropods within the feeding space of crabs, including Malacostraca (injured or newly molting crabs) (Li, 2006) and Insecta (aquatic nymphal insects) (Chen et al., 1989). This is different from the results of Jin et al. (2003), who studied the feeding habits of the second instar adult *E. sinensis*, and can be attributed to the different ecological niches and living environments of the crabs. The animal feed content of Malacostraca and Bivalvia in the stomach of juvenile crabs reared under the traditional feeding mode was the highest among the three feeding modes because the traditional feed is a plant-based feed mainly including bran, wheat, and soybean meal, which are insufficient to meet the nutritional needs of juvenile crabs (Veilleux and de Lafontaine, 2007), thus driving them to consume more animal feed. Under the formulated feeding mode, the plant feed content of Embryophyta and Trebouxiophyceae in the juvenile crab stomach was the highest among the three feeding modes, mainly because the formulated feed is mainly composed of animal feed, and to achieve nutritional balance (Chen et al., 1989), which are entwined and attached to the other feed sources of crabs (Vizzini and Mazzola, 2003). Under the mixed feeding mode, the animal and plant feed contents in the crab stomach were the lowest between the three feeding modes because the mixed feed contained both animal and plant feeds and provided balanced nutrition to crabs (Qian and Zhu, 1999).

Compared with zooplankton, fish, and macroinvertebrates in the near waters of Gouqi Island, the variation in the values of $\delta^{13}C$ and $\delta^{15}N$ were relatively large (Jiang et al., 2014), indicating that crabs have a wide range of feed sources. Previous studies have shown that the crab's $\delta^{13}C$ and $\delta^{15}N$ changes depending on what it eats (Zeng, 2010) and the $\delta^{13}C$ value of consumers is usually close to that of their feed, the $\delta^{13}C$ value of individuals does not change much in the transmission of the whole feed chain (Lin, 2013), which is in accordance with the results of this study. Animals reject $\delta^{15}N$ during exudation of substances; therefore, the higher the trophic level, the higher the content of $\delta^{15}N$ (McCutchan et al., 2010). However, the $\delta^{15}N$ values of *E. sinensis* crabs under traditional ($2.13 \pm 0.57‰$), formulated (3.37 ± 0.64 ‰), and mixed feeding (3.08 ± 0.37 ‰) modes in the present study were lower than those of *A. philoxeroides* ($5.63 \pm 0.37‰$), and this may be because $\delta^{15}N$ is poorly conserved and easily modified by biogeochemical processes, and an increase in the N content in the environment may increase the N content in plants (Lin, 2013; Yu et al., 2014).

Previous studies have shown that juvenile crabs with better growth performance can be obtained by feeding high quality feed (Wu et al., 2009), the content of fat and ash in formulated feed was higher than that of two other feeding modes indicating that formulated feed had higher energy level, this may be the reason why the growth performance of the crab fed with formulated feed is better. Under the traditional feeding mode, crabs mainly consumed POM as a feed supplement in addition to the traditional feed, which is consistent with the results of Paning (1934) and Rudnick et al. (2000), who reported that the stomach contents of *E. sinensis* contain a large amount of POM. POM is the substrate of complex biological communities, formed by the decomposition and mineralization of dead animals and plants, humus, and feces by microorganisms (Liu, 1999), and some of its components are the main feed sources for benthic invertebrates (Vizzini and Mazzola, 2003). Under the formulated feeding mode, apart from the formulated feed, the proportion of *A. philoxeroides* was the highest. In the presence of sufficient animal feed, crabs will still choose *A. philoxeroides* as a feed supplement (Li et al., 2018). This is because *A. philoxeroides* is rich in cellulase, which helps to improve the cellulase activity (Yang et al., 2014) and digestive ability of crabs. The mixed feed containing plant- and animal-based feeds had a balanced nutritional ratio (Qian and Zhu, 1999) and high palatability, and the proportion of mixed feed consumed by *E. sinensis* was the highest, resulting in the lowest feeding proportion of POM and *A. philoxeroides* between the three feeding modes. But the size, shape and palatability of the two diets were significantly different, and the physical difference of the mixed feeding group made the crab unable to make full use of the feed (Wang S. M. et al., 2017). Therefore, there was no significant difference in the weight change and WGR of juvenile crabs under the mixed feeding mode and the traditional feeding mode. The results of the stable isotope technique were consistent with those of the 18S rDNA analysis. Juvenile crabs feed on both plant- and animal-based feeds in an aquaculture pond, but they are not complete predators and selectively feed on animal or plant feeds as supplements of that which is deficient, in addition to their main feed (Jin, 2003).

Difference in Stomach Content Composition Between Male and Female *Eriocheir sinensis*

Various studies have shown that there are no differences in feed composition between male and female crabs, and they are, therefore, ecological equivalents (Spooner et al., 2007; Young and Elliott, 2020). In the present study, the diversity and abundance of female crabs were generally higher than those of male crabs, and female crabs had a higher rate of feeding on available feed sources in the pond, this may be because female crabs increase their feeding rate during October to November every year to meet their growth

and development needs (Chen et al., 1989), while promoting gonad maturation and transformation (Xu et al., 2019). However, no significant differences were observed in Simpson's diversity and Chao's abundance indices of male and female crabs under the same feeding mode as well as in the abundance of the three main feeding sources, Arthropoda, Phragmoplastophyta, and Diatomea (**Table 2**), indicating that male and female crabs showed similar feed composition under the same feeding mode. Cordone et al. (2020) studied the feeding habits of *C. maenas* and found that different living water depths and a wide range of available feed sources lead to differences in feed composition between male and female crabs, which is different from the results of this study. This may be because the ponds mainly contained artificial feeds for arthropods (including shrimp), mollusks (including snail), cultivated *A. philoxeroides*, Phragmoplastophyta dominated by Trebouxiophyceae species, and diatoms dominated by *Chlorella* (Lu et al., 2013). The pond was shallow, and the crabs can eat less feed than wild crabs, but the difference was minor.

CONCLUSION

The three different feeding modes and different genders had no effect on the feeding habits of crabs. But we concluded that feeding formulated feed is more favorable to the growth of juvenile crabs. The identified benthic organisms were the main food in the stomach contents of crabs under different feeding modes. In addition, the proportion of POM is similar to *Alternanthera philoxeroides* in three feeding modes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov, BioProject accession PRJNA759104.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committee, Shanghai Ocean University.

AUTHOR CONTRIBUTIONS

ZL and YS designed the experiments of this study. CX assisted ZL to complete several animal experiments. ZL performed results analysis and manuscript writing. YS reviewed and edited the manuscript. YS and YC provided the funding resources. All authors read and approved the final manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (No. 31802320) and the China Agriculture Research System (No. CARS-48).

REFERENCES

Bachy, C., Dolan, J. R., López-García, P., Deschamps, P., and David, M. (2013). Accuracy of protist diversity assessments: morphology compared with cloning and direct pyrosequencing of 18S rRNA genes and *ITS* regions using the conspicuous tintinnid ciliates as a case study. *ISME J.* 7, 244–255. doi: 10.1038/ismej.2012.106

Bade, L. M., Balakrishnan, C. N., Pilgrim, E. M., McRae, S. B., and Luczkovich, J. J. (2014). A genetic technique to identify the diet of cownose rays, *Rhinoptera bonasus*: analysis of shellfish prey items from North Carolina and Virginia. *Environ. Biol. Fishes* 97, 999–1012. doi: 10.1007/s10641-014-0290-3

Bokulich, N. A., and Mills, D. A. (2013). Improved selection of internal transcribed spacer-specific primers enables quantitative, ultra-high-throughput profiling of fungal communities. *Appl. Environ. Microbiol.* 79, 2519–2526. doi: 10.1128/AEM.03870-12

Caut, S., Angulo, E., and Courchamp, F. (2009). Variation in discrimination factors (Δ^{15}N and Δ^{13}C): the effect of diet isotopic values and applications for diet reconstruction. *J. Appl. Ecol.* 46, 443–453. doi: 10.1111/j.1365-2664.2009.01620.x

Chen, B., Du, N. S., and Ye, H. F. (1989). Analysis on the feeding habit of the mitten crab *Eriocheir Sisensis*. *Fish. Sci. Technol. Inf.* 01, 2–5. doi: 10.16446/j.cnki.1001-1994.1989.01.001

Cheng, Y. X., Wu, X. G., Yang, X. Z., and Anson, H. H. (2008). Current trends in hatcherytechniques and stock enhancement for Chinese mitten crab, *Eriocheir japonica sinensis*. *Rev. Fish. Sci.* 16, 377–384. doi: 10.1080/10641260701681698

Cordone, G., Lozada, M., Vilacoba, E., Thalinger, B., Bigatti, G., Lijtmaer, D., et al. (2020). Metabarcoding, direct stomach observation and stable isotope analysis reveal a highly diverse diet for the invasive green crab in Atlantic Patagonia. *Ecol. Environ. Conserv.* 489. doi: 10.1101/2020.08.13.249896

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

Fu, L. L., Li, Y. H., Lu, Q. P., Ding, S. Y., and Deng, Y. F. (2014). Comparative analysis of several breeding modes of 1st age crab species. *Aquaculture* 035, 39–42. doi: 10.3969/j.issn.1004-2091.2014.12.014

Han, W. F., Sun, Y. F., Liu, J., Zhang, Y., and Cheng, Y. X. (2021). Effect of different feeding modes on the growth, biochemical composition, and living environment of the juvenile Chinese mitten crab *Eriocheir sinensis*. *Aquaculture* 1:736687. doi: 10.1016/J.AQUACULTURE.2021.736687

He, J., Wu, X. G., Li, J. Y., Huang, Q., Huang, Z. F., and Cheng, Y. X. (2014). Comparison of the culture performance and profitability of wild-caught and captive pond-reared Chinese mitten crab (*Eriocheir sinensis*) juveniles reared in grow-out ponds: implications for seed selection and genetic selection programs. *Aquaculture* 434, 48–56.

Huang, J., Norgbey, E., Li, G., Wang, J., Rainizafy, M., Takyi-Annan, G. E., et al. (2019). Unraveling the feeding dynamics of chinese mitten crab-based ecosystems using carbon and nitrogen stable isotope techniques. *J. Consum. Prot. Food Saf.* 14, 251–261. doi: 10.1007/s00003-019-01220-w

Jiang, R. J., Wang, K., Zhou, X. J., and Zhao, J. (2014). Stable isotope analysis of food web in coastal waters of Gouqi Island. *Chin. J. Ecol.* 33, 930–938.

Jin, G. (2003). Advances in crab ecology. *J. Shenzhen Vocat. Tech. Coll.* 01, 28–34. doi: 10.3969/j.issn.1672-0318.2003.01.008

Jin, G., Xie, P., and Li, Z. J. (2003). Feeding habits of the second instar *Eriocheir sinensis* released from lakes. *Acta Hydrobiologica Sin.* 02, 29–35. doi: 10.3321/j.issn:1000-3207.2003.02.007

Lafontaine, Y. D., and Veilleux, É (2002). Biological synopsis of the Chinese mitten crab (*Eriocheir sinensis*). *Fish. Sci. China* 2812, 1–45. doi: 10.3724/SP.J.1011.2010.01351

Lan, S. Q., Zhang, L. W., Yi, H. P., Xu, C. L., Lu, F., Feng, G. H., et al. (2020). Food source and feeding habit of *Helice tientsinensis* from the common reed

vegetation in high marsh of Yellow River Delta, China. *Chin. J. Appl. Ecol.* 31, 319–325. doi: 10.13287/j.1001-9332.202001.034

Li, C., Cheng, Y. X., Guan, Q. Z., Xi, Y. W., and Li, J. Y. (2018). Analysis of rice shrimp system by stable isotope technique. *J. Fish. China* 42, 1778–1786.

Li, X. D. (2006). *New Technology of Northern River Crab Culture*. Beijing: China Agriculture Press.

Lin, G. H. (2013). *Stable Isotope Ecology*. Beijing: Higher Education Press.

Liu, J. K. (1999). *Advanced Aquatic Biology*. Beijing: Science Press.

Lu, D., Cheng, G. P., and Qin, M. L. (2013). Biological composition and dynamic changes of algae in different aquaculture ponds. *World Econ. Yearb. Chin. Bus. Urban Rural Constr.* 003, 336–336.

McCutchan, J. H., Lewis, W. M., Kendall, C., and Mcgrath, C. C. (2010). Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur. *Oikos* 102, 378–390. doi: 10.1034/j.1600-0706.2003.12098.x

MOAC (2020). *China Fisheries Yearbook*. Beijing: China Agriculture Press.

Mueller, R. C., Paula, F. S., Mirza, B. S., Rodrigues, J. L. M., Bohannan, B. J. M., and Nusslein, K. (2014). Links between plant and fungal communities across a deforestation chronosequence in the Amazon rainforest. *ISME J.* 8, 1548–1550. doi: 10.1038/ismej.2013.253

Ning, J. J., Du, F. Y., Wang, X. H., Gu, Y. G., Wang, G. L., and Li, Y. F. (2016). Analysis of feeding habits of mantis shrimps based on stable isotopes. *J. Fish. China* 6, 903–910. doi: 10.11964/jfc.20151110177

O'Rorke, R., Lavery, S., Chow, S., Takeyama, H., Tsai, P., Beckley, L. E., et al. (2012). Determining the diet of larvae of western rock lobster (*Panulirus cygnus*) using high-throughput DNA sequencing techniques. *PLoS One* 7:e42757. doi: 10.1371/journal.pone.0042757

Pan, J., Wu, X. G., Zhao, H. L., He, J., Jiang, X. D., Wang, Y. P., et al. (2016). Effects of three feeding modes on the culture performance of adult pond-reared Chinese mitten crab (*Eriocheir sinensis*) during the second year culture. *Freshw. Fish.* 46, 87–93.

Paning (1934). The Chinese mitten crab. *Nature* 134:17. doi: 10.1038/134017a0

Parnell, A., and Jackson, A. (2013). *Stable Isotope Analysis in R, Vol 4.2*. Vienna: R Foundation for Statistical Computing.

Parnell, A. C., Inger, R., Bearhop, S., and Jackson, A. L. (2010). Source partitioning using stable isotopes: coping with too much variation. *PloS One* 5:e9672. doi: 10.1371/journal.pone.0009672

Qian, G. Y., and Zhu, Q. H. (1999). The appropriate content of protein, fat and cellulose in the compound diet of chinese mitten crab. *J. Fish. Sci. China* 03, 61–65.

Que, Y. Q., Yang, Z. G., Ji, L. Y., He, J., Shao, L. C., Wang, C., et al. (2012). Effects of formulated dietary replacement of trash fish on growth performance, body composition and fatty acid composition of *Eriocheir sinensis*. *J. Fish. Sci. China* 036, 1612–1623. doi: 10.3724/SP.J.1231.2012.27912

R Development Core Team (2016). *R: A Language and Environment for Statistical Computing, Vol 3.3.2*. Vienna: R Foundation for Statistical Computing.

Riemann, L., Alfredsson, H., Hansen, M. M., Als, T. D., Nielsen, T. G., Munk, P., et al. (2010). Qualitative assessment of the diet of European eel larvae in the Sargasso Sea resolved by DNA barcoding. *Biol. Lett.* 6, 819–822. doi: 10.1098/rsbl.2010.0411

Rudnick, D., and Resh, V. (2005). Stable isotopes, mesocosms and gut content analysis demonstrate trophic differences in two invasive decapod crustacea. *Freshw. Biol.* 50, 1323–1336. doi: 10.1111/j.1365-2427.2005.01398.x

Rudnick, D. A., Halat, K. M., and Resh, V. H. (2000). *Distribution, Ecology and Potential Impacts of the Chinese Mitten Crab (Eriocheir sinensis) in San Francisco Bay*. Berkeley: University of California Water Resources Center, 206.

Shao, L. C., Wang, C., and He, J. (2013). Hepatopancreas and gonad quality of Chinese mitten crabs fattened with natural and formulated diets. *J. Food Qual.* 36, 217–227. doi: 10.1111/jfq.12030

Shao, L. C., Wang, C., He, J., Wu, X. G., and Cheng, Y. X. (2014). Meat quality of Chinese mitten crabs fattened with natural and formulated diets. *J. Aquat. Food Prod. Technol.* 23, 59–72. doi: 10.1080/10498850.2012.694583

Spooner, E. H., Coleman, R. A., and Attrill, M. J. (2007). Sex differences in body morphology and multitrophic interactions involving the foraging behaviour of the crab *Carcinus maenas*. *Mar. Ecol.* 28, 394–403. doi: 10.1111/j.1439-0485.2007.00186.x

Sun, Z. L. (2012). *Study on the Metabolic Process of Main Nutrient Elements in the Culture of Sea Cucumber*. Ph.D. thesis. Qingdao: Ocean University of China.

Veilleux, E., and de Lafontaine, Y. (2007). Biological synopsis of the Chinese mitten crab (*Eriocheir sinensis*). *Can. Manusc. Rep. Fish. Aquat. Sci.* 2812:45. doi: 10.3724/SP.J.1011.2010.01351

Vizzini, S., and Mazzola, A. (2003). Seasonal variations in the stable carbon and nitrogen isotope ratios ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) of primary producers and consumers in a western Mediterranean coastal lagoon. *Mar. Biol.* 142, 1009–1018. doi: 10.1007/s00227-003-1027-6

Wang, S. M., Lu, J., and Wang, P. P. (2017). A comparative experiment on the effect of whole-process feed feeding and traditional breeding method for *Eriocheir sinensis*. *Aquaculture* 38, 7–9.

Wang, W., and Wang, C. H. (2013). *Ecological Culture of River Crab*, 2nd Edn. Beijing: China Agriculture Press.

Wang, X. F., Lin, C. G., Xu, Q., Song, X. Y., Zhang, H. J., Ru, S. G., et al. (2017). Analysis of feeding changes of *Crassostrea gigas* during the transit of Enteromorpha green tide using 18S rDNA molecular method. *Oceanol. Limnol. Sin.* 048, 1362–1370.

Wu, X., Cheng, Y., Zeng, C., Sui, L., Southgate, P. C., Zhou, G., et al. (2009). Reproductive performance and offspring quality of Chinese mitten crab *Eriocheir sinensis* (H. Milne-Edwards) females fed an optimized formulated diet and the razor clam *Sinonovacula constricta*. *Aquac. Res.* 40, 1335–1349. doi: 10.1111/j.1365-2109.2008.02121.x

Xu, J. J., Geng, Z., and Feng, G. P. (2019). Feeding habits and feeding intensity of female crab *E.sinensis* in the Changjiang Estuary. *Mar. Fish.* 41, 397–407. doi: 10.13233/j.cnki.mar.fish.2019.04.002

Yang, L. L., Yang, X. Z., Zhao, L. L., Fan, P., Liang, P., and Cheng, Y. X. (2011). Effects of frozen fish and compound diet on growth, digestive enzyme activities and blood cells of juvenile Chinese mitten crab (*Eriocheir sinensis*). *J. Fudan Univ.* 50, 619–624.

Yang, X., Ye, J. Y., Zhang, Y. X., Wu, C. L., Liu, P., and Wang, W. (2014). Effects of replacement of fish meal by common cottonseed meal and fermented cottonseed meal on growth performance, body composition and hepatopancreas digestive enzyme activities of juvenile Chinese mitten crab (*Eriocheir sinensis*). *Chin. J. Anim. Nutr.* 26, 683–693. doi: 10.3969/j.issn.1006-267x.2014.03.018

Young, A. M., and Elliott, J. A. (2020). Life history and population dynamics of green crabs (*Carcinus maenas*). *Fishes* 5:4. doi: 10.3390/fishes5010004

Yu, H. Y., Yu, Z. M., Song, X. X., Liu, L. L., Cao, X. H., and Yuan, Y. Q. (2014). Seasonal distribution of stable nitrogen isotopes and key biogeochemical processes of suspended particulate organic matter in the Yangtze River estuary. *Acta Oceanol. Sin.* 36, 16–22. doi: 10.3969/j.issn.0253-4193.2014.02.002

Zeng, W. T. (2010). *Studies on the Growth, Feed Sources and Nutritional Niche of Eriocheir sinensis*. Ph.D. thesis. Beijing: University of Chinese Academy of Sciences.

Zhou, T. C., Hu, S. M., Lin, X. Z., Liu, S., and Huang, H. (2020). Analysis of feeding habits of *Tectus pyramis* in coral reef area based on 18S rDNA barcoding. *Mar. Sci.* 44:99.

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 in Marine Science

ORIGINAL RESEARCH
published: 30 November 2021
doi: 10.3389/fmars.2021.775575

Changes in Growth Performance, Nutrient Metabolism, Antioxidant Defense and Immune Response After Fishmeal Was Replaced by Low-Gossypol Cottonseed Meal in Golden Pompano (*Trachinotus ovatus*)

*Yawen Qin[1], Chaoqun He[1], Wenqiang Wang[1], Peng Yang[1], Jun Wang[2,3], Qinbo Qin[4], Kangsen Mai[1] and Fei Song[1,2,5]**

[1] Guangzhou Key Laboratory of Subtropical Biodiversity and Biomonitoring, Guangdong Provincial Key Laboratory for Healthy and Safe Aquaculture, College of Life Science, Institute of Modern Aquaculture Science and Engineering (IMASE), South China Normal University, Guangzhou, China, [2] Southern Marine Science and Engineering Guangdong Laboratory, Zhanjiang, China, [3] Key Laboratory of South China Sea Fishery Resources Exploitation and Utilization, Ministry of Agriculture, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou, China, [4] State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Sciences, Hunan Normal University, Changsha, China, [5] Southern Marine Science and Engineering Guangdong Laboratory (Zhuhai), Zhuhai, China

The present study was designed to elucidate the changing pattern of growth performance, nutrient metabolism, antioxidant defense, and immune response after fishmeal (FM) was replaced by cottonseed meal (CSM). Four isonitrogenous and isolipidic experimental diets (42.5% crude protein, 14.0% crude lipid) were formulated to replace 0% (CSM0 diet), 20% (CSM20 diet), 40% (CSM40 diet), and 60% (CSM60 diet) of the FM protein with CSM. Juvenile golden pompano (*Trachinotus ovatus*) with an initial body weight of 24.8 ± 0.02 g were fed each diet for 6 weeks. The results showed that compared with the control diet, significant differences were not found in the weight gain ratio, specific growth performance, and apparent digestibility of dry matter and protein after 20% FM was replaced by CSM. Higher CSM replacement markedly decreased the growth performance ($P < 0.05$). Moreover, the CSM20 and CSM40 diets did not depress the feed efficiency ratio and protein efficiency ratio compared with the CSM0 diet. Further exploring the physio-biochemical and molecular responses, the present study also showed that dietary 20–40% CSM replacement had no significant effect on altering the whole body, plasma nutrient contents, free amino acids, or whole-body fatty acid contents. Additionally, the CSM20 diet did not change the mRNA and protein phosphorylation levels of the key enzymes and regulators involved in target of rapamycin (TOR) and amino acid response (AAR) signaling, nutrient metabolism, immune response, and antioxidant defense. Higher CSM inclusion significantly varied the TOR and AAR

OPEN ACCESS

Edited by:
Kang-le Lu,
Jimei University, China

Reviewed by:
Cunxin Sun,
Chinese Academy of Fishery Sciences, China
M. Virginia Martín,
Instituto Español de Oceanografía (IEO), Spain
Shuyan Chi,
Guangdong Ocean University, China

***Correspondence:**
Fei Song
songfei@m.scnu.edu.cn

Specialty section:
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

Received: *14 September 2021*
Accepted: *22 October 2021*
Published: *30 November 2021*

Citation:
Qin Y, He C, Wang W, Yang P, Wang J, Qin Q, Mai K and Song F (2021) Changes in Growth Performance, Nutrient Metabolism, Antioxidant Defense and Immune Response After Fishmeal Was Replaced by Low-Gossypol Cottonseed Meal in Golden Pompano (Trachinotus ovatus). Front. Mar. Sci. 8:775575. doi: 10.3389/fmars.2021.775575

signaling response, nutrient metabolism, immune response, and antioxidant defense. Based on the results, CSM could successfully replace 20% FM in the formulated diets for golden pompano. This study proposes the optimal inclusion level of CSM in the diet, which has an important significance in optimizing aquafeeds formulation.

Keywords: fishmeal replacement, low-gossypol cottonseed meal, physio-biochemical changes, gene and protein expression, golden pompano (*Trachinotus ovatus*)

INTRODUCTION

Although researchers have been devoted to exploring fishmeal (FM) substitution for a few decades, seeking appropriate protein sources to replace FM is still an important mission to achieve sustainable development of aquaculture (Wacyk et al., 2012; Song et al., 2016). In recent decades, a large number of studies have been conducted to assess the efficiency of high-quality plant and animal protein sources in FM replacement (Gatlin et al., 2007; Lu et al., 2015). The results have proved that different protein sources showed diverse substitution efficiencies in different fish species (Song et al., 2014; Wang et al., 2015). In recent years, large quantities of alternative protein sources used in the aquafeeds industry resulted in a competition between humans and animals for food. This phenomenon will directly threaten the entire food security system of the world. Therefore, searching for new non-grain protein sources to replace FM represents a new research topic for aquaculture nutrition studies.

Previously, cottonseed meal (CSM) did not attract much attention as a potential FM substitution in the aquafeeds industry. Currently, an increasing number of researchers have realized that CSM is a special protein source for FM substitution because of its abundance throughout the world and also because it is considered as one of the most important non-grain protein sources for aquafeeds (Lim and Lee, 2009; Ye et al., 2020). The annual cottonseed production in China was more than 3.5 million tons (Hu et al., 2021). Moreover, compared with other alternative protein sources, CSM has relatively higher protein content, well-balanced amino acid (AA) profile and steady supply is a potential protein source in aquafeeds (Li and Robinson, 2006). However, CSM contains a high content of gossypol, and a large quantity of free gossypol in the diet is toxic to fish species, which restricts its utilization in aquafeeds (Zheng et al., 2012). Therefore, CSM in which gossypol was eliminated has become favored because of the improved efficiency of its application in diets.

Previous studies have demonstrated that low-gossypol CSM has different replacement proportions in different species. Research on southern flounder (*Paralichthys lethostigma*) suggested that low-gossypol CSM could substitute 75% FM without affecting the growth performance (Sullivan and Reigh, 1995). Anderson et al. (2016) also reported that CSM prepared by solvent extraction could successfully replace dietary 75% FM in black seabass (Anderson et al., 2016). In Pacific white shrimp (*Litopenaeus vannamei*), 130 g/kg of FM could be replaced by 150 g/kg of dephenolized cottonseed protein (Wan et al., 2018). However, a study conducted by Bu et al. (2017) demonstrated that CSM inclusion over 25.3% could significantly depress the growth performance, antioxidant capacity, and immune response in the Ussuri catfish *Pseudobagrus ussuriensis*. All of the literature has elucidated the varying toleration of CSM in different species.

Fishmeal substituted by other protein sources affects nutrient metabolism by regulating nutrient sensing signaling pathways. Our study showed the regulatory role of target of rapamycin (TOR) and amino acid response (AAR) signaling pathways on lipid and glucose metabolism (Song et al., 2016). Studies on the large yellow croaker (*Larimichthys crocea*) (Wei et al., 2019) and juvenile turbot (*Scophthalmus maximus* L.) (Wang et al., 2015) also showed that alternative protein meal replacement diets changed growth performance and nutrient metabolism by regulating the TOR signaling pathway. Moreover, FM replacement has also had a great effect on modifying the immune response in fish species (Dossou et al., 2018; Xie et al., 2019). In 2020, Yang et al. reported that largemouth bass fed a poultry by-product meal total substitution diet markedly altered their antioxidant defense and immune response compared with fish fed a FM diet (Yang et al., 2020). Moreover, researchers on red sea bream *Pagrus major* (Dossou et al., 2018) and hybrid grouper female *Epinephelus fuscoguttatus* × male *E. lanceolatus* (He et al., 2021) provided solid evidence that FM replacement impaired health status by damaging the immune system. Therefore, estimating the changes in nutrient metabolism, immune response, and antioxidant defense status after fish fed alternative protein sources was considered as another important evaluation indicator in assessing the optimal substitution ratio.

Golden pompano (*Trachinotus ovatus*) is a commercially important marine fish due to its advantage of growing fast, favorable taste, good nutrient profile, and suitability for culture (Tan et al., 2016). In China, it is widely distributed along the coastline of the South China Sea including Guangdong, Fujian, and Hainan provinces. Since it is a carnivorous fish species, golden pompano has a higher requirement for dietary protein (Tang et al., 2013; Xun et al., 2019). Therefore, searching new protein sources to optimize diet formulation has become the key way to achieve healthy and sustainable development of the golden pompano culture industry (Ma et al., 2014). The present study was designed to evaluate the optimal replacement ratio of CSM for FM in golden pompano based on the results of growth performance, physiological and biochemical indexes, metabolism changes, and molecular responses.

MATERIALS AND METHODS

Ethics Statement

The experimental procedures strictly complied with the regulations of the University Animal Care and Use Committee

of the South China Normal University (an approval reference number 1002019-02-0016).

Experimental Diet

Four isonitrogenous (approximately 42.5% crude protein) and isolipidic (approximately 14.0% crude lipid) diets were formulated mainly using FM, CSM, corn-gluten meal, poultry byproduct meal, soybean meal, and peanut meal as the main protein sources, and fish oil, soybean oil, and soybean lecithin as the lipid sources. The FM-based diet was regarded as the control diet (CSM0 diet), and the CSM gradient-substituted FM diet was named the CSM20 diet, CSM40 diet, and CSM60, respectively. Dietary lysine, methionine, and threonine were supplemented to meet the requirements of the golden pompano. The dry ingredients were finely ground into powder through a 320-μm screen. Afterward, all compositions used in the diets were individually blended in a mixer and further homogenized after oil and water were added. The pellets were then forced through a pelletizer (F-26, South China University of Technology, Guangzhou, China) and air-dried at 45°C to obtain moisture of approximately 10%. All the experimental diets were stored at −20°C until use. The experimental diet formulation is presented in **Table 1**, and the AA composition of the experimental diets is shown in **Table 2**.

TABLE 1 | Experimental diets formulations.

Ingredients	Diets (% dry weight)			
	CSM0	CSM20	CSM40	CSM60
Fishmeal	25.00	20.00	15.00	10.00
Cottonseed meal (CSM)	0.00	5.00	10.00	15.00
Corn gluten meal	13.00	13.00	13.00	13.00
Poultry by-product meal	11.00	11.00	11.00	11.00
Soybean meal	8.50	8.50	8.50	8.50
Peanut meal	6.50	6.50	6.50	6.50
Wheat meal	17.50	17.50	17.50	17.50
Fish oil	1.50	2.00	2.40	2.80
Soybean oil	5.00	5.00	5.00	5.00
Soybean lecithin	2.50	2.50	2.50	2.50
Monocalcium phosphate	1.50	1.70	1.90	2.10
Lysine	0.28	0.45	0.60	0.75
Methionine	0.10	0.15	0.20	0.25
Threonine	0.01	0.03	0.05	0.07
Squid paste	1.50	1.50	1.50	1.50
Mineral premix[a]	1.50	1.50	1.50	1.50
Vitamin premix[b]	0.50	0.50	0.50	0.50
Chromium trioxide	0.10	0.10	0.10	0.10
Lutein	0.10	0.10	0.10	0.10
Antioxidant	0.05	0.05	0.05	0.05
Mold inhibitor	0.10	0.10	0.10	0.10
Cellulose	3.76	2.82	2.00	1.18
Proximate composition				
DM (%)	90.24	89.98	89.91	90.12
Crude protein (%)	42.42	42.51	42.58	42.66
Crude lipid (%)	14.00	14.09	14.07	14.05

[a] *Mineral premix (mg/kg diet): NaF, 2 mg; KI, 0.8 mg; $CoCl_2 \cdot 6H_2O$ (10 g/kg), 50 mg; $CuSO_4 \cdot 5H_2O$, 10 mg; $FeSO_4 \cdot H_2O$, 80 mg; $ZnSO_4 \cdot H_2O$, 50 mg; $MnSO_4 \cdot H_2O$, 60 mg; $MgSO_4.7H_2O$, 1,200 mg; $Ca(H_2PO_4)_2 \cdot H_2O$, 3,000 mg; NaCl, 100 mg; zeolite, 15,447 mg.*

[b] *Vitamin premix (mg/kg diet): thiamin, 25 mg; riboflavin, 45 mg; pyridoxine HCl, 20 mg; vitamin B12, 0.1 mg; vitamin K3, 10 mg; inositol, 800 mg; pantothenic acid, 60 mg; niacin acid, 200 mg; folic acid, 20 mg; biotin, 1.20 mg; retinal acetate, 32 mg; cholecalciferol, 5 mg; α-to-copherol, 120 mg; ascorbic acid, 2,000 mg; choline chloride, 2,500 mg; ethoxyquin 150 mg; wheat middling, 14,012 mg.*

Fish and Experimental Conditions

Three hundred and sixty golden pompano were purchased from the Dayawan Fish Farm (Guangdong, China). All fish were fed the CSM0 diet two times per day for 2 weeks to apply experimental conditions before the start of the experiment. Afterward, golden pompano were weighed after 24 h of fasting. Thirst fish with an initial weight of 28.42 ± 0.02 g were randomly assigned to sea cages (1.5 m × 1.5 m × 1.5 m), and each diet was allocated in three replicates. Fish were fed two times (07:00 and 17:00) daily by hand to apparent satiation for 6 weeks. During the trial, the feed consumption, water temperature, and dissolved oxygen were recorded daily. Water temperature ranged from 28 to 30°C, and dissolved oxygen ranged from 5.0 to 6.0 mg/L.

Sample Collection

At the end of the feeding trial, the experimental fish were weighed and counted 24 h after the last feeding, and three fish from each cage were randomly selected for whole body biochemical and fatty acids content measurement. In addition, 12 fish (four per tank) from each treatment were anesthetized with 100 mg/L eugenol (Shanghai Medical Co., Ltd., Shanghai, China). Blood samples were obtained by using the caudal venipuncture method. All samples were put into the anticoagulant tubes and immediately centrifuged at 3,000 × *g* for 5 min at 4°C. The supernatant plasma was placed into liquid nitrogen and then kept at −80°C for analysis. At the same time, the liver, intestine, and kidney were dissected and pooled into RNAase-free tubes (Axygen), frozen in liquid N_2, and then stored at −80°C until gene expression analysis.

Digestibility Trial

A digestibility trial was conducted during the feeding trial period. Chromium trioxide (0.1%) (99.9%, Sigma Co., Ltd., MO, United States) was used as the indicator in the diets. The collected feces were dried for 6 h at 65°C and stored at −20°C until analysis. The content of chromium trioxide in the diet and feces was measured by perchloric acid digestion and determined by inductively coupled plasma-atomic emission spectrophotometry (ICP-OES, VISTA- MPX) (Bian et al., 2017).

Proximate Composition Analysis

A proximate composition analysis was conducted following previous studies (Cai et al., 2020). Moisture was measured by drying samples to a constant weight in an oven at 105°C. Crude protein was detected using the Dumas nitrogen determination apparatus (DT autosampler, Europe Gerhardt

TABLE 2 | The essential amino acids composition of the experimental diets.

Amino acids	Diets (% dry weight)			
	CSM0	CSM20	CSM40	CSM60
Lys	1.90	1.92	1.92	1.91
Met	0.70	0.70	0.70	0.70
Thr	0.88	0.88	0.88	0.88
Arg	1.29	1.51	1.73	1.95
His	0.47	0.49	0.51	0.53
Ile	0.84	0.81	0.79	0.77
Leu	1.51	1.48	1.45	1.42
Phe	0.89	0.94	0.99	1.04
Val	1.02	1.01	0.99	0.97
Cys	0.15	0.18	0.20	0.23
Tyr	0.66	0.65	0.65	0.64

Company, Germany). The Soxtec^{TM} 2055 extraction by using petroleum ether (B.P. 30–60°C for 3 h) was used for crude lipid determination. Ash was determined by combustion in a muffle furnace (FO610C, Yamato Scientific Co., Ltd., Tokyo, Japan) to a constant weight at 550°C. The ingredients and diets were dried by a freezer dryer (ALPHA1-2 LD plus, Christ Co., Ltd., Germany) to determine the AA concentrations. After digestion with 6 M HCl for 22 h, all the AA compositions in the ingredients and diets were measured by an L-8900 AA analyzer (Hitachi, Japan).

Analysis of Free Amino Acid and Fatty Acids

The free AA composition in plasma was analyzed using an auto AA analyzer (LA8080; Hitachi, Tokyo, Japan). The details were described by Wang et al. (2020). Briefly, 1.2 ml of 10% sulfosalicylic acid solution and 400 μl of each plasma sample were mixed well. After incubation for 5 min at 4°C, all the samples were centrifuged at 13,000 rpm for 15 min at 4°C. Supernatants were filtered through 0.22-μm filters for free AA concentration analysis. The fatty acid composition of the whole body was measured following the methodology of Mourente et al. (1999) with some modifications (Xu et al., 2010).

Determination of Plasma and Liver Biochemical Parameters

The measurement of plasma and liver biochemical parameters was followed by Yang et al. (2020). Around 200 μl of each plasma sample was used for this detection. The concentrations of total glucose (GLU), total protein (TP), triglycerides (TG), total cholesterol (CHOL), high-density lipoprotein (HDL), low-density lipoprotein (LDL), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and creatine kinase (CK) in plasma were measured by using an Automatic Biochemical Analyzer (Sysmex Corporation, CHEMIX-800, Kobe, Japan).

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted by using the TRIzol method (Vazyme Biotech Co., Ltd., China) following the manufacturer's instructions. After RNA eluted in diethyl pyrocarbonate (DEPC)-treated water, the quantity and quality of isolated RNA were measured using NanoDrop 2000 spectrophotometer (Thermo, NanoDrop Technologies, United States) and electrophoresis in a 1.2% agarose gel, respectively. One microgram of RNA was transcribed into cDNA by using the Prime Script RT reagent Kit (Vazyme Biotech Co., Ltd., China). The cDNA templates were diluted to 100 ng/μL with DEPC water before use for quantitative real-time PCR analysis.

Primers used in this study are shown in **Table 3**. Targeted gene expression levels were determined by quantitative RT-PCR carried out on a CFX96 real-time PCR machine (CFX96, BIO-RAD, United States) using Hiff® qPCR SYBR Green Master Mix (Yeasen, Shanghai, China). The thermal cycle program of qRT-PCR was as follows: 95°C for 2 min, then 40 cycles at 95°C for 15 s, 58°C–60°C for 30 s, and 72°C for 20 s. Melting curve analysis after the end of each PCR was executed to confirm that only one single product was present in these reactions. Relative expression levels of target genes were calculated using the comparative CT method ($2^{-\Delta\Delta Ct}$ method). Relatively gene expression is represented as the fold change relative to the control group (Zhou et al., 2019).

Western Blotting

The protein expression level of total TOR, phospho-TOR, total ribosomal protein S6 (S6), phospho-S6, totalith phosphatase inhibitor and protease cocktails (Bimake, TX, United States) for 1 h. The protein concentrations of all the samples were measured by using the BCA protein assay kit (Beyotime, Biotechnology, China). Samples were separated by sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to 0.45-μm PVDF membranes (Millipore). After blocking with 5% non-fat milk in TBST buffer (20 mM Tris-HCl, 500 mM NaCl, 0.1% Tween-20) for 1 h, the membranes were incubated with primary antibody (Cell Signaling Technology, MA, United States and Santa Cruz Biotechnology Inc., TX, United States) overnight at 4°C. All of the primary antibodies have been shown to be conserved in fish species and could be used successfully (Song et al., 2016; Wang et al., 2020). Then, the membranes were incubated in horseradish peroxidase-labeled secondary antibodies for 1 h and visualized using ECL reagents (Beyotime Biotechnology, China). The Western blot result bands were quantified with NIH Image 1.63 software. All the antibodies in the present study were confirmed to be conserved and successfully used in fish species.

Calculations and Data Analysis

Weight gain rate (WGR,%) = $100 \times (W_t - W_0)/W_0$;
Specific growth rate (SGR,%/day) = $100 \times (\text{Ln } W_t - \text{Ln } W_0)/t$;
Feed efficiency ratio (FER) = $(W_t - W_0)$/(dry feed intake);

TABLE 3 | The sequences of the primers used in the RT-PCR.

Target gene	Forward sequence(5′–3′)	Reverse sequence (5′–3′)
TOR	GGGTCTTATGAGCCAGTGC CAGG	CTTCAGGGTTGTCAGCGGA TTGT
S6	GCACTGTCCCTCGCCGTCTT	CTGGGCTTCTTGCCTTCTTT
4EBP1	ACACCCCAGCAGGAACTTT	GTGACCATCAACGACGCAG
eIF2α	TGTATTCCAGCACCTC AGCC	CGTGGTCGTCATCCGA GTAGA
ATF4	CTGCGTCACCCCTCAACTCC	CATTCGCTCCATCCACAACC
CHOP	CGGAGTTTCTGGATGTT TTGGA	AGGAGGAGGAAGAGGAG GATGA
REDD1	AGCCAAAGACTCAGAAT GCG	TGAAAGGTGGGGACAA GGTA
FAS	GATGGATACAAAGAGCAAGG	GTGGAGCCGATAAGAAGA
ACC	GTTGTCAATCCCAGCCGATC	ATCCACAATGTAGGCCCCAA
AGPAT3	CTTCCTGTTTTGGGCCACTC	GTCGCCATAACTTGAGCCTG
FAD2a	GAACAATCCCACTTCAACG	AGGAATCCCATACTTCTCACA
elovl5	TACATGGTCACGCTCATTA TCC	CCGTTCTGATGCTCCTTC TTTA
SREBP1	GAGCCAAGACAGAGGAGTGT	GTCCTCTTGTCTCCCAGCTT
LPL	TTTGTCCTTCCTCGTCACCA	AAGACAGCATCCTCTCCACC
HSL	TCATACCTCCACACCAACCC	GTCTCGCAGTTTCTTGGCAA
CPT1	CTTTAGCCAAGCCCTTCATC	CACGGTTACCTGTTCCCTCT
PPARγ	TCAGGGTTTCACTATGGCGT	CTGGAAGCGACAGTATTGGC
PPARα	AATCTCAGCGTGTCGTCTT	GGAAATGCTTCGGATACTTG
FABP1	CCAAGGACATCAAGCCAATTAC	TGGTGATTTCAGCCTCCTTAC
APRO B100	AAAAGCCACAAGACGAA AGCA	GAAGCAGCAAAAGGCA GAGC
g6pdh	CTGTGGCAAAAGTTGGTGTG	CCTGATGATGTGAGGGATGA
HK	CCTTCCTCGTCTTTGTCATTT	TGTCCGTCTCATCCTGGTG
PK	TTTGCCAGTTTCATCCGCT	CCATCACGCCATCGCTCT
pfk-1	TGGGTGGGACCGTGATT	AGGTTGGTGATGCCTTTCTT
pepck	TGGAGTGTTTGTTGGAG CAG	CGAAGTTGTAGCCGAA GAAG
glut2	TCCTGTTTGCTGTGCTGCTT	GTTTTCCGTCCCTTGCG
glut4	AATGGCTGTGGCTGGCTT	AGGTTTTTCCCCGTGTTTCT
IL-10	CTCCAGACAGAAGACTCC AGCA	GGAATCCCTCCACAAAA CGAC
IL-8	TGCATCACCACGGTG AAAAA	GCATCAGGGTCCAGACA AATC
TGF-β1	GAGATACGGAAAAGAGTGGGG	TGACAAAGCGGGAAGCAAG
C4	TGGAGAAAAAGTTAAAG GGGC	CAGGAAGGAAGTATGAGC GAGT
C-lyz	GGAGTCTGGTGTTTCTGCTC TTTG	GGTGGCTCTAGTGTTGTAG TTCG
HSP70	TTGAGGAGGCTGCGCACAGCT TGTG	ACGTCCAGCAGCAGCAGG TCCT
HSP90	GATGAAAAGGCGTTTGAGAAAA TGAT	TGTTGCAGGCTTGATGTT GAGTACAC
IκB-α	GCTGGTGAACGGGT TTGAG	GAGGAAGGGGTGATTG TGTGA
MnSOD	AGCCAGCCTCAGCCAAACT	GGCGGTGACATCTCCCTTT
CAT	AGTTTTACACCGAGGAGGGC	TGTGGGTTTGGGGATTGC
Nrf2	TTGCCTGGACACAACTGCTG TTAC	TCTGTGACGGTGGCAGT GGAC
Keap1	CAGATAGACAGCGTGGTGA AGGC	GACAGTGAGACAGGTTGAA GAACTCC
HO-1	AGAAGATTCAGACAGCAGCAG AACAG	TCATACAGCGAGCACAGGA GGAG
β-actin	TACGAGCTGCCTGACGGACA	GCTGTGATCTCCTTCTGC

Protein efficiency ratio (PER) = (W_t − W_0)/(dry feed intake × protein percent in dry diet);
Feed intake (FI,% body weight/d) = 100 × feed fed/[days × (initial weight + final weight)/2]
Protein productive value (PPV,%) = (W_t × P_t − W_0 × P_0)/(W_d × P_d) × 100;
Survival rate (SR,%) = 100 × final amount of fish/initial amount of fish;
Protein retention (PR,%) = 100 × (final body weight × final carcass protein content − initial body weight × initial carcass protein content)/protein intake;
Dry matter apparent digestibility (AD dry matter,%) = [1−chromium trioxide in feed/chromium trioxide in feces] × 100%;
Apparent digestibility of protein in feed (AD for protein,%) = [1−(chromium trioxide in diet/chromium trioxide in feces) (nutrient in feces/nutrient in diet)] × 100%.

W_0, W_t, and W_d represent the body weight (g) of initial fish, final fish, and diet dry weight, respectively, P_0, P_t, and P_d represent the body protein content (%) of initial fish, final fish, and diet dry matter, respectively, while t represents the rearing days of this experiment.

Data were expressed as means ± SEM. All statistical evaluations were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple range test with SPSS 19.0 software. Prior to the statistical tests, data in the present study were examined for homogeneity of variances. In cases in which data were non-parametric or not homoscedastic, data transformations (such as logarithms, square roots, and reciprocals) were used to meet ANOVA criteria. Differences were considered significant when $P < 0.05$.

RESULTS

Growth Performance, Apparent Digestibility, and Whole Body Proximate Composition

Growth performances were shown in **Table 4**. Compared with the CSM0 diet, the CSM20 diet did not significantly decrease the FBW, WGR, SGR, FER, PER, PR, AD (dry matter), or AD (protein) ($P > 0.05$). However, the CSM40 and CSM60 diet groups had markedly lower levels of FBW, WGR, SGR, PPV, PR, AD (dry matter), and AD (protein) compared with the control diet group ($P < 0.05$). Moreover, compared with the control diet, the CSM20 and CSM40 diets did not depress the FER, PER, or whole-body proximate crude fat composition ($P > 0.05$). The CSM60 diet significantly reduced the FER, PER, and crude fat composition compared with the CSM0 diet ($P < 0.05$). No significant differences in FI, SR, whole-body moisture, crude protein, or ash were found among the different treatments ($P > 0.05$).

Plasma Biochemical Index

At the end of the feeding trial, the effects of replacing FM with CSM on the plasma nutrient content were shown in **Table 5**.

TABLE 4 | Growth performance, feed utilization, apparent digestibility, and proximate composition of whole body of golden pompano fed the different experimental diets.

	Diet groups				*P*-value
	CSM0	CSM20	CSM40	CSM60	
IBW (g)	28.38 ± 0.04	28.40 ± 0.05	28.45 ± 0.02	28.44 ± 0.06	0.70
FBW (g)	92.67 ± 1.74^{a}	86.70 ± 1.79^{ab}	85.41 ± 0.79^{bc}	79.46 ± 1.20^{c}	0.00
WGR (%)	226.56 ± 6.61^{a}	205.32 ± 6.82^{ab}	195.70 ± 6.40^{b}	179.44 ± 4.40^{b}	0.04
SGR (%/d)	2.82 ± 0.05^{a}	2.66 ± 0.06^{ab}	2.58 ± 0.05^{b}	2.45 ± 0.04^{b}	0.04
FER	0.95 ± 0.02^{a}	0.90 ± 0.02^{ab}	0.87 ± 0.04^{ab}	0.82 ± 0.02^{b}	0.03
PER	2.24 ± 0.043^{a}	2.11 ± 0.033^{ab}	2.09 ± 0.047^{ab}	1.93 ± 0.050^{b}	0.007
FI (% body weight/d)	2.96 ± 0.025	2.98 ± 0.03	3.02 ± 0.08	3.06 ± 0.04	0.08
PPV (%)	43.41 ± 0.10^{a}	41.45 ± 0.01^{b}	40.33 ± 0.03^{c}	38.19 ± 0.23^{d}	0.00
SR (%)	99.0 ± 1.00	99.0 ± 1.00	100.0 ± 0.00	99.0 ± 1.00	0.36
PR (%)	39.68 ± 0.74^{a}	37.80 ± 0.49^{ab}	36.06 ± 0.33^{bc}	35.23 ± 0.28^{c}	0.01
AD (Dry mater)	84.47 ± 2.78^{a}	78.09 ± 3.87^{ab}	55.07 ± 8.78^{bc}	47.80 ± 6.24^{c}	0.06
AD (Protein)	92.35 ± 1.48^{a}	88.94 ± 1.98^{ab}	79.53 ± 4.17^{b}	76.80 ± 2.59^{b}	0.01
Proximate composition of whole body (wet weight%)					
Moisture	62.3 ± 0.54	62.7 ± 0.85	63.2 ± 0.24	63.5 ± 0.12	0.44
CP	18.2 ± 0.076	18.2 ± 0.21	17.6 ± 0.11	18.0 ± 0.19	0.06
CF	15.1 ± 0.37^{a}	14.5 ± 0.17^{ab}	14.3 ± 0.05^{ab}	13.6 ± 0.17^{b}	0.01
Ash	3.99 ± 0.086	3.93 ± 0.10	4.08 ± 0.10	3.98 ± 0.17	0.84

Data were expressed as means ± SEM (n = 3). Means ± SEM within the same row with the different superscript letters have significant difference among groups (P < 0.05).

IBW, initial body weight; FBW, final body weight; WGR, weight gain rate; SGR, specific growth rate; FER, feed efficiency ratio; PER, protein efficiency ratio; FI, feed intake; PPV, protein productive value; SR, survival rate; PR, protein retention; AD (Dry matter), dry matter apparent digestibility; AD (Protein), apparent digestibility of protein in feed; CP, crude protein; CF, crude fat.

TABLE 5 | Plasma biochemical index of golden pompano fed different experimental diets for 6 weeks.

	Diet groups				*P*-value
	CSM0	CSM20	CSM40	CSM60	
GLU(mmol/L)	12.31 ± 0.040^{b}	13.75 ± 0.13^{a}	10.15 ± 0.69^{c}	12.11 ± 0.23^{b}	0.000
TP (g/L)	42.30 ± 0.17^{a}	40.57 ± 0.60^{ab}	37.87 ± 0.080^{c}	39.40 ± 0.85^{bc}	0.000
TG (mmol/L)	9.66 ± 0.23^{a}	8.27 ± 0.19^{b}	6.90 ± 0.040^{c}	7.46 ± 0.16^{c}	0.000
CHOL (mmol/L)	6.50 ± 0.16^{a}	6.33 ± 0.13^{a}	5.57 ± 0.010^{b}	5.96 ± 0.13^{ab}	0.002
HDL (mmol/L)	2.94 ± 0.060^{a}	2.98 ± 0.040^{a}	2.75 ± 0.020^{b}	2.67 ± 0.040^{b}	0.002
LDL (mmol/L)	0.87 ± 0.00^{c}	1.02 ± 0.010^{b}	1.06 ± 0.00^{a}	1.02 ± 0.010^{b}	0.000

Data were expressed as means ± SEM (n = 6). Means ± SEM within the same row with the different superscript letters have significant difference among groups (P < 0.05).

GLU, total glucose; TP, total protein; TG, triglycerides; CHOL, total cholesterol; HDL, high-density lipoprotein; LDL, low density lipoprotein.

The CSM20 diet had no effect on decreasing the content of plasma TP, CHOL, and HDL compared with the CSM0 diet ($P > 0.05$). In contrast, the CSM20 diet elevated plasma GLU compared with the control diet ($P < 0.05$). However, the CSM40 diet group had lower plasma level of GLU, TP, TG, CHOL, and HLD, and the CSM60 diet group had lower plasma level of TP, TG, and HLD compared with the CSM0 diet group ($P < 0.05$).

The Plasma and Liver Enzyme Activities

The activities of markers indicating liver damage in plasma and liver were shown in **Table 6**. The activities of AST, ALT, ALP, and LDH were markedly increased in the liver with increasing CSM replacement in diets among groups ($P < 0.05$). Only 20% CSM replacing FM could markedly improve the activity of these four enzymes ($P < 0.05$). In plasma, the CSM20 diet group had no significant difference in the enzyme activities of ALT and ALP in the plasma compared with the CSM0 diet group ($P > 0.05$). Moreover, compared with the CSM0 diet, the CSM40 and CSM60 diets significantly elevated the activity of AST and inhibited the activity of ALP ($P < 0.05$). There was no significant difference in LDH activity among the different groups ($P > 0.05$).

Plasma Free Amino Acid Profile

The free AA profiles in plasma after fish were fed with different diets were shown in **Table 7**. Both the CSM20 and CSM40 diets had no effect on altering all the individual

TABLE 6 | The enzymes activities of golden pompano feed different experimental diets in the plasma and liver.

	Diet groups				*P*-value
	CSM0	**CSM20**	**CSM40**	**CSM60**	
Liver					
AST(U/g)	2039.95 ± 6.55^{d}	2420.81 ± 0.89^{c}	2587.73 ± 3.51^{b}	2728.93 ± 3.24^{a}	0.00
ALT(U/g)	297.87 ± 1.55^{d}	362.26 ± 1.48^{c}	423.39 ± 1.53^{a}	394.50 ± 0.87^{b}	0.00
ALP(U/g)	58.87 ± 0.22^{c}	65.22 ± 0.30^{b}	72.49 ± 0.30^{a}	72.44 ± 0.50^{a}	0.00
LDH(U/g)	75.46 ± 0.44^{b}	96.94 ± 0.51^{a}	104.84 ± 4.3^{a}	97.461 ± 0.29^{a}	0.00
Plasma					
AST (U/L)	46.00 ± 0.00^{c}	40.67 ± 0.33^{d}	54.67 ± 0.33^{a}	50.00 ± 0.00^{b}	0.00
ALT (U/L)	4.67 ± 0.33^{b}	5.00 ± 0.00^{b}	5.00 ± 0.00^{b}	7.00 ± 0.00^{a}	0.00
ALP (U/L)	51.67 ± 0.33^{a}	51.33 ± 0.33^{a}	46.00 ± 0.00^{c}	49.33 ± 0.67^{b}	0.00
LDH (U/L)	187.67 ± 17.23	164.00 ± 1.15	234.33 ± 30.67	189.33 ± 6.84	0.12

Data were expressed as means ± SEM (n = 6).
Means ± SEM within the same row with the different superscript letters have significant difference among groups (P < 0.05).
AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase.

TABLE 7 | The free amino acids concentration after golden pompano feed with different diets in the plasma (μg/ml).

	Diet groups				*P*-value
	CSM0	**CSM20**	**CSM40**	**CSM60**	
Met	24.71 ± 0.82	27.18 ± 1.02	24.85 ± 0.76	25.44 ± 0.41	0.18
Phe	22.61 ± 1.06	19.05 ± 0.86	21.52 ± 0.97	20.84 ± 0.76	0.12
Val	136.59 ± 4.51^{a}	116.59 ± 6.06^{ab}	120.04 ± 8.84^{ab}	99.4 ± 3.32^{b}	0.02
Ile	90.87 ± 3.94^{a}	75.55 ± 4.28^{ab}	77.40 ± 7.23^{ab}	64.10 ± 3.07^{b}	0.03
Leu	155.50 ± 7.26^{a}	127.01 ± 6.96^{ab}	132.74 ± 13.41^{ab}	113.02 ± 4.23^{b}	0.05
Thr	44.63 ± 2.01	50.09 ± 4.07	54.56 ± 2.47	46.91 ± 1.59	0.12
Lys	263.15 ± 25.28	222.67 ± 14.77	226.58 ± 16.14	210.76 ± 12.62	0.25
His	10.46 ± 0.22	10.78 ± 0.13	10.26 ± 0.25	10.62 ± 0.083	0.31
Arg	82.88 ± 6.50^{a}	72.62 ± 2.39^{ab}	71.18 ± 1.77^{ab}	62.06 ± 2.34^{b}	0.03
EAA	831.40 ± 12.88^{a}	721.53 ± 37.34^{ab}	755.09 ± 19.84^{ab}	644.21 ± 26.79^{b}	0.01
Glu	44.70 ± 5.24	38.93 ± 5.19	37.47 ± 4.53	43.65 ± 4.13	0.67
Gly	72.47 ± 2.14	69.60 ± 1.95	70.26 ± 0.96	75.77 ± 0.45	0.13
Ala	88.71 ± 3.68^{a}	78.91 ± 0.46^{b}	88.19 ± 0.54^{ab}	95.37 ± 2.02^{a}	0.00
Cys	1.83 ± 0.56	1.43 ± 0.23	1.54 ± 0.45	1.33 ± 0.43	0.87
Tyr	18.66 ± 0.39^{a}	15.99 ± 0.39^{b}	19.36 ± 0.59^{a}	18.84 ± 0.23^{a}	0.00
Ser	28.36 ± 0.30	27.85 ± 0.75	28.21 ± 0.94	31.16 ± 1.29	0.10
Tau	75.98 ± 2.17	81.29 ± 7.12	75.53 ± 0.62	83.52 ± 1.44	0.41
NEAA	318.46 ± 9.97	313.56 ± 5.60	320.56 ± 5.08	349.65 ± 8.64	0.06
TAA	1136.02 ± 51.75	1035.00 ± 31.76	1075.00 ± 18.25	993.86 ± 35.42	0.13

Data were expressed as means ± SEM (n = 6). Means ± SEM within the same row with the different superscript letters have significant difference among groups (P < 0.05).
EAA, total essential AA; NEAA, total non-essential AA; TAA, total AA.

EAAs and total EAA in plasma ($P > 0.05$). However, compared with the control diet, the CSM60 diet significantly decreased the concentrations of valine, isoleucine, leucine, arginine, and EAA in plasma ($P < 0.05$). Furthermore, the CSM40 and CSM60 diets also had no significant difference in changing the concentrations of alanine and tyrosine compared with the control diet ($P > 0.05$). No significant difference was detected in other AAs among the four different treatments ($P > 0.05$).

Whole Body Fatty Acid Profile

The changes in the whole body fatty acid profile after fish were fed with different diets were presented in **Table 8**. Compared with the control diet, the CSM20 diet had no significant difference in any of body fatty acid contents detected in the present study ($P > 0.05$). Compared with the fish fed with the CSM0 diets, fish fed with the 60% CSM replacement diets exhibited notably reduced whole body C18:2 and Σn-6 PUFA contents ($P < 0.05$). The CSM40 and CSM60 diets also significantly

TABLE 8 | Whole body fatty acids content (% total fatty acids).

	Diet groups				*P*-value
	CSM0	CSM20	CSM40	CSM60	
C14:0	1.61 ± 0.04	1.48 ± 0.04	1.59 ± 0.04	1.53 ± 0.06	0.26
C16:0	24.40 ± 0.20	24.80 ± 0.35	25.27 ± 0.44	24.23 ± 0.32	0.21
C18:0	6.73 ± 0.16	7.01 ± 0.18	6.75 ± 0.32	6.25 ± 0.17	0.18
C20:0	0.40 ± 0.02	0.39 ± 0.01	0.39 ± 0.02	0.38 ± 0.03	0.92
ΣSFA	33.14 ± 0.36	33.68 ± 0.57	34.00 ± 0.39	32.40 ± 0.55	0.17
C16:1	1.83 ± 0.04	1.67 ± 0.03	1.75 ± 0.02	1.76 ± 0.05	0.08
C18:1	27.30 ± 0.32	26.87 ± 0.43	27.23 ± 0.50	27.07 ± 0.44	0.89
C20:1	1.69 ± 0.05	1.66 ± 0.04	1.60 ± 0.03	1.56 ± 0.06	0.24
ΣMUFA	30.82 ± 0.34	30.20 ± 0.44	30.58 ± 0.55	30.39 ± 0.50	0.80
C18:2	23.17 ± 0.22^{a}	22.40 ± 0.17^{a}	21.63 ± 0.61^{ab}	20.33 ± 0.47^{b}	0.01
C20:4n-6	0.16 ± 0.002	0.13 ± 0.004	0.14 ± 0.007	0.13 ± 0.02	0.23
C20:3n-6	0.15 ± 0.004	0.16 ± 0.003	0.14 ± 0.01	0.13 ± 0.02	0.36
Σn-6 PUFA	23.47 ± 0.22^{a}	22.69 ± 0.17^{a}	21.91 ± 0.63^{ab}	20.60 ± 0.48^{b}	0.01
C18:3n-3	1.97 ± 0.03^{a}	1.81 ± 0.07^{a}	1.60 ± 0.03^{b}	1.35 ± 0.04^{c}	0.00
EPA (C20:5n-3)	0.20 ± 0.02	0.18 ± 0.004	0.16 ± 0.02	0.13 ± 0.02	0.09
DHA (C22:6n-3)	1.50 ± 0.08^{a}	1.59 ± 0.03^{a}	1.10 ± 0.04^{b}	0.77 ± 0.07^{c}	0.00
Σn-3 PUFA	3.66 ± 0.10^{a}	3.58 ± 0.05^{a}	2.85 ± 0.05^{b}	2.24 ± 0.09^{c}	0.00
Σn-3 HUFA	1.70 ± 0.09^{a}	1.77 ± 0.02^{a}	1.26 ± 0.05^{b}	0.90 ± 0.08^{c}	0.00
Σn-3/Σn-6	0.16 ± 0.005^{a}	0.16 ± 0.003^{a}	0.13 ± 0.002^{b}	0.11 ± 0.002^{c}	0.00
DHA/EPA	7.50 ± 0.27	8.68 ± 0.32	7.05 ± 0.66	6.31 ± 1.03	0.15

Data were expressed as means ± SEM (n = 3). Means ± SEM within the same row with the different superscript letters have significant difference among groups (P < 0.05).

ΣSFA: sum of C14:0, C16:0 and C18:0; ΣMUFA: sum of C16:1, C18:1 and C20:1; Σn-6 PUFA: sum of C18:2n-6 and C18:4n-6; Σn-3 PUFA: sum of C18:3n-3, C20:5 and C22:6. Σn-3/Σn-6:Σn-3 PUFA/Σ n-6 PUFA.

SFA, saturated fatty acids; MUFA, mono-unsaturated fatty acids; n-6 PUFA, n-6 poly-unsaturated fatty acids; n-3 PUFA, n-3 poly-unsaturated fatty acid.

decreased the whole body content of C18:3n-3, DHA (C22:6n-3), Σn-3 PUFA, Σn-3 HUFA, and Σn-3/Σn-6 compared with the control diet ($P < 0.05$). No significant effect was observed in the whole body C20:4n-6, C20:3n-6, EPA, and DHA/EPA among the different CSM gradient replacement diets ($P > 0.05$). Furthermore, CSM gradient replacements also had no significant effect on altering the whole body contents of individual and total saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) ($P > 0.05$).

Regulations of the Nutrient Sensing Signaling Pathways

Gene and protein phosphorylation levels of the key regulators involved in nutrient sensing signaling pathways in the liver after fish were fed different diets are presented in **Figure 1**. Compared with golden pompano fed with CSM0 diet, golden pompano fed with the CSM20 diet did not change the mRNA expression levels of TOR, S6, and 4EBP1 ($P > 0.05$). The CSM60 diet significantly reduced the gene expression level of TOR and S6 compared with the control diet ($P < 0.05$). On the other hand, CSM substitution markedly promoted the gene expression of eIF2α and REDD1 in the liver ($P < 0.05$). The CSM20 diet had no effect on altering the mRNA expression levels of ATF4 and CHOP compared with the control diet ($P > 0.05$). However, both the CSM40 and CSM60 diets increased the mRNA expression of ATF4 and CHOP in the liver ($P < 0.05$). Moreover, the CSM20 and CSM40 diets also had no effect in changing the protein phosphorylation levels of TOR and S6 compared with the CSM0 diet ($P > 0.05$). Conversely, the CSM60 diet decreased the protein phosphorylation level of S6 and increased the protein phosphorylation level of eIF2α than the control diet ($P < 0.05$).

Gene Expression Pattern of the Lipid Metabolism

The gene expression patterns of the key enzymes and regulators involved in lipid anabolism, catabolism, and transportation in the liver are shown in **Figures 2A–C**. Compared with the fish fed with CSM0 diets, the fish fed with CSM20 and CSM40 diets did not markedly decrease the gene expression of FAS, AGPAT3, SREBP1, and PPAR-γ ($P > 0.05$). In addition, the CSM20 diet also had no effect on reducing the mRNA expression of FAD, elovl5, and PPAR-α compared with the CSM0 diet ($P > 0.05$). In contrast, the CSM60 diets markedly lowered the gene expression levels of FAS, ACC, AGPAT3, FAD, elovl5, SREBP1, PPAR-α, and PPAR-γ ($P < 0.05$). For lipid catabolism, no changes were detected between the CSM0 and CSM20 diets for the gene expression of the key enzymes involved in lipid catabolism ($P > 0.05$). The CSM60 diet significantly increased the mRNA expression levels of LPL and CPT1 compared with the other three diets ($P < 0.05$). The gene expression patterns of FABP1 and APROB100 showed a decreasing trend with an increasing

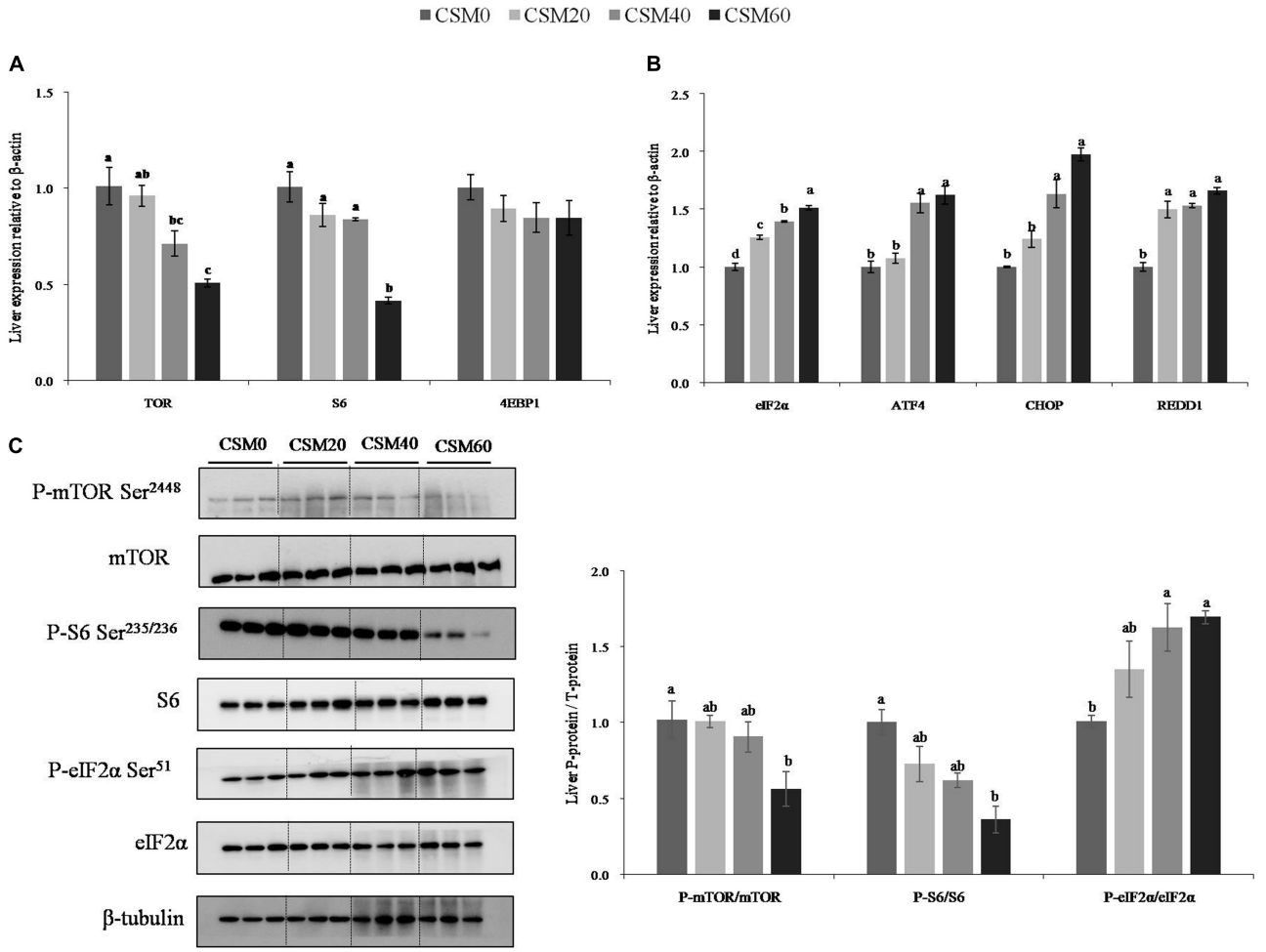


FIGURE 1 | The mRNA and protein phosphorylation level of key regulators in nutrient sensing signaling pathways **(A)** mRNA expression level of TOR signaling pathways. **(B)** mRNA expression level of AAR signaling pathway. **(C)** Protein phosphorylation level of TOR and AAR signaling pathways. In liver after juvenile golden pompano (*Trachinotus ovatus*) fed with CSM0, CSM20, CSM40, and CSM60 diets, respectively. Values with different superscripted small letters mean significant difference ($P < 0.05$). TOR, target of rapamycin; S6, S6 Ribosomal Protein; 4EBP1, 4 E-binding protein 1; eIF2α, initiation elongation factor alpha; ATF4, activating transcription factor 4; CHOP, channelopsin 2; REDD1, regulated in development and DNA damage responses 1.

CSM replacement ratio. The fish fed with 60% dietary CSM replacement diet showed significantly lower levels of FABP1 and APROB100 than the fish fed with the control diet ($P < 0.05$). However, the CSM20 diet did not change the gene expression of FABP1 and APROB100 compared with the control diet ($P > 0.05$).

mRNA Expression Level of the Key Enzymes and Transporters in Glucose Metabolism

As presented in **Figure 3**, the mRNA expression levels of the key enzymes and transporters in glucose metabolism showed a decreasing trend with increasing FM replacement. The CSM20 diet had no effect on inhibiting the mRNA expression levels of the g6pdh, pk, pepck, and glut4 compared with the control diet ($P > 0.05$). However, the CSM40 and CSM60 diets significantly decreased the mRNA expression levels of g6pdh, hk, pk, pfk-1, pepck, and glut2 ($P < 0.05$). Moreover, a marked decrease was observed in the gene expression of hk, pfk-1, and glut2 when only 20% FM was replaced by CSM ($P < 0.05$).

Cottonseed Meal Gradient Replacements Modulated the mRNA Expression Level of the Key Enzymes in Antioxidant Defense

Figure 4 described the mRNA expression level changes of the key enzymes in antioxidant defense after golden pompano were fed CSM gradient replacement diets. Compared with the fish fed with the CSM0 diets, the fish fed with the CSM20 diet did not show marked effects in the mRNA expression levels of MnSOD, Nrf2, and Keap1 in the intestine or CAT in the kidney ($P > 0.05$). Nevertheless, the CSM40 and CSM60 diets showed higher gene expression levels of the MnSOD, CAT, and Nrf2

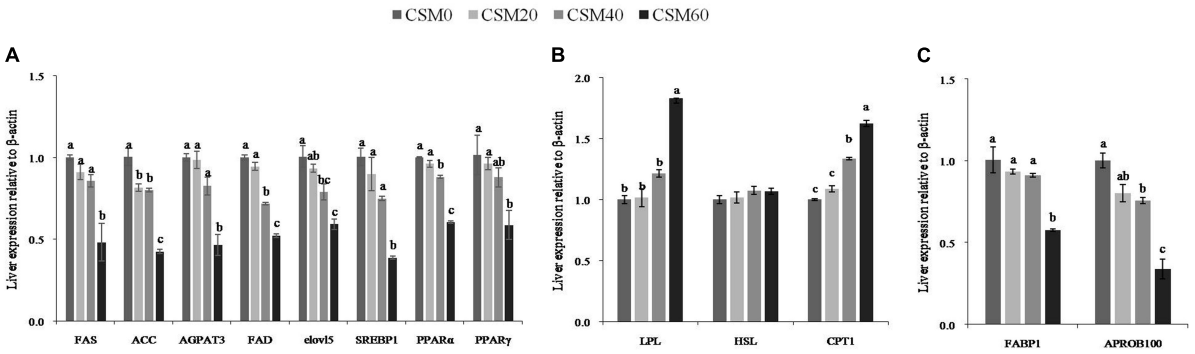


FIGURE 2 | Diagrammatic representation of the gene expression pattern of the key transcripts involved in lipid metabolism after juvenile golden pompano (*Trachinotus ovatus*) fed with CSM0, CSM20, CSM40, and CSM60 diets, respectively. **(A)** Lipid anabolism. **(B)** Lipid catamolism. **(C)** Lipid transporters. Values with different superscripted small letters mean significant difference ($P < 0.05$). FAS, fatty acid synthetase; ACC, acetyl-CoA carboxylase; AGPAT3, 1-acylglycerol-3-phosphate acyltransferase 3; FAD, fatty acyl desaturase; elovl5, elongase of very long-chain fatty acids 5; SREBP1, sterol regulatory element binding protein-1; PPARα, peroxisome proliferator activated receptors-alpha; PPARγ, peroxisome proliferator-activated receptors gamma; LPL, lipoprotein lipase; HSL, hormone-sensitive lipase; CPT1, carnitine palmitoyl transferase 1; FABP1, fatty acid binding protein 1; APROB100, apolipoprotein b 100.

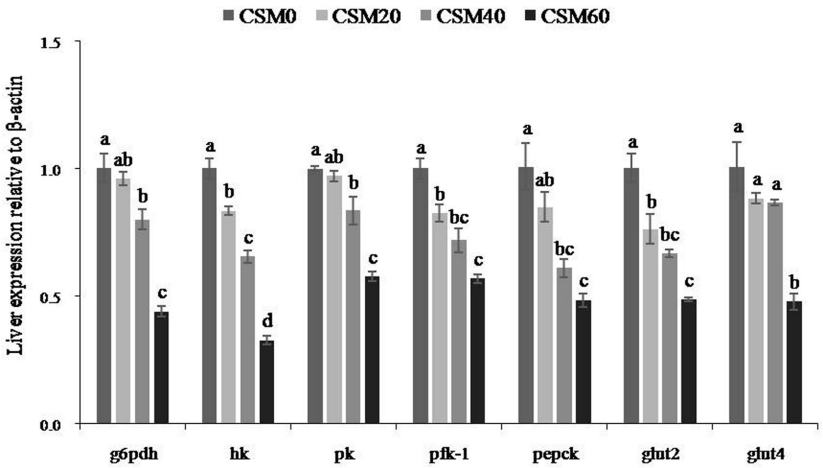


FIGURE 3 | RT-qPCR analysis of the key enzymes and transporters in glucose metabolism after juvenile golden pompano (*Trachinotus ovatus*) fed with CSM0, CSM20, CSM40, and CSM60 diets, respectively. Values with different superscripted small letters mean significant difference ($P < 0.05$). g6pdh, glucose-6-phosphate; hk, hexokinase; pk, pyruvate kinase; pfk-1, phospho fructokinase-1; pepck, phosphoenolpyruvate carboxykinase; glut2, glucose transport protein 2; glut4, glucose transport protein 4.

in the intestine, and MnSOD, CAT, and HO-1 in the kidney ($P < 0.05$). Moreover, the CSM60 diet presented lower Keap1 expression than the control diet in both the intestine and kidney ($P < 0.05$). CSM replacements also had no significant effect on changing the mRNA expression of HO-1 in the intestine and Nrf2 in the kidney ($P > 0.05$).

Cottonseed Meal Gradient Replacements Modified the Immune Response

Immune-response-related gene mRNA expression in the intestine and kidney is displayed in **Figure 5**. Compared with the CSM0 diet, the CSM20 diet had no effect on changing the gene expression of IL-10, TGFβ-1, C4, and Hsp90 in the intestine ($P > 0.05$). In addition, the CSM20 diet also did not alter the mRNA expression of IL-10, IL-8, TGFβ-1, C4, C-lyz, and Hsp90 in the kidney compared with the CSM0 diet ($P > 0.05$). However, compared with the control diet, the CSM40 and CSM60 diets had a marked effect on increasing the gene expression of IL-10, IL-8, C4,Hsp70, and Iκ-α in the intestine and IL-10, IL-8, C-lyz, and Hsp90 in the kidney ($P < 0.05$). On the other hand, CSM60 diets significantly inhibited the mRNA expression of TGFβ-1 in both the intestine and the kidney compared with the control diet ($P < 0.05$). There was no significant difference in the expression of C-lyz in the intestine or Hsp70 and IκB-α in the kidney among the different groups ($P > 0.05$).

DISCUSSION

The present study showed that CSM replaced with 40% FM protein did not decrease the FER and PER; however, based on the WGR and SGR, CSM could only substitute 20% of FM protein in

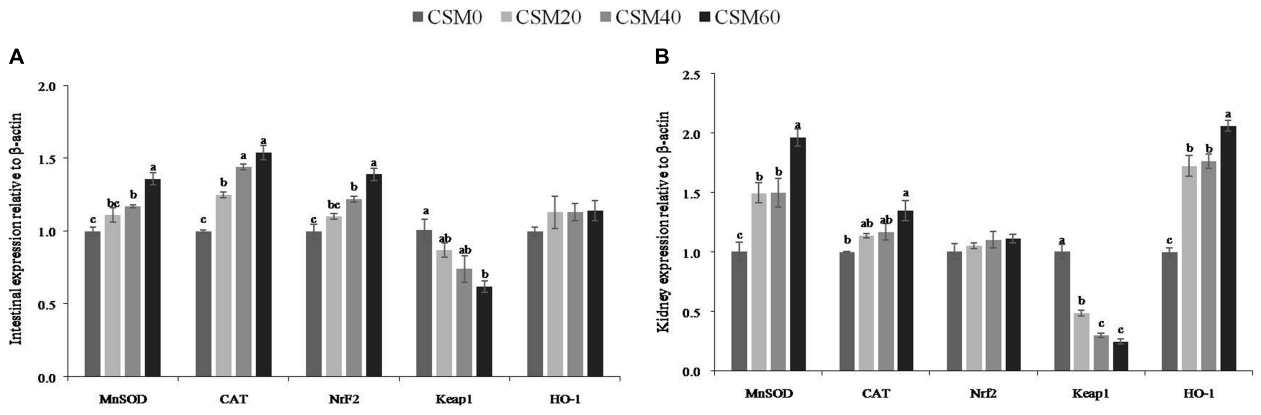


FIGURE 4 | The intestine and kidney gene expression pattern of the key regulators in antioxidant defense after juvenile golden pompano (*Trachinotus ovatus*) fed with CSM0, CSM20, CSM40 and CSM60 diets, respectively. **(A)** Intestine. **(B)** Kidney. Values with different superscripted small letters mean significant difference ($P < 0.05$). MnSOD, Manganese superoxide dismutase; CAT, catalase; Nrf2, NF-E2-related nuclear factor 2; Keap1, Kelch-like-ECH-associated protein 1; HO-1, heme oxygenase-1.

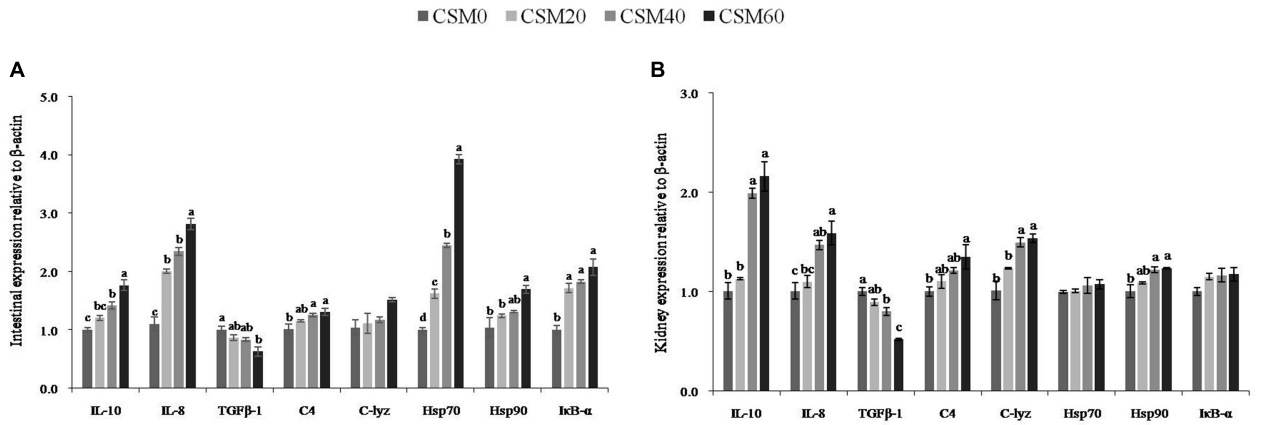


FIGURE 5 | mRNA expression levels of the key regulators in immune response after juvenile golden pompano (*Trachinotus ovatus*) fed with CSM0, CSM20, CSM40 and CSM60 diets, respectively. **(A)** Intestine. **(B)** Kidney. Values with different superscripted small letters mean significant difference ($P < 0.05$). IL-10, interleukin 10; IL-8, interleukin 8; TGFβ-1, transforming growth factor β1; C4, complement 4; C-lyz, c-type lysozyme; Hsp70, heat shock protein 70; Hsp90, heat shock protein 90; IκB-α, NF-kappa-B inhibitor alpha.

the golden pompano diet. Moreover, compared with the CSM0 diet, only the CSM20 diet did not decrease the dry matter and protein apparent digestibility compared with the control diet. However, higher CSM inclusion significantly decreased growth performance and feed utilization. All the results were consistent with previous studies on juvenile black sea bass *Centropristis striata* (Anderson et al., 2016), juvenile hybrid grouper (Ye et al., 2020), and crucian carp (*Carassius auratus gibelio*) (Gui et al., 2010). Golden pompano, a carnivorous fish species, has a limited ability to utilize dietary plant protein sources. Wu et al. (2014) reported that soy protein concentrate alone could substitute 20% FM without supplemental dietary taurine in golden pompano. Similarly, in a carnivorous fish, dietary replacement of over 25.3% FM with CSM could markedly inhibit the growth performance of Ussuri catfish (Bu et al., 2017). The formulation of the present diet was designed based on the commercial diet of golden pompano. A variety of plant protein sources including corn gluten meal, soybean meal, and peanut meal were used as the main protein sources in the diet, and the supplemental amount of FM in the control group was only 25%. Therefore, the replacement of > 20% FM with CSM inhibited growth performance.

Understanding the changes in physio-biochemical and molecular responses after fish were fed a CSM substitution diet is also an important way to improve the substitution efficiency (Dai et al., 2015; Xu et al., 2016). Plasma nutrient and free AA contents were intuitive indexes reflecting the situation of feed ingestion (Wang W.Q. et al., 2021; Wang Z. et al., 2021). In the present study, CSM replacement had a profound effect on altering the plasma biochemical index. CSM substitution at 40% and 60% markedly decreased the total TP, TG, and HLD, which was consistent with a study on largemouth bass and turbot (Wang et al., 2020). The plasma-free AA concentration was

considered the main driving force determining the metabolic rate (Liao et al., 2015; Wei et al., 2020). Higher plasma-free AA concentrations provided abundant substrates that could improve protein anabolism. Previous studies using Antarctic krill as a FM substitution in large yellow croaker and poultry by-product meal as a FM substitution in largemouth bass showed lower levels of individual AAs after FM was substituted by other protein sources (Wei et al., 2019; Wang et al., 2020). This study also presented a declining trend of plasma individuals and total EAA after CSM replaced 60% of FM in the diet. Compared with NEAA, the concentrations of some EAAs were more sensitive to dietary composition. Lower plasma nutrient and free AA concentrations decreased the metabolic rate of nutrients and further limited the deposition of body nutrients.

The fatty acid content profile of the whole body is another important indicator to reflecting the optimal substitution ratio of alternative protein sources (Hill et al., 2019). From a human nutritive perspective, fish with a high n-3 PUFA conent are regarded as more valuable than those with a high n-6 PUFA content (Simopoulos, 2002). The present study demonstrated that the inclusion of CSM in the diet had no significant effect on regulating the content of SFA and MUFA. In contrast, the CSM 40 and CSM60 replacement diets decreased the contents of DHA, Σn-3 PUFA, Σn-3 HUFA, and Σn-3/Σn-6 compared with the control diet. Moreover, decreased profiles of whole body C18:2,Σn-6 PUFA, and C18:3n-3 were observed only when CSM was replaced at a higher ratio (CSM60 diet). A similar suppression of whole body Σn-3 PUFA and Σn-3 HUFA was observed in a study using a poultry by-product meal ingredient replacement diet in the hybrid grouper (Wang Z. et al., 2021). The results of whole body fatty acid contents indicated that CSM could substitute 20–40% FM without affecting the fatty acid compositions of golden pompano.

To illustrate the underlying mechanisms of the changing growth performance and nutrient metabolism after CSM replaced FM, the present study also focused on the molecular response of nutrient deposition and metabolism. TOR and AAR are two complementary signaling pathways that respond to dietary nutrient levels (Kimball, 2007; Hietakangas and Cohen, 2009). The two signaling pathways were more sensitive to the dietary AA profile (Gallinetti et al., 2013). An unbalanced AA profile was the first limiting factor restricting the utilization of alternative protein sources in aquafeeds (Li et al., 2009). Our previous study on turbot and largemouth bass demonstrated that FM replaced by other protein sources with or without AA supplementation significantly varied the mRNA and protein expression levels of the key regulators in the TOR and AAR signaling pathways (Song et al., 2016; Wang W.Q. et al., 2021). Consistent with our previous studies, the present study on golden pompano also revealed a lower expression level of the key regulators involved in TOR signaling and a higher expression level of the key regulators involved in AAR signaling at both the mRNA and protein phosphorylation levels after FM was replaced by CSM at 40–60%. The liver is the main organ that regulates metabolism overall. The TOR signaling pathway is the regulating center for nutrient metabolism (Hayashi and Proud, 2007; Irm et al., 2020). Therefore, on further exploring the mRNA regulation of glucose and lipid metabolism, we found that higher CSM replacement significantly inhibited the gene expression level of the key enzymes and regulators in lipid anabolism and lipid transporters, and elevated the gene expression level of the key enzymes in lipid catabolism. Similarly, CSM substitution also suppressed the mRNA expression level of the key enzymes and transporters involved in glucose metabolism. Inhibited nutrient anabolism and activated nutrient catabolism may provide a reasonable explanation for the reduced growth performance and nutrient metabolism after FM was replaced by CSM.

In addition to affecting nutrient metabolism, alternative protein source-substituted FM also has a profound effect on health status by regulating antioxidant defense and immune response (He et al., 2021). AST, ALT, ALP, and LDH are important indicators that reflect liver function (Hyder et al., 2013). The increasing activities of these enzymes in plasma and liver imply liver damage (Regmi et al., 2017). Bu et al. (2017) reported that the activities of plasma ALT and LDH significantly increased when the CSM substitution level was >40% in Ussuri catfish. The present study also found that the activities of AST, ALT, ALP, LDH, and CK in the plasma and liver of juvenile golden pompano that was fed a large proportion of CSM inclusion diets (CSM40 and CSM60 diets) were higher than those in fish fed the CSM0 diet. These results confirmed liver damage when a higher ratio of CSM was supplemented in the diet of golden pompano. Moreover, the intestine and kidney are considered to be the key organ responses for the body's immune status (Awad et al., 2015; Lauriano et al., 2016; Wu et al., 2017). The intestine is not only considered the main site of the response to nutrient digestion and absorption but also the largest part of the immune system, which contains many lymphoid cells, macrophages, eosinophils, and neutrophilic granulocytes (Rombout et al., 2011). The present study was conducted to understand the overall immune response that focused on both the intestine and the kidney. The immune status was closely associated with inflammation which was mainly regulated by inflammatory cytokines. TNF, IL, and TGF are cytokines that play a vital role in body immunity (Delcenserie et al., 2008). In this study, a high level of CSM (CSM60 diet) in the diet markedly elevated the mRNA expression of IL-10, IL-8, C4, Hsp70, and IκB-α in the intestine and kidney. Moreover, data on the gene expression level in the intestine and kidney also demonstrated that CSM replacement activated the antioxidant defense. These results suggested that proportionate CSM substitution (20–40%) had no effects on activating the immune response and antioxidant defense. However, higher CSM substitution induced the immune response and antioxidant defense and further damaged the health status. Overall, based on the immune response, CSM could substitute 20–40% FM in the present diet formulation.

CONCLUSION

Based on the results, the present study demonstrated that CSM could successfully substitute 20% dietary FM in the diet of the golden pompano with an initial body weight of approximately 28.4 g. Using CSM as a substitute for FM has promising prospects

in golden pompano diets. However, higher CSM inclusion could affect growth performance by regulating TOR and AAR signaling and further modifying the nutrient metabolism. Additionally, higher CSM replacement could also alter the fish health status by regulating the immune response and antioxidant defense. This study proposes the optimal inclusion level of CSM in the diet and also contribute to understanding the mechanisms of dietary CSM in growth performance, nutrient metabolism, and immune response in golden pompano.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the University Animal Care and Use Committee of the South China Normal University (an approval reference number 1002019-02-0016).

AUTHOR CONTRIBUTIONS

FS and KM designed the study. YQ and CH performed the study. PY and WW analyzed the data. FS, JW, and QQ revised the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by the Fund of Southern Marine Science and Engineering Guangdong Laboratory (Zhanjiang) (ZJW-2019-06), the Foundation and Applied Basic Research Fund Project of Guangdong Province (2019A1515110606), the State Key Laboratory of Developmental Biology of Freshwater Fish and Research (201KF007), and the Research and Development Fund for Young Teachers of South China Normal University (19KJ03).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.775575/full#supplementary-material

REFERENCES

Anderson, A. D., Alam, M. S., Watanabe, W. O., Carroll, P. M., Wedegaertner, T. C., and Dowd, M. K. (2016). Full replacement of menhaden fish meal protein by low-gossypol cottonseed flour protein in the diet of juvenile black sea bass *Centropristis striata*. *Aquaculture.* 464, 618–628. doi: 10.1016/j.aquaculture.2016.08.006

Awad, E., Awaad, A. S., and Esteban, M. A. (2015). Effects of dihydroquercetin obtained from deodar (*Cedrus deodara*) on immune status of gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol.* 43, 43–50. doi: 10.1016/j.fsi.2014.12.009

Bian, F. Y., Zhou, H. H., He, G., Wang, C., Peng, H., Pu, X., et al. (2017). Effects of replacing fishmeal with different cottonseed meals on growth, feed utilization, haematological indexes, intestinal and liver morphology of juvenile turbot (*Scophthalmus maximus* L.) Aquacult. *Nutr.* 23, 1429–1439. doi: 10.1111/anu.12518

Bu, X. Y., Chen, A. J., Lian, X. Q., Chen, F. Y., Zhang, Y., Muhammad, I., et al. (2017). An evaluation of replacing fish meal with cottonseed meal in the diet of juvenile ussuri catfish *Pseudobagrus ussuriensis*: growth, antioxidant capacity, nonspecific immunity and resistance to *Aeromonas hydrophila*. *Aquaculture.* 479, 829–837. doi: 10.1016/j.aquaculture.2017.07.032

Cai, L. S., Wang, L., Song, K., Lu, K. L., Zhang, C. X., and Rahimnejad, S. (2020). Evaluation of protein requirement of spotted seabass (*Lateolabrax maculatus*) under two temperatures, and the liver transcriptome response to thermal stress. *Aquaculture* 516, 734615. doi: 10.1016/j.aquaculture.2019.734615

Dai, W. W., Panserat, S., Plagnes-Juan, E., Seiliez, I., and Skiba-Cassy, S. (2015). Amino acidsattenuate insulin action on gluconeogenesis and promote fattyacid biosynthesis *via* mTORC1 signaling pathway in trouthepatocytes. *Cell. Physiol. Biochem.* 36, 1084–1100. doi: 10.1159/000430281

Delcenserie, V., Martel, D., Lamoureux, M., Amiot, J., Boutin, Y., and Roy, D. (2008). Immunomodulatory effects of probiotics in the intestinal tract. *Curr. Issues Mol. Biol.* 10, 37–54. doi: 10.21775/cimb.010.037

Dossou, S., Koshio, S., Ishikawa, M., Yokoyama, S., Dawood, M. A. O., Basuini, M. F. E., et al. (2018).). Effect of partial replacement of fish meal by fermented rapeseed meal on growth, immune response and oxidative condition of red sea bream juvenile, *Pagrus major*. *Aquaculture* 490, 228–235.

Gallinetti, J., Harputlugil, E., and Mitchell, J. R. (2013). Amino acid sensing in dietary restriction-mediated longevity: roles of signal-transducing kinases GCN2 and TOR. *Biochem. J.* 449, 1–10. doi: 10.1042/BJ20121098

Gatlin, D. M., Barrows, F. T., Brown, P., Dabrowski, K., Gaylord, T. G., Hardy, R. W., et al. (2007). Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquac. Res.* 38, 551–579. doi: 10.1111/j.1365-2109.2007.01704.x

Gui, D., Liu, W. B., Shao, X. P., and Xu, W. N. (2010). Effects of different dietary levels of cottonseed meal protein hydrolysate on growth, digestibility, body composition and serum biochemical indices in crucian carp (*Carassius auratus gibelio*). *Anim. Feed Sci. Technol* 156, 112–120. doi: 10.1016/j.anifeedsci.2010.01.012

Hayashi, A. A., and Proud, C. G. (2007). The rapid activation of protein synthesis by growth hormone requires signaling through mTOR. *Am. J. Physiol. Endocrinol. Metab.* 292, 1647–1655. doi: 10.1152/ajpendo.00674.2006

He, Y. F., Guo, X. W., Tan, B. P., Dong, X. H., Yang, Q. H., Liu, H. Y., et al. (2021). Partial fishmeal protein replacement with peptides from swine blood modulates the nutritional status, immune response, and intestinal microbiota of hybrid groupers (female *Epinephelus fuscoguttatus*× male *E.lanceolatus*). *Aquaculture* 533, 736154. doi: 10.1016/j.aquaculture.2020.736154

Hietakangas, V., and Cohen, S. M. (2009). Regulation of tissue growth through nutrient sensing. *Annu. Rev. Genet.* 43, 389–410. doi: 10.1146/annurev-genet-102108-134815

Hill, J. C., Alam, M. S., Watanabe, W. O., Carroll, P. M., Seaton, P. J., and Bourdelais, A. J. (2019). Replacement of menhaden fish mealby poultry by-product meal in the diet of juvenile red porgy. *N. Am. J. Aquac.* 81, 81–93. doi: 10.1002/naaq.10074

Hu, C. L., Li, X. H., He, X. Y., Yuan, X. L., Wang, R. X., Han, J., et al. (2021). Industry development report on cotton processing industry 2020. *Cotton Processing in China.* 2, 4–15.

Hyder, M. A., Hasan, M., and Mohieldein, A. (2013). Comparative levels of ALT, AST, ALPand GGT in liver associated diseases. *Eur. J. Exp. Biol.* 3, 280–284.

Irm, M., Taj, S., Jin, M., Andriamialinirina, H. J. T., Cheng, X., and Zhou, Q. C. (2020). Influence of dietary replacement of fish meal with fish soluble meal on growth and TOR signaling pathway in juvenile black sea bream (*Acanthopagrus schlegelii*). *Fish Shellfish Immunol.* 101, 269–276. doi: 10.1016/j.fsi.2020.03.053

Kimball, S. R. (2007). The role of nutrition in stimulating muscle protein accretion at the molecular level. *Biochem.Soc. Trans* 35, 1298–1301.

Lauriano, E. R., Pergolizzi, S., Capillo, G., Kuciel, M., Alesci, A., and Faggio, C. (2016). Immunohistochemical characterization of Toll-like receptor 2 in gut epithelial cells and macrophages of goldfish *Carassius auratus* fed with a high-cholesteroldiet. *Fish Shellfish Immunol.* 59, 250–255. doi: 10.1016/j.fsi.2016.11.003

Li, M. H., and Robinson, E. H. (2006). Use of cottonseed meal in aquatic animal diets: a review. *N. Am. J. Aquac.* 68, 14–22. doi: 10.1577/A05-028.1

Li, P., Mai, K. S., Trushenski, J., and Wu, G. Y. (2009). New developments in fish amino acid nutrition: towards functional and environmentally oriented aquafeeds. *Amino Acids.* 37, 43–53. doi: 10.1007/s00726-008-0171-1

Liao, S. F., Wang, T. J., and Regmi, N. (2015). Lysine nutrition in swine and the related monogastric animals: muscle protein biosynthesis and beyond. *Springer Plus.* 4, 147–158. doi: 10.1186/s40064-015-0927-5

Lim, S. J., and Lee, K. J. (2009). Partial replacement of fish meal by cottonseed meal and soy-bean meal with iron and phytase supplementation for parrot fish *Oplegnathus fasciatus*. *Aquaculture.* 290, 283–289. doi: 10.1016/j.aquaculture.2009.02.018

Lu, F., Haga, Y., and Satoh, S. (2015). Effects of replacing fish meal with rendered animal protein and plant protein sources on growth response, biological indices, and amino acid availability for rainbow trout *Oncorhynchus mykiss*. *Fish. Sci.* 81, 95–105. doi: 10.1007/s12562-014-0818-7

Ma, X. Z., Wang, F., Han, H., Wang, Y., and Lin, Y. Y. (2014). Replacement of dietary fish meal with poultry by-product meal and soybean meal for golden pompano, *Trachinotus ovatus*, reared in net pens. *J. World Aquacult. Soc.* 45, 662–671. doi: 10.1111/jwas.12154

Mourente, G., Tocher, D. R., Diaz-Salvago, E., Grau, A., and Pastor, E. (1999). Study of the n-3 highly unsaturated fatty acids requirement and antioxidant status of *Dentex dentex*larvae at the *Artemia* feeding stage. *Aquaculture* 179, 291–307. doi: 10.1016/S0044-8486(99)00166-0

Regmi, N., Wang, T., Crenshaw, M. A., Rude, B. J., and Liao, S. F. (2017). Effects of dietary lysinelevels on the concentrations of selected nutrient metabolites in blood plasma of late-stage finishing pigs. *J. Anim. Physiol. Anim. Nutr.* 102, 403–409. doi: 10.1111/jpn.12714

Rombout, J. H. W. M., Abelli, L., Picchietti, S., Scapigliati, G., and Kiron, V. (2011). Teleost intestinal immunology. *Fish Shellfish Immunol* 31, 616–626.

Simopoulos, A. P. (2002). The importance of the ratio of omega-6/omega-3 essential fattyacids. *Biomed. Pharmacother* 56, 365–379.

Song, F., Xu, D. D., Mai, K. S., Zhou, H. H., Xu, W., and He, G. (2016). Comparative study on the cellular and systemic nutrient sensing and intermediary metabolism after partial replacement of fishmeal by meat and bone meal in the diet of Turbot (*Scophthalmus maximus* L.). *PLoS One.* 11:e0165708. doi: 10.1371/journal.pone.0165708

Song, Z. D., Li, H. Y., Wang, J. Y., Li, P. Y., Sun, Y. Z., and Zhang, L. M. (2014). Effects of fishmeal replacement with soy protein hydrolysates on growth performance, blood biochemistry, gastrointestinal digestion and muscle composition of juvenile starry flounder (*Platichthys stellatus*). *Aquaculture* 426–427, 96–104.

Sullivan, J. A., and Reigh, R. C. (1995). Apparent digestibility of selected feedstuffs in diets for hybrid striped bass (*Morone saxatilis* ♀x *Morone chrysops* ♂). *Aquaculture.* 138, 313–322. doi: 10.1016/0044-8486(95)01071-8

Tan, X. H., Lin, H. Z., Huang, Z., Zhou, C. P., Wang, A. L., Qi, C. L., et al. (2016). Effects of dietary leucine on growth performance,feed utilization, non-specific immune responses and gut morphology of juvenilegolden pompano *Trachinotus ovatus*. *Aquaculture.* 465, 100–107. doi: 10.1016/j.aquaculture.2016.08.034

Tang, Y. Y., Zhang, J. N., Ai, C. X., and Hu, B. (2013). Review of nutrient requirements and formuladietary for *Trachinotus ovatus*. *Feed Indust.* 34, 46–50.

Wacyk, J., Powell, M., Rodnick, K., Overturf, K., Hill, R. A., and Hardy, R. (2012). Dietary protein source significantly alters growth performance, plasma variables and hepatic gene expression in rainbow trout (*Oncorhynchus mykiss*) fed amino acid balanced diets. *Aquaculture.* 35, 223–234. doi: 10.1016/j.aquaculture.2012.05.013

Wan, M. G., Yin, P., Fang, W. P., Xie, S. W., Chen, S. J., Tian, L. X., et al. (2018). The effect of replacement of fishmeal by concentrated dephenolization cottonseed protein on the growth, body composition, haemolymph indexes and haematological enzyme activities of the Pacific white shrimp (*Litopenaeus vannamei*). *Aquac. Nutr.* 24, 1845–1854. doi: 10.1111/anu.12823

Wang, Q., He, G., Mai, K. S., Xu, W., and Zhou, H. H. (2015). Fishmeal replacement by mixed plantproteins and maggot meal on growth performance, target of rapamycin signalling andmetabolism in juvenile turbot (*Scophthalmus maximus* L.). *Aquac. Nutr.* 22, 752–758. doi: 10.1111/anu.12296

Wang, W. Q., Xu, Y. J., Chi, S. Y., Yang, P., Mai, K. S., and Song, F. (2020). Dietary lysine regulates bodygrowth performance *via* the Nutrient-sensing signaling pathways in largemouth bass (*Micropterus salmoides*). *Front. Mar. Sci.* 7:595682.

Wang, W. Q., Yang, P., He, C. Q., Chi, S. Y., Li, S. L., Mai, K. S., et al. (2021). Effects of dietary methionine on growth performance and metabolism through modulating nutrient-related pathways in largemouth bass (*Micropterus salmoides*). *Aquacult. Rep.* 20, 100642. doi: 10.1016/j.aqrep.2021.100642

Wang, Z., Qian, X. Q., Xie, S. Q., and Yun, B. (2021). Changes of growth performance and plasma biochemical parameters of hybrid grouper (*Epinephelus lanceolatus* ♂× *Epinephelus fuscoguttatus* ♀) in response to substitution of dietary fishmeal with poultry by-product meal. *Aquacult. Rep.* 18, 100516. doi: 10.1016/j.aqrep.2020.100516

Wei, Y. L., Li, B. X., Xu, H. G., and Liang, M. Q. (2020). Effects of lysine and leucine in free and differentdipeptide forms on the growth, amino acid profile and transcription of intestinalpeptide, and amino acid transporters in turbot (*Scophthalmus maximus*). *Fish Physiol.Biochem.* 46, 1795–1807. doi: 10.1007/s10695-020-00828-2

Wei, Y. T., Shen, H. H., Xu, W. Q., Pan, Y., Chen, J., Zhang, W. B., et al. (2019). Replacement of dietary fishmeal by antarctic krill meal on growth performance, intestinal morphology, body composition and organoleptic quality of large yellow croaker *Larimichthys crocea*. *Aquaculture* 512, 734281.

Wu, P., Liu, Y., Jiang, W. D., Jiang, J., Zhang, Y. A., Zhou, X. Q., et al. (2017). Intestinal immune responses of jian carp against *Aeromonas hydrophila* depressed by choline deficiency: varied change patterns of mRNA levels of cytokines, tight junction proteins and related signaling molecules among three intestinal segments. *Fish Shellfish Immunol.* 65, 34–41. doi: 10.1016/j.fsi.2017.03.053

Wu, Y., Han, H., Qin, J., and Wang, Y. (2014). Replacement of fishmeal by soy protein concentrate with taurine supplementation in diets for golden pompano (*Trachinotus ovatus*). *Aquac. Nutr.* 21, 214–222. doi: 10.1111/anu.12161

Xie, S. W., Wei, D., Yin, P., Zheng, L., Guo, T. Y., Liu, Y. J., et al. (2019). Dietary replacement of fish-meal impaired protein synthesis and immune response of juvenile pacific white shrimp, *Litopenaeus vannamei* at low salinity. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* 228, 26–33. doi: 10.1016/j.cbpb.2018.11.002

Xu, D. D., He, G., Mai, K. S., Zhou, H. H., Xu, W., and Song, F. (2016). Postprandial nutrient-sensing and metabolic responsesafter partial dietary fishmeal replacement by soybean meal in turbot (*Scophthalmus maximus* L.). *Br. J.Nutr.* 115, 379–388. doi: 10.1017/S0007114515004535

Xu, H. G., Ai, Q. H., Mai, K. S., Xu, W., Wang, J., Ma, H. M., et al. (2010). Effects of dietary arachidonic acid on growth performance, survival, immune response and tissue fatty acid composition of juvenile Japanese seabass, *Lateolabrax japonicus*. *Aquaculture.* 307, 75–82. doi: 10.1016/j.aquaculture.2010.07.001

Xun, P. W., Lin, H. Z., Wang, R. X., Huang, Z., Zhou, C. P., Yu, W., et al. (2019). Effects of dietary vitamin B1 on growth performance, intestinal digestion and absorption, intestinal microflora and immune response of juvenile golden pompano (*Trachinotus ovatus*). *Aquaculture.* 506, 75–83. doi: 10.1016/j.aquaculture.2019.03.017

Yang, P., Wang, W. Q., Chi, S. Y., Mai, K. S., Song, F., and Wang, L. (2020). Effects of dietary lysine on regulating GH-IGF system, intermediate metabolism and immune response in largemouth bass (*Micropterus salmoides*). *Aquacult. Rep.* 17, 100323. doi: 10.1016/j.aqrep.2020.100323

Ye, G. L., Dong, X. H., Yang, Q. H., Chi, S. Y., Liu, H. Y., Zhang, H. T., et al. (2020). Low-gossypol cottonseed protein concentrate used as a replacement of fish meal for juvenile hybrid grouper (*Epinephelus fuscoguttatus* ♀×*Epinephelus lanceolatus* ♂): Effects on growth performance, immune responses and intestinal microbiota – science dire. *Aquaculture* 524, 735309. doi: 10.1016/j.aquaculture.2020 735309

Zheng, Q. M., Wen, X. B., Han, C. Y., Li, H. B., and Xie, X. H. (2012). Effect of replacing soybean meal withcottonseed meal on growth, hematology, antioxidant enzymes activity and expressionfor juvenile grass carp, *Ctenopharyngodon idellus*. *Fish Physiol. Biochem.* 38, 1059–1069. doi: 10.1007/s10695-011-9590-0

Zhou, W., Rahimnejad, S., Lu, K., Wang, L., and Liu, W. (2019). Effects of berberine on growth, liver histology, and expression of lipid-related genes in blunt snout bream (*Megalobrama amblycephala*) fed high-fat diets. *Fish Physiol Biochem* 45, 83–91.

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 in Marine Science

ORIGINAL RESEARCH
published: 08 February 2022
doi: 10.3389/fmars.2022.828497

Phytogenics From Sage and Lemon Verbena Promote Growth, Systemic Immunity and Disease Resistance in Atlantic Salmon (*Salmo salar*)

Ricardo Salomón[1,2], M. Dolors Furones[1], Felipe E. Reyes-López[3,4], Lluis Tort[3], Joana P. Firmino[1†], José C. Quintela[5], José M. Pinilla-Rosas[5], Eva Vallejos-Vidal[4,6] and Enric Gisbert[1]**

[1] IRTA, Centre de Sant Carles de la Ràpita (IRTA-SCR), Aquaculture Program, Sant Carles de la Ràpita, Spain, [2] Ph.D. Program in Aquaculture, Universitat Autònoma de Barcelona, Bellaterra, Spain, [3] Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, Bellaterra, Spain, [4] Centro de Biotecnología Acuícola, Facultad de Química y Biología, Universidad de Santiago de Chile, Santiago, Chile, [5] Natac Biotech, Madrid, Spain, [6] Facultad de Medicina Veterinaria y Agronomía, Universidad de Las Américas, Santiago, Chile

The transcriptomic response of the head kidney, the main lymphohematopoietic tissue of the body, was evaluated in Atlantic salmon (*Salmo salar*) smolts fed a functional feed containing a phytogenic rich in verbascoside and triterpenic compounds like ursolic acid. Fish (initial body weight = 55.0 ± 0.1 g) were fed two experimental diets (40% crude protein, 22% crude fat; 21.6 MJ/kg gross energy) that only differed in the phytogenic content (0.1% inclusion). Each diet has six replicates and was tested over a period of 133 days. The tested zootechnical feed additive a medicinal plant leaf extract (MPLE) obtained from sage (*Salvia officinalis*) and lemon verbena (*Lippia citriodora*). At the end of the trial, smolts fed the MPLE diet were heavier than their congeners from the control group (271.5 ± 7.9 g vs. 240.2 ± 19.3 g, respectively; $P < 0.05$). Feed conversion ratio (FCR) values in fish fed the control diet were higher than those in fish fed the MPLE diet ($FCR_{controldiet}$ = 1.27 ± 0.08 vs. $FCR_{0{\cdot}1\%MPLEdiet}$ = 1.08 ± 0.05; $P < 0.05$). The immunomodulatory properties of the functional diet were evaluated by means of an *in vivo* challenge with *Aeromonas salmonicida* subsp. *salmonicida* (1×10^7 CFU mL^{-1}). The microarray analysis of head kidney samples from both dietary groups revealed 1,178 differentially expressed genes (802 upregulated and 376 downregulated). Among them, several biological processes related to immunity were identified in fish fed the MPLE diet (*i.e.*, interferon-gamma-mediated signaling pathway, antigen processing and presentation of peptide antigen *via* MHC class II, autophagy, regulation of i-kappaB kinase/NF-kappaB signaling, and leukocyte activation). Results from the bacterial challenge showed that survival rates were higher in smolts from the MPLE group (90.6 ± 6.4%) in comparison to the control group (60.7 ± 13.5%), confirming the functional benefits of the phytogenic in terms of host's immunity and disease resistance. Biological processes such as cytoskeleton organization and regulation of cellular protein metabolic process detected in fish fed the MPLE diet supported the metabolic changes related to increased somatic growth promoted. The present findings showed that the

OPEN ACCESS

Edited by:
Kang-le Lu,
Jimei University, China

Reviewed by:
Patricia Diaz-Rosales,
Centro de Investigación en Sanidad Animal, CISA (INIA-CSIC), Spain
Seyyed Morteza Hoseini,
Iranian Fisheries Science Research Institute (IFSRI), Iran

***Correspondence:**
Enric Gisbert
enric.gisbert@irta.cat
Eva Vallejos-Vidal
eva.vallejosv@usach.cl

†Present address:
Joana P. Firmino,
TECNOVIT-FARMFAES S.L., Alforja, Spain

Specialty section:
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

Received: *03 December 2021*
Accepted: *04 January 2022*
Published: *08 February 2022*

Citation:
Salomón R, Furones MD, Reyes-López FE, Tort L, Firmino JP, Quintela JC, Pinilla-Rosas JM, Vallejos-Vidal E and Gisbert E (2022) Phytogenics From Sage and Lemon Verbena Promote Growth, Systemic Immunity and Disease Resistance in Atlantic Salmon (Salmo salar). Front. Mar. Sci. 9:828497. doi: 10.3389/fmars.2022.828497

inclusion at 0.1% of the tested MPLE obtained from sage and lemon verbena in diets for Atlantic salmon smolts promoted somatic growth, and enhanced their systemic immune response and reduced mortality when fish were challenged with *A. salmonicida* cumulative, the causative agent of furunculosis in salmonids.

Keywords: feed additive, aquaculture, systemic immunity, Atlantic salmon, *Aeromonas salmonicida*, phytogenics

INTRODUCTION

Aquaculture is predicted to be the main source of aquatic dietary protein sources by 2050, playing a relevant role in food security and supply, as well as in poverty alleviation (Stentiford et al., 2020). The Atlantic salmon (*Salmo salar*) is the most important fish species consumed in the countries of the first world, whose production has strongly increased due to the development of this industry in the northern Europe and in North and South America, with Norway and Chile as the main world producers (FAO, 2021). Despite the sector's efforts focused on competitiveness and sustainable development to build this thriving sector, this rapid and continuous growth of the salmon farming has some side effects. In this sense, under intensive farming, fish can be influenced by various environment-related biotic and abiotic factors that can have potentially harmful or stressful effects (Taranger et al., 2015). All these factors have a negative impact on fish welfare and overall rearing performance, increasing susceptibility to disease; thus, negatively impacting the industry by causing health crises and economic losses (Tort, 2011; Taranger et al., 2015). This makes aquatic animal diseases one of the main factors limiting the growth of aquaculture and its sustainability (Reverter et al., 2020; Naylor et al., 2021).

Despite the fact that in 2022 several countries, including the EU, will ban the regular administration of antimicrobial agents in farming, including preventive group treatments (More, 2020), the general use of antibiotics for prophylactic purposes linked to intensive aquaculture activities can still be detected in some of the major aquaculture producing countries (Lulijwa et al., 2020; Schar et al., 2020). Thus, the need to develop health preventive treatments is becoming more of a necessity than an option. Thus, among the repertoire of tested strategies related to health management (Barrett et al., 2020; Miccoli et al., 2021), functional feeds are reputed as one of the most affordable solutions in terms of their prophylactic application. These diets are formulated for supporting the nutritional and physiological requirements of fish, as well as providing protection in front of biotic and abiotic stressors that are intrinsic to aquaculture rearing conditions (Waagbø and Remø, 2020). In this regard, the development and application of functional feeds represent a sound strategy for the aquaculture industry, as they provide functional benefits for animal health beyond their nutritional value, taking into account the purpose of their use either as nutritional, sensory, or functional additives (Vallejos-Vidal et al., 2016; Dawood et al., 2018). Thus, feed additives promoting immunity and enhancing stress and disease resistance in farmed fish have received notorious attention by the industry and academia as environmentally-friendly health management strategies. Therefore, phytogenics are among others, one of the most widely evaluated and recognized zootechnical feed additives with immunomodulatory properties (Encarnação, 2016). Phytogenics are defined as environmentally friendly plant-derived bioactive compounds that show positive effects on animal growth and health-promoting, antimicrobial, antiparasitic, immunostimulant, antioxidant, and anti-inflammatory properties (Firmino et al., 2020; Dawood, 2021; Reverter et al., 2021).

Functional feeds based on phytogenics have been the focus of attention for the industry during the last decade due to their antimicrobial, immunostimulant, antioxidant, anti-stress, and growth-promoting functions (Awad and Awaad, 2017; Sutili et al., 2018; Hernández-Contreras and Hernández, 2020). Furthermore, phytogenics have shown to enhance both humoral and cellular immune response in teleosts (Elumalai et al., 2020; Firmino et al., 2021a,b), whereas other studies have also demonstrated their antimicrobial activity against a wide range of pathogenic organisms (Vaseeharan and Thaya, 2014; Firmino et al., 2021a). These functional properties make them very attractive for the industry as potential prophylactic dietary treatments. In particular, the current investigation endeavors to explore the dietary effects of phytogenics derived from a mixture of medicinal plants, the sage (*Salvia officinalis*, Lamiaceae) and the lemon verbena (*Lippia citriodora*, Verbenaceae), both recognized for their health and growth-promoting properties for aquatic species (Elumalai et al., 2020; Salomón et al., 2020, 2021a). In particular, in a previous study from our group, we showed that a medicinal plant leaf extract (MPLE) from sage and lemon verbena promoted an improvement in the classical key performance indicators (KPIs) linked to somatic growth and feed efficiency. These effects were coupled with a tightly controlled systemic immune response in an *ex vivo* assay using gilthead seabream (*Sparus aurata*) splenocytes stimulated by lipopolysaccharide (LPS; Salomón et al., 2020). In addition, we have recently reported that this MPLE obtained from sage and lemon verbena promoted gut integrity and immunity; particularly, T cell activation and differentiation (Salomón et al., 2021a).

In traditional medicine, *S. officinalis* has long enjoyed a reputation for its health benefits and for treating all kinds of ailments. Sage is a common herbal plant widely cultivated in various parts around the world, but it is native to the Mediterranean region. In addition, is known to be rich in phenolic compounds such as flavonoids, tannins, coumarins, and triterpenes (Ghorbani and Esmaeilizadeh, 2017), which are a highly diverse group of natural components widely found in a variety of common European plants and fruits (Vincken et al., 2007; Babalola and Shode, 2013).

Thus, its content in functional compounds has attracted the attention within livestock and aquaculture industry. For instance, Simonová et al. (2010) reported that diets containing sage increased the energy content and amino acid profile in rabbit meat, in addition to promoting a good health condition of the animals. Similarly, Placha et al. (2015) demonstrated that sage promoted the integrity of the duodenal wall in laying hens. Regarding aquatic species, Sönmez et al. (2015) reported a positive effect of sage on growth performance and antioxidant enzyme activities in juvenile rainbow trout (*Oncorhynchus mykiss*). In this sense, several bioactive compounds have been identified in plants from the genus *Salvia*, such as flavonoids (Lu and Foo, 2000), phenolic acids (Wang et al., 1999), and pentacyclic triterpenes (Mašterová et al., 1989), among others. For instance, one of these triterpenic acids is ursolic acid, which is a pentacyclic terpenoid that has shown many beneficial properties effects on human health (Woźniak et al., 2015), and even in teleosts (Ding et al., 2015; Li et al., 2019). In zebrafish (*Danio rerio*), ursolic acid was reported to have anti-inflammatory activity (Ding et al., 2015), whereas, in rainbow trout, a strong antiviral activity was reported both *in vitro* and *in vivo* (Li et al., 2019).

Lippia citriodora, colloquially known as lemon verbena, is a plant species of the *Verbenaceae* family that mostly grows in South America and is cultivated in northern Africa and southern Europe. Lemon verbena leaf extract contains polyphenols, including phenylpropanoids such as verbascoside, iridoids like gardoside, and flavonoids such as luteolin-7-diglucoronide, among which verbascoside acid is the most abundant compound in lemon verbena leaves, so most of its beneficial effects are attributed to this phytochemical (Sánchez-Marzo et al., 2019). Several studies have indicated that verbascoside acid is responsible for multiple beneficial properties of lemon verbena like its antioxidant (Mosca et al., 2014; Martino et al., 2016), anti-inflammatory, and antineoplastic properties in addition to numerous wound-healing and neuroprotective properties (Funes et al., 2009; Caturla et al., 2011; Alipieva et al., 2014). The use of lemon verbena in juvenile sheep has been reported to promote embryo development by protecting the oocyte against oxidative stress (Martino et al., 2016). In addition, another study showed that pigs fed with a diet enriched with verbascoside rich showed an improvement in their growth and feed efficiency performances (Corino et al., 2007; Pastorelli et al., 2012). Despite the existing literature, information on the function of these bioactive compounds of plant origin, such as sage and lemon verbena, is still scarce regarding their applications in animal production, especially their immunomodulatory effects on the systemic immune response and their potential use as a functional feed additive to promote disease resistance in fish.

Under this context, the present study aimed to evaluate the transcriptional responses of the head kidney in Atlantic salmon smolts fed a functional feed containing a mixture of MPLE obtained from sage and lemon verbena. At the end of the nutritional trial, disease resistance in smolts was evaluated by means of a bacterial challenge with the causative agent of furunculosis (*Aeromonas salmonicida* subsp. *salmonicida*).

MATERIALS AND METHODS

This study was divided into two different stages. Firstly, a nutritional trial during 133 days was conducted in order to evaluate the effects of the phytogenic on growth performance and transcriptomic analysis in head kidney of Atlantic salmon. The nutritional trial encompassed different periods, the parr phase (47 days; 19th December – 04th February); the smoltification phase, which started on the 5th of February and lasted 10 days; and the full seawater transfer stage that started on the 14th February until the end of the nutritional assay. In the second stage, fish from the nutritional trial were used in a bacterial challenge in order to test whether the tested MPLE diet provided protection to the host in front of a pathogenic bacteria responsible for furunculosis in salmonid fish.

Diets

Table 1 describes the ingredient list and proximate composition of the two experimental diets used in the current study. Diets were named as control and MPLE, and only differed in the level of inclusion of the MPLE obtained from *S. officinalis* and *L. citriodora*, which was 0.1% in the MPLE diet. The tested phytogenic was provided by NATAC Biotech SL and obtained as described in Salomón et al. (2020). The proximate composition of tested MPLE was: 73% carbohydrates, 2% crude lipids, <1% crude proteins, 5% salts and 4% water. In terms of phytogenic bioactive compounds, the MPLE contained: 10% ursolic acid

TABLE 1 | List of ingredients and proximal composition of experimental diets; control and a basal diet supplemented with MPLE tested in Atlantic salmon (*Salmo salar*).

Ingredients, %	Control diet	MPLE diet
Fishmeal LT70	17.5	17.5
Soy protein concentrate	20.0	20.0
Fish protein concentrate	2.5	2.5
Wheat gluten	9.0	9.0
Corn gluten	5.0	5.0
Faba beans	5.0	5.0
Wheat meal	16.23	16.13
Fish oil	12.0	12.0
Vitamin and mineral premix	1.0	1.0
Soy lecithin	0.5	0.5
Vitamin C35%	0.07	0.07
Monocalcium phosphate	3.0	3.0
Rapeseed oil	7.0	7.0
Betaine HCl	1.0	1.0
DL-Methionine	0.2	0.2
MPLE	–	0.10
Proximate composition		
Crude protein, %	40.03	40.02
Crude fat, %	22.15	22.15
Fiber, %	1.75	1.74
Starch, %	13.02	12.93
Ash, %	8.74	8.89
Gross energy, MJ/kg	21.60	21.58

(100 ppm), 3% other triterpenic compounds (30 ppm), 2% verbascoside (60 ppm) and <1% polyphenols (<10 ppm). Diets were manufactured by Sparos Lda (Olhão, Portugal). All powder ingredients were mixed accordingly to the target formulation in a double-helix mixer (model 500L, TGC Extrusion, France) and ground (below 400 μm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Diets (pellet size: 2 and 3 mm) were manufactured with a twin-screw extruder (model BC45, Clextral, France) with a screw diameter of 55.5 mm. Extrusion conditions: feeder rate (80–85 kg/h), screw speed (247–266 rpm), water addition in barrel 1 (345 ml/min), temperature barrel 1 (32–34°C), temperature in barrel 2 (59–62), and temperature barrel 3 (111–114°C). Extruded pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). After cooling, oils were added by vacuum coating (model PG-10VCLAB, Dinnissen, Netherlands). Coating conditions were: pressure (700 mbar); spraying time under vacuum (approximately 90 s), return to atmospheric pressure (120 s). Feeds were stored at 4°C during the experimental period (133 days) in order to prevent their oxidation.

Fish and Experimental Design

Unvaccinated Atlantic salmon parrs (n = 1,550) were purchased from SARL SALMO (Gonneville-Le Thiel, France) and transported by road to IRTA-Sant Carles de la Ràpita research facilities (Sant Carles de la Ràpita, Spain). Once there, parrs were acclimated in two 2,000-L tanks connected to an open-flow system (12.0 ± 1.5°C) for 2 weeks under natural photoperiod and fed *ad libitum* a commercial feed (T2-2 Royal Optime, Skretting; proximate composition: 44% crude protein; 21% crude fat; 6.9% crude ash; 2.9% crude fiber).

Before the onset of the nutritional trial, parrs (n = 696) were gently anesthetized (50 mg L^{-1} tricaine methane sulfonate. MS-222, Sigma-Aldrich, Madrid, Spain) and individually measured in body weight (BW) and standard length (SL) to the nearest 0.1 g and 1 mm, respectively. Fish (55.0 ± 0.1 g and 16.2 ± 0.2 mm in BW and SL, respectively) were distributed among twelve experimental tanks (n = 58 fish per tank; 6 replicate tanks per experimental diet). Both experimental diets were offered to parrs at a daily feeding rate of 3.0% of the stocked biomass as described in Salomón et al. (2021b). In addition, feed utilization was evaluated by the following formula: feed conversion ratio (FCR) = feed intake (g)/increase of fish biomass (g).

During the parr phase that lasted 47 days, rearing conditions were as follows: water temperature and pH (pH meter 507; Crison Instruments, Barcelona, Spain), salinity (MASTER-20T; ATAGO Co., Ltd., Tokyo, Japan), and dissolved oxygen (OXI330; Crison Instruments) were 12.2 ± 1.0°C, 7.4 ± 0.3 and 9.4 ± 0.8 mg L^{-1} (mean ± SD), respectively. The water flow rate in experimental tanks was maintained at approximately 9.0 L min^{-1} (open-flow system), which guaranteed two full tank's water renewal per hour. Photoperiod was 8 h light: 16 h darkness.

Smoltification started on the 5th of February and lasted 10 days. During this period, water salinity was increased progressively at *ca*. 3 ppt per day until reaching 35 ppt using filtered seawater according to SARL SALMO recommendations. Water temperature, pH, and oxygen levels during this period were 12 ± 0.1°C, 7.4 ± 0.3 and 9.6 ± 0.2 mg L^{-1}. The photoperiod during the smoltification period was 24 h light, 0 h darkness. Once fish were transferred to seawater 14th February, water quality and temperature were maintained by means of a water recirculation system (IRTAmar®; Spain) that maintained adequate water quality through UV, biological, and mechanical filtration. Water quality parameters during the rest of the trial were 12.1 ± 0.2°C, 7.4 ± 0.3 and 9.5 ± 0.2 mg L^{-1}. Ammonia and nitrite were ≤0.07 and 0.14 mg L^{-1}, respectively. Ammonia and nitrites were measured twice per week by means of a portable spectrophotometer (LOVIBOND MD600, Tintometer GmbH, Germany) using the Vario Ammonia Salicyklate F 10 mL (Tintometer GmbH, Germany) and Nitriver® 3 Nitrite reagent (Permachem® Reagent, HACH Lange, GmbH) assays. The photoperiod during the smolt stage was 24 h light: 0 h darkness. The illumination system for the smolt phase consisted of a led illumination system (Celer, Spain) with a light temperature of 4.000 K and light intensity of 1.540 lumens. At the end of the trial, all fish were netted, anesthetized with MS-222 as previously described and individually weighted.

Pathogenic Bacterial Challenge

At the end of the nutritional trial, smolts fed both diets were exposed to a bacterial challenge with the causative agent of furunculosis (*A. salmonicida* subsp. *salmonicida*). The internal coding for this pathogenic bacterial strain is IRTA-17-44, a strain available for courtesy of HIPRA (Amer, Spain). In brief, the bacterial inoculum was grown on TSA at 23.0 ± 1.0°C for 48 h. The inoculum was prepared to an optical density (OD) = 1.2 measured at λ = 550 nm, which corresponded to 1 × 10^8 CFU mL^{-1}. The bacterial suspension was ten-fold serially diluted in sterile PBS to prepare the desired inoculum density, which was confirmed by CFU's plate counting. Prior to the challenge trial, the lethal dose of 50% (LD_{50}) for *A. salmonicida* was determined for the experimental conditions established. For this purpose, thirty smolts (n = 10 per dose) were intraperitoneally injected (0.2 mL) at three different concentrations of the pathogenic bacteria (1 × 10^6, 1 × 10^7 and 1 × 10^8 CFU mL^{-1}). Ten additional fish were injected with PBS as methodological control. The LD_{50} was established at 1 × 10^7 CFU mL^{-1} (data not shown). For the challenge trial, 32 Atlantic salmon smolts (BW = 194.0 ± 29.1 g) per each dietary treatment were randomly distributed[1] into quadruplicate tanks (4 tanks per dietary treatment), with eight fish per tank (stocking density = 14–16 kg m^{-3}). During the acclimation period (5 days), fish were fed *ad libitum* with the same experimental diets used in the nutritional assay. After acclimation, fish were anesthetized and IP injected with 0.2 mL of 10^7 CFU/mL of *A. salmonicida* (IRTA-17-44).

Both the establishment of the *A. salmonicida* LD_{50} and the challenge trial were performed at IRTA's biosafety challenge room, in 32 cylindrical tanks (100 L) connected to a RAS unit (IRTAmar®) equipped with real-time control of oxygen and temperature, mechanical filtration, biofiltration, and ultraviolet disinfection of the water. The outflow water was chlorinated,

[1]https://www.randomizer.org

followed by ozone treatment before being discharged. Water quality conditions in terms of temperature and salinity were 13.1 ± 1.1°C and 32.3 ± 0.4 ppt, respectively. Mortality occurring after the first 12 h post-injection (hpi) was considered to be induced *A. salmonicida* rather than handling stress, since no mortality was found in the control group injected with PBS.

During the duration of the challenge (12 days), smolts were supervised every 2 h, six times per day, including weekends. Following the ethical guidelines for the use of animals in research, when fish became moribund, they were euthanized with an overdose of MS-222 (>150 mg L^{-1}). At the end of the challenge, all fish were sacrificed following the same procedure. A species-specific PCR (Beaz-Hidalgo et al., 2008) was performed from DNA of bacterial colonies recovered from head kidney smears of all moribund fish in order to confirm the cause of death. For this purpose, animals were aseptically dissected and a sample from the head kidney was taken and plated on TSA, incubated at 23°C for 72 h. Confluent pure bacterial growth was found from all samples, from which *A. salmonicida* was confirmed by means of PCR as described in Salomón et al. (2021b).

Transcriptional Analysis

RNA Isolation and Quality Control

At the end of the nutritional assay, three fish from each tank (n = 18 fish per diet) were sacrificed with an overdose of MS-222 (>150 mg L^{-1}). Then, head kidney was removed and fixed in RNAlater® (Sigma-Aldrich, Sant Louis, MO, United States), incubated overnight (4°C), and stored at −80°C. Total RNA from the head kidney of individual fish was extracted using TRI reagent (Sigma-Aldrich, Sant Louis, MO, United States) following the guidelines provided by the manufacturer. Total RNA concentration and purity were quantified using a Nanodrop-2000® spectrophotometer (Thermo Scientific™, United States) and stored at −80°C for further analysis. To check RNA integrity, samples were diluted (133.33 ng μL^{-1}) and the RNA Integrity Number (RIN) determined by means of an Agilent 2100 Bioanalyzer (Agilent Technologies, Spain). Only samples with a RIN value higher than 8.5 were selected for further microarray analysis. For each dietary group, we used for microarray analysis three head kidney pooled samples. Each pool consisted of one fish from each tank replicate (n = 18 fish per dietary group); thus, data regarding individual variability was lost with this analysis.

Microarray Design and Analysis

Gene expression analysis from head kidney samples was performed using the custom-commercial *Salmo salar* oligonucleotide microarray platform (AMADID 084881; Gene Expression Omnibus (GEO) access number: GPL28080; Agilent Technologies, United States). Data from this study are available in the GEO accession number GSE184485.

RNA handling and the microarray analysis of samples were conducted as described by Salomón et al. (2021b). Total RNA (200 ng) was reverse transcribed (Agilent One-Color RNA spike-in kit; Agilent Technologies) and used as a template for Cyanine-3 (Cy3) labeled cRNA synthesis and amplification (Quick Amp Labeling kit, Agilent Technologies). The RNeasy micro kit (Qiagen) was used for cRNA purification. Dye incorporation and cRNA yield were checked with the NanoDrop ND-2000® spectrophotometer. Then, Cy3-labeled cRNA (1.5 mg) with specific activity >6.0 pmol Cy3/mg cRNA was fragmented (60°C, 30 min), and then mixed with the hybridization buffer and hybridized to the array (ID 084881, Agilent Technologies) at 65°C for 17 h (Gene expression hybridization kit; Agilent Technologies). The microarray was washed as indicated by the manufacturer (Gene expression wash buffers; Agilent Technologies), followed by the application of stabilizing and drying solutions (Agilent Technologies). Microarray slides were scanned (Agilent Technologies Scanner, model G2505B) and spot intensities and other quality control features were extracted with Agilent's Feature Extraction software version 10.4.0.0 (Agilent Technologies). Quality reports were checked for each array. The identification of differentially expressed genes was done as described by Reyes-López et al. (2015). Data processing and mining were performed by means of the package STARS (NOFIMA, Norway) (Krasnov et al., 2011). Lowess normalization of log2-expression ratios (ER) was performed after removing the low-quality spots formerly identified. The selection of differentially expressed genes (DEGs) was done considering the difference between both diets following an unpaired t-test ($P < 0.05$).

Functional Network Analyses: Transcripteractomes

The transcripteractome analysis was conducted according to Reyes-López et al. (2021) using the Search Tool for the Retrieval of Interacting Genes (STRING) public repository version 10.0[2] (Szklarczyk et al., 2019). A protein-protein interaction (PPI) network for DEGs was done with a high-confidence interaction score (value = 0.4). The mechanisms of response in which DEGs are involved were obtained from a comparative analysis using *Homo sapiens* as a reference organism. Thus, an *H. sapiens* acronym was assigned based on *S. salar* transcript annotation using Uniprot (2019) and Genecards (Stelzer et al., 2016) databases. When genes with no annotation match were found for Atlantic salmon, we assigned an orthologue *H. sapiens* Entrez Gene based on the homology between sequences using the best tBlastX (NCBI) hit. Matches with at least E value $\leq 1e^{-10}$ were only considered, whereas the Uniprot and Genecards databases were used to confirm the match of the gene acronym tag between both species. Gene ontology (GO) pathway enrichment analysis for biological processes (GO_BiologicalProcess-EBI-UniProt-GOA-ACAP-ARAP_10.11.2020_00h00) was obtained using ClueGO v2.5.7 (Bindea et al., 2009) through Citoscape 3.8.2 (Shannon et al., 2003). The enrichment and depletion of GO categories (two-sided hypergeometric test; $P < 0.05$) using the Benjamini-Hochberg correction. Furthermore, a GO Fusion was run in order to avoid redundant terms with a Kappa Score Threshold of 0.4 in order to propose more stringent GO terms associated to the mechanism of response for the MPLE diet. GO terms grouping was performed when the sharing group's percentage was above 50 ($P < 0.05$). The statistically significant GOs obtained from the enrichment analysis were assigned to each one of the nodes represented in the functional

[2]https://string-db.org

network. The ClueGo v2.5.7 a Cytoscape plug-in was used for visualizing nodes classified in different clusters based on their functionality. Hub genes of PPI networks were calculated by Cytoscape plug-in, *cytoHubba* (version 0.1), predicted the top 10 nodes using analysis algorithms including Maximum Clique Centrality (MCC; Chin et al., 2014).

Ethics Statement

Experimental procedures were conducted following the Guiding Principles for Biomedical Research Involving Animals (EU2010/63) and the guidelines of the Spanish laws (law 32/2007 and RD 1201/2015) and authorized by the Ethical Committee of IRTA (FUE-2020-01314717).

Statistics

Data in terms of somatic growth performance was compared between the control and the MPLE diets by means of a *t*-test ($P < 0.05$). Regarding the bacterial challenge, mortality rates were registered in both groups and data were depicted using Kaplan–Meier survival curves (Kaplan and Meier, 1958). Survival rates were calculated using the Mantel–Cox log-rank test. All the statistical analyses were conducted using SPSS for Windows® (version 15.0, SPSS Inc., Chicago, IL, United States). The Heatmapper server was used for constructing the hierarchical heatmap of DEGs (Babicki et al., 2016).

RESULTS

Survival and Growth Performance

At the end of the study, no significant differences in survival were found between Atlantic salmon smolts fed the control (98.9 ± 1.9%) and MPLE (99.4 ± 1.3%) diets ($P > 0.05$). Smolts fed the MPLE diet (271.5 ± 7.9 g) were 11.5% heavier than those fed the control diet (240.2 ± 19.3 g) ($P < 0.05$). Values of FCR were lower in fish fed the 0.1% MPLE diet (1.08 ± 0.05) than in those fed the control diet (1.27 ± 0.08) ($P < 0.05$).

Head Kidney Transcriptomic Results

A total of 1,178 DEGs were found in the head kidney of smolts fed the MPLE diet compared to the control group (**Figure 1**). The complete list of DEGs may be found in **Supplementary Table 1**. Most of the upregulated genes ($n = 523$) were found within the 0.8 < log2 absolute fold-change (| log2 FC|) < 1.4 interval. In addition, 243 genes were identified in the 1.4 < | log2 FC| < 2.5 interval, 35 transcripts in the 2.5 < | log2 FC| < 5.0, and only one single gene in the | log2 FC| > 5.0. Regarding downregulated genes, 289 transcripts were found in the 0.8 < | log2 FC| < 1.4 interval; other 76 transcripts were grouped in the 1.4 < | log2 FC| < 2.5 interval, whereas only 11 DEGs were included in the 2.5 < | log2 FC| < 5.0 expression interval. The detailed analysis of gene absolute log2 fold-change (| log2 FC|) revealed that genes were mostly upregulated in fish fed the MPLE diet (68.1% of DEGs), while its gene modulation was moderate in terms of FC intensity (**Figure 1A**). Results from the PCA are shown in **Figure 1B**, whereas those related to the hierarchical clustering heatmap of DGEs from both diets are depicted in **Figure 1C**.

Enrichment Analyses and Transcripteractome Results

The analysis of the transcripteractome showed the presence of thirty-four clusters (**Table 2**). The complete list of them is detailed in **Supplementary Table 2**. Among them, eleven clusters were identified with only one-single node, four clusters were constituted by two nodes, four clusters by three nodes, and four clusters by four nodes (**Figure 2**). Only one cluster was identified composed by five (GO:0042770: "signal transduction in response to DNA damage"), six (GO:0007492: "endoderm development"), seven (GO:0071901: "negative regulation of protein serine/threonine kinase activity"), and nine nodes (GO:0043122: "regulation of I-kappaB kinase/NF-kappaB signaling"). Other seven clusters contained more than ten nodes, including the "activation of cysteine-type endopeptidase activity involved in apoptotic process" (GO:0006919; eleven nodes), "response to organonitrogen compound" (GO:0010243; eleven nodes), "leukocyte activation involved in immune response" (GO:0002366; thirteen nodes), "intracellular signal transduction" (GO:0035556; thirteen nodes), "Autophagy" (GO:0006914; sixteen nodes). Importantly, two clusters registered more than thirty nodes including the "regulation of DNA-binding transcription factor activity" (GO:0051090; thirty-seven nodes), and the "actin filament organization" (GO:0007015; forty-one nodes) (**Figure 2**).

Through plugin *cytoHubba* in Cytoscape software, we evaluated the degree and betweenness centrality in the PPI network and screening the hub genes. Thus, the top ten hub genes with a high level of correlation for the selected clusters related to immunity and obtained from the enriched biological functions were selected for further consideration. From the "Antigen processing and presentation of peptide antigen *via* MHC class II", we identified six upregulated transcripts (*hla-dqa1*, *cd74*, *ctsl*, *ctsd*, *kif23*, *dync1li2*) and four downregulated hub genes (*klc1*, *hla-dmb*, *kif2a*, *sptbn2*) (**Figure 3A**). Considering the cluster "Interferon-gamma-mediated signaling pathway", among the top 10 hub genes, six transcripts were upregulated (*camk2α*, *hla-dqa1*, *trim21*, *trim22*, *med1*, *trim68*), and four of them downregulated (*jak1*, *jak2*, *tp53bp1*, *ncam1*) (**Figure 3B**). For the cluster "Regulation of I-kappaB kinase/NF-kappaB signaling", we identified five upregulated transcripts (*notch1*, *cebpb*, *smad3*, *sirt1*, *cd40*), and five others were downregulated (*gapdh*, *jak2*, *spi1*, *smad4*, *brd4*) (**Figure 3C**). Considering the cluster "Leucocyte activation involved in immune response", most of the hub genes were upregulated (*fn1*, *notch1*, *grb2*, *rac2*, *rdx*, *ezr*, *smad3*, *abl1*) compared to the two downregulated hub genes (*actb*, *jak2*) (**Figure 3D**). Regarding the cluster "Cytoskeleton organization", seven hub genes were upregulated (*fn1*, *notch1*, *itgb4*, *itgb5*, *itga11*, *col2a1*, *col4a5*), and three downregulated (*actb*, *gapdh*, *itga10*) (**Figure 3E**). Considering the cluster "Regulation of cellular protein metabolic process", just three genes were upregulated (*fn1*, *irs1*, *sirt1*), whereas seven genes showed a downregulation (*actb*, *gapdh*, *jak2*, *ptpn1*,

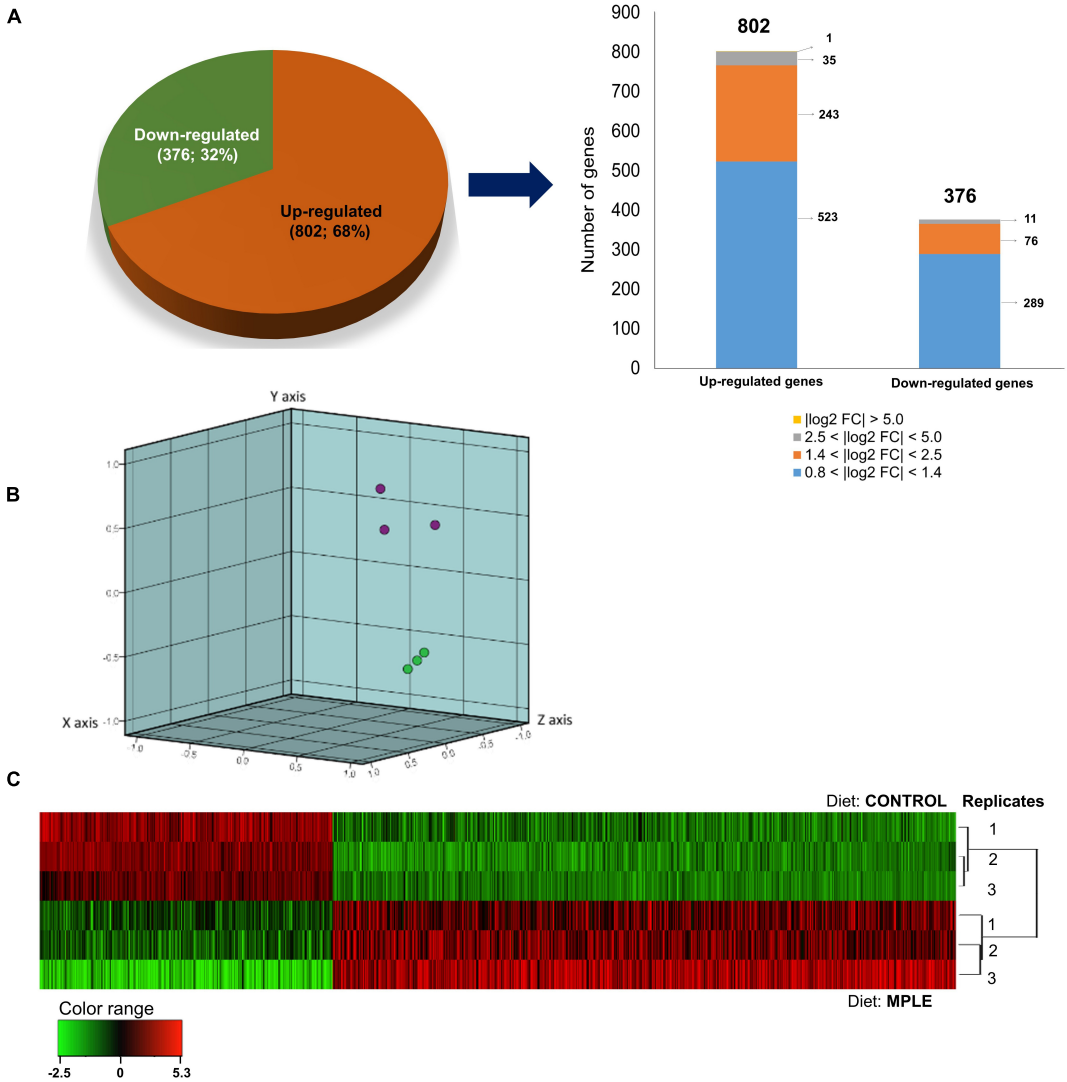


FIGURE 1 | Differential expression analysis of the Atlantic salmon (*S. salar*) head kidney transcriptomic response to MPLE diet. **(A)** Distribution (pie-chart) of the differential expressed genes (DEGs) obtained from the microarray-based transcriptomic analysis response fed a diet supplemented with phytochemicals from sage and lemon verbena. Absolute log2 fold-change (log2 FC) indicates the magnitude interval of response. **(B)** Principal component analysis (PCA) of the DEGs for the Atlantic salmon head kidney in response to the control (purple node) and phytogenic-supplemented diet (green node). **(C)** Hierarchical clustering of the Atlantic salmon head kidney transcriptomic response for the control and MPLE diet, based on similitude patterns of the DEGs detected from three sample pools per dietary group. Data of the six microarrays are depicted. The normalized intensity values (log2) obtained for each microarray analyzed for control (replicate 1, 2, and 3) and MPLE group (replicate 1, 2, and 3) are shown.

jak1, *smad4*, *insr*) (**Figure 3F**). The "Cellular biosynthetic process" cluster showed also ten hub genes. From them, seven genes were upregulated (*rps6*, *rpl19*, *rpl26*, *rpl12*, *rps7*, *rps17*, *dock4*), and three of them were downregulated (*rps10*, *rpl3l*, *eef1d*) (**Figure 3G**). For the GO "Autophagy," five genes were upregulated (*sirt1*, *prkaa2*, *slc38a9*, *trim21*, *abl1*), and other five genes were downregulated (*gapdh*, *ube2v1*, *ube2n*, *tp53bp1*, *gba*) (**Figure 3H**).

Bacterial Challenge Test

Results regarding the Kaplan-Meier survival rates curves of Atlantic salmon smolts injected intraperitoneally with *A. salmonicida* (1×10^7 UFC mL^{-1}) showed significant differences between the control and MPLE diets (**Figure 4**; $P < 0.05$). Smolts fed the MPLE diet showed higher survival rates (90.6 ± 6.4%, mean ± standard deviation) compared to those smolts fed the control diet (60.7 ± 13.5%).

DISCUSSION

Nowadays, transcriptome-based functional network analyses on teleost fish fed functional feeds have gained attention, since they provide further insight into the mode of action

TABLE 2 | List of the 34 total clusters related to representative biological processes identified by the transcripteractome in Atlantic salmon smolts fed the MPLE diet.

Cluster term	% Terms per group	No. GO	No. Genes
Actin cytoskeleton organization	18.98	41	300
Cellular biosynthetic process	17.13	37	556
Autophagy	7.41	16	230
Regulation of cellular metabolic process	6.02	13	407
Leukocyte activation involved in immune response	6.02	13	171
Response to organic substance	5.09	11	184
Activation of cysteine-type endopeptidase activity involved in apoptotic process	5.09	11	155
Regulation of I-kappaB kinase/NF-kappaB signaling	4.17	9	130
Negative regulation of protein serine/threonine kinase activity	3.24	7	178
Endoderm development	2.78	6	49
Signal transduction in response to DNA damage	2.31	5	38
Intracellular signal transduction	1.85	4	215
Regulation of histone methylation	1.85	4	32
Cytoskeleton organization	1.85	4	307
Regulation of intracellular steroid hormone receptor signaling pathway	1.85	4	79
Regulation of viral life cycle	1.39	3	24
Positive regulation of cell death	1.39	3	93
Regulation of cellular protein metabolic process	1.39	3	373
Positive regulation of neuron death	1.39	3	26
Sphingolipid metabolic process	0.93	2	18
Regulation of cellular protein localization	0.93	2	40
Carbohydrate derivative metabolic process	0.93	2	50
Chondrocyte differentiation	0.93	2	21
Protein localization to nucleus	0.46	1	22
Maintenance of protein location	0.46	1	11
Actin cytoskeleton reorganization	0.46	1	11
Regulation of cell shape	0.46	1	14
Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	0.46	1	12
Antigen processing and presentation of peptide antigen *via* MHC class II	0.46	1	11
Cell-cell adhesion	0.46	1	56
Hepaticobiliary system development	0.46	1	15
Stem cell differentiation	0.46	1	21
Interferon-gamma-mediated signaling pathway	0.46	1	10
Negative regulation of transcription by RNA polymerase II	0.46	1	52

of zootechnical additives with immunomodulatory properties on the host (Firmino et al., 2021a; Reyes-López et al., 2021; Salomón et al., 2021a,b). In this sense, this study was performed to gain insight into the potential immunomodulatory, disease resistance, and other biological effects of a phytogenic feed additive obtained from sage and lemon verbena in Atlantic salmon smolts. These bioactive compounds were chosen due to their health and growth-promoting properties in aquatic species (Elumalai et al., 2020; Salomón et al., 2020, 2021a) and screened as a potential additive for a functional feed in order to promote host's immunity and enhance disease resistance. In this context, we found that the inclusion of MPLE at 0.1% in Atlantic salmon smolts exerted a positive effect on somatic growth, being fish fed the diet containing the MPLE 11.5% heavier than the control group. Similar results have been observed in higher vertebrates (Corino et al., 2007; Pastorelli et al., 2012; Casamassima et al., 2013) and in fish species like gilthead sea bream fed the same feed additive (Salomón et al., 2020) and other species like rainbow trout or beluga fed functional diets containing sage and/or lemon verbena phytogenics (Sönmez et al., 2015; Dadras et al., 2020). These findings might be partially attributed to the potential effects of triterpenoid compounds, such the ursolic acid, which has been reported to promote muscular growth by the hypertrophy of muscular fibers in mice (Kunkel et al., 2012) and rainbow trout (Fernández-Navarro et al., 2006). Furthermore, the growth-promoting effects of polyphenolic compounds like verbascoside present in *L. citriodora* have also been observed in several studies (Corino et al., 2007; Pastorelli et al., 2012; Casamassima et al., 2013).

Regulation of Cellular Protein Metabolic and Cytoskeleton Organization

The transcripteractome results showed the enrichment of the biological processes related to "Regulation of cellular protein metabolic process (GO:0031323)", "Positive regulation

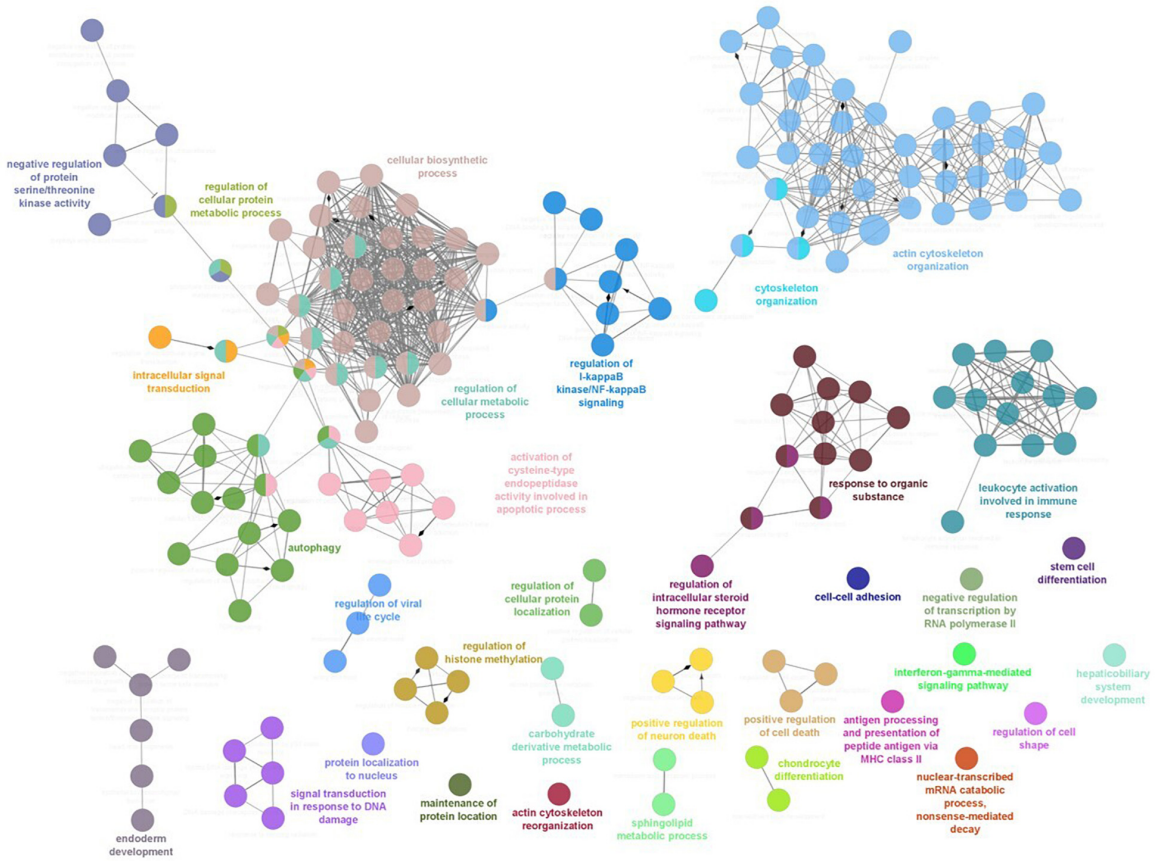


FIGURE 2 | Functional enrichment network analysis for biological processes based on the total number of differential expressed genes (DEGs) in head kidney of Atlantic salmon smolts fed the MPLE-supplemented diet. Each node represents a specific enriched biological process from the total number of DEGs. Each color indicates a cluster of closely related biological processes. The lines into the cluster indicated a closed relationship between biological processes. The details of each node are shown in **Supplementary Table 2**.

of metabolic process (GO:0009893)", and "Regulation of catabolic process (GO:0009894)". The identification of the above-mentioned biological processes in the head kidney of smolts fed the functional diet may be attributed to their higher somatic growth performance, which in turn may increase the metabolism of the body, and in particular, that of this lymphoid tissue (Shved et al., 2011; Khansari et al., 2017), as different gene expression patterns between both dietary groups indicated. For instance, among several hub genes identified by the *cytoHubba* analysis tool (Chin et al., 2014), *sirt1* was one of the hub genes that showed upregulation in the cluster named "Regulation of cellular protein metabolic". In mammals, its protein product (Sirtuin 1) play a vital role in metabolism as a mediator of endocrine function of several hormones modulating energy balance (Quiñones et al., 2014). Thus, our results are also in accordance with those of Lagouge et al. (2006) and Milne et al. (2007), who reported an upregulation of *sirt1* due to the dietary administration of resveratrol, a natural polyphenol found in several plants; results that confirmed the role of sirtuin 1 as key regulator of energy and metabolic homeostasis. Other hub genes related to "Regulation of cellular protein metabolic" process were the insulin receptor substrate 1 (*irs1*) and insulin receptor (*insr*), both transcripts were upregulated in the head kidney of smolts fed the MPLE diet. Their protein products are known as insulin receptor substrates, which are mediators of insulin signaling, and have a significant role in maintaining growth and metabolic cell functions (Caruso and Sheridan, 2011; Shved et al., 2011). This is relevant since insulin plays a fundamental role in the regulation of somatic growth and metabolism of all vertebrates (Hernández-Sánchez et al., 2006). Thus, the upregulation of genes associated to insulin receptors reinforces the hypothesis of a growth-promoting effect of the tested MPLE in Atlantic salmon smolts.

The cytoskeleton is the cellular structure that helps cells maintain their shape and internal organization; in particular, it spatially organizes the contents of the cell; it connects the cell physically and biochemically to the external environment; and it generates coordinated forces that enable the cell to move and change shape while providing cellular homeostasis and survival (Fletcher and Mullins, 2010). In this sense, we found the modulation of transcripts associated to the biological process "Cytoskeleton organization (GO:0007010)". Interestingly, several genes related to cell structure and morphogenesis (extracellular

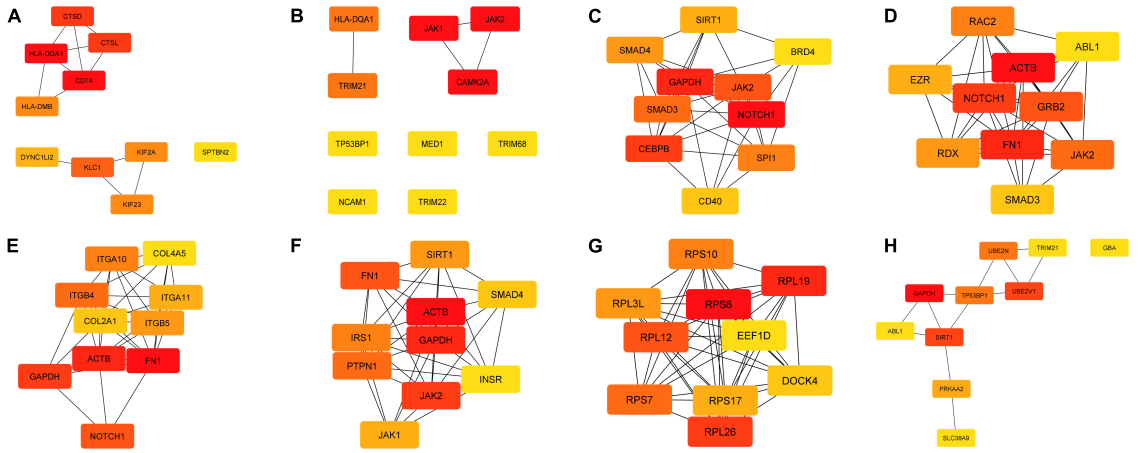


FIGURE 3 | Hub genes in each of the cluster terms identified from the enriched biological functions in the head kidney of Atlantic salmon smolts fed the MPLE supplemented diet. The principal cluster shows the ten-hub genes and the closest neighbor's genes for each network. The color intensity from yellow to red (i.e., yellow, orange, red) represents the degree of the hub gene (i.e., the relevance degree) in each network, being red those most relevant hub genes. **(A)** Antigen processing and presentation of peptide antigen *via* MHC class II. **(B)** Interferon-gamma-mediated signaling pathway. **(C)** Regulation of I-kappaB kinase/NF-kappaB signaling. **(D)** Leucocyte activation involved in immune response. **(E)** Cytoskeleton organization. **(F)** Regulation of cellular protein metabolic process. **(G)** Cellular biosynthetic process. **(H)** Autophagy.

matrix and cytoskeleton) presented a higher expression level in fish fed the MPLE-supplemented diet. Among the structural genes, several components of the extracellular matrix and cytoskeleton organization were upregulated in fish fed the MPLE diet, including genes of the integrin family (*itgb4*, *itgb5*, *itga10*, *itga11*) and fibronectin (*fn1*). In this context, *itgb4*, *itgb5*, *itga10* and *itga11* were identified as hub genes. Integrins link the extracellular matrix to the cytoskeleton, regulating signal transduction pathways intracellularly (Hynes, 2002). Furthermore, integrins also participate in the immune response (Han et al., 2016). Particularly, the upregulation of *fn1*, whose protein product (Fibronectin 1) is an important acute phase protein required for the protection and repair of the extracellular matrix (Jessen, 2015). Even more interesting, it has been shown that there is an interaction between fibronectin and integrins, which may induce cytoskeleton reorganization, focal adhesion formation, and importantly, cell-generated tension to unfold cryptic fibronectin, which is critical for fibronectin matrix assembly (Xu and Mosher, 2011).

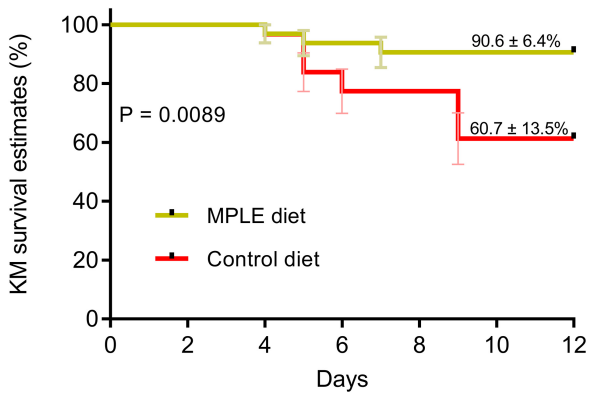


FIGURE 4 | Results of the bacterial challenge conducted in Atlantic salmon smolts intraperitoneally injected with 10^7 CFU mL^{-1} of *A. salmonicida*. Kaplan-Meier (KM) survival curves (%) for Atlantic salmon smolts intraperitoneally injected with *A. salmonicida* subsp. *salmonicida* (10^7 CFU mL^{-1}) during the 12 days challenge trial period. Data correspond to the mean ± standard error (4 replicates tanks per experimental diet; n = 8 fish per tank).

Immunomodulatory Effects of the Dietary Medicinal Plant Leaf Extract

The GO enrichment analysis of all DEGs indicated that the biological processes related to immunity in smolts were significantly modulated by the tested phytogenics in the head kidney, which were consistent with previous results on this additive at systemic and local levels (Salomón et al., 2020, 2021a). This organ undertakes immune functions similarly to the mammalian bone marrow, *i.e.*, hematopoiesis (Tort, 2011). In addition, the head kidney in fish is a basic organ forming blood elements; thus, it is potentially useful for identifying new immune-related genes (Gerdol et al., 2015). The immunomodulatory action at cellular and humoral levels of the tested phytogenic in the head kidney of Atlantic salmon smolts is further discussed as follows.

Antigen Processing and Presentation of Peptide Antigen *via* MHC Class II

Diet supplemented with MPLE modulated several biological processes related to immune effector cells in Atlantic salmon smolts. One of them was the cluster "Antigen processing and presentation of peptide antigen *via* MHC class II (GO:0002495)". Antigen processing and presentation are essential for triggering cellular and humoral immune responses, which are mediated

by T and B lymphocytes (Vyas et al., 2008). Thus, one of the major functions of MHC Class II molecules is presenting antigens derived from extracellular proteins for their recognition by $CD4^+$ T cells, being critical for the initiation of the antigen-specific immune response (Yagamuchi and Dijkstra, 2019). Furthermore, the α-and β-chain of MHC class II molecules are synthesized in the endoplasmic reticulum and associated with the class II invariant chain (also known as CD74) for proper folding, trafficking, and providing protection of the antigen-binding groove (Bryant and Ploegh, 2004). In our study, *cd74* was upregulated in fish fed the MPLE-supplemented diet. This gene is a cell-surface receptor for the cytokine known as macrophage migration inhibitory factor (MIF), which plays a specific role as an important component in the functional presentation of MHC class II restricted antigens (Gil-Yarom et al., 2017; Wang et al., 2017). Similar results regarding *cd74* expression were observed in virus-challenged Atlantic salmon, results that were correlated to an increased resistance to pancreas disease caused by salmonid alphavirus (Hillestad et al., 2020). In addition, several other genes like *hla-dqa1*, *ctsl*, *ctsd*, *kif23,* and *dync1li2* were upregulated in the cluster associated to this biological process. The major histocompatibility complex, class II, DQ alpha 1 (*hla-dqa1*), which is one of the MHC Class II family members was one of the upregulated hub genes involved in the above-mentioned cluster. This gene plays a central role helping the immune system to distinguish the host's own proteins from proteins made by viruses and bacteria (Lipton et al., 2011). This is of special relevance since proteins produced by the MHC class II are presented to the immune system, and if the immune system recognizes these peptides as foreign, it triggers a response to attack the invading viruses or bacteria (Mack et al., 1999). Furthermore, other hub genes like those belonging to the cathepsin family were also differentially transcribed between both experimental groups. This family of proteins are known to play important roles in antigen processing and presentation through the MHC II complex, being involved in adaptive immune responses (Conus and Simon, 2010). Under present experimental conditions, two cathepsins (*ctsd*, *ctsl*) were upregulated in smolts fed the phytogenic-supplemented diet. Cathepsin D (*ctsd*) is a lysosomal endoproteolytic aspartic proteinase that is involved in the presentation of antigenic peptides (Deussing et al., 1998), among other functions (Benes et al., 2008). It has also been shown that deficiency in cathepsin D may cause a delay in the innate immune response during both bacterial infection and septic shock (Cha et al., 2012). Similar to cathepsin D, cathepsin L is also described to be important in the innate response of teleost, playing key roles in host immune defense *via* the antigen processing and presentation, through the MHC II-associated presentation and regulation of $CD4^+$ T lymphocyte (Chen et al., 2020). Therefore, our data indicated that the regulation of several genes related to the antigen processing and presentation of peptide antigen *via* MHC class II pathway suggested that the tested phytogenics might be involved in the regulation of lymphocytes activity trough above-mentioned hub genes; thus, suggesting the stimulation of both innate and adaptive immune responses.

Interferon Gamma Mediated Signaling Pathway and Autophagy

As it was previously discussed, the dietary administration of MPLE modulated the biological process linked to "Antigen processing and presentation of peptide antigen *via* MHC class II". Interestingly, interferon gamma (IFN-γ) signaling has been shown to influence the entire process of antigen processing and presentation by inducing MHC class II; thus, contributing to immunity through the enhancement of pathogen-specific T cell responses (Decker et al., 2005). IFN-γ is mainly produced by activated T cells, natural killer cells, and antigen-presenting cells and it acts on many types of immune cells, regulating both innate and cell-mediated immune responses (Araki et al., 2013). Thus, IFN-γ plays critical roles not only in orchestrating both innate and adaptive immune responses against viruses and bacteria, but also in promoting inflammation (Zou and Secombes, 2011). Although, the activities mediated by this molecule are well known in mammals, several aspects of the IFN-γ system in teleosts remain a riddle to scientists (Pereiro et al., 2019). Recently, Hu et al. (2021) have demonstrated that the number of IFN-γ producing cells increased in rainbow trout challenged with *A. salmonicida*, results that were associated to an enhanced immune protection. These results may be of particular relevance under the present experimental condition, since Atlantic salmon smolts fed the MPLE diet showed higher survival rates (90.6 ± 6.4%) in comparison to those fed the control diet (60.7 ± 13.5%). Furthermore, another hub gene of relevance found in this biological process is the calcium/calmodulin-dependent protein kinase II alpha (*camk2α*). This gene is involved in the production of cytokines such interleukin-6, tumor necrosis factor-α and interferons in macrophages (Liu et al., 2008). In addition, the interaction between two other hub genes such janus kinase 1 (*jak1*) and janus kinase 2 (*jak2*) are required for association with the IFN-γ receptor chains and downstream signaling. Jak kinase function encompassed components of diverse signal transduction pathways that govern cellular survival, proliferation, differentiation, and apoptosis (Rane and Reddy, 2000; Yamaoka et al., 2004), being involved from disease resistance to maintaining immune tolerance (Villarino et al., 2015).

Another biological process that was modulated by the tested feed additive is "Autophagy" (GO:0006914), which is also modulated by IFN-γ (Pereiro et al., 2019). Autophagy is a highly conserved pathway that plays an important role in cellular physiology, adaptive responses to stress, and the immune response (Kuballa et al., 2012). Autophagy as a defense mechanism in teleost in front of intracellular bacterial and viral infections has been well documented (Meijer and van der Vaart, 2014; Pereiro et al., 2017, Yin et al., 2021). In this way, our results showed that "Autophagy (GO:0006914)" was regulated in the head kidney of the Atlantic salmon fed with the functional diet. According to the existing literature, studies have reported that autophagy as well as IFN-γ play a specific role against opportunistic pathogens such as *Aeromonas* spp. in farmed fish (Pereiro et al., 2016; Yin et al., 2021); thus, opening the possibility to understanding and relating our results to the increase in

disease resistance obtained from the bacterial challenge. For instance, Pereiro et al. (2016) reported a reduction in mortality in turbot (*Scophthalmus maximus*) when they were challenged with *A. salmonicida*. These results were attributed to the effect of IFN-γ in bacterial infections, and the participation of this protein in the inflammatory response (Pereiro et al., 2016). On the other hand, Yin et al. (2021) was able to demonstrate the role of autophagy in grass carp (*Ctenopharyngodon idella*) monocytes/macrophages, which lead to a promote innate defense against *Aeromonas hydrophila*.

Regulation of I-kappaB Kinase/NF-kappaB Signaling

NF-κB is an important factor for the maintenance of the immune homeostasis, by modulating the transcription of a diverse group of genes involved in many biological processes such as development, immunity, apoptosis, and cell differentiation in different cell types such as B and T cells, monocytes, chemokines, cytokines, among others (Dorrington and Fraser, 2019). In our study, we observed a modulation of the biological process "Regulation of I-kappaB kinase/NF-kappaB signaling (GO:0043122)". This modulation is in line with a recent study from our group, in which we demonstrated that a phytogenic feed additive from the olive fruit (*Olea europaea*), with biochemical and functional properties similar to MPLE, was also able to modulate biological process such as "Regulation of I-kappaB kinase/NF-kappaB signaling" (Salomón et al., 2021b).

Among the DEGs participating in this GO, the hub gene notch receptor 1 (*notch1*) is of special relevance. NOTCH proteins are transmembrane receptors of critical importance for several biological functions (Shin et al., 2014). In particular, *notch1* upregulates the expression of the cytokine IFN-γ in T cells through activation of NF-κB (Shin et al., 2014), which highlights the connection between NF-κB and IFN-γ biological processes. Moreover, we found another hub gene which is involved in the regulation of the NF-κB pathway; in particular, CCAAT enhancer-binding protein beta (*cebpb*) was upregulated in the head kidney of Atlantic salmon smolts fed the MPLE-supplemented diet. This gene has been well documented in direct association with NF-κB, whose function has been associated to the activation of pro-inflammatory mediators, such as chemokines, being also involved in migration, maturation, and activation of immune cells (Rebl et al., 2014). Last but not least, it has been demonstrated that the hub gene named *cd40* and its protein product (TNF receptor superfamily member 5), which is upregulated in our study, has been shown to be capable of stimulating the NF-κB pathway, as well as playing an essential role for the cooperation of T and B cells in responses to protein antigens (Gong et al., 2009; Hayden and Ghosh, 2014).

Leukocyte Activation Involved in Immune and B Cells Signaling Response

A successful immune response involves the tight control of a wide repertoire of processes such the leukocyte-mediated cells, among others (Rieger and Barreda, 2011). Leukocyte activation is mediated by several signaling pathways that interact to produce changes in binding protein affinity on the surface of neutrophils that mobilize the cytoskeleton for chemotaxis and phagocytosis, ultimately triggering a respiratory burst and degranulation (Rieger and Barreda, 2011). In our study, the "Leukocyte activation involved in immune response (GO:0002366)" was enriched. In this context, eight of the ten hub genes related to leukocyte activation (*fn1, notch1*, *grb2*, *rac2*, *rdx*, *ezr*, *smad3*, *abl1*) were upregulated. Similarly, a modulation of immune-related GOs such as "Leukocyte activation involved in immune response" were enriched in Atlantic salmon smolts fed a feed additive AQUOLIVE® as we mentioned above from a phytogenic derived from olive (*O. europaea*) fruit with similar biochemical and functional properties to the MPLE phytogenic tested in the current study (Salomón et al., 2021b). For instance, *notch1* a gene we have previously discussed highlighting its connection between NF-κB and IFN-γ as biological processes that have a key role in the differentiation of granulocytes, macrophages, and dendritic cells (Schroeder et al., 2003; Monsalve et al., 2009). This is relevant since, granulocytes, macrophages, dendritic cells, and B cells are recognized as a critical component of the innate and adaptive immunity against pathogens (Secombes and Wang, 2012). These results are in agreement with our transcriptomic data, since biological processes like "Neutrophil mediated immunity (GO:0002446)" and "Lymphocyte activation involved in immune response (GO:0002285)" were also enriched. In addition, we also found the upregulation of *rac2* among hub genes. In teleosts, RAC2 (Rac family small GTPase 2) is a member of the Rho family that plays an important role in the host defense-mediated neutrophil response, which is a critical first step in innate immunity (Deng et al., 2011; Tell et al., 2012). Moreover, loss of RAC2 activity has been shown to cause severe bacterial infections and deficits in neutrophil function in humans and mice (Tell et al., 2012).

Furthermore, some of these genes involved in the GO "Leukocyte activation involved in immune response" were also associated with the innate and adaptive immunity modulated by the MPLE-supplemented. For instance, fish fed with the MPLE diet showed an increase of the expression in the growth factor receptor bound protein 2 (*grb2*), which is an essential signal integrator that can interact with multiple genes to regulate signal transduction; thus, playing an important role in regulating B cells activation (Neumann et al., 2009). This is relevant, as B cell activation is essential the development of an effective antigen-specific humoral immune response (Scapigliati et al., 2018). Moreover, two hub genes (*rdx*, *ezr*), which are part of the family of proteins called ezrin-radixin-moesin (ERM), were also upregulated in our study. Several studies have demonstrated that ERM proteins are involved in the regulation of B cell function under healthy and disease conditions (Pore and Gupta, 2015). Therefore, the upregulation of these two genes, *rdx* and *ezr*, might confirm the immunomodulatory properties of the MPLE in Atlantic salmon. Therefore, the regulation of genes involved in leukocyte activation and B cells might suggest an increased systemic specific immune capacity promoted by the tested phytogenic feed additive. Thus, our transcriptional analysis seemed to indicate that the tested phytogenic activated leukocytes, which would promote host's disease resistance as the *in vivo* challenge with a pathogenic bacteria confirmed.

CONCLUSION

Phytogenics obtained from sage and lemon verbena included at 0.1% in diets promoted somatic growth and improved FCR in Atlantic salmon smolts. In addition, data from the microarray analysis of the head kidney samples indicated this feed additive enhanced the host's systemic immune response through the transcriptional regulation of innate immunity processes like leukocytes' activation. We showed that other pathways related to immunity were also enhanced by the tested functional feed additive, such as interferon-gamma-mediated signaling pathway, antigen processing and presentation of peptide antigen *via* MHC class II, autophagy, and regulation of i-kappaB kinase/NF-kappaB, promoting disease resistance when challenged with *A. salmonicida*. Altogether, this study indicated that the tested feed additive promotes systemic immunity and protects Atlantic salmon smolts against bacterial infections like *A. salmonicida*. According to these findings, we suggest that the combination of current vaccination practices coupled with the administration of MPLE may be a good strategy against furunculosis in salmonids. Furthermore, this phytogenic may be also of interest for other marine species like European sea bass (*Dicentrarchus labrax*) also suffering from emerging *A. salmonicida* infections in the Mediterranean basin.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Experimental procedures were conducted following the Guiding Principles for Biomedical Research Involving Animals (EU2010/63), the guidelines of the Spanish laws (law 32/2007 and RD 1201/2015) and authorized by the Ethical Committee of IRTA (FUE-2020-01314717).

AUTHOR CONTRIBUTIONS

EG: conceptualization, resources, project administration, and funding acquisition. MF, EV-V, and FER-L: methodology. RS, FER-L, JF, and EV-V: formal analysis. RS: writing—original draft. MF, EV-V, RS, FER-L, EG, and LT: writing—review and editing. RS, FER-L, and EV-V: visualization. EG and EV-V: supervision. All authors have read and agreed to the published version of the manuscript.

FUNDING

This work has been financially supported by the project "AQUOLIVE by NATAC," funded by European Union's Horizon 2020 Research and Innovation Program (Grant Agreement No. 830202) and partially by the Agencia Española de Investigación (grant AGL2016-76069-C2-2-R to Universitat Autònoma de Barcelona). FER-L and EV-V thank the support of Fondecyt Regular (No. 1211841) and Fondecyt iniciación grants (No. 11221308), respectively (ANID; Government of Chile). RS was supported by a Ph.D. grant from the government of Paraguay (BECAL). JF has been subsidized by the Industrial Ph.D. program of *Generalitat de Catalunya* and TECNOVIT-FARMFAES S.L. (No. 2017 DI 017). Collaboration between Ibero-American researchers has been done under the framework of the network LARVAplus "Strategies for the development and improvement of fish larvae production in Ibero-America" (117RT0521) funded by the Ibero-American Program of Science and Technology for Development (CYTED, Spain).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.828497/full#supplementary-material

REFERENCES

Alipieva, K., Korkina, L., Orhan, I. E., and Georgiev, M. I. (2014). Verbascoside - a review of its occurrence, (bio)synthesis and pharmacological significance. *Biotechnol. Adv.* 32, 1065–1076. doi: 10.1016/j.biotechadv.2014.07.00

Araki, K., Takizawa, F., Yamasaki, M., Esumi, M., Moritomo, T., Ototake, M., et al. (2013). Expression profiles of interferon gamma genes in response to immunostimulants and alloantigen in ginbuna crucian carp *Carassius auratus* langsdorfi. *Fish. Sci.* 79, 213–220. doi: 10.1007/s12562-012-0590-5

Awad, E., and Awaad, A. (2017). Role of medicinal plants on growth performance and immune status in fish. *Fish Shellfish Immunol.* 67, 40–54. doi: 10.1016/j.fsi.2017.05.034

Babalola, I. T., and Shode, F. O. (2013). Ubiquitous ursolic acid: a potential pentacyclic triterpene natural product. *J. Pharmacogn. Phytochem.* 2, 214–222.

Babicki, S., Arndt, D., Marcu, A., Liang, Y., Grant, J. R., Maciejewski, A., et al. (2016). Heatmapper: web-enabled heat mapping for all. *Nucleic Acids Res.* 44, 147–153. doi: 10.1093/nar/gkw419

Barrett, L. T., Oppedal, F., Robinson, N., and Dempster, T. (2020). Prevention not cure: a review of methods to avoid sea lice infestations in salmon aquaculture. *Rev. Aquac.* 12, 2527–2543. doi: 10.1111/raq.12456

Beaz-Hidalgo, R., Magi, G. E., Balboa, S., Barja, J. L., and Romalde, J. L. (2008). Development of a PCR protocol for the detection of Aeromonas salmonicida in fish by amplification of the fstA (ferric siderophore receptor) gene. *Vet. Microbiol.* 128, 386–394. doi: 10.1016/j.vetmic.2007.10.004

Benes, P., Vetvicka, V., and Fusek, M. (2008). Cathepsin D–many functions of one aspartic protease. *Crit. Rev. Oncol. Hematol.* 68, 12–28. doi: 10.1016/j.critrevonc.2008.02.008

Bindea, G., Mlecnik, B., Hackl, H., Charoentong, P., Tosolini, M., Kirilovsky, A., et al. (2009). ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics* 25, 1091–1093. doi: 10.1093/bioinformatics/btp101

Bryant, P., and Ploegh, H. (2004). Class II MHC peptide loading by the professionals. *Curr. Opin. Immunol.* 16, 96–102. doi: 10.1016/j.coi.2003.11.011

Caruso, M. A., and Sheridan, M. A. (2011). New insights into the signaling system and function of insulin in fish. *Gen. Comp. Endocrinol.* 173, 227–247. doi: 10.1016/j.ygcen.2011.06.014

Casamassima, D., Palazzo, M., D'Alessandro, A. G., Colella, G. E., Vizzarri, F., and Corino, C. (2013). The effects of lemon verbena (*Lippia citriodora*) verbascoside on the productive performance, plasma oxidative status, and some blood metabolites in suckling lambs. *J. Anim. Feed Sci.* 22, 204–212. doi: 10.22358/jafs/65989/2013

Caturla, N., Funes, L., Pérez-Fons, L., and Micol, V. (2011). A randomized, double-blinded, placebo-controlled study of the effect of a combination of lemon verbena extract and fish oil omega-3 fatty acid on joint management. *J. Altern. Comp. Med.* 17, 1051–1063. doi: 10.1089/acm.2010.0410

Cha, I. S., Kwon, J., Mun, J. Y., Park, S. B., Jang, H. B., Nho, S. W., et al. (2012). Cathepsins in the kidney of olive flounder, *Paralichthys olivaceus*, and their responses to bacterial infection. *Dev. Comp. Immunol.* 38, 538–544. doi: 10.1016/j.dci.2012.08.00

Chen, J., Zhang, L., Yang, N., Cao, M., Tian, M., Fu, Q., et al. (2020). Characterization of the immune roles of cathepsin L in turbot (*Scophthalmus maximus* L.) mucosal immunity. *Fish Shellfish Immunol.* 97, 322–335. doi: 10.1016/j.fsi.2019.12.005

Chin, C. H., Chen, S. H., Wu, H. H., Ho, C. H., Ko, M. T., and Lin, C. Y. (2014). cytoHubba: identifying hub objects and sub-networks from complex interactome. *BMC Syst. Biol.* 8:S11. doi: 10.1186/1752-0509-8-S4-S11

Conus, S., and Simon, H. U. (2010). Cathepsins and their involvement in immune responses. *Swiss Med. Wkly.* 140:w13042. doi: 10.4414/smw.2010.13042

Corino, C., Rossi, R., Musella, M., Cannata, S., and Pastorelli, G. (2007). Growth performance and oxidative status in piglets supplemented with verbascoside and teupolioside. *Ital. J. Anim. Sci.* 6, 292–294. doi: 10.4081/ijas.2007.1s.292

Dadras, H., Hayatbakhsh, M. R., and Golpour, A. (2020). Dietary administration of common sage (*Salvia officinalis*) and coneflower (*Echinacea angustifolia*) extracts affects growth, blood parameters and immune responses of beluga, *Huso huso*. *Turk. J. Fish. Aquatic Sci.* 20, 367–374. doi: 10.4194/1303-2712-v20_5_05

Dawood, M. A. O., Koshio, S., and Esteban, M. A. Á. (2018). Beneficial roles of feed additives as immunostimulants in aquaculture: a review. *Rev. Aquac.* 10, 950–974. doi: 10.1111/raq.12209

Dawood, M. A. O. (2021). Nutritional immunity of fish intestines: important insights for sustainable aquaculture. *Rev. Aquac.* 13, 642–663. doi: 10.1111/raq.12492

Decker, T., Müller, M., and Stockinger, S. (2005). The Yin and Yang of type I interferon activity in bacterial infection. *Nat. Rev. Immnuol.* 5, 675–687. doi: 10.1038/nri1684

Deng, Q., Yoo, S. K., Cavnar, P. J., Green, J. M., and Huttenlocher, A. (2011). Dual roles for Rac2 in neutrophil motility and active retention in zebrafish hematopoietic tissue. *Dev. Cell* 21, 735–745. doi: 10.1016/j.devcel.2011.07.013

Deussing, J., Roth, W., Saftig, P., Peters, C., Ploegh, H. L., and Villadangos, J. A. (1998). Cathepsins B and D are dispensable for major histocompatibility complex class II-mediated antigen presentation. *Proc. Nat. Acad. Sci. U.S.A.* 95, 4516–4521. doi: 10.1073/pnas.95.8.4516

Ding, Y. J., Sun, C. H., Wen, C. C., and Chen, Y. H. (2015). Nephroprotective role of resveratrol and ursolic Acid in aristolochic acid intoxicated zebrafish. *Toxins* 7, 97–109. doi: 10.3390/toxins7010097

Dorrington, M. G., and Fraser, I. D. C. (2019). NF-κB signaling in macrophages: Dynamics, Crosstalk, and Signal Integration. *Front. Immunol.* 10:705. doi: 10.3389/fimmu.2019.00705

Elumalai, P., Kurian, A., Lakshmi, S., Faggio, C., Esteban, M. A. Á., and Ringø, E. (2020). Herbal immunomodulators in aquaculture. *Rev. Fish. Sci. Aquac.* 29, 33–57. doi: 10.1080/23308249.2020.1779651

Encarnação, P. (2016). "Functional feed additives in aquaculture feeds," in *Aquafeed Formulation*, ed. S. F. Nates (London: Academic Press), 217–237. doi: 10.1016/B978-0-12-800873-7.00005-1

FAO (2021). *Fishery and Aquaculture Statistics. Global aquaculture production 1950-2019 (FishstatJ)*. Rome: FAO.

Fernández-Navarro, M., Peragón, J., Esteban, F. J., de la Higuera, M., and Lupiáñez, J. A. (2006). Maslinic acid as a feed additive to stimulate growth and hepatic protein-turnover rates in rainbow trout (*Onchorhynchus mykiss*). *Comp. Biochem. Physiol.* 144, 130–140. doi: 10.1016/j.cbpc.2006.07.006

Firmino, J. P., Galindo-Vallegas, J., Reyes-López, F. E., and Gisbert, E. (2021a). Phytogenic bioactive compounds shape fish mucosal immunity. *Front. Immunol.* 12:695973. doi: 10.3389/fimmu.2021.695973

Firmino, J. P., Vallejos-Vidal, E., Balebona, M. C., Ramayo-Caldas, Y., Cerezo, I. M., Salomón, R., et al. (2021b). Diet, immunity, and microbiota interactions: an integrative analysis of the intestine transcriptional response and microbiota modulation in gilthead seabream (*Sparus aurata*) fed an essential oils-based functional diet. *Front. Immunol.* 12:625297. doi: 10.3389/fimmu.2021.625297

Firmino, J. P., Vallejos-Vidal, E., Sarasquete, C., Ortiz-Delgado, J. B., Balasch, J. C., Tort, L., et al. (2020). Unveiling the effect of dietary essential oils supplementation in Sparus aurata gills and its efficiency against the infestation by *Sparicotyle chrysophrii*. *Sci. Rep.* 10:17764. doi: 10.1038/s41598-020-74625-5

Fletcher, D. A., and Mullins, D. (2010). Cell mechanics and the cytoskeleton. *Nature* 463, 485–492. doi: 10.1038/nature08908

Funes, L., Fernández-Arroyo, S., Laporta, O., Pons, A., Roche, E., and Segura-Carretero, A. (2009). Correlation between plasma antioxidant capacity and verbascoside levels in rats after oral administration of lemon verbena extract. *Food Chem.* 117, 589–598. doi: 10.1016/j.foodchem.2009.04.059

Gerdol, M., Buonocore, F., Scapigliati, G., and Pallavicini, A. (2015). Analysis and characterization of the head kidney transcriptome from the Antarctic fish *Trematomus bernacchii* (Teleostea, Notothenioidea): a source for immune relevant genes. *Mar. Genomics* 20, 13–15. doi: 10.1016/j.margen.2014.12.005

Ghorbani, A., and Esmaeilizadeh, M. (2017). Pharmacological properties of Salvia officinalis and its components. *J. Tradit. Comp. Med.* 7, 433–440. doi: 10.1016/j.jtcme.2016.12.014

Gil-Yarom, N., Radomir, L., Sever, L., Kramer, M. P., Lewinsky, H., Bornstein, C., et al. (2017). CD74 is a novel transcription regulator. *Proc. Nat. Acad. Sci. U.S.A.* 114, 562–567. doi: 10.1073/pnas.1612195114

Gong, Y. F., Xiang, L. X., and Shao, J. Z. (2009). CD154-CD40 interactions are essential for thymus-dependent antibody production in zebrafish: insights into the origin of costimulatory pathway in helper T cell-regulated adaptive immunity in early vertebrates. *J. Immunol.* 182, 7749–7762. doi: 10.4049/jimmunol.0804370

Han, M. M., Lu, J. G., Bin, S., Peng, L. N., Mahboob, S., Al-Ghanim, K. A., et al. (2016). Integrins contributes to innate immune response in *Pelteobagrus fulvidraco*. *Biochem. Physiol. Open Access.* 5:2. doi: 10.4172/2168-9652.1000204

Hayden, M. S., and Ghosh, S. (2014). Regulation of NF-κB by TNF family cytokines. *Semin. Immunol.* 26, 253–266. doi: 10.1016/j.smim.2014.05.004

Hernández-Contreras, Á., and Hernández, M. D. (2020). "Chapter 14 - Application of aromatic plants and their extracts in aquaculture," in *Feed Additives*, eds P. Florou-Paneri, E. Christaki, and I. Giannenas (London: Academic Press), 239–259. doi: 10.1016/B978-0-12-814700-9.00014-5

Hernández-Sánchez, C., Mansilla, A., de la Rosa, E. J., and de Pablo, F. (2006). Proinsulin in development: new roles for an ancient prohormone. *Diabetologia* 49, 1142–1150. doi: 10.1007/s00125-006-0232-5

Hillestad, B., Makvandi-Nejad, S., Krasnov, A., and Moghadam, H. K. (2020). Identification of genetic loci associated with higher resistance to pancreas disease (PD) in Atlantic salmon (*Salmo salar* L.). *BMC Genomics* 21:388. doi: 10.1186/s12864-020-06788-4

Hu, Y., Alnabulsi, A., Alnabulsi, A., Scott, C., Tafalla, C., Secombes, C. J., et al. (2021). Characterisation and analysis of IFN-gamma producing cells in rainbow trout *Oncorhynchus mykiss*. *Fish Shellfish Immunol.* 117, 328–338. doi: 10.1016/j.fsi.2021.07.022

Hynes, R. O. (2002). Integrins: bidirectional, allosteric signaling machines. *Cell* 110, 673–687. doi: 10.1016/S0092-8674(02)00971-6

Jessen, J. R. (2015). Recent advances in the study of zebrafish extracellular matrix proteins. *Dev. Biol.* 401, 110–121. doi: 10.1016/j.ydbio.2014.12.022

Kaplan, E. L., and Meier, P. (1958). Nonparametric Estimation from Incomplete Observations. *J. Am. Stat. Assoc.* 53, 457–481. doi: 10.2307/2281868

Khansari, A. R., Balasch, J. C., Reyes-López, F. E., and Tort, L. (2017). Stressing the inflammatory network: immuno-endocrine responses to allostatic load in fish. *Mar. Sci. Res. Technol.* 1, 856–862.

Krasnov, A., Timmerhaus, G., Afanasyev, S., and Jørgensen, S. M. (2011). Development and assessment of oligonucleotide microarrays for Atlantic salmon (*Salmo salar* L.). *Comp. Biochem. Physiol. Part D Genomics Proteomics* 6, 31–38. doi: 10.1016/j.cbd.2010.04.006

Kuballa, P., Nolte, W. M., Castoreno, A. B., and Xavier, R. J. (2012). Autophagy and the immune system. *Annu. Rev. Immunol.* 30, 611–646. doi: 10.1146/annurev-immunol-020711-074948

Kunkel, S. D., Elmore, C. J., Bongers, K. S., Ebert, S. M., Fox, D. K., Dyle, M. C., et al. (2012). Ursolic acid increases skeletal muscle and brown fat and decreases diet-induced obesity, glucose intolerance and fatty liver disease. *PLoS One* 7:e39332. doi: 10.1371/journal.pone.0039332

Lagouge, M., Argmann, C., Gerhart-Hines, Z., Meziane, H., Lerin, C., Daussin, F., et al. (2006). Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1α. *Cell* 127, 1109–1122. doi: 10.1016/j.cell.2006.11.013

Li, B. Y., Hu, Y., Li, J., Shi, K., Shen, Y. F., Zhu, B., et al. (2019). Ursolic acid from *Prunella vulgaris* L. efficiently inhibits IHNV infection *in vitro* and *in vivo*. *Virus Res.* 273:197741. doi: 10.1016/j.virusres.2019.19774

Lipton, R. B., Drum, M., Greeley, S. A. W., Danielson, K. K., Bell, G. I., and Hagopian, W. A. (2011). HLA-DQ haplotypes differ by ethnicity in patients with childhood-onset diabetes. *Pediatr. Diabetes* 12, 388–395. doi: 10.1111/j.1399-5448.2010.00712.x

Liu, X., Yao, M., Li, N., Wang, C., Zheng, Y., and Cao, X. (2008). CaMKII promotes TLR-triggered proinflammatory cytokine and type I interferon production by directly binding and activating TAK1 and IRF3 in macrophages. *Blood* 112, 4961–4970. doi: 10.1182/blood-2008-03-144022

Lu, Y., and Foo, L. Y. (2000). Flavonoid and phenolic glycosides from Salvia officinalis. *Phytochemistry* 55, 263–267. doi: 10.1016/s0031-9422(00)00309-5

Lulijwa, R., Rupia, E. J., and Lfaro, A. C. (2020). Antibiotic use in aquaculture, policies and regulation, health and environmental risks: a review of the top 15 major producers. *Rev. Aquac.* 12, 640–663. doi: 10.1111/raq.12344

Mack, D. G., Johnson, J. J., Roberts, F., Roberts, C. W., Estes, R. G., David, C., et al. (1999). HLA-class II genes modify outcome of Toxoplasma gondii infection. *Int. J. Parasitol.* 29, 1351–1358. doi: 10.1016/s0020-7519(99)00152-6

Martino, N. A., Ariu, F., Bebbere, D., Uranio, M. F., Chirico, A., Marzano, G., et al. (2016). Supplementation with nanomolar concentrations of verbascoside during in vitro maturation improves embryo development by protecting the oocyte against oxidative stress: a large animal model study. *Reprod. Toxicol.* 65, 204–211. doi: 10.1016/j.reprotox.2016.08.004

Mašterová, I., Uhrín, D., Kettmann, V., and Suchı, V. (1989). Phytochemical study of *Salvia officinalis* L. *Chem. Pap.* 43, 797–803.

Meijer, A. H., and van der Vaart, M. (2014). DRAM1 promotes the targeting of mycobacteria to selective autophagy. *Autophagy* 10, 2389–2391. doi: 10.4161/15548627.2014.984280

Miccoli, A., Manni, M., Picchietti, S., and Scapigliati, G. (2021). State-of-the-art vaccine research for aquaculture use: the case of three economically relevant fish species. *Vaccines* 9:140. doi: 10.3390/vaccines9020140

Milne, J. C., Lambert, P. D., Schenk, S., Carney, D. P., Smith, J. J., Gagne, D. J., et al. (2007). Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. *Nature* 450, 712–716. doi: 10.1038/nature06261

Monsalve, E., Ruiz-García, A., Baladrón, V., Ruiz-Hidalgo, M. J., Sánchez-Solana, B., Rivero, S., et al. (2009). Notch1 upregulates LPS-induced macrophage activation by increasing NF-kappaB activity. *Eur. J. Immunol.* 39, 2556–2570. doi: 10.1002/eji.200838722

More, S. J. (2020). European perspectives on efforts to reduce antimicrobial usage in food animal production. *Ir. Vet. J.* 73:2. doi: 10.1186/s13620-019-0154-4

Mosca, M., Ambrosone, L., Semeraro, F., Casamassima, D., Vizzarri, F., and Costagliola, C. (2014). Ocular tissues and fluids oxidative stress in hares fed on verbascoside supplement. *Int. J. Food Sci. Nutr.* 65, 235–240. doi: 10.3109/09637486.2013.836742

Naylor, R. L., Hardy, R. W., Buschmann, A. H., Bush, S. R., Cao, L., Klinger, D. H., et al. (2021). A 20-year retrospective review of global aquaculture. *Nature* 591, 551–563. doi: 10.1038/s41586-021-03308-6

Neumann, K., Oellerich, T., Urlaub, H., and Wienands, J. (2009). The B-lymphoid Grb2 interaction code. *Immunol. Rev.* 232, 135–149. doi: 10.1111/j.1600-065X.2009.00845.x

Pastorelli, G., Rossi, R., and Corino, C. (2012). Influence of *Lippia citriodora* verbascoside on growth performance, antioxidant status, and serum immunoglobulins content in piglets. *Czech J. Anim. Sci.* 57, 312–322. doi: 10.17221/6006-CJAS

Pereiro, P., Figueras, A., and Novoa, B. (2019). Insights into teleost interferon-gamma biology: an update. *Fish Shellfish Immunol.* 90, 150–164. doi: 10.1016/j.fsi.2019.04.002

Pereiro, P., Forn-Cuni, G., Figueras, A., and Novoa, B. (2016). Pathogen-dependent role of turbot (*Scophthalmus maximus*) interferon-gamma. *Fish Shellfish Immunol.* 59, 25–35. doi: 10.1016/j.fsi.2016.10.021

Pereiro, P., Romero, A., Rosales-Díaz, P., Estepa, A., Figueras, A., and Novoa, B. (2017). Nucleated teleost erythrocytes play an Nk-lysin- and autophagy-dependent role in antiviral immunity. *Front. Immunol.* 8:1458. doi: 10.3389/fimmu.2017.01458

Placha, I., Ryzner, M., Cobanova, K., Faixova, Z., and Faix, S. (2015). Effects of dietary supplementation with sage (*Salvia officinalis* L.) essential oil on antioxidant status and duodenal wall integrity of laying strain growers. *Pol. J. Vet. Sci.* 18, 741–749. doi: 10.1515/pjvs-2015-0096

Pore, D., and Gupta, N. (2015). The ezrin-radixin-moesin family of proteins in the regulation of B-cell immune response. *Crit. Rev. Immunol.* 35, 15–31. doi: 10.1615/CritRevImmunol.2015012327

Quiñones, M., Al-Massadi, O., Fernø, J., and Nogueiras, R. (2014). Cross-talk between SIRT1 and endocrine factors: effects on energy homeostasis. *Mol. Cell. Endocrinol.* 397, 42–50. doi: 10.1016/j.mce.2014.08.002

Rane, S. G., and Reddy, E. P. (2000). Janus kinases: components of multiple signaling pathways. *Oncogene* 19, 5662–5679. doi: 10.1038/sj.onc.1203925

Rebl, A., Rebl, H., Korytáø, T., Goldammer, T., and Seyfert, H. M. (2014). The proximal promoter of a novel interleukin-8-encoding gene in rainbow trout (Oncorhynchus mykiss) is strongly induced by CEBPA, but not NF-κB p65. *Dev. Comp. Immunol.* 46, 155–164. doi: 10.1016/j.dci.2014.03.024

Reverter, M., Sarter, S., Caruso, D., Avarre, J. C., Combe, M., Pepey, E., et al. (2020). Aquaculture at the crossroads of global warming and antimicrobial resistance. *Nat. Commun.* 11:1870. doi: 10.1038/s41467-020-15735-6

Reverter, M., Tapissier-Bontemps, N., Sarter, S., Sasal, P., and Caruso, D. (2021). Moving towards more sustainable aquaculture practices: a meta-analysis on the potential of plant-enriched diets to improve fish growth, immunity and disease resistance. *Rev. Aquac.* 13, 537–555. doi: 10.1111/raq.12485

Reyes-López, F. E., Ibarz, A., Ordóñez-Grande, B., Vallejos-Vidal, E., Andree, K. B., Balasch, J. C., et al. (2021). Skin Multi-Omics-Based interactome analysis: integrating the tissue and mucus exuded layer for a comprehensive understanding of the teleost mucosa functionality as model of study. *Front. Immunol.* 11:613824. doi: 10.3389/fimmu.2020.613824

Reyes-López, F. E., Romeo, J. S., Vallejos-Vidal, E., Reyes-Cerpa, S., Sandino, A. M., Tort, L., et al. (2015). Differential immune gene expression profiles in susceptible and resistant full-sibling families of Atlantic salmon (Salmo salar) challenged with infectious pancreatic necrosis virus (IPNV). *Dev. Comp. Immunol.* 53, 210–221. doi: 10.1016/j.dci.2015.06.017

Rieger, A. M., and Barreda, D. R. (2011). Antimicrobial mechanisms of fish leukocytes. *Dev. Comp. Immunol.* 35, 1238–1245. doi: 10.1016/j.dci.2011.03.009

Salomón, R., Firmino, J. P., Reyes-López, F. E., Andree, K. B., González-Silvera, D., Esteban, M. A. Á, et al. (2020). The growth promoting and immunomodulatory effects of a medicinal plant leaf extract obtained from *Salvia officinalis* and *Lippia citriodora* in gilthead seabream (*Sparus aurata*). *Aquaculture* 524:735291. doi: 10.1016/j.aquaculture.2020.735291

Salomón, R., Reyes-López, F. E., Tort, L., Firmino, J. P., Sarasquete, C., Ortiz-Delgado, J. B., et al. (2021a). Medicinal plant leaf extract from sage and lemon verbena promotes intestinal immunity and barrier function in gilthead seabream (*Sparus aurata*). *Front. Immunol.* 12:670279. doi: 10.3389/fimmu.2021.670279

Salomón, R., Furones, M. D., Reyes-López, F. E., Tort, L., Firmino, J. P., Esteban, M. A. Á., et al. (2021b). A bioactive extract rich in triterpenic acid and polyphenols from *Olea europaea* promotes systemic immunity and protects Atlantic salmon smolts against furunculosis. *Front. Immunol.* 12:737601. doi: 10.3389/fimmu.2021.737601

Sánchez-Marzo, N., Lozano-Sánchez, J., de la Luz Cádiz-Gurrea, M., Herranz-López, M., Micol, V., and Segura-Carretero, A. (2019). Relationships between chemical structure and antioxidant activity of isolated phytocompounds from lemon verbena. *Antioxidants* 8:324. doi: 10.3390/antiox8080324

Scapigliati, G., Fausto, A. M., and Picchietti, S. (2018). Fish lymphocytes: An evolutionary equivalent of mammalian innate-like lymphocytes? *Front. Immunol.* 9:971. doi: 10.3389/fimmu.2018.00971

Schar, D., Klein, E. Y., Laxminarayan, R., Gilbert, M., and Van Boeckel, T. P. (2020). Global trends in antimicrobial use in aquaculture. *Sci. Rep.* 10:1878. doi: 10.1038/s41598-020-78849-3

Schroeder, T., Kohlhof, H., Rieber, N., and Just, U. (2003). Notch signaling induces multilineage myeloid differentiation and up-regulates PU.1 *expression*. *J. Immunol.* 170, 5538–5548. doi: 10.4049/jimmunol.170.11.5538

Secombes, C. J., and Wang, T. (2012). "Chapter 1 - The innate and adaptive immune system of fish," in *Infectious Disease in Aquaculture: Prevention and Control*, ed. B. Austin (Amsterdam: Elsevier Inc), 3–68.

Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., et al. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* 13, 2498–2504. doi: 10.1101/gr.1239303

Shin, H. M., Tilahun, M. E., Cho, O. H., Chandiran, K., Kuksin, C. A., Keerthivasan, S., et al. (2014). NOTCH1 can initiate NF-kB activation via cytosolic interactions with components of the T cell signalosome. *Front. Immunol.* 5:249. doi: 10.3389/fimmu.2014.00249

Shved, N., Berishvili, G., Mazel, P., Baroiller, J. F., and Eppler, E. (2011). Growth hormone (GH) treatment acts on the endocrine and autocrine/paracrine GH/IGF-axis and on TNF-α expression in bony fish pituitary and immune organs. *Fish Shellfish Immunol.* 31, 944–952. doi: 10.1016/j.fsi.2011.08.012

Simonová, M. P., Chrastinová, L., Mojto, J., Lauková, A., Szabóová, R., and Rafay, J. (2010). Quality of rabbit meat and phyto-additives. *Czech J. Food Sci.* 28, 161–167. doi: 10.17221/49/2008-CJFS

Sönmez, A. Y., Bilen, S., Alak, G., Hisar, O., Yanık, T., and Biswas, G. (2015). Growth performance and antioxidant enzyme activities in rainbow trout (*Oncorhynchus mykiss*) juveniles fed diets supplemented with sage, mint and thyme oils. *Fish Physiol. Biochem.* 41, 165–175. doi: 10.1007/s10695-014-0014-9

Stelzer, G., Rosen, N., Plaschkes, I., Zimmerman, S., Twik, M., Fishilevich, S., et al. (2016). The GeneCards suite: from gene data mining to disease genome sequence analyses. *Curr. Protoc. Bioinf.* 54, 1.30.1–1.30.33. doi: 10.1002/cpbi.5

Stentiford, G. D., Bateman, I. J., Hinchliffe, S. J., Bass, D., Hartnell, R., Santos, E. M., et al. (2020). Sustainable aquaculture through the One Health lens. *Nat. Food* 1, 468–474. doi: 10.1038/s43016-020-0127-5

Sutili, F. J., Gatlin, D. M. III, Heinzmann, B. M., and Baldisserotto, B. (2018). Plant essential oils as fish diet additives: benefits on fish health and stability in feed. *Rev. Aquac.* 10, 716–726. doi: 10.1111/raq.12197

Szklarczyk, D., Gable, A. L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., et al. (2019). STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* 47, 607–613. doi: 10.1093/nar/gky1131

Taranger, G. L., Karlsen, Ø., Bannister, R. J., Glover, K. A., Husa, V., Karlsbakk, E., et al. (2015). Risk assessment of the environmental impact of Norwegian Atlantic salmon farming. *ICES J. Mar. Sci.* 72, 997–1021. doi: 10.1093/icesjms/fsu132

Tell, R. M., Kimura, K., and Paliæ, D. (2012). Rac2 expression and its role in neutrophil functions of zebrafish (*Danio rerio*). *Fish Shellfish Immunol.* 33, 1086–1094. doi: 10.1016/j.fsi.2012.07.020

Tort, L. (2011). Stress and immune modulation in fish. *Dev. Comp. Immunol.* 35, 1366–1375. doi: 10.1016/j.dci.2011.07.002

Uniprot (2019). UniProt: a worldwide hub of protein knowledge. *Nucleic Acids Res.* 47, 506–515. doi: 10.1093/nar/gky1049

Vallejos-Vidal, E., Reyes-López, F. E., Teles, M., and MacKenzie, S. (2016). The response of fish to immunostimulant diets. *Fish Shellfish Immunol.* 56, 34–69. doi: 10.1016/j.fsi.2016.06.028

Vaseeharan, B., and Thaya, R. (2014). Medicinal plant derivatives as immunostimulants: an alternative to chemotherapeutics and antibiotics in aquaculture. *Aquac. Int.* 22, 1079–1091. doi: 10.1007/s10499-013-9729-3

Villarino, A. V., Kanno, Y., Ferdinand, J. R., and O'Shea, J. J. (2015). Mechanisms of Jak/STAT signaling in immunity and disease. *J. Immunol.* 194, 21–27. doi: 10.4049/jimmunol.1401867

Vincken, J. P., Heng, L., de Groot, A., and Gruppen, H. (2007). Saponins, classification and occurrence in the plant kingdom. *Phytochemistry* 68, 275–297. doi: 10.1016/j.phytochem.2006.10.008

Vyas, J. M., Van der Veen, A. G., and Ploegh, H. L. (2008). The known unknowns of antigen processing and presentation. *Nat. Rev. Immunol.* 8, 607–618. doi: 10.1038/nri2368

Waagbø, R., and Remø, S. C. (2020). "Functional diets in fish health management," in *Aquaculture Health Management*, eds F. S. B. Kibenge and M. D. Powell (London: Academic Press), 187–234. doi: 10.1016/B978-0-12-813359-0.00007-5

Wang, M., Shao, Y., Li, J., Zhu, N., Rangarajan, M., LaVoie, A. J., et al. (1999). Antioxidative phenolic glycosides from sage (*Salvia officinalis*). *J. Nat. Prod.* 62, 454–456. doi: 10.1021/np980436g

Wang, Z. Q., Milne, K., Webb, J. R., and Watson, P. H. (2017). CD74 and intratumoral immune response in breast cancer. *Oncotarget* 8, 12664–12674. doi: 10.18632/oncotarget.8610

Woźniak, L., Skąpska, S., and Marszałek, K. (2015). Ursolic acid–a pentacyclic triterpenoid with a wide spectrum of pharmacological activities. *Molecules* 20, 20614–20641. doi: 10.3390/molecules201119721

Xu, J., and Mosher, D. (2011). "Fibronectin and other adhesive glycoproteins," in *The Extracellular Matrix: An Overview*, ed. R. P. Mecham (Berlin: Springer), 41–75. doi: 10.1007/978-3-642-16555-9_2

Yagamuchi, T., and Dijkstra, J. M. (2019). Major histocompatibility complex (MHC) genes and disease resistance in fish. *Cells* 8:378. doi: 10.3390/cells8040378

Yamaoka, K., Saharinen, P., Pesu, M., Holt, V. E. III, Silvennoinen, O., and O'Shea, J. J. (2004). The Janus kinases (Jaks). *Genome Biol.* 5:253. doi: 10.1186/gb-2004-5-12-253

Yin, L., Xu, W., Liu, X., Wang, Y., Ge, P., Wang, X., et al. (2021). Autophagy promotes innate defense against *Aeromonas hydrophila* in grass carp (*Ctenopharyngodon idella*) monocytes/macrophages. *Aquaculture* 535:736391. doi: 10.1016/j.aquaculture.2021.736391

Zou, J., and Secombes, C. J. (2011). Teleost fish interferons and their role in immunity. *Dev. Comp. Immunol.* 35, 1376–1387. doi: 10.1016/j.dci.2011.07.001

Conflict of Interest: JQ and JP-R are current NATAC BIOTECH S.L. employers.

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

Publisher's Note: 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 in Marine Science

ORIGINAL RESEARCH
published: 14 February 2022
doi: 10.3389/fmars.2022.788455

Effect of Greater Duckweed *Spirodela polyrhiza* Supplemented Feed on Growth Performance, Digestive Enzymes, Amino and Fatty Acid Profiles, and Expression of Genes Involved in Fatty Acid Biosynthesis of Juvenile Common Carp *Cyprinus carpio*

*Avanish Kumar Shrivastav[1], Guddu Kumar[2], Prabhat Mittal[3], Douglas R. Tocher[4,5], Brett D. Glencross[4], Rina Chakrabarti[2] and JaiGopal Sharma[1]**

[1] Department of Biotechnology, Delhi Technological University, New Delhi, India, [2] Aqua Research Lab, Department of Zoology, University of Delhi, New Delhi, India, [3] Satyawati College, University of Delhi, New Delhi, India, [4] Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Stirling, United Kingdom, [5] Guangdong Provincial Key Laboratory of Marine Biotechnology, Shantou University, Shantou, China

OPEN ACCESS

Edited by:
Songlin Li,
Shanghai Ocean University, China

Reviewed by:
Omid Safari,
Ferdowsi University of Mashhad, Iran
Sudhir Garg,
Chaudhary Charan Singh Haryana Agricultural University, India
Jingjing Tian,
Chinese Academy of Fishery Sciences, China

***Correspondence:**
JaiGopal Sharma
sharmajaigopal@yahoo.com

Specialty section:
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

Received: 02 October 2021
Accepted: 03 January 2022
Published: 14 February 2022

Citation:
Shrivastav AK, Kumar G, Mittal P, Tocher DR, Glencross BD, Chakrabarti R and Sharma JG (2022) Effect of Greater Duckweed Spirodela polyrhiza Supplemented Feed on Growth Performance, Digestive Enzymes, Amino and Fatty Acid Profiles, and Expression of Genes Involved in Fatty Acid Biosynthesis of Juvenile Common Carp Cyprinus carpio. Front. Mar. Sci. 9:788455. doi: 10.3389/fmars.2022.788455

The greater duckweed *Spirodela polyrhiza* (Lemnaceae) is a free-floating freshwater macrophyte. The effect of dietary inclusion of duckweed in the feed of common carp *Cyprinus carpio* fry was evaluated. The control feed (SP0) contained soybean meal as the primary protein source. In four experimental feeds, greater duckweed was incorporated at levels of 5% (SP5), 10% (SP10), 15% (SP15), and 20% (SP20) replacing soybean meal. Broken-line regression showed that incorporation of greater duckweed at 10 and 13.4% levels were the breakpoint for final weight and specific growth rate (SGR) of fish, respectively. The final weight and SGR of common carp fed diet SP20 were significantly higher compared with those of others. The feed conversion ratio was lowest in SP20 treatment. The inclusion of greater duckweed in the fish feeds showed linear relationships with amylase, trypsin, chymotrypsin, and lipase activities. The content of crude protein was significantly higher in SP10, SP15, and SP20 treatments compared with that of others. Significantly higher crude lipid and ash contents were found in SP20 diet-fed fish compared with other diet-fed fish. The essential amino acids composition was similar in five different diet-fed fish. The greater duckweed supplemented feeds influenced the fatty acid contents of fish. The monounsaturated fatty acids (MUFA) showed an inverse relationship with the inclusion level of greater duckweed in the feed. The highest MUFA content was found in fish fed SP0 diet. The highest level of linoleic acid was found in SP20 diet fed fish. The n-3 PUFA contents of fish showed an increasing trend with the increasing inclusion of greater duckweed, and a significantly higher level was found in SP20 compared with that of others. A significantly higher

expression of *fas* was found in SP5 and *fads2d6* in SP5 and SP10 compared with that of others. The expressions of *elovl2* and *elovl5* were significantly higher in SP5, SP10, and SP15 diet-fed fish compared with other diet-fed fish. The incorporation of greater duckweed in diets improved the growth performance and nutritional value of common carp.

Keywords: *Cyprinus carpio, Spirodela polyrhiza,* digestive enzymes, linoleic acid, eicosapentaenoic acid, docosahexaenoic acid, *fads2d6, elovl2*

INTRODUCTION

The application of freshwater macrophytes as fish feed ingredients is an emerging area of research. There is an increasing demand for quality ingredients that can replace fishmeal and fish oil without affecting the survival, growth performance, and quality of the farmed products. As an alternative to fish meal, plant protein is widely used in aquaculture as well as the poultry and swine feed industries (Hardy, 2010). The nutritional value (e.g., amino acid and fatty acid compositions, fiber content, and flavorings) of ingredients should be considered during fish feed formulation (Gatlin et al., 2007; Glencross et al., 2020). Meals and other products of soybean are the most commonly used plant-based ingredients in the aqua feed industry. However, soybean meal has great market demand as it is also used extensively by other animal feed industries. Therefore, there is a need to find other, non-conventional ingredients that have less or no use in other feed sectors, but that still have high-quality nutritional profiles with all the required amino acids and fatty acids. The greater duckweed *Spirodela polyrhiza* (family: Lemnaceae) is a free-floating freshwater macrophyte that has been considered as a suitable feed ingredient for both fish and livestock (FAO, 2001; Hasan and Chakrabarti, 2009; Cruz-Velásquez et al., 2014; Chakrabarti, 2017).

The study of the proximate composition showed that the crude protein content of soybean meal (460.7 g/kg) is higher compared with greater duckweed (366.5 g/kg), whereas crude lipid and ash levels are higher in greater duckweed (crude lipid: 76.2 g/kg, ash: 181.9 g/kg) compared with soybean meal (crude lipid: 11.0 g/kg, ash: 71.1 g/kg) (Lee et al., 2013). However, greater duckweed is a rich source of essential and non-essential amino acids (Sharma et al., 2019). The amino acid profile of greater duckweed fulfills all the recommended essential amino acid requirements of common carp *Cyprinus carpio* and Nile tilapia *Oreochromis niloticus* (NRC, 1998, 2011). Duckweeds are also known to be good sources of vitamins and fatty acids (Appenroth et al., 2017), with the fatty acid profile of greater duckweed being favorable in comparison with soybean meal. The n-3 PUFA content is 7.5-fold higher in greater duckweed than in soybean meal, with α-linolenic acid (ALA, 18:3n-3) being the predominant fatty acid in greater duckweed contributing 35.75% of total fatty acids (Sharma et al., 2019).

Supply of sufficient amounts of the essential n-3 long-chain PUFA (LC-PUFA), specifically eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), to support optimal human nutrition is a global problem that transcends geographical and political boundaries. While the n-3 LC-PUFA is completely absent in conventional plant meals and vegetable oils, their precursor ALA can be abundant in terrestrial and freshwater plants. The conversion of ALA (and linoleic acid, LOA, 18:2n-6) to LC-PUFA requires a series of fatty acyl desaturase (*fads*) and elongation of very long-chain fatty acid (*elovl*) enzymes such as *elovl5* and *elovl2* (Kuhajda et al., 1994; Torstensen and Tocher, 2010; Castro et al., 2016; Monroig et al., 2016; Xie et al., 2021). The products of the Δ*6fads* and *elovl5* genes are key enzymes in the biosynthesis of EPA and DHA (Fonseca-Madrigal et al., 2005; Torstensen and Tocher, 2010). Importantly, many freshwater fishes including common carp and Nile tilapia have the metabolic capacity to convert dietary ALA to the n-3 LC-PUFA, EPA, and DHA (Tocher et al., 2002; Glencross, 2009; Tocher, 2010; Taşbozan and Gökçe, 2017). Therefore, supplementation of the greater duckweed *S. polyrhiza* as a rich source of ALA in the feed of freshwater carp is a useful and cost-effective way to increase the n-3 LC-PUFA content of farmed fish for human consumption.

The omnivore common carp *C. carpio* (family: Cyprinidae) is the fourth most cultured freshwater fish and contributed 7% of total aquaculture (fish) production in 2018 (FAO, 2020) and is extensively used in composite fish culture in India (Rathore et al., 2005). The digestibility of ingredients plays a very significant role in the overall bioavailability of the nutrients present in feed (Chakrabarti and Rathore, 2009), and, recently, an *in vitro* digestibility study showed the potential suitability of greater duckweed as an ingredient in fish feed (Sharma et al., 2016). Rathore et al. (2005) have reported the variations in the activities of digestive enzyme in common carp during ontogenic development and observed significantly increased amylase activity in 30-day-old fish. This finding confirmed the capacity of common carp to digest plant-based feed. The presence of anti-nutritional factors is a major constraint to the application of plant-based ingredients in aquafeeds (Alarcón et al., 1998; Olsen et al., 2007; Hansen and Hemre, 2013).

Several studies have investigated the effects of freshwater macrophytes in feeds for different fish species. Dietary supplementation of *Lemna minor* (20%) and *Azolla pinnata* had no negative impacts on growth performance or feed utilization of common carp (Yılmaz et al., 2004; Gangadhar et al., 2017). Rohu *Labeo rohita*-fed diets containing 20 and 30% *L. minor* showed highest weight gain, SGR, and lowest FCR compared with the control diet without duckweed (Bairagi et al., 2002; Mer et al., 2016). The supplementation of duckweed in the diet of common carp increased the antioxidant capacity as evidenced by enhanced activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) and reduced levels of malondialdehyde, MDA (Yang et al., 2019). The aim of the

present study was to investigate the effects of feeding diets containing greater duckweed *S. polyrhiza* on the survival, growth performance, digestive enzyme activities, and biochemical composition of common carp *C. carpio*. The greater duckweed was included in a graded manner at levels of 0%, 5%, 10%, 15%, and 20% of total diet in carp feeds. The expression of genes involved in the metabolic conversion of ALA was also studied to determine impacts on n-3 LC-PUFA biosynthesis.

MATERIALS AND METHODS

Feed Formulation

The greater duckweed *S. polyrhiza* was cultured using organic manures *viz*., cattle manure mustard oil-cake and poultry dropping (Sharma et al., 2019). These manures are cheap and easily available. The production cost of greater duckweed in this study was around Rs.14.0/kg. The greater duckweed was collected, cleaned, dried, and ground. The meal was stored at 4°C prior to further use. The moisture, crude protein, crude lipid, carbohydrate, and ash contents of the greater duckweed meal were 75.2, 366.5, 76.2, 300.2, and 181.9 g/kg, respectively. Five isoproteic, isolipidic, and isoenergetic experimental feeds were prepared with graded inclusion of *S. polyrhiza* meal replacing soybean meal as the primary protein source (**Table 1**). Fish feeds were formulated using the Winfeed 2.8 software package (WinFeed UK Limited, Cambridge, United Kingdom). The control feed (SP0) contained soybean meal as the only primary source of protein, while in the four experimental feeds, greater duckweed was incorporated at the levels of 5%, 10%, 15%, and 20% of total feed at the expense of soybean meal, wheat flour, corn meal, and sunflower oil (to maintain constant crude protein, crude lipid, and gross energy levels) to produce feeds SP5, SP10, SP15, and SP20. The soybean meal was replaced in a graded manner, which resulted in changes in the proportions of amino acids in the feeds. Therefore, some specific amino acids such as histidine, methionine, lysine, and threonine were supplemented to the feeds based on the reported requirements of common carp (NRC, 2011). The inclusion levels of these four amino acids were determined using the Winfeed software to ensure the requirements of the fish were satisfied. All dry feed ingredients were blended for 10 min and mixed with the oil before warm water was added slowly and everything mixed thoroughly. The entire mixture was placed in the hopper of the twin-screw-extruder (Basic Technology Private Limited, Kolkata, India), and feed pellets were formed with extrusion conditions as follows: cutter 134 rpm; feeder 10 rpm; extrusion 190 rpm; extrusion torque 9.22; heater 1 temperature 65°C; heater 2 temperature 70°C; and final mass temperature 75°C. The diameter of the produced pellets was 1 mm. All feeds were stored at 4°C prior to use. A common difficulty in the use of feeds based on plant ingredients is their palatability to the fish (Rodriguez et al., 1996), but this can be mitigated by the extrusion process. Antinutritional factors such as trypsin inhibitor, phytic acid tannins, oxalates etc., are found in greater duckweed (Cruz et al., 2011). However, the preparation of the feeds by the extrusion technique helped to mitigate the impact of antinutritional factors as high temperature and pressure inactivate many of these factors and control enzymatic rancidity of nutrients (Rokey, 2004; Stadtlander et al., 2019). In this study, the preparation of feed using the extrusion technology improved the digestibility of proteins and starches and destroyed the antinutritional factors present in the feed. The amino acid and fatty acid compositions of the feeds were measured and are presented in **Supplementary Tables 1**, **2**.

Culture of Fish and Sampling

Common carp were cultured and sampled following the guidelines of the University of Delhi Institutional Animal Ethics Committee (DU/ZOOL/IAEC-R/2015/07). The fish were collected from a local fish farm and acclimated in the aquarium at the University of Delhi for 1 week during which time the fry were fed the soybean-based control feed. The fry (0.473–0.479 g) were then distributed randomly into 15 glass aquaria (50 L each) with 30 fry per aquarium. The fry of common carp was selected to determine the influence of the plant-based diet on the digestive physiology of fish and to understand the suitability of these diets for early life stages. Each aquarium was connected to an external filtration unit (Sera fil bioactive 130, Germany). Water from the fish culture units was constantly filtered through the filtration unit to maintain ammonia levels of the units. The dissolved oxygen level of water was maintained with the help of an aerator. The carp were then fed one of the five different feeds SP0, SP5, SP10, SP15, and SP20 with three replicate aquaria per dietary treatment. The feeds were distributed *ad libitum* two times daily at 09:00 and 17:00 h and the weight of feed measured before distribution. Excess (uneaten) feed was collected from each aquarium 1 h after feeding, oven drying, and recording weight. Water quality parameters, namely, temperature, pH, dissolved oxygen, and conductivity were monitored in each aquarium using a probe connected to a portable meter (HQ40d Multiparameter, Hach, United States). The ammonia (NH_3) level was estimated using a probe, connected to Orion Versastar (Thermo Scientific, United States). The nitrite (NO_2^-), nitrate (NO_3^-), and phosphate ($PO4^{3-}$) contents were analyzed regularly (APHA, 2017). There was no significant difference in water quality parameters among the dietary treatments throughout the culture period. Temperature, pH, and dissolved oxygen ranged from 25.8 to 28.1°C, 6.66 to 7.56, and 6.45 to 7.48 mg/L, respectively, during the experimental period. The range of ammonia, nitrite, nitrate, and phosphate levels were 0.003–0.0530, 0.291–0.906, 0.956–3.84, and 0.013–0.097 mg/L, respectively, in different treatments and conductivity ranged from 636–811 μS/cm.

After 60 days of culture, the feeding experiment was terminated, and fish was sampled. Fish were starved for 24 h before harvesting, and then, all fish were euthanized with tricaine methane sulfonate (MS222, Sigma, United States), and the weight of individual fish was measured. Four fish from each tank were pooled (four fish/replicate) and stored at -80°C for the assay of whole-body proximate, amino acid, and fatty acid compositions. Three tank replicates were used for all assays (three replicates per diet, $n = 3$). The digestive tracts of two individual fish per aquarium were collected (two fish/replicate, three replicates; $2 \times 3 = 6$ fish/diet) for the assay of digestive

TABLE 1 | Formulations and measured proximate compositions of experimental diets.

Ingredients (g/Kg)	SP0	SP5	SP10	SP15	SP20
Soybean meal	500	475	450	425	400
Wheat flour	245	224	204	183	162
Corn flour	147	144	141	138	135
Sunflower oil	62	60	57	55	52
S. polyrhiza powder	0	50	100	150	200
Vitamin/minerals premix	5	5	5	5	5
Mono calcium phosphate	20	20	20	20	20
Choline chloride	1	1	1	1	1
Histidine	1	1	2	2	2
Methionine	12	12	12	12	12
Lysine	5	6	6	7	7
Threonine	2	3	3	4	4
Proximate composition (g/kg)					
Moisture	63.3 ± 0.81	63.6 ± 0.20	63.2 ± 0.63	63.3 ± 0.84	62.5 ± 0.42
Crude Protein	317.6 ± 0.50	318.4 ± 2.20	318.2 ± 3.10	322.2 ± 3.61	322.0 ± 0.70
Crude Lipid	73.3 ± 0.81	73.4 ± 0.91	73.7 ± 0.25	73.2 ± 0.10	72.8 ± 0.17
Carbohydrate	474.6 ± 4.10	474.3 ± 1.72	472.0 ± 0.25	464.2 ± 2.21	460.6 ± 3.1
Ash	71.2 ± 0.28	70.3 ± 0.40	72.9 ± 0.60	77.1 ± 0.21	84.1 ± 1.1

SP0, soybean meal only; SP5, soybean meal + 5% S. polyrhiza; SP10, soybean meal + 10% S. polyrhiza; SP15, soybean meal + 15% S. polyrhiza; SP20, soybean meal + 20% S. polyrhiza.

TABLE 2 | Initial weight, survival rate, final weight, specific growth rate, feed conversion ratio, and digestive enzyme activities of *Cyprinus carpio* fed five different diets.

Parameters	SP0	SP5	SP10	SP15	SP20	ANOVA		Linear regression R^2 value
						P value	*F* value	
Initial weight (g)	0.473 ± 0.001^a	0.479 ± 0.001^a	0.473 ± 0.003^a	0.473 ± 0.001^a	0.477 ± 0.002^a	0.093	2.692	0.518
Survival (%)	100	100	100	100	100	-	-	-
Final weight (g)	1.60 ± 0.004^e	1.88 ± 0.012^d	2.15 ± 0.007^c	2.49 ± 0.003^b	2.74 ± 0.013^a	< 0.01	7,507.44	0.999
Specific growth rate (SGR% BW/day)	2.01 ± 0.002^e	2.30 ± 0.011^d	2.52 ± 0.007^c	2.75 ± 0.001^b	2.93 ± 0.001^a	< 0.01	3,419.05	0.999
Feed conversion ratio (FCR)	1.25 ± 0.010^a	1.23 ± 0.012^a	1.11 ± 0.011^b	1.06 ± 0.013^{bc}	1.01 ± 0.010^c	< 0.01	74.394	0.967
Amylase (mU/mg protein/min)	126.48 ± 3.80^a	98.91 ± 2.30^b	102.28 ± 2.13^b	125.41 ± 1.87^a	125.14 ± 1.82^a	< 0.01	30.70	0.925
Protease (Fluorescence change/unit)	249.95 ± 1.40^b	247.31 ± 0.88^b	265.35 ± 5.30^a	264.78 ± 1.75^a	278.82 ± 6.28^a	0.091	2.715	0.521
Trypsin (μM AMC/mg protein/min)	799.93 ± 4.11^c	588.87 ± 6.18^d	768.03 ± 9.42^c	$1,073.91 \pm 6.72^b$	$1,335.45 \pm 9.52^a$	< 0.01	1,532.15	0.998
Chymotrypsin (μM AMC/mg protein/min)	683.17 ± 17.86^b	813.10 ± 10.84^a	617.5 ± 4.30^c	477.24 ± 10.24^d	406.38 ± 15.20^e	< 0.01	147.52	0.983
Lipase (μM 4-MU/mg protein/min)	$1,000.97 \pm 17.26^c$	950.40 ± 10.80^c	992.27 ± 14.82^c	$1,137.10 \pm 16.30^a$	$1,074.27 \pm 15.32^b$	< 0.01	27.50	0.917

Values (n = 3) with different letters in the same row are significantly different ($p < 0.05$).
SP0, soybean meal only; SP5, soybean meal + 5% S. polyrhiza; SP10, soybean meal + 10% S. polyrhiza; SP15, soybean meal + 15% S. polyrhiza; SP20, soybean meal + 20% S. polyrhiza; BW, body weight.

enzyme activities. The hepatopancreas from individual fish was collected (100 mg) and stored in 1 ml of TRIzol reagent (Ambion, Life Technologies, United States) for the gene expression analysis (four fish/treatment; from two aquaria 1 + 1 fish and two fish from the third aquarium). Specific growth rate (SGR) and feed conversion ratio (FCR) of fish were calculated as follows.

SGR (% body weight/day) = (In final body mass - In initial body mass) × 100/duration of experiment (days).

FCR = feed (dry weight) consumed by individual fish during feeding trial/weight gain (wet weight) of individual fish.

Digestive Enzymes Activities

Intestinal samples were freeze-dried and homogenized in ice-cold Milli-Q® water (1:10) to maintain neutral pH of the extracts. Homogenates were centrifuged for 30 min at 10,000 × *g* at 4°C, and supernatants were collected for the assay of digestive enzyme activities using fluorimetry (Multimode reader, BioTek Synergy H1 Hybrid, United States) using three replicates per dietary treatment. The amylase activity was determined using an assay kit (E33651; Invitrogen, United States) with fluorescence measured at 485 (i.e., excitation) and 520 nm (i.e., emission). The enzyme activity was expressed as mU/mg protein/min. A protease kit (E6638; Invitrogen) was used to measure the total protease activity with fluorescence measured at 485 (i.e., excitation) and 530 nm (i.e., emission). The protease activity was expressed as fluorescence change/unit. The substrate *N*-benzoyl-L-arginine-methyl-coumarinylamide (Sigma-Aldrich) was used for the estimation of serine proteases trypsin (Ueberschär, 1988). The fluorescence was measured at 380 (i.e., excitation) and 440 nm (i.e., emission). The chymotrypsin was measured using succinyl-Leu-Val-Tyr-4-methyl-coumaryl-7-amide (Sigma-Aldrich) as the substrate (Cao et al., 2000). The fluorescence was measured at 380 (i.e., excitation) and 450 nm (i.e., emission). The enzyme activities were expressed as μM 7-amino-4-methylcoumarin (AMC)/mg protein/min. The neutral lipase activity was determined following the method of Roberts (1985) using the substrate 4-methylumbelliferyl butyrate, 4-MU (Sigma-Aldrich). The fluorescence was measured at 365 nm (excitation) and 450 nm (emission). The enzyme activity was expressed as μM 4-MU/mg protein/min. Protein content was estimated using bovine serum albumin (BSA, Sigma-Aldrich) as standard (Bradford, 1976).

Proximate Composition

Samples of feed were ground, and the fish samples (four fish pooled per replicate) were blended to form homogeneous paste prior to biochemical composition analyses. Proximate compositions of feeds and cultured fish were determined (three replicates for each assay) following the standard protocols of the Association of Official Analytical Chemists International (AOAC, 2000). The moisture level was calculated after drying samples at 110°C for 24 h, and ash content was measured after incineration of samples at 600°C for 16 h. The nitrogen content was first assayed using micro Kjeldahl method, and then, crude protein was calculated ($N \times 6.25$). The crude lipid content was measured gravimetrically following extraction of total lipid using chloroform/methanol (2:1, v/v) (Folch et al., 1957). The subtraction method was applied for the calculation of carbohydrate levels in the feeds (Aksnes and Opstvedt, 1998).

Amino Acid Analysis

Feed and whole fish samples were processed as described above and amino acid compositions assayed with an Automatic Amino Acid Analyzer L-8900 (Hitachi Co., Ltd., Tokyo, Japan) using three replicates for each sample. For all amino acids other than cysteine, methionine, and tryptophan, samples were hydrolyzed using 6 N HCl at 110°C for 24 h (Bassler and Buchholz, 1993; Chakrabarti et al., 2018). The sulfur-containing amino acids were analyzed after oxidizing the sample with performic acid prior to treating the sample with 6 N HCl. For the estimation of tryptophan, the sample was hydrolyzed using 4 M methanesulfonic acid with 0.2% 3-(2-aminoethyl). A nitrogen evaporator (PCi Analytic Private Limited, Maharashtra, India) was used to dry the digested samples, and 0.02 N HCl was added to obtain a protein concentration of 0.5 mg/ml in the sample, and 1.5 ml was placed in a glass vial of the auto sampler. A cation-exchange resin column (4.6 mm ID × 60 mm L) with 3 μm particle size was used for the separation of amino acids with the following analytical conditions: column temperature 30-70°C, reaction temperature 135°C, and a ninhydrin flow rate of 0.35 ml/min. All amino acids were monitored at 570 nm, except proline and hydroxyproline that were monitored at 440 nm. The concentration of individual amino acids was compared with a standard solution (Wako Pure Chemical Industries Limited, United States) and expressed as g/kg.

Fatty Acid Analysis

After processing feed and whole fish samples as described above, fatty acid compositions were analyzed by gas chromatography and flame ionization detection (GC-FID) using a Clarus 580 (PerkinElmer, Waltham, United States). In brief, crude lipid was extracted from the samples using chloroform/methanol (2:1, v/v) following the protocol of Folch et al. (1957) with three replicates per dietary treatment. Fatty acid methyl esters (FAME) were prepared from crude lipid extracts by acidic transesterification, treating the lipid with 1% sulfuric acid in methanol for 16 h at 50°C (Christie, 2003). After extraction and purification of FAME (Tocher and Harvie, 1988), a 1 ml aliquot was placed in a glass vial of the GC autosampler. Fatty acids were separated using a 60 m ZB-wax GC column, internal diameter of 0.32 mm, and film thickness of 0.25 μm (Phenomenex, Hyderabad, India). Data were collected using preinstalled programmed software (TotalChrom Workstation Ver6.3; PerkinElmer, United States). The FAME were identified and quantified by the comparison with standards (Supelco FAME 37 mix; Sigma-Aldrich, United States) and published data (Tocher and Harvie, 1988) and concentration expressed as mg/100 g.

Gene Expression

The levels of mRNA expression of delta-6 fatty acyl desaturase (*fads2d6*), elongation of very-long-chain fatty acids protein 2 (*elovl2*), elongation of very-long-chain fatty acids protein 5 (*elovl*5), and fatty acid synthase (*fas*) genes were determined in the hepatopancreas of common carp. Total RNA was extracted using the TRIzol reagent (Ambion, Life Technologies, United States) following the protocol of the manufacturer. The absorbance of extracted RNA was examined at 260 and 280 nm using a Nanodrop spectrophotometer (Thermo Scientific, United States) to determine the concentration and quality. The extracted RNA was treated with 1 U of DNase I

TABLE 3 | Proximate compositions (g/kg, wet weight) of five different diets-fed *Cyprinus carpio*.

Parameters	SP0	SP5	SP10	SP15	SP20	ANOVA		Linear Regression R^2 Value
						P value	*F* value	
Moisture	756.4 ± 3.6^{a}	752.8 ± 3.7^{a}	750.7 ± 1.02^{a}	750.5 ± 5.7^{a}	747.6 ± 1.2^{a}	0.524	0.852	0.254
Crude Protein	150.3 ± 0.66^{c}	152.34 ± 0.25^{b}	156.73 ± 0.25^{a}	156.79 ± 0.12^{a}	157.06 ± 0.1^{a}	<0.01	81.09	0.970
Crude Lipid	64.7 ± 0.1^{c}	64.4 ± 0.10^{c}	65.2 ± 0.1^{bc}	65.9 ± 0.2^{b}	68.1 ± 0.5^{a}	<0.01	33.949	0.931
Ash	19.6 ± 0.3^{c}	21.4 ± 0.1^{b}	21.8 ± 0.2^{b}	21.9 ± 0.3^{b}	22.5 ± 0.3^{a}	<0.01	28.401	0.919

Values (n = 3) with different letters in the same row are significantly different ($p < 0.05$).
SP0, soybean meal only; SP, soybean meal + 5% S. polyrhiza; SP10, soybean meal + 10% S. polyrhiza; SP15, soybean meal + 15% S. polyrhiza; SP20, soybean meal + 20% S. polyrhiza.

(Sigma-Aldrich, United States) to avoid DNA contamination, and the quality of RNA treated with DNase was checked with 1% agarose gel electrophoresis. Subsequently, total RNA was reverse transcribed into cDNA by the reverse transcription reaction using high-capacity cDNA reverse transcription kit (Applied Biosystems, United States), using the protocol provided by the manufacturer.

Quantification of gene expression was carried out by using quantitative reverse transcription polymerase chain reaction (qRT-PCR) using a Quant Studio 6 Flex system (Applied Biosystems) and PowerUp SYBR^{TM} Green Master Mix (Applied Biosystems). Primers were designed using the online primer design tool of NCBI with β−actin used as the reference (housekeeping) gene (**Supplementary Table 3**). The efficiency of primers was evaluated by using the melt curve and standard curve analysis using the QuantStudio 6 Flex Real-Time PCR system software v1 (Applied Biosystems). The 10 μl reaction mixture for qRT-PCR was composed of 0.25 μl PCR forward primer (2.5 μM), 0.25 μl PCR reverse primer (2.5 μM), 1 μl of cDNA (1:3), 5 μl of 2 × PowerUp^{TM} SYBR^{TM} Green PCR Master Mix (Applied Biosystems), and nuclease-free water (3.5 μl). Samples were run in duplicate for each target gene with non-template control (NTC). The thermal cycling conditions were as follows: predenaturation of nucleic acid at 95°C for 10 min followed by either 40 cycles of 15 s at 95°C and 1 min at 60°C (primer Tm 60°C) or 40 cycles of 15 s at 95°C, 15 s at 55°C, and 1 min at 72°C for (primer Tm < 60°C). The data of qRT−PCR were calculated using the $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001) method with β−actin as the internal control.

Statistical Analysis

Data are presented as means with standard error (SEM) with *n* values as stated. The IBM SPSS 25.0 software (SPSS Inc., Michigan Avenue, Chicago, IL, United States) was used for the statistical analysis. Data were analyzed using one-way analysis of variance, and the Tukey's test was performed to compare the differences among experimental groups. The linear regression analysis was performed to check the effect of inclusion level of *S. polyrhiza* in the diets on growth performance, digestive enzyme activities, proximate composition, and amino acid and fatty acid profiles of fish. The broken-line regression analysis was performed for average weight and SGR to determine the breakpoint, BP (Muggeo, 2008) with final BP estimated based on the least sum of squares of deviation (LS method) using R package. The significance was accepted at $p < 0.05$ level.

RESULTS

Performance of Fish

After 60 days of culture, the number of common carp in each aquarium was recorded. There was no mortality of fish; all fish survived (**Table 2**). The broken-line regression analysis showed the impact of greater duckweed on the growth performance and SGR of common carp. The estimated break point for final weight and SGR were 10 and 13.4% of greater duckweed, respectively. The final weight of SP20 diet-fed common carp was significantly higher compared with other diet-fed fish. Minimum weight was found in the SP0 treatment. Similar trend was also found with SGR. SGR was maximum in SP20 diet-fed carp. FCR was minimum and maximum in SP20 and SP0 treatment, respectively.

Digestive Enzymes

The amylase activity ranged from 98.91 to 126.48 mU/mg protein/min in five different diet-fed common carp with a minimum amylase activity in SP5 diet-fed fish (**Table 2**). The amylase activity was significantly higher in SP0, SP15, and SP20 diet-fed fish compared with other diet-fed fish. The total protease activity was significantly higher in SP10, SP15, and SP20 diet-fed fish compared with other diet-fed fish. Trypsin activity was significantly higher in SP20 diet-fed fish compared with other diet-fed fish. Significantly higher chymotrypsin and lipase activities were found in SP5 and SP15 diet-fed common carp, respectively, compared with other treatments. The inclusion of greater duckweed in the carp diet showed linear relationships (R^2 = 0.917–0.998) with amylase, trypsin, chymotrypsin, and lipase activities.

Biochemical Composition of Fish

Proximate Composition of the Whole Body

The moisture contents of fish varied from 747.6 to 756.4 g/kg in five different diet-fed common carp (**Table 3**). The crude protein content was significantly higher in SP10, SP15, and SP20 diet-fed common carp compared with other diet-fed fish. Significantly

TABLE 4 | Amino acid compositions (g/kg, wet weight) of five different diets-fed *Cyprinus carpio*.

Amino acids	SP0	SP5	SP10	SP15	SP20	ANOVA		Linear regression R^2 value
Essential						***P* value**	***F* value**	
Arginine (Arg)	6.77 ± 0.2^{c}	6.40 ± 0.1^{d}	10.7 ± 0.1^{a}	10.21 ± 0.03^{b}	9.94 ± 0.04^{b}	<0.01	905.511	0.997
Histidine (His)	3.72 ± 0.1^{a}	3.64 ± 0.2^{a}	2.85 ± 0.05^{b}	2.87 ± 0.06^{b}	2.73 ± 0.03^{b}	<0.01	156.009	0.984
Isoleucine (Ile)	5.13 ± 0.12^{ab}	5.39 ± 0.07^{a}	4.98 ± 0.01^{b}	4.25 ± 0.1^{c}	4.44 ± 0.13^{c}	<0.01	67.119	0.964
Leucine (Leu)	9.59 ± 0.19^{a}	10.12 ± 0.33^{a}	8.59 ± 0.04^{b}	8.28 ± 0.11^{bc}	8.01 ± 0.25^{c}	<0.01	53.555	0.955
Lysine (Lys)	11.54 ± 0.04^{b}	12.75 ± 0.08^{a}	10.1 ± 0.1^{c}	9.15 ± 0.05^{d}	9.47 ± 0.24^{d}	<0.01	323.243	0.992
Methionine (Met)	2.94 ± 0.04^{c}	3.37 ± 0.04^{ab}	3.63 ± 0.21^{a}	2.77 ± 0.09^{c}	3.01 ± 0.30^{c}	<0.01	12.611	0.835
Phenylalanine (Phe)	5.52 ± 0.12^{ab}	5.83 ± 0.1^{a}	5.16 ± 0.01^{bc}	4.91 ± 0.20^{c}	4.99 ± 0.14^{a}	<0.01	14.657	0.854
Threonine (Thr)	4.73 ± 0.01^{d}	5.16 ± 0.03^{c}	6.20 ± 0.1^{b}	6.12 ± 0.1^{b}	6.67 ± 0.08^{a}	<0.01	293.479	0.992
Tryptophan (Trp)	2.27 ± 0.03^{c}	1.60 ± 0.05^{d}	1.56 ± 0.003^{d}	2.32 ± 0.01^{b}	2.48 ± 0.002^{a}	<0.01	1,705.8	0.998
Valine (Val)	5.98 ± 0.05^{d}	6.49 ± 0.05^{bc}	7.14 ± 0.06^{a}	6.25 ± 0.01^{c}	6.63 ± 0.17^{b}	<0.01	69.776	0.965
Σ Essential	**58.2 ± 1.1**^{b}	**60.8 ± 0.1**^{a}	**61.1 ± 0.4**^{a}	**57.1 ± 0.7**^{b}	**58.4 ± 0.4**^{b}	<0.01	21.402	0.895
Non-essential	**SP0**	**SP5**	**SP10**	**SP15**	**SP20**	**ANOVA**		**Linear regression R^2 value**
						***P* value**	***F* value**	
Alanine (Ala)	8.03 ± 0.03^{b}	7.65 ± 0.03^{c}	8.03 ± 0.06^{b}	9.85 ± 0.07^{a}	9.94 ± 0.15^{a}	<0.01	363.836	0.993
Aspartate (Asp)	11.7 ± 0.03^{b}	11.4 ± 0.12^{b}	12.4 ± 0.36^{a}	11.3 ± 0.02^{b}	12.7 ± 0.12^{a}	<0.01	33.477	0.931
Cysteine (Cys)	1.14 ± 0.002^{c}	1.69 ± 0.07^{a}	1.12 ± 0.07^{c}	1.30 ± 0.17^{bc}	1.39 ± 0.02^{b}	<0.01	18.725	0.882
Glutamic Acid (Glu)	20.3 ± 0.08^{a}	18.8 ± 0.10^{c}	17.3 ± 0.01^{d}	17.3 ± 0.05^{d}	19.6 ± 0.17^{b}	<0.01	535.013	0.995
Glycine (Gly)	8.91 ± 0.09^{d}	10.0 ± 0.3^{c}	8.58 ± 0.01^{d}	10.80 ± 0.14^{b}	11.62 ± 0.03^{a}	<0.01	77.612	0.969
Proline (Pro)	27.8 ± 0.1^{c}	29.0 ± 0.1^{b}	31.37 ± 0.01^{a}	31.10 ± 0.20^{a}	25.1 ± 0.2^{d}	<0.01	387.422	0.991
Serine (Ser)	4.83 ± 0.02^{c}	4.81 ± 0.07^{c}	5.62 ± 0.01^{b}	5.48 ± 0.03^{b}	6.32 ± 0.08^{a}	<0.01	221.76	0.989
Tyrosine (Tyr)	4.10 ± 0.2^{a}	3.67 ± 0.34^{ab}	3.57 ± 0.02^{ab}	3.24 ± 0.16^{b}	3.34 ± 0.04^{b}	<0.01	7.311	0.745
Phosphoserine (p-Ser)	0.06 ± 0.00^{bc}	0.05 ± 0.00^{c}	0.10 ± 0.05^{bc}	0.28 ± 0.06^{a}	0.15 ± 0.01^{b}	<0.01	28.851	0.920
Taurine (Tau)	1.76 ± 0.03^{bc}	1.51 ± 0.15^{c}	1.74 ± 0.16^{bc}	1.96 ± 0.13^{b}	2.73 ± 0.04^{a}	<0.01	48.115	0.951
α Amino -n- butyric acid (α-ABA)	–	–	0.19 ± 0.02^{a}	0.20 ± 0.03^{a}	0.18 ± 0.00^{a}	0.221	1.961	0.395
Citrulline (Cit)	0.13 ± 0.01^{b}	0.15 ± 0.03^{b}	0.44 ± 0.00^{a}	0.54 ± 0.20^{a}	0.56 ± 0.00^{a}	<0.01	16.081	0.865
Cystathionine (Cysthi)	0.28 ± 0.05^{d}	0.11 ± 0.01^{d}	2.13 ± 0.14^{b}	2.60 ± 0.01^{a}	1.38 ± 0.04^{c}	<0.01	301.177	0.992
β -Alanine (β -Ala)	–	0.08 ± 0.02^{a}	0.06 ± 0.00^{ab}	0.11 ± 0.001^{a}	0.11 ± 0.05^{a}	0.185	2.051	0.435
β Amino isobutyric acid (β-AiBA)	0.04 ± 0.00^{b}	–	–	–	0.08 ± 0.00^{a}	-	-	–
γ-Amino-n-butyric acid (γ-ABA)	0.04 ± 0.00^{a}	–	–	–	–	-	-	–
Hydroxylysine (Hylys)	0.16 ± 0.05^{b}	–	–	0.24 ± 0.002^{a}	–	<0.01	518.664	0.992
3 Methyl histidine(3 Mehis)	–	–	0.07 ± 0.000^{a}	– –	0.06 ± 0.000^{a}	0.02	13.914	0.777
Hydroxy proline (Hypro)	1.58 ± 0.09^{c}	2.28 ± 0.08^{b}	2.91 ± 0.22^{a}	3.13 ± 0.002^{a}	3.07 ± 0.16^{a}	<0.01	301.177	0.992
Σ Non- essential	**90.95 ± 0.31**^{c}	**91.51 ± 0.21**^{c}	**95.76 ± 0.4**^{b}	**99.56 ± 0.50**^{a}	**98.16 ± 0.06**^{a}	**<0.01**	**136.54**	**0.982**

Values (n = 3) with different letters in the same row are significantly different ($p < 0.05$).
SP0, soybean meal only; SP5, soybean meal + 5% S. polyrhiza; SP10, soybean meal + 10% S. polyrhiza; SP15, soybean meal + 15% S. polyrhiza; SP20, soybean meal + 20% S. polyrhiza. The bold values means summation/total value.

higher crude lipid and ash contents were found in SP20 diet-fed fish compared with other diet-fed fish.

Amino Acid Composition

The essential amino acids composition was similar in common carp cultured in five different feeding schemes (**Table 4**). The arginine level was 1.55- to 1.67-fold higher in 10–20% greater duckweed supplemented diet-fed common carp compared with SP0 and SP5 diet-fed fish. Histidine (3.64–3.72 g/kg), isoleucine (5.13–5.39 g/kg), leucine (9.59–10.12 g/kg), and lysine (11.54–12.75 mg/kg) contents were significantly higher in SP0 and SP5 treatments compared with other diet-fed fish. Significantly higher levels of methionine and valine were recorded in SP10; threonine and tryptophan levels were maximum in SP20 diet-fed common carp.

Among non-essential amino acids, alanine, aspartate, glycine, serine, taurine, and β-alanine contents were maximum in SP20 diet-fed common carp. Highest levels of glutamic acid and tyrosine were observed in control diet (SP0)-fed common carp. Aspartate contents were significantly higher in SP10 and SP20 diet-fed common carp. Highest levels of phosphoserine, cystathionine, and hydroxyproline were found in SP15 diet-fed fish. The α-amino-n-butyric acid content was highest in SP15, and the citrulline content was maximum in SP15 and SP20

TABLE 5 | Fatty acid compositions (mg/100 g, wet weight) of five different diets-fed *Cyprinus carpio*.

Fatty acids	SP0	SP5	SP10	SP15	SP20	ANOVA		Linear regression R^2 value
						P value	*F* value	
14:0	22.92 ± 1.23^{b}	30.64 ± 1.00^{a}	22.80 ± 0.34^{b}	22.42 ± 0.11^{b}	15.78 ± 0.07^{c}	< 0.01	156.593	0.984
15:0	4.21 ± 0.23^{c}	6.52 ± 0.25^{b}	4.89 ± 0.61^{c}	6.21 ± 0.34^{b}	7.29 ± 0.29^{a}	< 0.01	22.074	0.898
16:0	374.00 ± 9.01^{d}	548.07 ± 20.3^{a}	507.88 ± 6.26^{b}	435.9 ± 1.41^{c}	369.36 ± 2.75^{d}	< 0.01	176.633	0.986
18:0	1.90 ± 0.01^{a}	2.81 ± 0.68^{a}	3.00 ± 0.20^{a}	2.05 ± 0.29^{a}	1.24 ± 0.21^{a}	0.081	**2.856**	0.533
24:0	10.53 ± 0.01^{b}	14.68 ± 2.17^{a}	12.49 ± 0.04^{a}	7.21 ± 0.77^{c}	9.25 ± 0.11^{b}	< 0.01	79.245	0.969
Σ SFA	**413.58 ± 10.00**^{d}	**602.75 ± 22.66**^{a}	**551.09.1 ± 7.05**^{b}	**473.8 ± 3.20**^{c}	**403.20 ± 3.50**^{d}	**< 0.01**	**165.49**	**0.985**
16:1 n-9	26.44 ± 0.02^{a}	14.10 ± 1.0^{b}	12.25 ± 2.35^{b}	24.44 ± 0.72^{a}	4.46 ± 0.73^{c}	< 0.01	164.648	0.985
18:1 n-9	1, 013.68 ± 0.02^{a}	963.71 ± 46.81^{a}	889.60 ± 5.90^{b}	905.3 ± 1.80^{b}	699.36 ± 10.84^{c}	< 0.01	91.965	0.974
24:1	2.51 ± 0.04^{b}	3.86 ± 0.44^{a}	2.33 ± 0.29^{b}	–	–	< 0.01	22.290	0.881
Σ MUFA	**1,042.6 ± 1.75**^{a}	**981.688 ± 46.26**^{b}	**904.18 ± 3.83**^{c}	**929.79 ± 2.52**^{bc}	**703.82 ± 11.57**^{d}	**< 0.01**	**107.854**	**0.977**
18:2 n-6	911.99 ± 8.58^{b}	989.75 ± 9.10^{a}	1, 030.2 ± 5.18^{a}	1, 016.81 ± 3.76^{a}	1, 053.6 ± 0.06^{a}	< 0.01	11.145	0.817
20:2 n-6	32.67 ± 1.24^{b}	20.60 ± 0.03^{d}	32.55 ± 0.05^{b}	37.51 ± 1.82^{a}	24.48 ± 2.07^{c}	< 0.01	72.707	0.967
20:3 n-6	13.44 ± 0.22^{b}	11.91 ± 0.78^{c}	10.77 ± 0.02^{d}	14.60 ± 0.30^{a}	10.85 ± 0.52^{d}	< 0.01	55.671	0.957
20:4 n-6	26.89 ± 0.22^{b}	22.96 ± 0.32^{d}	28.17 ± 0.83^{a}	21.75 ± 0.15^{d}	25.12 ± 0.38^{c}	< 0.01	102.122	0.976
22:5 n-6	71.09 ± 0.22^{ab}	66.27 ± 0.17^{c}	46.22 ± 0.002^{d}	69.43 ± 2.17^{b}	72.80 ± 0.144^{a}	< 0.01	282.796	0.991
Σ n-6 PUFA	**1,056.1 ± 11.25**^{b}	**1,111.5 ± 10**^{ab}	**1,147.96 ± 62.51**^{a}	**1,160.11 ± 8.01**^{a}	**1,186.95 ± 2.2**^{a}	**< 0.01**	**9.048**	**0.784**
18:3 n-3	15.10 ± 0.115^{d}	13.65 ± 0.18^{e}	18.09 ± 0.30^{c}	19.53 ± 0.40^{b}	32.63 ± 0.19^{a}	< 0.01	2,563.494	0.999
20:4 n-3	126.51 ± 0.07^{c}	137.58 ± 0.33^{b}	144.32 ± 0.56^{a}	125.9 ± 3.42^{c}	145.15 ± 0.80^{a}	< 0.01	111.460	0.978
20:5 n-3	3.94 ± 0.06^{c}	5.01 ± 0.33^{bc}	4.09 ± 1.04^{c}	8.99 ± 0.01^{a}	6.00 ± 0.40^{b}	< 0.01	46.860	0.949
22:5 n-3	5.39 ± 0.01^{c}	9.29 ± 0.311^{b}	11.32 ± 1.36^{a}	13.00 ± 0.58^{a}	8.06 ± 0.16^{b}	< 0.01	51.941	0.954
22:6 n-3	48.03 ± 0.04^{d}	63.07 ± 0.240^{c}	65.64 ± 0.30^{c}	74.05 ± 0.41^{b}	81.92 ± 1.00^{a}	< 0.01	315.64	0.992
Σ n-3 PUFA	**198.9 7 ± 2.31**^{d}	**228.6 ± 0.54c**	**243.46 ± 3.52**^{b}	**241.47 ± 5.69**^{b}	**273.77 ± 0.45**^{a}	**< 0.01**	**473.046**	**0.995**
EPA + DHA	**51.97 ± 2.19**^{d}	**68.08 ± 0.75c**	**69.73 ± 1.28**^{c}	**83.04 ± 1.29**^{b}	**87.93 ± 0.60**^{a}	**< 0.01**	**598.073**	**0.996**

Values (n = 3) with different letters in the same row are significantly different (p < 0.05).
SP0, soybean meal only; SP5, soybean meal + 5% S. polyrhiza; SP10, soybean meal + 10% S. polyrhiza; SP15, soybean meal + 15% S. polyrhiza; SP20, soybean meal + 20% S. polyrhiza. The bold values means summation/total value.

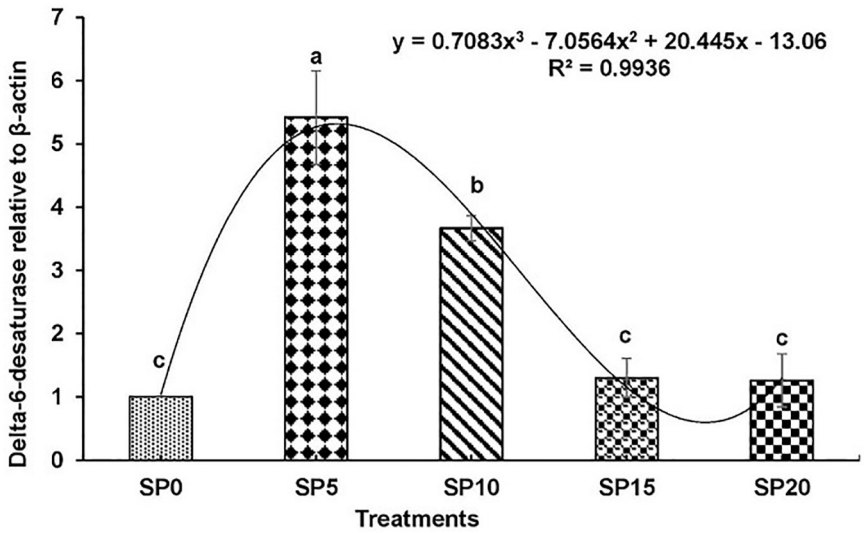


FIGURE 1 | Expression of delta-6-desaturase (*fads2d6)* relative to β-actin in hepatopancreas of five different diet-fed *Cyprinus carpio*. Bars with different superscripts are significantly different (*n* = 3). A polynomial (order 3) relationship was found between the diet and expression of *fads2d6* (R^2 = 0.994).

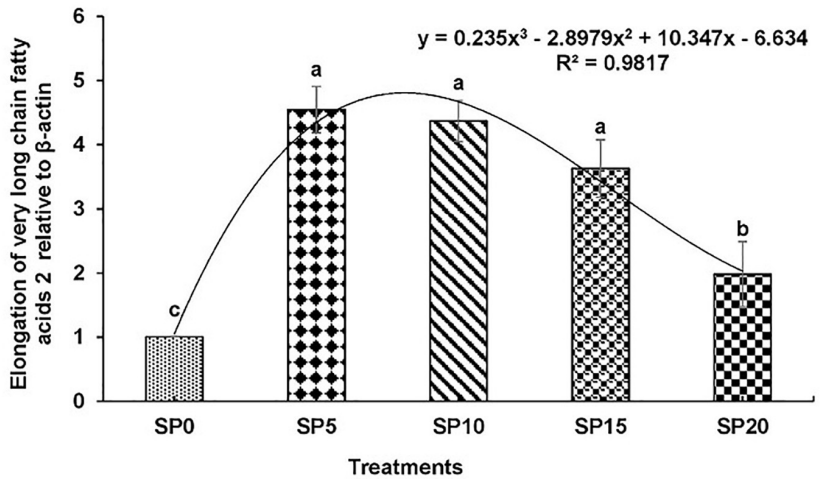


FIGURE 2 | Expression of elongation of very long chain fatty acids protein 2 (*elovl2*) relative to β-actin in hepatopancreas of five different diet-fed *Cyprinus carpio*. Bars with different superscripts are significantly different (n = 3). A polynomial (order 3) relationship was found between the diet and expression of *fads2d6* (R^2 = 0.982).

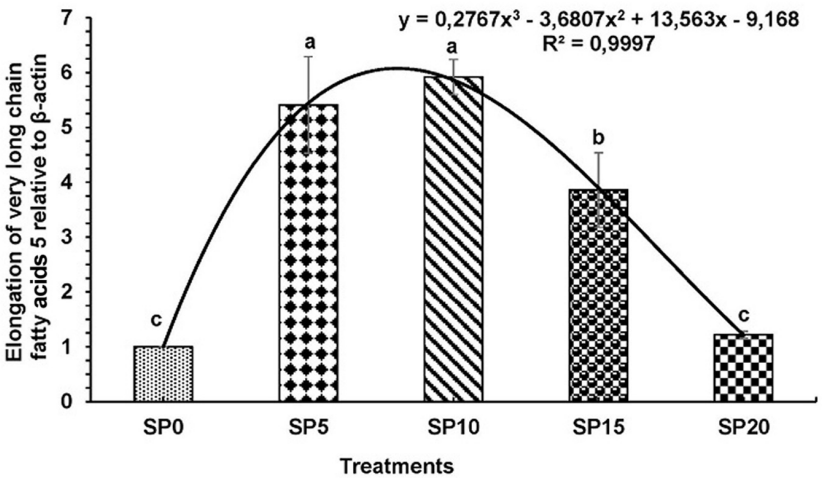


FIGURE 3 | Expression of elongation of very long chain fatty acids protein 5 (*elovl5*) relative to β-actin in hepatopancreas of five different diet-fed *Cyprinus carpio*. Bars with different superscripts are significantly different (n = 3). A polynomial (order 3) relationship was found between the diet and expression of *fads2d6* (R^2 = 0.999).

treatments. The α-amino-n-butyric acid and 3-methyl histidine were absent in SP0 and SP5 diet-fed fish. The β-amino-isobutyric and γ-amino-butyric acids were absent in SP5, SP10, and SP15 diet-fed common carp. The inclusion of greater duckweed in the carp diet showed linear relationships (R^2 = 0.745–0.998) with different amino acids, except α-amino-*n*-butyric acid and β -alanine.

Fatty Acid Composition

The feeding of common carp with greater duckweed supplemented diets influenced the fatty acid composition of fish (**Table 5**). Among saturated fatty acids (SFA), palmitic acid (16:0) was the dominant one regardless of treatments with myristic acid (14:0), the second most dominant SFA. Highest SFA was found in carp fed the SP5 diet. Monounsaturated fatty acids (MUFA) showed an inverse relationship with the inclusion level of greater duckweed in the diet. Significantly higher MUFA content was found in the control diet (SP0)-fed common carp compared with that of others. Among MUFA, oleic acid (18:1n-9) was the dominant one in all treatments. Nervonic acid (24:1) was absent in fish fed diets SP15 and SP20. The greater duckweed supplemented diets enhanced the n-6 PUFA and LOA (18:2 n-6) content in fish. The highest level of LOA was found in SP20 diet-fed fish. The n-3 PUFA contents of fish showed an increasing trend with the increasing inclusion of greater duckweeds in the diet. The ALA (18:3n-3), EPA (20:5n-3), docosapentanoic acid (22:5 n-3, DPA), and DHA (22: 6n-3) contents were significantly lower in SP0 diet fed common carp. The n-3 PUFA content was

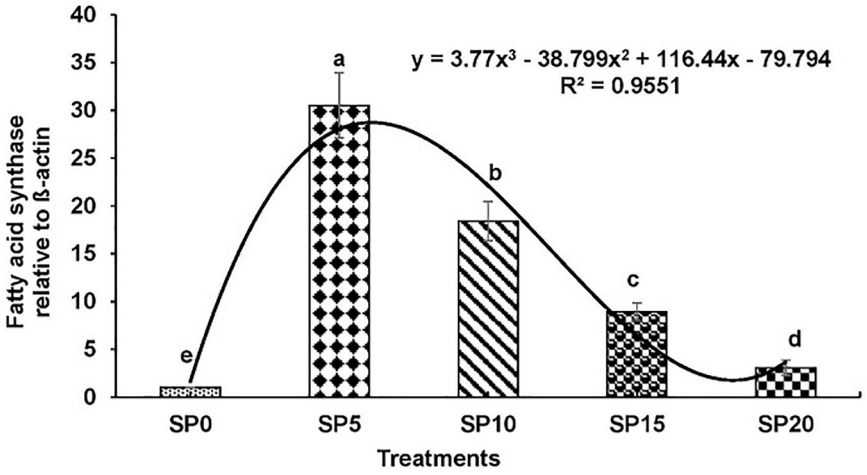


FIGURE 4 | Expression of fatty acid synthase (*fas*) relative to β-actin in hepatopancreas of five different diet-fed *Cyprinus carpio*. Bars with different superscripts are significantly different (n = 3). A polynomial (order 3) relationship was found between the diet and expression of *fads2d6* (R^2 = 0.955).

significantly higher in SP20 diet-fed common carp compared with other diet-fed fish. The inclusion of greater duckweed in the carp diet showed linear relationships (R^2 = 0.745–0.998) with different fatty acids, except stearic acid (18:0).

Gene Expression

Expressions of various genes involved in the metabolism of fatty acid were recorded in the hepatopancreas of common carp fed the five diets. The expression of *fads2d6 was* significantly higher in fish fed diet SP5 compared with others (**Figure 1**). The expression levels of *elovl2* and *elovl5* were significantly lower in SP20 diet-fed common carp compared with others (**Figures 2**, **3**). The significantly higher expression of *fas* was observed in SP5 compared with others (**Figure 4**). The mRNA expressions showed polynomial 3 order relationships with different treatments.

DISCUSSION

Performance of Common Carp

The effect of dietary inclusion of greater duckweed at four different levels (replacing soybean meal) on the performance of common carp was recorded in this experiment. The survival rate of fish was not affected with the inclusion of greater duckweed in the diets. The earlier study showed mixed results. The inclusion of greater duckweed more than 20% in diet resulted into mortality of tilapia (Fasakin et al., 1999, 2001), supplementation of *L. minor* (20%) in diets affected the survival rate of common carp (Yılmaz et al., 2004). El-Shafai et al. (2004) reported that the inclusion of duckweed in the feed of tilapia improved the survival rate of fish. The incorporation canola meal at 50% level in the diet was not affecting the survival rate of tilapia (Iqbal et al., 2021).

The broken-line regression showed that incorporation of greater duckweed at 10% level as a breakpoint for final weight of common carp, and it was 13.4% for SGR of fish. Highest growth performance was observed in SP20 diet-fed fish. Food was efficiently utilized in this treatment as minimum FCR was recorded. In *L. minor* supplemented diet-fed common carp and tilapia, similar trends of growth performance, SGR, and FCR were found (El-Shafai et al., 2004; Yılmaz et al., 2004). The inclusion of *L. polyrhiza* in the diets of mrigal *Cirrhinus mrigala* and rohu *Lebeo rohita* improved the weight gain, SGR, and FCR (Bairagi et al., 2002; Ghosh and Ray, 2014). Inclusion of fermented *L. minor* at 2.5% level and canola meal at 50% level increased the growth of tilapia (Herawati et al., 2020; Iqbal et al., 2021). In this study, the SGR of common carp (initial weight: 0.473–0.479 g) ranged from 2.01 to 2.93%. Similar results were reported in earlier study like, in *L. minor* supplemented diet-fed common carp (initial weight: 0.283–0.295 g), SGR ranged from 1.96 to 2.26% (Yılmaz et al., 2004), and in soy protein concentrate (SPC)-incorporated diet-fed common carp (initial weight: 2.43–2.47 g), SGR was 2.01–2.93% (Zhu et al., 2020). Xie et al. (2021) reported that feeding of common carp with diets containing fishmeal and ultra-micro-ground mixed plant proteins (uPP)-based diets resulted in 540–560% growth of fish after 112 days of culture. In this study with common carp, 238–474% weight gain of fish was recorded after 60 days of culture.

Digestive Enzyme Activities

The study of digestive enzyme activities in five different diet-fed common carp explained the reason of efficient utilization of consumed diet in SP20. Total protease and trypsin activities were maximum in SP20 diet-fed fish; considerable amylase and lipase activities were also found in SP20 diet-fed common carp. The digestive enzymes, namely, protease, lipase, and amylase played a significant role in digestion and absorption of nutrient (Zhou et al., 2010). Fish fed with different diets are able to adjust the activity of their digestive enzymes (Shiping and Zhao, 2005). The inclusion of duckweed in the diets of rohu and tilapia enhanced digestive enzyme activities like amylase, trypsin, and chymotrypsin (Goswami et al., 2020; Zhao et al., 2020).

Biochemical Composition of Fish

The proximate composition study showed that the inclusion (10–20%) of greater duckweed enhanced the crude protein content of common carp in this study. The crude lipid and ash contents of common carp increased in a graded manner with the enhanced inclusion of greater duckweed in the diet. An earlier study showed that supplementation of duckweed improved the crude protein and crude lipid contents in fish (El-Shafai et al., 2004; Yılmaz et al., 2004; Fasakin, 2008; Abou et al., 2011). Aslam et al. (2021) reported significantly higher crude protein contents in *L. minor*-incorporated diet-fed grass carp *Ctenopharyngodon idella* and silver carp *Hypophthalmichthys molitrix* compared with the *soybean*-supplemented diet-fed fishes. The inclusion of duckweed increased the ash content in fish (Fasakin et al., 1999; El-Shafai et al., 2004; Fasakin, 2008). This indicated that greater duckweed-supplemented diets fulfilled the nutritional requirements of common carp. The proximate composition study showed that the crude protein, crude lipid, and ash contents of greater duckweed were 36.65, 7.62, and 18.19 g/100 g (Sharma et al., 2019). The amino acid profile of greater duckweed is comparable with *soybean* meal. Feeding with greater duckweed-supplemented diets improved the non-essential amino acid contents in the common carp. The supplementation of fermented *L. minor* in the diet enhanced the lysine content in tilapia (Herawati et al., 2020).

In this study, fatty acid composition of fish was influenced by the supplementation of greater duckweed. Highest and lowest SFA contents were found in SP5 and SP20 diet-fed common carp, respectively. MUFA content showed an inverse relationship with the increased inclusion of greater duckweed in diet of common carp. A direct relationship was found between the amount of greater duckweed in the diet and n-6 PUFA and n-3 PUFA contents in common carp. Inclusion of greater duckweed in the diet enhanced the ALA, DHA, and EPA contents in common carp. The duckweeds are a rich source of fatty acids (Appenroth et al., 2017; Chakrabarti et al., 2018; Sharma et al., 2019). The feeding of *Azolla filiculoides* enhanced the total n-3 PUFA (especially EPA and DHA) content (Abou et al., 2011), and fermented *L. minor* enhanced LOA (Herawati et al., 2020) in Nile tilapia. Similarly, the contents of EPA and DHA in common carp increased linearly with increasing greater duckweed level in the diet.

Expression of Genes Involved in the Biosynthesis of Fatty Acids

In this study, the expression levels of key genes involved in the biosynthesis of fatty acids like *fads2d6*, *elovl2*, *elovl5,* and *fas* were evaluated in the common carp. Upregulation of all these genes was found in fish fed greater duckweed supplemented diets compared with control diet-fed fish. This might be due to the presence of LOA and ALA in the experimental diets. Earlier study showed that the higher contents of LOA and ALA upregulated the expression of desaturases/elongases (Tocher et al., 2004; Turchini et al., 2006; Francis et al., 2007; Li et al., 2008). However, an excess of ALA in diet can inhibit the transcription of *fads2d6* gene (Bell et al., 1993). In this study, among the fish fed the experimental diets, the highest expression levels of genes were recorded in the SP5 treatment, and then the expression gradually decreased. The ALA content increased with increasing inclusion of greater duckweed in diet. EPA and DHA contents increased with decreasing expression of genes *elovl2, elovl5,* and *fads2d6*. Similar results were found in common carp and rainbow trout *Oncorhynchus mykiss* where the expressions of desaturases and elongases were higher in fish with lower contents of EPA and DHA (Ren et al., 2012; Lazzarotto et al., 2018).

CONCLUSION

Greater duckweed (*S. polyrhiza*) may replace soybean meal up to 20% in the diet of *C. carpio* without affecting the digestive physiology and growth performance of fish even at an early life stage. Inclusion of greater duckweed enhanced growth performance and improved the quality of fish in terms of amino acids and n-3 PUFA, especially EPA and DHA.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Ethics Committee, University of DelhiDU/ZOOL/IAEC-R/2015/07.

AUTHOR CONTRIBUTIONS

RC, DT, JS, and BG designed the study. AS, RC, JS, and GK cultured the fish and analyzed the samples. JS, RC, PM, DT, BG, and AS performed statistical analysis and wrote the manuscript. AS, RC, JS, and GK prepared graphs and tables. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

The authors would like to thank the Department of Biotechnology (DBT), Government of India, New Delhi, India (Dy. No. 102/IFD/SAN/4678/2015-2016, dated 28.3.2016) and the Biotechnology and Biological Science Research Council (BBSRC) Newton Fund Global Research Partnership Project (BB/N005031/1) for providing financial support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.788455/full#supplementary-material

REFERENCES

Abou, Y., Fiogbé, E. D., Beckers, Y., and Micha, J. C. (2011). Approximate compositional values and tissue fatty acid profiles of Nile tilapia (*Oreochromis niloticus* L.) fed *Azolla*-diets in earthen ponds. *Food Nutr. Sci.* 2, 964–973. doi: 10.4236/fns.2011.29131

Aksnes, A., and Opstvedt, J. (1998). Content of digestible energy in fish feed ingredients determined by the ingredient-substitution method. *Aquaculture* 161, 45–53. doi: 10.1016/S0044-8486(97)00255-X

Alarcón, F. J., Díaz, M., Moyano, F. J., and Abellán, E. (1998). Characterization and funtional properties of digestive proteases in two sparids; gilthead sea bream (*Sparus aurata*) and common dentex (*Dentex dentex*). *Fish Physiol. Biochem.* 19, 257–267.

AOAC (2000). *Official Methods of Analysis*. Washington, DC: Association of Official Analytical Chemists Inc.

APHA (2017). *Standard Methods for the Examination of Water and Waste Water*, 22nd Edn. Washington DC: American Public Health Association, American Water Works Association, Water Environment Federation.

Appenroth, K. J., Sree, K. S., Böhm, V., Hammann, S., Vetter, W., Leiterer, M., et al. (2017). Nutritional value of duckweeds (*Lemnaceae*) as human food. *Food Chem.* 217, 266–273. doi: 10.1016/j.foodchem.2016.08.116

Aslam, S., Zuberi, A., Chan, M. W. H., and Mustaquim, J. (2021). Effect of *Lemna minor* and *Glycine max* on haematological parameters, glucose level, total protein content and anti-oxidant enzyme activities in *Ctenopharyngodon idella* and *Hypophthalmichthys molitrix*. *Aquac. Rep.* 19:100616. doi: 10.1016/j.aqrep.2021.100616

Bairagi, A., Ghosh, K. S., Sen, S. K., and Ray, A. K. (2002). Duckweed (*Lemna polyrhiza*) leaf meal as a source of feedstuff in formulated diets for rohu (*Labeo rohita* Ham.) fingerlings after fermentation with a fish intestinal bacterium. *Bioresour. Technol.* 85, 17–24. doi: 10.1016/s0960-8524(02)00067-6

Bassler, R., and Buchholz, H. (1993). *Amino Acid Analysis Methodenbuch, vol III, 4.1 1.1. Die chemische Untersuchung von Futtermitteln*. Darmstadt: VDLUFA-Verlag, 1–5.

Bell, J. G., Dick, J. R., Vicar, M. C., Sargent, J. R., and Thompson, K. D. (1993). Dietary sunflower, linseed and fish oils affect phospholipid fatty acid composition, development of cardiac lesions, phospholipase activity and eicosanoid production in Atlantic salmon. *Prostaglandins Leukot. Essent. Fatty Acids* 49, 665–673. doi: 10.1016/0952-3278(93)90075-8

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *J. Anal. Biochem.* 72, 248–254. doi: 10.1016/0003-2697(76)90527-3

Cao, M. J., Osatomi, K., Suzuki, M., Hara, K., Tachibana, K., and Ishihara, T. (2000). Purification and characterization of two anionic trypsins from the hepatopancreas of carp. *Fish. Sci.* 66, 1172–1179. doi: 10.1046/j.1444-2906.2000.00185.x

Castro, L. F. C., Tocher, D. R., and Monroig, Ó (2016). Long-chain polyunsaturated fatty acid biosynthesis in chordates: insights into the evolution of *fads* and *elovl* gene repertoire. *Prog. Lipid Res.* 62, 25–40. doi: 10.1016/j.plipres.2016.01.001

Chakrabarti, R. (2017). "Culture of zooplankton and aquatic macrophytes as non-conventional livelihood," in *Aquaculture for Nutritional and Livelihood Security*, eds R. Dhanze, A. S. Ninawe, and J. R. Dhanze (New Delhi: Narendra Publishing House), 189–203.

Chakrabarti, R., Clark, W. D., Sharma, J. G., Goswami, R. K., Shrivastav, A. K., and Tocher, D. R. (2018). Mass production of *Lemna minor* and its amino acid and fatty acid profiles. *Front. Chem.* 6:479. doi: 10.3389/fchem.2018.00479

Chakrabarti, R., and Rathore, R. M. (2009). Ontogenic changes in the digestive enzyme patterns and characterization of proteases in Indian major carp *Cirrhinus mrigala*. *Aquac. Nutr.* 16, 569–581. doi: 10.1111/j.1365-2095.2009.00694.x

Christie, W. W. (2003). *Lipid Analysis*, 3rd Edn. Bridgewater, UK: Oily Press.

Cruz, Y., Kijora, C., Wedler, E., Danier, J., and Schulz, C. (2011). Fermentation properties and nutritional quality of selected aquatic macrophytes as alternative fish feed in rural areas of the neotropics. *Livest. Res. Rural Dev.* 23, 239–246.

Cruz-Velásquez, Y., Kijora, C., Vergara-Hernández, W., and Schulz, C. (2014). On-farm evaluation of Cachama blanca and Nile tilapia fed fermented aquatic plants in a polyculture. *Orinoquia* 18, 269–277. doi: 10.1016/j.aquaculture.2020.735176

El-Shafai, S. A., El-Gohary, F. A., Verreth, J. A. J., Schrama, J. W., and Gijzen, H. J. (2004). Apparent digestibility coefficient of duckweed (*Lemna minor*), fresh and dry for Nile tilapia (*Oreochromis niloticus* L.). *Aquac. Res.* 35, 574–586. doi: 10.1111/j.1365-2109.2004.01055.x

FAO (2001). *Duckweed: a Tiny Aquatic Plant with Enormous Potential for Agriculture and Environment*. Geneva: Food and Agricultural Organization of the United Nations.

FAO (2020). *The State of World Fisheries and Aquaculture 2020. Sustainability in action*. Rome: Food and Agricultural Organization of the United Nations, doi: 10.4060/ca9229en

Fasakin, E. A. (2008). *Fish as food yesterday, today and forever. Inaugural Lecture Series 48*. Akure: The Federal University of Technology, 52.

Fasakin, E. A., Balogun, A. M., and Fagbenro, O. A. (2001). Evaluation of sun-dried water fern, *Azolla africana* and duckweed, *Spirodela polyrrhiza* in practical diets for Nile tilapia, *Oreochromis niloticus* fingerlings. *J. Appl. Aquac.* 11, 83–92. doi: 10.1300/J028v11n04_09

Fasakin, E. A., Balogun, A. M., and Fasuru, B. E. (1999). Use of duckweed, *Spirodela polyrrhiza* L. Schleiden, as a protein feedstuff in practical diets for tilapia, *Oreochromis niloticus* L. *Aquac. Res.* 30, 313–318. doi: 10.1046/j.1365-2109.1999.00318.x

Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226, 497–509. doi: 10.1007/s11745-002-1004-1

Fonseca-Madrigal, J., Karalazos, V., Campbell, P. J., Bell, J. G., and Tocher, D. R. (2005). Influence of dietary palm oil on growth, tissue fatty acid compositions and fatty acid metabolism in liver and intestine in rainbow trout (*Oncorhynchus mykiss*). *Aquac. Nutr.* 11, 241–250. doi: 10.1111/j.1365-2095.2005.00346.x

Francis, D. S., Turchini, G. M., Jones, P. L., and De Silva, S. S. (2007). Dietary lipid source modulates in vivo fatty acid metabolism in the freshwater fish, Murray cod (*Maccullochella peelii peelii*). *J. Agric. Food Chem.* 55, 1582–1591. doi: 10.1021/jf062153x

Gangadhar, B., Umalatha, H., Hegde, G., and Sridhar, N. (2017). Digestibility of dry matter and nutrients from three ingredients by the carps *Labeo fimbriatus* (Bloch, 1795) and *Cyprinus carpio* Linnaeus, 1758 with a note on digestive enzyme activity. *Indian J. Fish.* 64, 75–84. doi: 10.21077/ijf.2017.64.3.69091-11

Gatlin, D. M., Barrows, F. T., Brown, P., Dabrowski, K., Gaylord, T. G., Hardy, R. W., et al. (2007). Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquac. Res.* 38, 551–579. doi: 10.1111/j.1365-2109.2007.01704.x

Ghosh, P., and Ray, A. K. (2014). Effects of duckweed (*Lemna polyrhiza*) meal incorporated diet on enzyme producing autochthonous gut bacteria in fingerling mrigal, *Cirrhinus mrigala* (Hamilton). *Int. J. Fish. Aquat. Stud.* 2, 72–78.

Glencross, B. D. (2009). Exploring the nutritional demand for essential fatty acids by aquaculture species. *Rev. Aquac.* 1, 71–124. doi: 10.1111/j.1753-5131.2009.01006.x

Glencross, B. D., Baily, J., Berntssen, M. H. G., Hardy, R. W., Mackenzie, S., and Tocher, D. R. (2020). Risk assessment of the use of alternative animal and plant raw material resources in aquaculture feeds. *Rev. Aquac.* 12, 703–758. doi: 10.1111/raq.12347

Goswami, R. K., Shrivastav, A. K., Sharma, J. G., Tocher, D. R., and Chakrabarti, R. (2020). Growth and digestive enzyme activities of rohu *Labeo rohita* fed diets containing macrophytes and almond oil-cake. *Anim. Feed Sci. Technol.* 263:114456. doi: 10.1016/j.anifeedsci.2020.114456

Hansen, A. C., and Hemre, G. I. (2013). Effects of replacing fish meal and oil with plant resources in on-growing diets for Atlantic cod *Gadus morhua* L. *Aquac. Nutr.* 19, 641–650. doi: 10.1111/anu.12078

Hardy, R. W. (2010). Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. *Aquac. Res.* 41, 770–776. doi: 10.1111/j.1365-2109.2009.02349.x

Hasan, M. R., and Chakrabarti, R. (2009). *Use of Algae and Aquatic Macrophytes as Feed in Small-Scale Aquaculture. A Review. Food and Agricultural Organization Fisheries Technical Paper, 531*. Geneva: Food and Agricultural Organization.

Herawati, V. E., Pinandoyo, P., Darmanto, Y., Rismaningsih, N., and Radjasa, O. K. (2020). The effect of fermented duckweed (*Lemna minor*) in feed on growth and nutritional quality of tilapia (*Oreochromis niloticus*). *Biodivers. J.* 21, 3350–3358. doi: 10.13057/biodiv/d210759

Iqbal, M., Yaqub, A., and Ayub, M. (2021). Partial and full substitution of fishmeal and soybean meal by canola meal in diets for genetically improved farmed tilapia (*O. niloticus*): growth performance, carcass composition, serum biochemistry, immune response, and intestine histology. *J. Appl. Aquac.* 1–26. doi: 10.1080/10454438.2021.1890661

Kuhajda, F. P., Jenner, K., Wood, F. D., Hennigar, R. A., Jacobs, L. B., Dick, J. D., et al. (1994). Fatty acid synthesis: a potential selective target for antineoplastic therapy. *Proc. Natl. Acad. Sci. U. S. A.* 91, 6379–6383. doi: 10.1073/pnas.91.14.6379

Lazzarotto, V., Me'dale, F., Larroquet, L., and Corraze, G. (2018). Long-term dietary replacement of fishmeal and fish oil in diets for rainbow trout (*Oncorhynchus mykiss*): effects on growth, whole body fatty acids and intestinal and hepatic gene expression. *PLoS One* 13:190730. doi: 10.1371/journal.pone.0190730

Lee, J. W., Kil, D. Y., Keever, B. D., Killefer, J., McKeith, F. K., Sulabo, R. C., et al. (2013). Carcass fat quality of pigs is not improved by adding corn germ, beef tallow, palm kernel oil, or glycerol to finishing diets containing distillers dried grains with solubles. *J. Anim. Sci.* 91, 2426–2437. doi: 10.2527/jas.2012-5328

Li, Y., Hu, C., Zheng, Y., Xia, X., Xu, W., Wang, S., et al. (2008). The effects of dietary fatty acids on liver fatty acid composition and delta-6 desaturase expression differ with ambient salinities in *Siganus canaliculatus*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 151, 183–190. doi: 10.1016/j.cbpb.2008.06.013

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Mer, R. R., Vadher, K. H., and Patel, M. R. (2016). Effect of partial supplementation of duckweed (*Lemna minor*) on growth and survival of *Labeo rohita* (Hamilton, 1822) fry. *Bioscan Int. Q. J. Life Sci.* 11, 101–106.

Monroig, Ó, Lopes-Marques, M., Navarro, J. C., Hontoria, F., Ruivo, R., Santos, M. M., et al. (2016). Evolutionary wiring of the polyunsaturated fatty acid biosynthetic pathway. *Sci. Rep.* 6:20510. doi: 10.1038/srep20510

Muggeo, V. M. (2008). Segmented: an R package to fit regression models with broken-line relationships. *R News* 8, 20–25.

NRC (1998). *National Research Council. Nutrient Requirements of Swine*. Washington DC, USA: The National Academic Press.

NRC (2011). *Nutrient Requirements of Fish and Shrimp*. Washington, DC, USA: The National Academic Press.

Olsen, R. E., Hansen, A. C., Rosenlund, G., Hemre, G. I., Mayhew, T. M., Knudsen, D. L., et al. (2007). Total replacement of fish meal with plant proteins indiets for Atlantic cod (*Gadus morhua* L.) II-Health aspects. *Aquaculture* 272, 612–624. doi: 10.1016/j.aquaculture.2007.05.010

Rathore, R. M., Kumar, S., and Chakrabarti, R. (2005). Digestive enzyme profile of *Cyprinus carpio* during ontogenic development. *J. World Aquac. Soc.* 36, 37–39. doi: 10.1016/j.cbpc.2005.06.007

Ren, H. T., Yu, J. H., Xu, P., and Tang, Y. K. (2012). Influence of dietary fatty acids on muscle fatty acid composition and expression levels of Δ6 desaturase-like and elovl5-like elongase in common carp (*Cyprinus carpio* var. Jian). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 163, 184–192. doi: 10.1016/j.cbpb.2012.05.016

Roberts, I. M. (1985). Hydrolysis of 4-methylumbelliferyl butyrate: a convenient and sensitive fluorescent assay for lipase activity. *Lipids* 20, 243–247. doi: 10.1007/bf02534195

Rokey, G. (2004). "Raw material for extrusion processing," in *Feeds and Pet Food Extrusion Manual*, eds M. N. Riaz and M. Barron (College Station, TX: Taxas A & M University), 5.

Rodriguez, S. M., Olvera, N. M. A., and Carmona, O. C. (1996). Nutritional value of animal by-product meal in practical diets for Nile tilapia, *Oreochromis niloticus* (L) fry. *Aquac. Res.* 27, 67–73. doi: 10.1111/j.1365-2109.1996.tb00967.x

Sharma, J., Clark, W. D., Shrivastav, A. K., Goswami, R. K., Tocher, D. R., and Chakrabarti, R. (2019). Production potential of greater duckweed *Spirodela polyrhiza* (L. Schleiden) and its biochemical composition evaluation. *Aquaculture* 513:734419. doi: 10.1016/j.aquaculture.2019.734419

Sharma, J. G., Kumar, A., Saini, D., Targay, N. L., Khangembam, B. K., and Chakrabarti, R. (2016). *In vitro* digestibility study of some plant protein sources as aquafeed for carps *Labeo rohita* and *Cyprinus carpio* using pH-stat method. *Indian J. Exp. Biol.* 54, 606–611.

Shiping, S. U., and Zhao, X. W. (2005). The influence of different diets on the digestive enzyme activities of Amur sturgeon *Acipenseride schrencki Brandt* larvae. *J. Biol.* 22, 27–29.

Stadtlander, T., Förster, S., Rosskothen, D., and Leiber, F. (2019). Slurry-grown duckweed (*Spirodela polyrhiza*) as a means to recycle nitrogen into feed for rainbow trout fry. *J. Clean. Prod.* 228, 86–93. doi: 10.1016/j.jclepro.2019.04.196

Taşbozan, O., and Gökçe, M. A. (2017). "Fatty Acids in Fish," in *Fatty Acids*, ed. A. Catala (London: IntechOpen), 143–159. doi: 10.5772/68048

Tocher, D. R. (2010). Fatty acid requirements in ontogeny of marine and freshwater fish. *Aquac. Res.* 41, 717–732. doi: 10.1111/j.1365-2109.2008.02150.x

Tocher, D. R., Agaba, M., Hastings, N., Bell, J. G., Dick, J. R., and Teale, A. J. (2002). Nutritional regulation of hepatocyte fatty acid desaturation and polyunsaturated fatty acid composition in zebrafish (*Danio rerio*) and tilapia (*Oreochromis niloticus*). *Fish Physiol. Biochem.* 24, 309–320.

Tocher, D. R., Fonseca-Madrigal, J., Dick, J. R., Ng, W., Bell, J. G., and Campbell, P. J. (2004). Effects of water temperature and diet containing palm oil on fatty acid desturation and oxidation in hepatocytes and intestinal enterocytes of rainbow trout (*Onchorhynchus mykiss*). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 137, 49–63. doi: 10.1016/j.cbpc.2003.10.002

Tocher, D. R., and Harvie, D. G. (1988). Fatty acid compositions of the major phosphoglycerides from fish neural tissues; (n-3) and (n-6) polyunsaturated fatty acids in rainbow trout (*Salmo gairdneri*) and cod (*Gadus morhua*) brains and retinas. *Fish Physiol. Biochem.* 5, 229–239. doi: 10.1007/BF01874800

Torstensen, B. E., and Tocher, D. R. (2010). "The effects of fish oil replacement on lipid metabolism of fish," in *Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds*, eds G. M. Turchini, W. Ng, and D. R. Tocher (Boca Raton, Florida: Taylor & Francis, CRC Press), 405–437

Turchini, G. M., Francis, D. S., and De Silva, S. S. (2006). Fatty acid metabolism in the freshwater fish Murray cod (*Maccullochella peelii*) deduced by the whole-body fatty acid balance method. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 144, 110–118. doi: 10.1016/j.cbpb.2006.01.013

Ueberschär, B. (1988). Determination of the nutritional condition of individual marine fish larvae by analyzing their proteolytic enzyme activities with a highly sensitive fluorescence technique. *Meeresforschung* 32, 144–154.

Xie, D., Chen, C., Dong, Y., You, C., Wang, S., Monroig, Ó, et al. (2021). Regulation of long-chain polyunsaturated fatty acid biosynthesis in teleost fish. *Prog. Lipid Res.* 82:101095. doi: 10.1016/j.plipres.2021.101095

Yang, S., Luo, J., Long, Y., Du, J., Xu, G., Zhao, L., et al. (2019). Mixed diets reduce the oxidative stress of common carp (*Cyprinus carpio*): based on microRNA sequencing. *Front. Physiol.* 10:631. doi: 10.3389/fphys.2019.00631

Yılmaz, E., Akyurt, I., and Gunal, G. (2004). Use of duckweed *Lemna minor* as a protein feedstuff in practical diets for common carp, *Cyprinus carpio*, fry. *Turkish J. Fish. Aquat. Sci.* 4, 105–109.

Zhao, L., Luo, J., Liu, Q., Du, J., Yang, H., Li, B., et al. (2020). Different diets can affect the digestion and immunity of common carp (*Cyprinus carpio*) according to enzyme activity assay and transcriptome sequencing. *Aquaculture* 523:735176.

Zhou, X. Q., Zhao, C. R., and Lin, Y. (2010). Compare the effect of diet supplementation with uncoated or coated lysine on juvenile Jian carp (*Cyprinus carpio* Var. Jian). *Aquac. Nutr.* 13, 457–461. doi: 10.1111/j.1365-2095.2007.00498.x

Zhu, R., Lia, L., Lia, M., Yua, Z., Wanga, H., and Wu, L. (2020). The effects of substituting fish meal with soy protein concentrate on growth performance, antioxidant capacity and intestinal histology in juvenile golden crucian carp, *Cyprinus carpio* × *Carassius auratus*. *Aquac. Rep.* 18:100435. doi: 10.1016/j.aqrep.2020.100435

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 in Marine Science

ORIGINAL RESEARCH
published: 18 February 2022
doi: 10.3389/fmars.2022.814233

Spray-Dried Porcine Plasma Promotes the Association Between Metabolic and Immunological Processes at Transcriptional Level in Gilthead Sea Bream (*Sparus aurata*) Gut

Eva Vallejos-Vidal[1,2,3†], Sebastián Reyes-Cerpa[4,5†], Lluis Tort[1], Javier Polo[6], Felipe E. Reyes-López[1,2] and Enric Gisbert[7]*

[1] Department of Cell Biology, Physiology, and Immunology, Universitat Autònoma de Barcelona, Barcelona, Spain, [2] Centro de Biotecnología Acuícola, Facultad de Química y Biología, Universidad de Santiago de Chile, Santiago, Chile, [3] Facultad de Medicina Veterinaria y Agronomía, Universidad de Las Américas, Santiago, Chile, [4] Centro de Genómica y Bioinformática, Facultad de Ciencias, Universidad Mayor, Santiago, Chile, [5] Escuela de Biotecnología, Facultad de Ciencias, Universidad Mayor, Santiago, Chile, [6] APC Europe SL, Granollers, Spain, [7] Aquaculture Program, Centre de Sant Carles de la Ràpita (IRTA-SCR), Institut de Recerca i Tecnologia Agroalimnetaries, Sant Carles de la Rápita, Spain

The spray-dried porcine plasma (SDPP) is an abattoir by-product used in animal nutrition with beneficial effects reported in livestock and commercial aquatic species. Previous results have found that the dietary inclusion of SDPP in gilthead sea bream (*Sparus aurata*) increased the density of intestinal goblet cells, and it did not result in significant changes in the autochthonous microbiota. However, there is no comprehensive data on the mechanisms that could take place on the intestine of gilthead sea bream fed with an SDPP-supplemented diet. For this reason, this study aimed to unveil the biological mechanisms modulated in response to the dietary administration of SDPP in the gilthead sea bream gut. To achieve this goal, we made a microarrays-based transcriptomic approach in gut samples from gilthead sea bream fed with an SDPP-supplemented diet for 95 days. As control diet, we used a protein-rich commercial feed (51% crude protein, 17% crude fat, and 20.6 MJ/kg gross energy) which was supplemented with 3% SDPP at the expense of LT70 fishmeal. The microarray analyses showed a total of 803 (468 up- and 335 down-regulated) differential expressed genes (DEGs). The functional network analysis revealed that dietary inclusion of SDPP induced sustained changes in 120 biological processes, grouped in 12-clusters. Among them, the metabolic-related process (cellular catabolic process, organic substance catabolic process, protein metabolism process), protein transport, and leukocyte mediated immunity interacted in the leading interactome network. This evidence confirms the previous evidence of the enhancement of the mucosal health status in response to the dietary administration of SDPP and provides further understanding of the mode of action of this ingredient in aquafeeds.

Keywords: teleost fishes, dietary additives, immune response, mucosal-associated lymphoid tissue (MALT), spray-dried porcine plasma (SDPP), aquaculture

OPEN ACCESS

Edited by:
Samad Rahimnejad, University of South Bohemia in České Budějovice, Czechia

Reviewed by:
Omid Safari, Ferdowsi University of Mashhad, Iran
Francisco Javier Moyano, University of Almería, Spain
Jaume Pérez-Sánchez, Spanish National Research Council (CSIC), Spain

****Correspondence:***
Felipe E. Reyes-López
felipe.reyes.l@usach.cl;
felipe.reyes@uab.cat

† These authors share first authorship

Specialty section:
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

Received: *13 November 2021*
Accepted: *07 January 2022*
Published: *18 February 2022*

Citation:
Vallejos-Vidal E, Reyes-Cerpa S, Tort L, Polo J, Reyes-López FE and Gisbert E (2022) Spray-Dried Porcine Plasma Promotes the Association Between Metabolic and Immunological Processes at Transcriptional Level in Gilthead Sea Bream (Sparus aurata) Gut. Front. Mar. Sci. 9:814233. doi: 10.3389/fmars.2022.814233

INTRODUCTION

In fish, the intestine is a complex multifunctional organ with critical physiological roles, including water and electrolyte homeostasis, endocrine modulation, metabolism and immune regulation, and commensal microbiota balance (Salinas and Parra, 2015; Khansari et al., 2018; Firmino et al., 2021c; Salomón et al., 2021). Notably, the intestine is one of the leading portals of pathogens' entry into the organism. Accordingly, the intestine has a mucus layer that acts as a physical and chemical barrier (Parra et al., 2015); thus, protecting the gut epithelium by defense mechanisms that help for maintaining the tissue homeostasis (Ellis, 2001; Gomez et al., 2013). The intestine is considered a mucosal-associated lymphoid tissue (MALT) because of its capacity for controlling the innate and adaptive immune response at the mucosal site [also called gut-associated lymphoid tissue (GALT)] (Parra et al., 2015).

Functional feeds are achieving significant attention globally due to their growth-promoting, antimicrobial, immunostimulant, antioxidant, anti-inflammatory, and sedative properties (Reyes-Cerpa et al., 2018; Firmino et al., 2021b). Accordingly, the nutritional requirements must be fully integrated into management strategies for aquaculture (Tacchi et al., 2011). Considerable advances have been made in studying nutritional requirements and sustainable feeding in fish, with deep knowledge about the optimal dietary composition for most important cultured fish species. Furthermore, the aquaculture industry has developed functional feeds to improve growth, immune response; physiological functions, and health performance in fishes that promote specific benefits to the host beyond the primary nutritional requirement. Functional feed additives include prebiotics, probiotics, vitamins, minerals, and plant, animal, or algae extracts (Parra et al., 2015; Salinas and Parra, 2015; Khansari et al., 2018; Salomón et al., 2021). The spray-dried porcine plasma (SDPP) is an abattoir by-product used in animal nutrition due to its excellent amino acid profile and digestibility close to 99% (Bureau et al., 1999). Several studies in livestock have shown that SDPP has positive effects on productivity and physiological parameters, immunity, and gut health in pigs and poultry (Ellis, 2001; Gomez et al., 2013; Reyes-Cerpa et al., 2018; Firmino et al., 2021b). In addition, its beneficial effect has also been reported in commercial aquatic species such as rainbow trout (*Oncorhynchus mykiss*) (Campbell et al., 2003), gilthead seabream (*Sparus aurata)* (Gisbert et al., 2015; Reyes-López et al., 2021), and Nile tilapia (*Oreochromis niloticus*) (de Araújo et al., 2017). In these fish species, SDPP has shown a repertoire of beneficial effect attributes on the host, including an improvement in somatic growth performance, digestibility, feed intake, feed efficiency, immunity, and improved antioxidant system capacity (Johnson and Summerfelt, 2000; Gisbert et al., 2015; de Araújo et al., 2017; Reyes-López et al., 2021). Furthermore, the use of porcine blood derivatives has environmental benefits for the sustainability and circular economy associated with animal production. It helps to provide added value to the industry and has less carbon footprint than plant protein sources that may be used in aquafeeds.

The importance of the diet lies not only in its nutritional contribution to the host but also in protecting the fish by maintaining intestinal health and improving digestion and immunity (Dawood, 2021). A previous report showed the inclusion of SDPP in the diet for gilthead sea bream promoted their somatic growth and increased intestinal goblet cells' density, and it did not result in major changes in autochthonous microbiota (Tapia-Paniagua et al., 2020). However, there is no comprehensive data on the mechanisms that could take place on the intestine of gilthead sea bream fed with an SDPP-supplemented diet. For that reason, this study aimed to unveil the biological mechanisms modulated by the dietary administration of SDPP in the gilthead sea bream gut. To do it, we used a microarrays-based transcriptomic approach to increase the coverage of those modulated processes by a high throughput screening strategy.

MATERIALS AND METHODS

Ethics Statement

Animal experimental procedures were conducted in compliance with the research protocol approved by the IRTA's Committee of Ethics and Animal Experimentation and following the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals.

Diets

Two diets were formulated as follows: (1) a control diet (Diet C) containing 51% crude protein, 17% crude fat, and 20.6 MJ/kg gross energy that fulfill the nutritional requirements of juvenile sea bream; (2) the SDPP diet was manufactured using this basal formulation, substituting Fishmeal LT70 by 3% SDPP (Apetein GS, APC Europe SL, Granollers, Spain; **Table 1**). The inclusion of 3% SDPP was used in this study because the evidence from a previous study showing an improved fish growth performance in terms of BWf, SLf, K, and SGRBW compared to 6% SDPP inclusion (Gisbert et al., 2015). The diets were manufactured by extrusion at SPAROS LDA (Olhão, Portugal). All powder ingredients were mixed accordingly to the target formulation in a double-helix mixer (model 500L, TGC Extrusion, France) and ground (<400 μm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Diets (pellet size: 2.5 mm) were manufactured with a twin-screw extruder (model BC45, Clextral, France) with a screw diameter of 55.5 mm. Extrusion conditions were as follows: feeder rate (80–85 kg/h), screw speed (247–266 rpm), water addition in barrel 1 (345 mL/min), temperature in barrel 1 (32–34°C), temperature in barrel 2 (59–62°C) and temperature in barrel 3 (111–114°C). Extruded pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). After cooling, oils were added by vacuum coating (model PG-10VCLAB, Dinnissen, Netherlands). Coating conditions were: pressure (700 mbar), spraying time under vacuum (approximately 90 s), and return to atmospheric pressure (120 s). Immediately after coating, diets were packed in sealed plastic buckets and shipped to IRTA research facilities (Sant Carles de la Ràpita, Spain).

TABLE 1 | Ingredient list and proximate composition on a dry weight basis (%) of experimental diets.

	Diets	
Ingredients (%)	**Control**	**SDPP**
Fishmeal LT 70	36.90	33.35
Fishmeal 60	12.50	12.50
P 90	4.00	4.00
Squid meal	6.00	6.00
Appetein GS		3.00
Wheat Gluten	7.60	7.60
Soybean meal 48 (micronized)	7.00	7.00
Wheat meal	7.70	7.70
Pea starch	4.50	4.80
Fish oil	11.20	11.45
Vitamin and Mineral Premix PV01	1.00	1.00
Choline chloride	0.10	0.10
Soy lecithin	0.50	0.50
Binder (guar gum)	1.00	1.00
Total	**100.00**	**100.00**
Proximate composition basis	**CTRL**	**SDPP**
Crude protein (%)	51.09	51.11
Crude fat (%)	17.17	17.16
Fiber (%)	0.51	0.51
Ash (%)	11.75	11.38
Gross Energy (MJ/kg) *	20.56	20.69
Amino acid composition (as feed basis)		
Histidine	1.05	1.08
Isoleucine	2.00	2.02
Leucine	3.76	3.85
Lysine	3.52	3.60
Threonine	2.35	2.40
Tryptophan	0.54	0.56
Valine	2.32	2.39
Methionine + Cysteine	1.82	1.84
Phenylalanine + Tyrosine	4.32	4.42
Taurine	0.16	0.15

**Gross energy content was estimated by using the following: total carbohydrate = 17.2 J/kg, fat = 39.5 J/kg and protein = 23.5 J/kg.*

Fish and Experimental Design

Gilthead seabream fry (average body size 9.5 g) were obtained from a commercial hatchery (Piscimar SL, Andromeda Group, Spain) and transported by road to IRTA-Sant Carles de la Ràpita research facilities (Sant Carles de la Ràpita, Spain). Fish were acclimated for 2 weeks into 2,000-L tanks. After their acclimation period, all fish were anesthetized [tricaine methanesulfonate (MS-222), 150 mg/L] and individually weighed for initial body weight (BWi) and measured for standard length (SLi) to the nearest 0.1 g and 1 mm, respectively. Then, fish were distributed into eight 500-L cyclindroconical tanks at a density of 50 fish per tank (4 tanks/replicates per diet).

Fish (BWi = 10.6 ± 0.1 g, n = 400, mean ± standard deviation, SD) were fed during 95 days with C and SDPP diets using automatic feeders (ARVO-TEC T Drum 2000; Arvotec, Huutokosk, Finland) at a rate of 2.5% of the stocked biomass, which approached apparent satiation. The daily feed ratio was evenly distributed in 7 meals per day from 08:00 to 18:00 h. During the trial, water temperature and pH (pH meter 507; Crison Instruments, Barcelona, Spain), salinity (MASTER-20T; ATAGO Co., Ltd., Tokyo, Japan), and dissolved oxygen (OXI330; Crison Instruments) were 22.1 ± 0.4°C, 7.0 ± 0.01, 36 mg/L, and 7.2 ± 0.3 mg/L (mean ± SD), respectively. The water flow rate was maintained at approximately 9.0–10.1 L/min in a recirculation system (IRTAmar®; Spain) that provided the experimental tanks with adequate water quality (total ammonia and nitrite were ≤ 0.15 and 0.6 mg/L, respectively) through UV, biological, and mechanical filtration. Photoperiod followed natural changes according to the season of the year (November to February; 40°37′41″ N). Fish were regularly sampled monthly to evaluate their growth in BW and adjust the feeding ratio to stocked biomass.

TABLE 2 | Somatic growth and feed efficiency in gilthead sea bream (*Sparus aurata*) fed diets containing spray-dried porcine plasma (SDPP).

	Control diet	SDPP diet (3.0%)
Body weight (BW) (g)	82.7 ± 3.2^{b}	88.2 ± 1.6^{a}
Standard length (cm)	14.6 ± 0.2	14.8 ± 0.1
Fulton's condition factor	2.66 ± 0.6	2.72 ± 0.3
Specific growth rate (% BW/day)	1.63 ± 0.03^{b}	1.70 ± 0.04^{a}
Food conversion ratio	1.21 ± 0.05^{a}	1.09 ± 0.07^{b}

Different letters within the same row indicate the presence of statistically significant differences between two experimental groups (t-test; $p < 0.05$). Data are expressed as mean ± standard deviation (n = 4).

At the end of the trial, fish were anesthetized as described above. All fish in experimental tanks were measured for final BW and SL as indicated. Then, 10 fish per tank were sacrificed with an overdose of MS-222 (200 mg/L) for tissue sampling purposes. The intestinal cavity was exposed, and the intestine was removed with a scalpel. A small piece of the anterior intestine (0.5 cm) was dissected and immediately transferred into RNAlater (Ambion®) incubated at 4°C overnight and frozen at −80°C until further RNA extraction. The anterior intestine was chosen because of its immunological relevance in comparison with other intestinal sections (Oehlers et al., 2011; Salomón et al., 2021). Fish growth and feed utilization from Control and SDPP groups were evaluated using the following indices: Fulton's condition factor (K) = $(BW/SL^3) \times 100$; specific growth rate in BW (SGR,%) = [(ln BWf −ln BWi) × 100]/time (d) and feed conversion ratio (FCR, g/g) = FI/(Bf −Bi), where FI was the total feed intake during the experimental period (g) and, Bi and Bf were the initial and final biomass (g), respectively. In particular, BWf and SGR values were 6.2 and 4.1% higher in fish fed the SDPP diet compared to the control group ($p < 0.05$), respectively. No significant differences were found in terms of standard length (SL) and the condition factor K ($p > 0.05$). In addition, FCR values were lower in fish fed the SDPP diet in comparison to those fed the control diet ($p < 0.05$) (**Table 2**). These data have been previously reported elsewhere (Reyes-López et al., 2021).

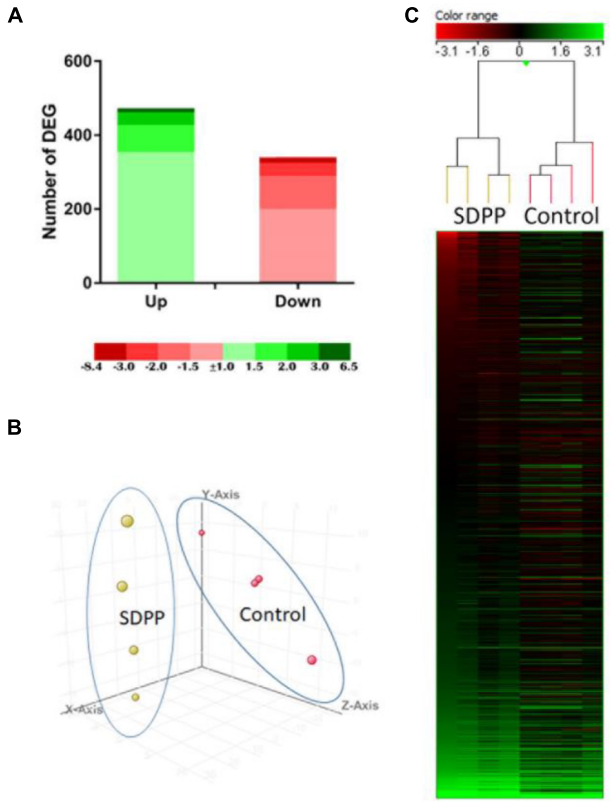


FIGURE 1 | Anterior intestine microarrays-based transcriptomic analysis for gilthead sea bream fed the SDPP diet. **(A)** Number of total differential expressed genes (DEGs). The green (upregulation) and red (downregulation) color scheme indicate the gene modulation according to its magnitude interval (fold-change). **(B)** Principal component analysis (PCA). Control (red spheres) and SDPP groups (yellow spheres) are represented. Each array included in the study is represented by one sphere. **(C)** Hierarchical clustering heatmap representing the 803 DEGs. The results of each microarray analyzed for the control and SDPP group are shown. The green (up-regulation) and red (down-regulation) color scheme indicates the gene modulation according to its magnitude interval (fold-change).

Transcriptional Analysis

Transcriptomic analysis was conducted to determine differences in gene expression level in the gut of the fish feed the control and SDPP diets at the end of the feeding trial (95 days). In particular, total RNA was extracted individually from fish gut using QIAGEN RNeasy® Mini Kit following the manufacturer's recommendations. Total RNA concentration was quantified using a NanoDrop ND-2000 (Thermo Fisher Scientific), and RNA quality and integrity were checked with Experion (Automated Electrophoresis Station, Bio-Rad) using the Experion Standard Sens RNA chip (Bio-Rad). Samples with an RNA integrity number (RIN) $>$ 8.0 were chosen for further analysis. Transcriptional analysis was carried out using the AquaGenomic *Sparus aurata* oligonucleotide microarray (SAQ) platform. The complete information on this platform is available through the public repository Gene Expression Omnibus (GEO) (accession numbers GPL13442) at the United States National Center for Biotechnology Information (NCBI). For each experimental group, total RNA samples were pooled (n = 4 pools each group; n = 4 fish each pool, including one fish randomly taken from each experimental tank) at a final concentration (133 ng/μL each pool). This methodological approach of pooling RNA from four different specimens in each replicate allowed authors to evaluate the population's variability. However, the information regarding individual variability was lost with this choice. One-color microarray was carried out according to the manufacturer's protocols and as previously described by our group (Reyes-López et al., 2021). Microarray slides were scanned with Agilent Technologies Scanner model G2505B. Spot intensities and other quality control features were extracted with Agilent's Feature Extraction software version 10.4.0.0 (Agilent Technologies). Quality reports were checked for each array. Although validation by mean of qPCR is required when there is a high risk of obtaining paralog genes and unspecific hybridization; in our study, we used an oligonucleotide-based microarray, which has probes with a reduced number of bases and high affinity, which avoids the necessity of conducting the validation of gene expression results by qPCR.

The extracted raw data were imported and analyzed with GeneSpring (version 14.5 GX software, Agilent Technologies). To standardize the arrays intensity, 75% percentile normalization was used, and data were filtered by expression levels. The differential expressed genes (DEGs) were obtained from a gene-level differential expression analysis. Expression values with a p-value $<$ 0.05 were considered statistically significant. The DEGs were grouped according to their fold-change value (p-value $<$ 0.05) and represented using the GraphPad software v7.0 for Windows. The Principal Component Analysis (PCA) was carried out using GeneSpring software (Agilent), four eigenvectors were calculated to describe the aggrupation of the control and SDPP groups in a 3D plot. The gene expression values (log2-expression ratios) were represented by a hierarchical clustering heatmap analysis using GeneSpring software.

Functional Network Analyses: Interactomes

The complete map of interactions that can occur in a living organism (interactome) was obtained from the DEGs obtained in the microarrays-based transcriptomics analysis. For this purpose, the Search Tool for the Retrieval of Interacting Genes (STRING) public repository version 10.0[1] was used. A Protein-Protein interaction (PPI) network for the differentially expressed genes was conducted with a high-confidence interaction score (value = 0.4). The mechanisms of response in which DEGs are involved were obtained from a comparative analysis based on *Homo sapiens* as a reference organism to extract the maximum information currently available. Thus, an ortholog *H. sapiens* Entrez Gene acronym was assigned based on sequence homology. Briefly, we selected the best tBlastX (NCBI) hit between the DEGs and DSPs query sequence for *Sparus aurata* and the human transcriptome database. We only consider those matches with at least E value $\leq$ 1e-10. The Uniprot and Genecards

[1]https://string-db.org

databases were used to confirm the gene acronym tag match between both species. Gene ontology (GO) pathway enrichment analysis for biological processes (GO_BiologicalProcess-EBI-UniProt-GOA-ACAP-ARAP_10.11.2020_00h00) was obtained using ClueGO v2.5.7 app through Cytoscape 3.8.2 platform. The Statistical analysis used was Enrichment/Depletion (two-sided hypergeometric test) with a *P*-value cut-off = 0.05 and corrected by Benjamin-Hochberg. A GO Fusion was performed to avoid redundant terms with a Kappa Score Threshold = 0.4 to propose more stringent GO terms associated with the mechanism of response for the experimental diet. In addition, grouping the GO terms was conducted when the sharing groups' percentage was above 50, a *P*-value of < 0.05 was considered significant. The statistically significant GOs obtained from the enrichment analysis were assigned to each one of the nodes represented in the functional network. The nodes classified in different clusters according to their functionality were represented with ClueGo v2.5.7. Later, the MCC (Maximal Clique Centrality) of each node was calculated by CytoHubba (version 0.1) to find hub nodes (Chin et al., 2014). Those genes with the top 10 MCC values were considered hub genes.

Statistical Analysis

An unpaired *t*-test was conducted for microarrays data using the GeneSpring software GX 14.5 to detect DEGs ($p < 0.05$) between the control and SDPP groups. The DEGs were represented with GraphPad software version 7.0. The PCA analysis and the heatmap for DEGs were obtained from GeneSpring software version 14.5 (Agilent Technologies). The hierarchical map was clustered by the normalized intensity values ($p < 0.05$), with Euclidean distance and Ward linkage.

RESULTS

Intestine Transcriptional Response From Fish Fed Spray-Dried Porcine Plasma -Supplemented Diet

The transcriptional analysis of the anterior intestine was carried out using the *S. aurata* oligonucleotide custom microarray, and the results are shown in **Figure 1**. A total of 803 DEGs were found in the anterior intestine of fish fed the SDPP diet. From the total number of DEGs, 441 DEGs were annotated, and 362 were unknown genes. Among them, 468 DEGs were up-regulated; in particular, 350 DEGs showed a modulation between 1.0- and 1.5-fold change (FC), 73 DEGs a modulation between 1.5 and 2.0 FC, 34 DEGs a modulation between 2.0 and 3.0, and 11 DEGs with a modulation higher than 3.0 FC. On the other hand, a total of 335 DEGs were down-regulated with 195 DEGs with modulation between −1.0 and −1.5 FC, showing 90 DEGs a modulation between −1.5 and −2.0 FC, 35 DEGs with modulation between −2.0 and −3.0, and 15 DEGs with a modulation lower than −3.0 FC (**Figure 1A**). The PCA analysis divided the dataset into three principal components and revealed a clear grouping between the microarrays for each of the tested diets (**Figure 1B**). The hierarchical clustering heatmap found a clear differential expression profile with a clear set of DEGs of fish fed with the control diet (**Figure 1C**; the top half of the panel) compared to those fed the SDPP diet (**Figure 1C**; bottom half).

In addition, a functional network analysis was carried out based on the DEGs obtained from the transcriptional analysis of gut samples from fish fed with the SDPP diet. From the 441 annotated DEGs, we got a functional association between 430 DEGs with 2,688 interactions (edges). In particular, 393 DEGs belonged to the central core interaction network (233 up-regulated DGEs; 160 down-regulated DEGs), while 37 DEGs (20 up-regulated DEGs; 17 down-regulated DEGs) did not have direct interaction with the central core (**Figure 2**).

To identify the function of these DEGs, a gene ontology (GO) pathway enrichment analysis was performed with the ClueGO platform. The results showed that the dietary inclusion of SDPP induced changes in 120 biological processes, grouped in 12 clusters. The sharing nodes between clusters are considered evidence of the interacting networks within biological processes. The most significant biological term names each cluster. For instance, the "Cellular catabolic process (GO:0044248)" is the most abundant category, with 69 terms corresponding to 42.3% of the total retrieved terms. Other biological processes clusters were the "Energy derivation by oxidation of organic compounds (GO:0015980)" with 16 terms corresponding to 9.8% of the total; the "Response to organic substance (GO:0010033)" with 15 terms related to 9.2%; the "Protein transport (GO:0015031)" with 12 terms corresponding to 74.4%; the "Leukocyte mediated immunity (GO:0002443)" with 11 terms related to a 6.8%; the "Organic substance catabolic process (GO:1901575)" and "Protein metabolic process (GO:0019538)" both with 10 terms corresponding to a 6.1%. Biological processes representing less than 5% of the total terms obtained were: the "Carboxylic acid metabolic process (GO:0019752)" with 6 GO terms corresponding to 3.7%; the "Generation of precursor metabolites and energy (GO:0006091)" and the "Protein catabolic process (GO:0030163)" both with five terms corresponding to 3.1%; the "Protein containing complex subunit organization (GO:0043933)" with three terms related to 1.8%; and the "G protein-coupled receptor signaling pathway (GO:0007186)" with one-term corresponding to a 0.6% of the total of enriched biological process (**Figures 3**, **4**).

According to the data obtained from biological processes GO terms, the functional network showed a strong association between leukocyte-mediated immunity, protein transport, cellular catabolic process, protein metabolic process and organic substance catabolic process. Another association was also registered between carboxylic acid metabolic processes, energy derivation by oxidation of organic compounds, and generation of precursor metabolites and energy (**Figure 3**).

From the enrichment analysis performed by the ClueGO platform, those DEGs belonging to the most representative GO terms from the biological process were represented in an individual functional network (**Figure 5**). The diet containing SDPP resulted in the positive regulation for the "G protein-coupled receptor signaling pathway" (7 up-regulated DEGs; 4 down-regulated DEGs) (**Figure 5L**), the "Protein containing

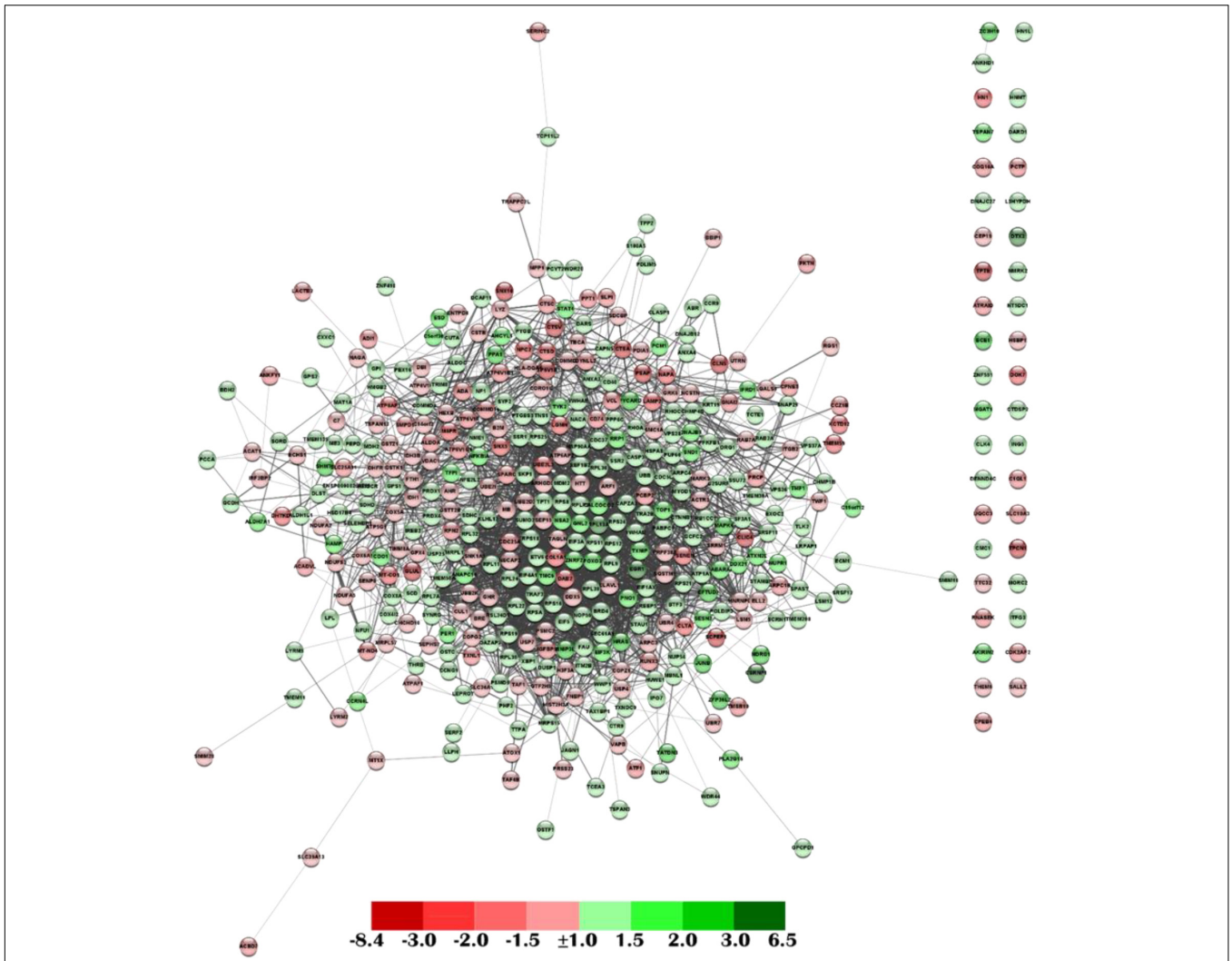


FIGURE 2 | Functional network analysis from the anterior intestine of the gilthead sea bream fed the SDPP diet. Each node represents one differential expressed gene (DEG) obtained from the anterior intestine transcriptomic analysis. The DEG modulatory profile is represented with green (up-regulated) and red (down-regulated) into each node according to its fold-change magnitude interval. The green (up-regulation) and red (down-regulation) color scheme indicates the gene modulation according to its magnitude interval (fold-change).

complex subunit organization" (45 up-regulated DEGs; 37 down-regulated DEGs) (**Figure 5K**), the "Protein metabolic process" (121 up-regulated DEGs; 65 down-regulated DEGs) (**Figure 5G**), the **"**Organic substance catabolic process" (80 up-regulated DEGs; 45 down-regulated DEGs) (**Figure 5F**), the "Protein transport" (66 up-regulated DEGs; 34 down-regulated DEGs) (**Figure 5D**), the **"**Response to organic substance" (80 up-regulated DEGs; 61 down-regulated DEGs) (**Figure 5C**), and the "Cellular catabolic process" (80 up-regulated DEGs; 43 down-regulated DEGs) (**Figure 5A**). In addition, the diet containing SDPP resulted in a balanced regulation (i.e., similar number of up- and down-regulated DEGs) for the "Energy derivation by oxidation of organic compounds" (14 up-regulated DEGs; 14 down-regulated DEGs) (**Figure 5B**), the "Leukocyte mediated immunity" (23 up-regulated DEGs; 27 down-regulated DEGs) (**Figure 5E**), the "Carboxylic acid metabolic process" (29 up-regulated DEGs; 23 down-regulated DEGs) (**Figure 5H**), the "Generation of precursor metabolites and energy" (20 up-regulated DEGs; 18 down-regulated DEGs) (**Figure 5I**), and the "Protein catabolic process" (22 up-regulated DEGs; 27 down-regulated DEGs) (**Figure 5J**). Notably, in almost all the functional networks identified for the DEGs belonging to the selected GO enrichment from the biological process, the up-regulated genes show a close interaction (**Figures 5A,D,F,G,J,K**).

The top 10 hub genes with a high level of correlation for the 12 clusters obtained from the enriched biological functions were selected with CytoHubba software. Thus, from the "Cellular catabolic process", 10 transcripts have a higher level of connection in the network (*rps8, rps24, rps18, rps16, rps11,rps12, rpl7a, rpl11, rpl13A, rpl29*). Importantly, all these transcripts were up-regulated (**Figure 6A**). For the cluster "Energy derivation

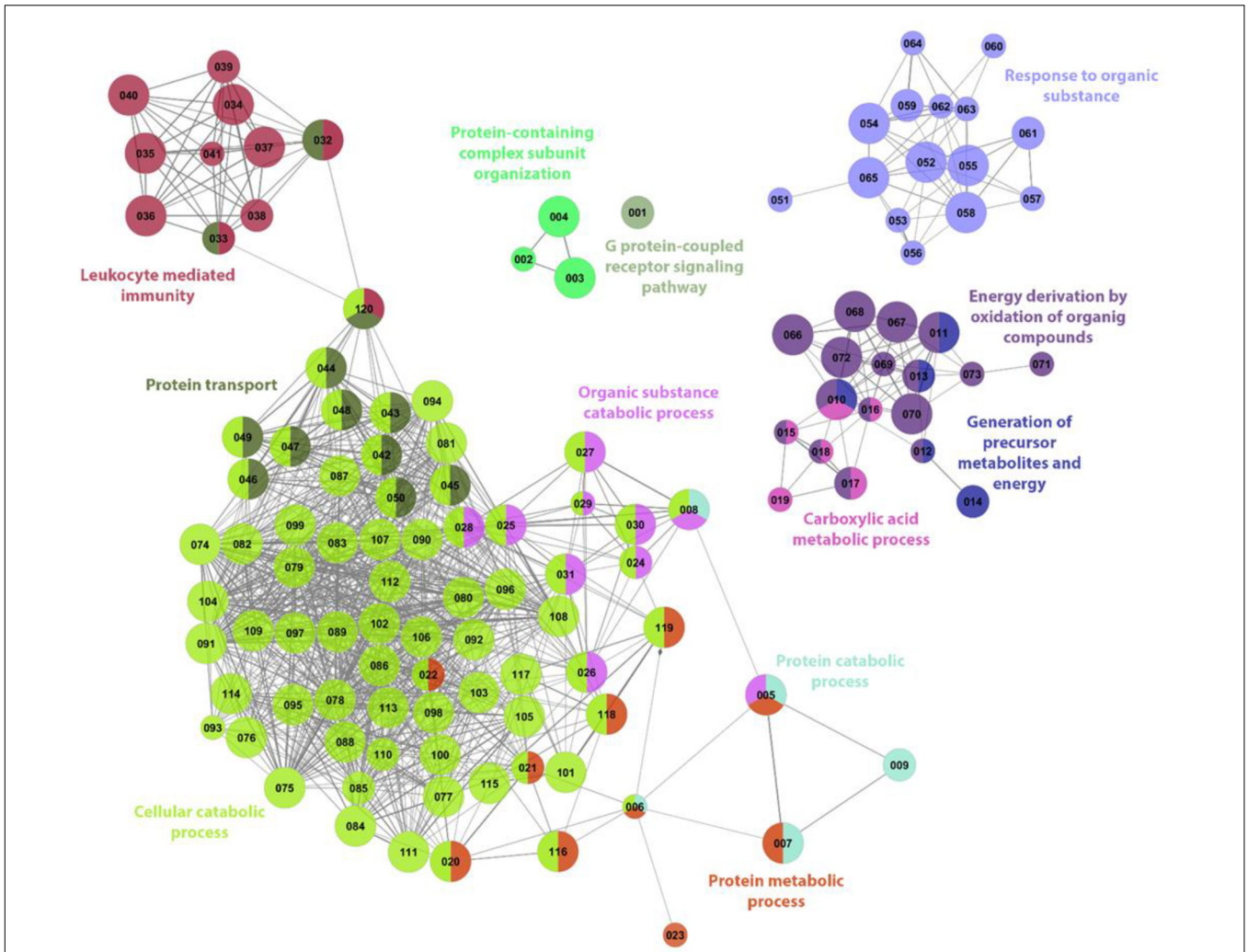


FIGURE 3 | ClueGo analysis of significantly enriched terms based on the biological functions in the anterior intestine of gilthead sea bream fed the SDPP diet. Each node represents one GO enrichment pathway (please refers to **Supplementary Table 1** for details). The size of the node means its significance. Each color indicates a cluster of closely related biological processes. The colored biological term denominates the leading group term. The lines into the cluster showed a closed relationship between biological processes.

by oxidation of organic compounds", among the top 10 hub genes, seven transcripts were down-regulated (*ndufs3*, *cox5a*, *cox6a1*, *ndufa5*, *ndufa2*, *mt-nd4*, *mt-co1*), and three of them up-regulated (*sdhc*, *sdhd*, *cox4i2*) (**Figure 6B**). For the cluster "Response to organic substance", most of the hub genes were up-regulated (*rpsa*, *ubb*, *rps16*, *skp1*, *ctnnb1*, *hspa8*, *hsp90aa1*, *eftud2*) compared to the two down-regulated hub genes (*psmc2*, *cul1*) (**Figure 6C**). Considering the cluster "Protein transport", all ten hub genes were up-regulated (*rps8*, *rps24*, *rps18*, *rps16*, *rps11*, *rplp2*, *rpl9*, *rpl7a*, *rpl13*, *rpl11*) (**Figure 6D**). For the "Leukocyte mediated immunity" cluster, we identified three up-regulated transcripts (*pycard*, *anxa2*, *pygb*) and seven down-regulated hub genes (*sdcbp*, *lyz*, *hexb*, *ctsc*, *ctsd*, *npc2*, *ctsa*) (**Figure 6E**). Regarding the clusters "Organic substance catabolic process" and "Protein metabolic process", the same hub genes were identified (*rps8*, *rps24*, *rps18*, *rps16*, *rps12*, *rps11*, *rpl9*, *rpl7a*, *rpl13a*, *rpl11*). Remarkably, all of them were up-regulated genes (**Figures 6F,G**). For the cluster "Carboxylic acid metabolic process", seven hub genes were up-regulated (*shmt1*, *sord*, *me3*, *aldoc*, *gpi*, *mdh2*, *pepd*), and three down-regulated (*idh1*, *aldoa*, *glul*) (**Figure 6H**). Considering the cluster "Generation of precursor metabolites and energy", just two genes were up-regulated (*sdhd* and *sdhc*), whereas eight genes showed a down-regulation (*ndufs3*, *atp5g1*, *cox6a1*, *ndufa2*, *cox5a*, *ndufa5*, *mt-nd4*, *mt-co1*) (**Figure 6I**). The "Protein catabolic process" cluster showed nine hub genes. From them, five genes were upregulated (*wwp1*, *mdm2*, *hwew1*, *skp1*, *ubb*), and four of them were downregulated (*ube2d2*, *cul1*, *ubr4*, and *ube2l3*) (**Figure 6J**). For the cluster "Protein containing complex subunit organization", all hub genes were upregulated (*eif3k*, *rsl24d1*, *eif3a*, *mrpl1*, *mrps15*, *eif5*, *rps19*, *rpl11*, *rpl24*, *rpsa*) (**Figure 6K**). For the cluster "G protein-coupled receptor signaling pathway", six hub genes were up-regulated (*ahcyl1*, *abr*, *rhoa*, *ccr9*, *mdh2*, *rhoc*), and four down-regulated (*grk6*, *rgs1*, *gnai2*, *psap*) (**Figure 6L**).

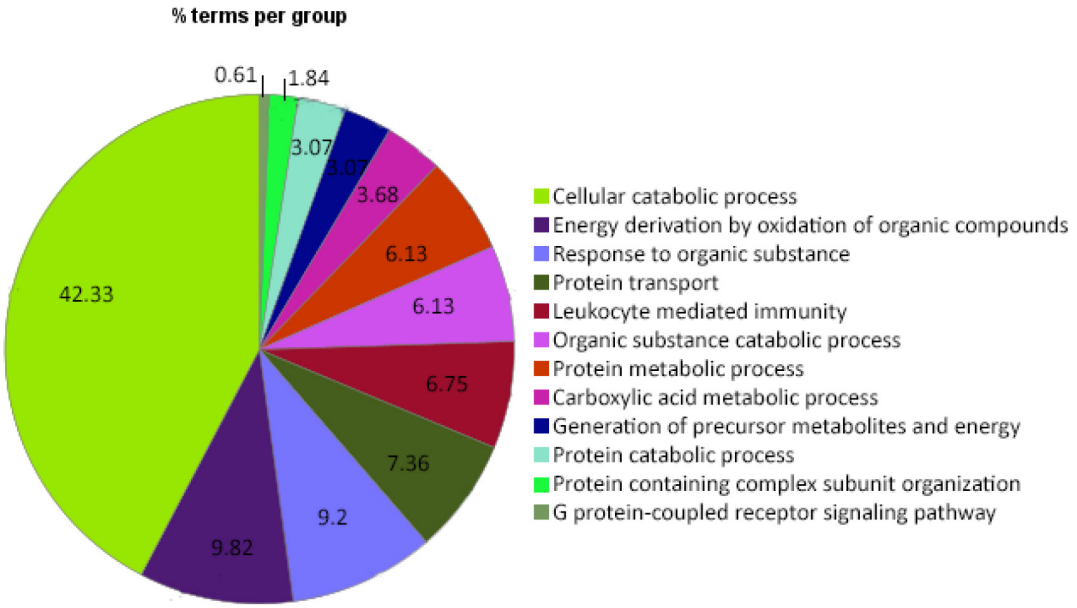


FIGURE 4 | Pie chart distribution of significantly enriched terms based on the biological functions in the anterior intestine of gilthead sea bream fed the SDPP diet. It is represented the percentage of the significantly differentially expressed genes in each principal cluster.

Collectively, the transcriptomic data showed a strong association between the genes associated with the catabolic process, protein transport, and leukocyte mediated immunity, suggesting that SDPP favors digestive- and defensive-related functions in the gilthead sea bream intestine.

DISCUSSION

In the present study, we evaluated the potential benefits of the dietary administration of SDPP in the gut of a marine teleost species by a detailed transcripteractome built from the enriched biological processes obtained to elucidate how this functional ingredient acted upon the maintenance of the gut mucosal tissue. Transcripteractome-based functional network analysis on teleost fish has gained attention in the last years (Firmino et al., 2020, 2021a,c; Reyes-López et al., 2021; Salomón et al., 2021). Previously, a similar strategy was followed by Reyes-López et al. (2021) to analyze the effects of SDPP at skin level. In our study, the transcriptomic analysis revealed that DEGs obtained corresponded mainly to biological processes related to "Cellular catabolic process (GO:0044248)", "Energy derivation by oxidation of organic compounds (GO:0015980)", and "Response to organic substance (GO:0010033)". These processes are probably correlated with the previous reported improved growth performance, feed intake, and feed efficiency promoted by dietary inclusion of SDPP in this species (Gisbert et al., 2015; Reyes-López et al., 2021), rainbow trout (Campbell et al., 2003), Nile tilapia (de Araújo et al., 2017), and higher vertebrates including pigs (Angulo and Cubiló, 1998; Van Dijk et al., 2002; Kósa et al., 2019), poultry (Beski et al., 2016; Dabbou et al., 2020), and cats (Rodríguez et al., 2016). In 2001, van Dijk et al. (2001) reported that dietary SDPP levels up to 6% increased both average daily gain and feed intake in the first 2 weeks in a dose-dependent fashion and improved feed conversion ratio (FCR) in piglets. Similar results were reported by Gisbert et al. (2015) in gilthead sea bream fed diets containing 3% SDPP in diets for gilthead sea bream fingerlings in terms of these two common key performance indicators related to somatic growth and feed efficiency. Remarkably, the ten transcripts that showed the higher level of connection in the "Cellular catabolic process" network were up-regulated, including mostly ribosomal proteins, such as *rps8, rps24, rps18, rps16, rps11,rps12, rpl7a, rpl11, rpl13A,* and *rpl29*. Ribosome biogenesis is a highly regulated process associated directly with cell growth and the most active proteins in the cell. In the case of rapidly growing yeast cells, the rRNA constitutes approximately 80% of total cellular nucleic acid, being 50% of all RNA polymerase II transcription initiation events that occur in ribosomal protein genes (Warner, 1999; Lempiäinen and Shore, 2009). Previously, in a similar experimental dietary approach, Reyes-López et al. (2021) evaluated the skin multi-omics interactome merging the transcripteractomic response with a proteomic profile at mucosal skin level in this species, showing a significant linkage for "cellular catabolic process", "biogenesis", and "protein transport" processes. Additionally, the GO analysis revealed that several biological processes were up-regulated, including "ribonucleoprotein complex biogenesis"; and "ribosome biogenesis." The authors suggested that the results may be attributed to the higher digestibility of protein in SDPP, which may promote protein turnover and exudation machinery in mucosal tissues (Reyes-López et al., 2021). Protein catabolism is the breakdown of proteins into absorbable monomers, which may be the only source of essential amino acids (Gurina and Mohiuddin, 2021), reflecting the relationship

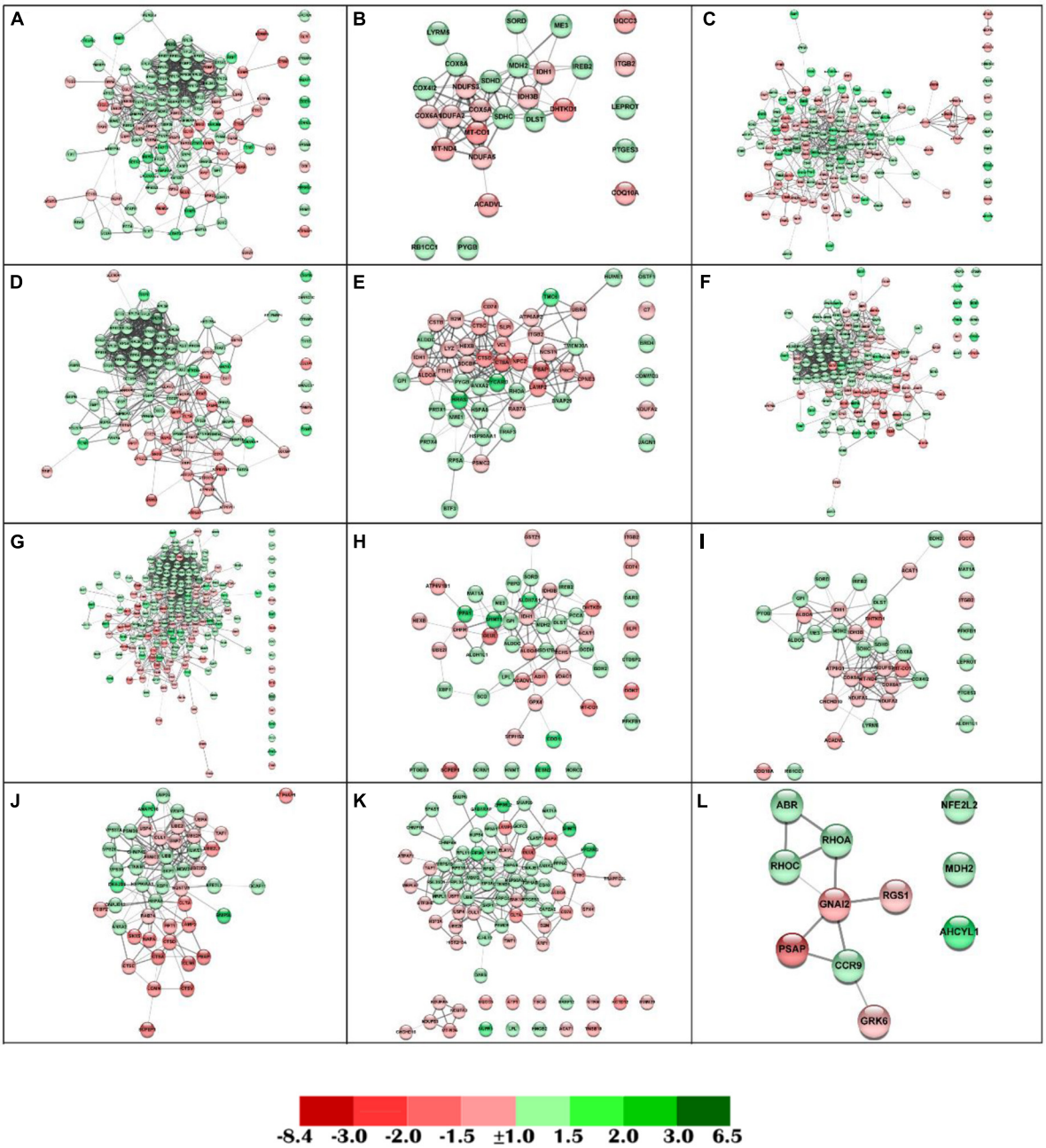


FIGURE 5 | Detailed functional networks for those DEGs belonging to the most representative GO terms for each enrichment cluster for biological functions. Each node represents one differential expressed gene (DEG) obtained from the anterior intestine transcriptomic analysis. **(A)** Cellular catabolic process. **(B)** Energy derivation by oxidation of organic compounds. **(C)** Response to organic substance. **(D)** Protein transport. **(E)** Leukocyte mediated immunity. **(F)** Organic substance catabolic process. **(G)** Protein metabolic process. **(H)** Carboxylic acid metabolic process. **(I)** Generation of precursor metabolites and energy. **(J)** Protein catabolic process. **(K)** Protein containing complex subunit organization. **(L)** G protein-coupled receptor signaling pathway. The DEG modulatory profile is represented with green (up-regulated) and red (down-regulated) into each node according to its fold-change magnitude interval scheme (detailed at the bottom of the figure).

between the cellular catabolic process and the biogenesis process. However, among the 10 up-regulated ribosomal proteins found in the gut under current experimental conditions, *rps8*, *rps18*, and *rps16* were differentially down-regulated in the skin mucosa (Reyes-López et al., 2021). Differences in the gene expression profile between mucosal tissues to the same stimulus have been described elsewhere (Khansari et al., 2018). The scope of these differences remains to be elucidated in further studies, although it may be related to differences in tissue function, physiology, and cellular turnover.

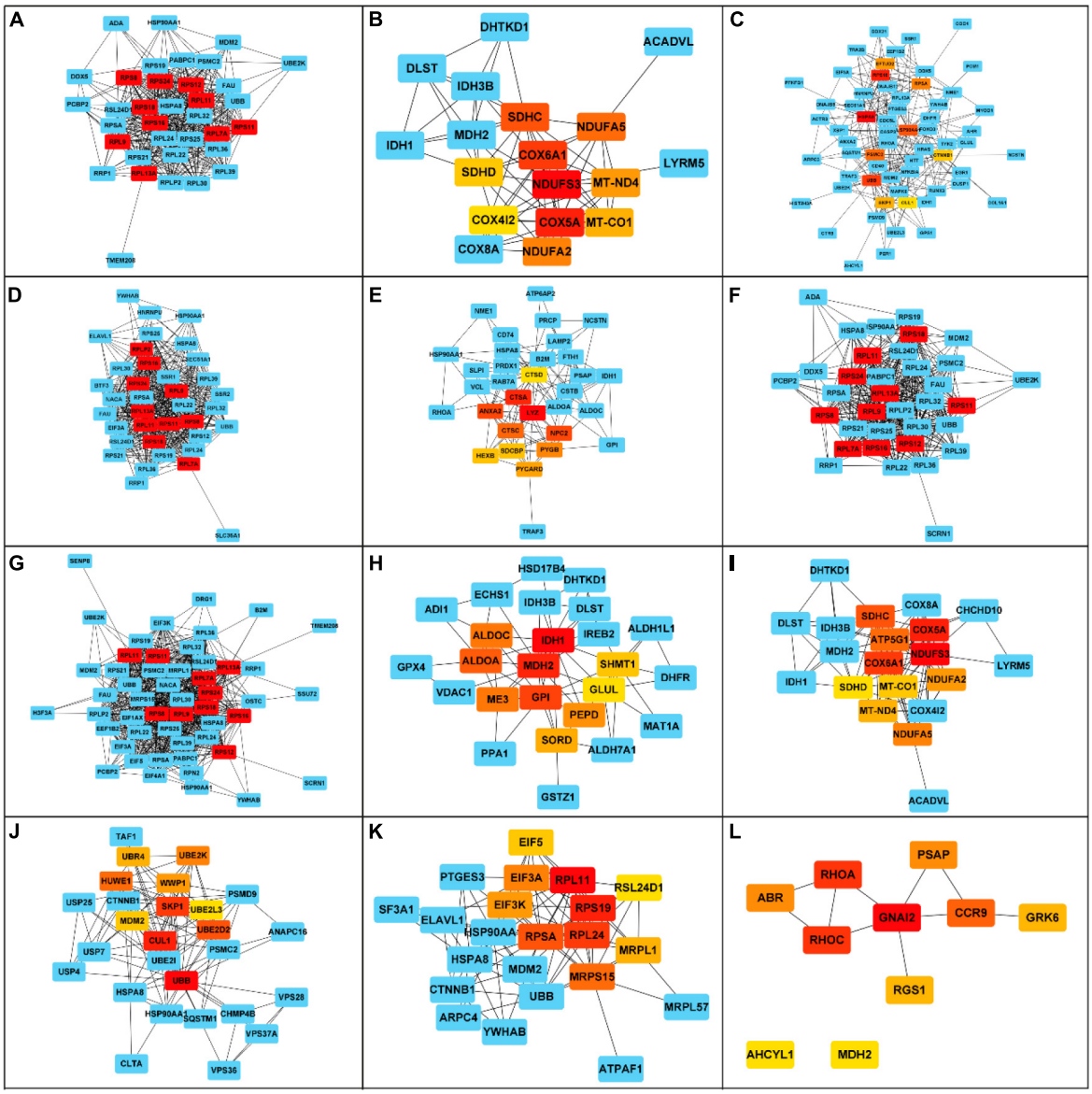


FIGURE 6 | Hub genes in each cluster term identified from the enriched biological functions in the anterior intestine of gilthead sea bream fed the SDPP diet. The principal cluster shows the 10-hub genes and the closest neighbor's genes for each network. The color intensity from yellow to red (i.e., yellow, orange, red) represents the degree of the hub gene (i.e., the relevance degree) in each network, being red those most relevant hub genes. The blue nodes represent the closest neighbors. **(A)** Cellular catabolic process. **(B)** Energy derivation by oxidation of organic compounds. **(C)** Response to organic substance. **(D)** Protein transport. **(E)** Leukocyte mediated immunity. **(F)** Organic substance catabolic process. **(G)** Protein metabolic process. **(H)** Carboxylic acid metabolic process. **(I)** Generation of precursor metabolites and energy. **(J)** Protein catabolic process. **(K)** Protein containing complex subunit organization. **(L)** G protein-coupled receptor signaling pathway.

Blood plasma is a complex fluid that contains a wide variety of proteins, cytokines, growth factors, hormones, bioactive peptides, and amino acids that are involved in the health benefits found at systemic and different mucosal levels when SDPP is included in animal diets (Pérez-Bosque et al., 2016). The exact mechanism of action of SDPP is not fully understood. Some authors suggest that immunoglobulins are the main proteins involved in the growth rate and feed intake enhancement, together with an improved immune and intestinal function (Pierce et al., 2005; Petschow et al., 2014). However, its mechanism of action is not fully understood (Petschow et al., 2014). In addition, blood plasma contains more than 695 proteins in its composition (Schenk et al., 2008), and probably some of these proteins are also involved in the mechanism of action of SDPP. Although the number of peptides in blood plasma is relatively scarce about the number of proteins with MW > 10 kDa, some authors suggest that bioactive peptides present in plasma proteins may have beneficial effect after enzymatic hydrolysis at the intestinal level

and may partially explain the improved performance and health benefits associated with the inclusion of SDPP in animal diets (Bah et al., 2013).

In the present study, we identified the modulation of DEGs associated with the energy derivation by oxidation of organic compounds. Most DEGs were down-regulated (*ndufs3*, *cox5a*, *cox6a1, ndufa5, ndufa2, mt-nd4,* and *mt-co1*) in comparison to the up-regulated ones (*sdhc, sdhd,* and *cox4i2*). Importantly, all these transcripts are related to mitochondrial processes associated with obtaining energy. In this way, NDUFS3 is one of the central subunits in the mitochondrial complex I. NDUFS3 is a catalytic subunit that has been shown to play a vital role in the proper assembly of functional complex I in the human mitochondrial respiratory chain (Suhane et al., 2011). On the other hand, NDUFA5 and NDUFA2 are accessory subunits of the mitochondrial complex I (Brandt, 2006). Similarly, MT-ND4 is also a central subunit of the mitochondrial complex 1 encoded in the mitochondrial genome (Brandt, 2006). In addition, COX5A (Cytochrome c oxidase 5A) and COX6A1, and COX4I2 are subunits of the cytochrome c oxidase (complex IV), which is the terminal complex of eukaryotic oxidative phosphorylation in mitochondria (Sommer et al., 2017; Watson and McStay, 2020). MT-CO1 is also part of the complex IV but is encoded in the mitochondrial genome (Carr and Winge, 2003). Finally, SDHC and SDHD are subunits of the Succinate dehydrogenase (SDH or Complex II), which is a heterotetrameric complex that links the tricarboxylic acid cycle (TCA) with the electron transport chain (Van Vranken et al., 2015). Taking together, the up- and down-regulatory expression profile of these genes in response to the SDPP administration showed that this ingredient resulted in the controlled activation of this very dynamic and strictly regulated cooperation interaction between nuclear and mitochondrial DNA for orchestrating a coordinated biogenesis of the mitochondrial complexes; thus, preserving the mitochondrial activity and homeostasis (Piomboni et al., 2012; Eisenberg-Bord and Schuldiner, 2017; Gómez-Serrano et al., 2018).

In the present study, the protein transport process was also enriched. The up-regulation of all protein transport hub genes identified (*rps8, rps24, rps18, rps16, rps11,* and *rpl7a*) coincided with the higher level of connection in the functional network between cellular catabolic processes and protein transport. In a previous study focused on the skin mucosa, a strong relationship between protein transport and the cellular catabolic process was also described in gilthead sea bream fed the SDPP (Reyes-López et al., 2021), results that may be attributed to the nutritional profile of this functional ingredient (Almeida et al., 2013). Such association was shown to be related to enhanced integrity of the mucosal tissue by the enhancement of tight junctions at the cell-cell level and/or between the cell and the extracellular matrix (Reyes-López et al., 2021), thus reinforcing the physical barrier favoring the protection of the organism in front of an environment fluctuation (Esteban, 2012; Parra et al., 2015). According to this idea, in our study we also found the up-regulation of syntenin. This protein has been associated with the cytoskeletal-membrane organization, cell adhesion, protein trafficking, and the activation of transcription factors. Importantly, this protein is primarily localized to membrane-associated adherens junctions and focal adhesion, reinforcing the idea of higher mucosal robustness by administrating SDPP. In the same line, in our study the up-regulation of *rhoa* was also registered, and whose function is related to the organization of the cytoskeleton, cell cycle progression, and cell adhesion by the maintenance of adherents and tight junctions (Terry et al., 2010; Citalán-Madrid et al., 2013). Taking together, these antecedents suggest that SDPP improves the intestinal barrier function, which is supported by previous results of SDPP tested in terrestrial animals (Peace et al., 2011; Pérez-Bosque et al., 2016).

In the context of a higher mucosal robustness, there is evidence that the SDPP oral administration also improved the activity of the intestinal antioxidant defense system and increased the density of goblet cells in intestinal mucosa; thus, favoring the intestinal health and innate immune function (Gisbert et al., 2015). The intestine plays a vital role in fish health as a physiological and immunological barrier to a wide range of microorganisms and foreign substances (Lalles, 2010; Gisbert et al., 2015). Similar results have been reported in pigs (Peace et al., 2011; Tran et al., 2014) and poultry (Beski et al., 2016), being the intestinal mucosa one of the best-studied tissues under the administration of SDPP. In fact, SDPP has been recommended for livestock nutrition as a source of immunological support due to its content in immunoglobulins and bioactive peptides (Bureau et al., 1999; Pérez-Bosque et al., 2010, 2016; Peace et al., 2011; Chin et al., 2014). That composition is related to enterocyte nutrition, mucin production, energetic resources, nucleotides synthesis, mechanical barrier stimulation, and innate immune response stimulation through the increase in leukocytes and immunoglobulins (Pérez-Bosque et al., 2006; Gao et al., 2011; Gisbert et al., 2015; de Araújo et al., 2017). In this way, in this study we also observed a strong relationship between the genes associated with immunity and protein transport, as it was previously reported for the skin mucosa (Reyes-López et al., 2021). At the innate immune level, lysozyme expression was down-regulated in our study. By contrast, it was previously reported that oral administration of SDPP enhanced the serum immune function in fish, increasing the serum lysozyme levels (Gisbert et al., 2015). This apparent discrepancy between the response observed for serum and intestine could be strictly associated with a specific response pattern for each tissue, even coming from the same organism. In fact, there is evidence in gilthead sea bream that the same stimulus may provoke different response patterns between tissues (Khansari et al., 2019), even at the mucosal level (Khansari et al., 2018). Lysozyme is a crucial antibacterial defense molecule of the innate immune system produced by leukocytes. Certain nutrients and compounds present in the diet might modulate the lysozyme activity in fish, which is generally associated with an increase in the protection against various bacterial infections (Saurabh and Sahoo, 2008; Gisbert et al., 2015). This aspect reaches particular relevance considering the high protein content in our diet. In mammals, a diet rich in animal protein promotes a gut pro-inflammatory response (Jantchou et al., 2010; Kostovcikova et al., 2019). In fish, there is evidence of gut inflammation

by the use of soybean protein diet, inducing enteropathy and secretions that lead to greater immune sensitivity with migration of eosinophilic granulocytes, high levels of lysozyme, and IgM (Krogdahl et al., 2000; Uran et al., 2009; Hedrera et al., 2013; Estruch et al., 2015). Importantly, a previous report revealed an average intestinal mucosa organization in gilthead sea bream fed with an SDPP supplemented diet (Tapia-Paniagua et al., 2020). Similarly, in our study, *ctsa*, *ctsc*, and *ctsd* were also down-regulated. Their protein products are cathepsins, lysosomal proteases active in a slightly acidic environment involved at different levels in the process of the innate and adaptive immune responses (Turk et al., 2012; Perisic Nanut et al., 2014) that appears to be a central coordinator for activation of many serine proteinases in cells of the immune system. Thus, the down-regulation of genes associated with pro-inflammatory promotion reinforces the hypothesis of tight regulation of the inflammatory response in gilthead sea bream gut fed with the SDPP diet.

According to the tight regulation of the immune response, we observed in our transcripteractome analysis just 6.8% of the up-regulated transcripts associated with the biological process "Leukocyte mediated immunity (GO:0002443)." Among them, it stands out the gene *pycard* and whose protein product, called ASC [apoptosis-associated speck-like protein containing a caspase-recruitment domain (CARD)], is an adaptor protein involved in the formation of inflammasomes (Li et al., 2018). The inflammasome activation is a tightly regulated process, including transcriptional and post-transcriptional regulation mechanisms of their components, including upstream regulators and downstream effectors. Remarkably, the expression of ASC has been suggested to be constitutive in many cells and does not appear to be inducible by inflammatory signaling (Christgen et al., 2020). However, cells stimulated with LPS in the human intestinal mucosa, ASC can be up-regulated but posteriorly repressed (Masumoto et al., 2006). Similarly, in peripheral blood human neutrophils, ASC expression was transiently up-regulated by the pro-inflammatory cytokines IL-1α, IL-1β, IFNα, IFNγ, TNFα, and the stimulus by LPS (Shiohara et al., 2002). Recently, ASC was described in the inflammasome activation pathway on zebrafish with conserved and unique structural/functional features (Li et al., 2018). However, more studies are required to characterize the expression of ASC in gilthead sea bream and the scope of its up-regulation in response to the dietary administration of SDPP.

In the same context of the tight control of the immune system for a successful response, we also found the up-regulation of *sdcbp*. In mammals, its protein product (Syntenin-1) is an important positive regulator of transforming growth factor-beta (TGF-β) signaling (Hwangbo et al., 2016). At the same time, it has a role as a negative regulator of intestinal immunoglobulin production to maintain intestinal homeostasis *in vivo* (Tamura et al., 2015). In teleost, information about the role of Syntenin in the immune response is scarce. Currently, in zebrafish it is associated with epiboly and gastrulation movements (Lambaerts et al., 2012), the exosome biogenesis and release from yolk syncytial layer (Verweij et al., 2019), and in adults to the regeneration after traumatic insult to the central nervous system (Yu and Schachner, 2013). In kuruma shrimp (*Marsupenaeus japonicus*), there is some evidence of syntenin-like genes in antibacterial response (Liu et al., 2015), increasing a conserved evolutionary role in immunity.

CONCLUSION

Taking together, in this study we observed a strong association between immunity, protein transport, and process related to metabolism-associated processes. This evidence confirms the previous evidence of the immune, metabolic, and protein transport processes that take place at the mucosal tissue level in response to SDPP (Reyes-López et al., 2021). Thus, the integrative analysis of gilthead sea bream fed with SDPP supported the better key performance indicators related to growth and feed performances of gilthead sea bream, which are also associated with a balanced immune response and an enhancement of the mucosal health status.

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: Gene Expression Omnibus (GEO) (accession number GSE193972) at the National Center for Biotechnology Information (NCBI).

ETHICS STATEMENT

Animal experimental procedures were conducted in compliance with the research protocol approved by the IRTA's Committee of Ethics and following the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals.

AUTHOR CONTRIBUTIONS

JP and EG designed the study. EG carried out the nutritional trial. LT, FR-L, and EG supervised the study. EV-V, SR-C, and FR-L made microarrays hybridizations, raw data output and processing and transcriptomic data analysis and co-wrote the original draft, were in charge of the conceptualization, methodology, and interpretation of the integrative analysis, including interactomes, GO terms enrichment, and cluster analysis, and made the conceptualization and design of figures. All authors provided critical feedback and approved the final manuscript.

FUNDING

This work has been financially supported by the project "AQUOLIVE by NATAC," funded by European Union's Horizon 2020 Research and Innovation Program (Grant Agreement No. 830202) and partially by the Agencia Española de Investigación (grant AGL2016-76069-C2-2-R to UAB).

ACKNOWLEDGMENTS

We thank (1) DIETAplus project of the JACUMAR (Junta de Cultivos Marinos, MAPAMA; Spanish government), which is co-funded with FEMP funds (EU); (2) ERC (European Research Council) in MedAID project (Mediterranean Aquaculture Integrated Development; Grant Agreement no. 727315); (3) Fondecyt regular [project number 1211841; Agencia. Nacional de Investigación y Desarrollo de Chile (ANID); Government of Chile]; and (4) Fondecyt iniciación grant (project number 11221308; ANID, Government of Chile). The funder bodies were not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.814233/full#supplementary-material

REFERENCES

Almeida, F. N., Htoo, J. K., Thomson, J., and Stein, H. H. (2013). Comparative amino acid digestibility in US blood products fed to weanling pigs. *Anim. Feed Sci. Technol.* 181, 80–86. doi: 10.1016/j.anifeedsci.2013.03.002

Angulo, E., and Cubiló, D. (1998). Effect of different dietary concentrations of spray-dried porcine plasma and a modified soyprotein product on the growth performance of piglets weaned at 6 kg body weight. *Anim. Feed Sci. Technol.* 72, 71–79.

Bah, C. S. F., Bekhit, A. E. D. A., Carne, A., and Mcconnell, M. A. (2013). Slaughterhouse blood: an emerging source of bioactive compounds. *Compr. Rev. Food Sci. Food Saf.* 12, 314–331. doi: 10.1111/1541-4337.12013

Beski, S. S., Swick, R. A., and Iji, P. A. (2016). Effect of dietary inclusion of spray-dried porcine plasma on performance, some physiological and immunological response of broiler chickens challenged with *Salmonella sofia*. *J. Anim. Physiol. Anim. Nutr.* 100, 957–966. doi: 10.1111/jpn.12414

Brandt, U. (2006). Energy converting NADH:quinone oxidoreductase (complex I). *Annu. Rev. Biochem.* 75, 69–92. doi: 10.1146/annurev.biochem.75.103004.142539

Bureau, D., Harris, A., and Cho, C. (1999). Apparent digestibility of rendered animal protein ingredients for rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 180, 345–358. doi: 10.1016/s0044-8486(99)00210-0

Campbell, J. M., Quigley, J. D., Russell, L. E., and Kidd, M. T. (2003). Effect of spray-dried bovine serum on intake, health, and growth of broilers housed in different environments. *J. Anim. Sci.* 81, 2776–2782. doi: 10.2527/2003.81112776x

Carr, H. S., and Winge, D. R. (2003). Assembly of cytochrome c oxidase within the mitochondrion. *Acc. Chem. Res.* 36, 309–316. doi: 10.1021/ar0200807

Chin, C. H., Chen, S. H., Wu, H. H., Ho, C. W., Ko, M. T., and Lin, C. Y. (2014). cytoHubba: identifying hub objects and sub-networks from complex interactome. *BMC Syst. Biol.* 8:S11. doi: 10.1186/1752-0509-8-S4-S11

Christgen, S., Place, D. E., and Kanneganti, T. D. (2020). Toward targeting inflammasomes: insights into their regulation and activation. *Cell Res.* 30, 315–327. doi: 10.1038/s41422-020-0295-298

Citalán-Madrid, A. F., García-Ponce, A., Vargas-Robles, H., Betanzos, A., and Schnoor, M. (2013). Small GTPases of the Ras superfamily regulate intestinal epithelial homeostasis and barrier function via common and unique mechanisms. *Tissue Barriers* 1:e26938. doi: 10.4161/tisb.26938

Dabbou, S., Trocino, A., Xiccato, G., Nery, J., Madrid, J., Martinez, S., et al. (2020). The effect of dietary supplementation with globin and spray-dried porcine plasma on performance, digestibility and histomorphological traits in broiler chickens. *J. Anim. Physiol. Anim. Nutr.* 105(Suppl. 2), 42–51. doi: 10.1111/jpn.13356

Dawood, M. A. O. (2021). Nutritional immunity of fish intestines: important insights for sustainable aquaculture. *Rev. Aquac.* 13, 642–663. doi: 10.1111/raq.12492

de Araújo, E. P., de Carvalho, P. L. P. F., de Freitas, J. M. A., da Silva, R. L., Rocha, M. K. H. R., Teixeira, C. P., et al. (2017). Dietary spray-dried plasma enhances the growth performance, villus:crypt ratio and cold-induced stress resistance in *Nile tilapia* (*Oreochromis niloticus*). *Aquaculture* 479, 675–681. doi: 10.1016/j.aquaculture.2017.07.003

Eisenberg-Bord, M., and Schuldiner, M. (2017). Ground control to major TOM: mitochondria–nucleus communication. *FEBS J.* 284, 196–210. doi: 10.1111/febs.13778

Ellis, A. E. (2001). Innate host defense mechanisms of fish against viruses and bacteria. *Dev. Comp. Immunol.* 25, 827–839. doi: 10.1016/s0145-305x(01)00038-6

Esteban, M. Á (2012). An overview of the immunological defenses in fish skin. *Int. Sch. Res. Netw. Immunol.* 2012:853470. doi: 10.5402/2012/853470

Estruch, G., Collado, M. C., Penaranda, D. S., Tomas Vidal, A., Jover Cerda, M., Perez Martinez, G., et al. (2015). Impact of fishmeal replacement in diets for gilthead sea bream (*Sparus aurata*) on the gastrointestinal microbiota determined by pyrosequencing the 16S rRNA Gene. *PLoS One* 10:e0136389. doi: 10.1371/journal.pone.0136389

Firmino, J. P., Fernández-Alacid, L., Vallejos-Vidal, E., Salomón, R., Ignasi Sanahuja, Tort, L., et al. (2021a). Carvacrol, thymol, and garlic essential oil promote skin innate immunity in gilthead seabream (*Sparus aurata*) through the multifactorial modulation of the secretory pathway and enhancement of mucus protective capacity. *Front. Immunol.* 12:633621. doi: 10.3389/fimmu.2021.633621

Firmino, J. P., Galindo-Villegas, J., Reyes-López, F. E., and Gisbert, E. (2021b). Phytogenic bioactive compounds shape fish mucosal immunity. *Front. Immunol.* 12:695973. doi: 10.3389/fimmu.2021.695973

Firmino, J. P., Vallejos-Vidal, E., Balebona, M. C., Ramayo-Caldas, Y., Cerezo, I. M., Salomón, R., et al. (2021c). Diet. *Front. Immunol.* 12:625297. doi: 10.3389/fimmu.2021.625297

Firmino, J. P., Vallejos-Vidal, E., Sarasquete, C., Delgado, J. B. O., Balasch, J. C., Tort, L., et al. (2020). Unveiling the effect of dietary essential oils supplementation in Sparus aurata gills and its efficiency against the infestation by *Sparicotyle chrysophrii*. *Sci. Rep.* 10:17764. doi: 10.1038/s41598-020-74625-5

Gao, Y. Y., Jiang, Z. Y., Lin, Y. C., Zheng, C. T., Zhou, G. L., and Chen, F. (2011). Effects of spray-dried animal plasma on serous and intestinal redox status and cytokines of neonatal piglets. *J. Anim. Sci.* 89, 150–157. doi: 10.2527/jas.2010-2967

Gisbert, E., Skalli, A., Campbell, J., Solovyev, M. M., Rodríguez, C., Dias, J., et al. (2015). Spray-dried plasma promotes growth, modulates the activity of antioxidant defenses, and enhances the immune status of gilthead sea bream (*Sparus aurata*) fingerlings. *J. Anim. Sci.* 93, 278–286. doi: 10.2527/jas2014-7491

Gomez, D., Sunyer, J. O., and Salinas, placeI. (2013). The mucosal immune system of fish: the evolution of tolerating commensals while fighting pathogens. *Fish Shellfish Immunol.* 35, 1729–1739. doi: 10.1016/j.fsi.2013.09.032

Gómez-Serrano, M., Camafeita, E., Loureiro, M., and Peral, B. (2018). Mitoproteomics: tackling mitochondrial dysfunction in human disease. *Oxid. Med. Cell. Longev.* 2018:1435934. doi: 10.1155/2018/1435934

Gurina, T., and Mohiuddin, S. (2021). *Biochemistry, Protein Catabolism*. Treasure StateFL: StatPearls Publ.

Hedrera, M. I., Galdames, J. A., Jimenez-Reyes, M. F., Reyes, A. E., Avendano-Herrera, R., Romero, J., et al. (2013). Soybean meal induces intestinal inflammation in zebrafish larvae. *PLoS One* 8:e69983. doi: 10.1371/journal.pone.0069983

Hwangbo, C., Tae, N., Lee, S., Kim, O., Park, O. K., Kim, J., et al. (2016). Syntenin regulates TGF-beta1-induced Smad activation and the epithelial-to-mesenchymal transition by inhibiting caveolin-mediated TGF-beta type I receptor internalization. *Oncogene* 35, 389–401. doi: 10.1038/onc.2015.100

Jantchou, P., Morois, S., Clavel-Chapelon, F., Boutron-Ruault, M. C., and Carbonnel, F. (2010). Animal protein intake and risk of inflammatory bowel disease: the E3N prospective study. *Am. J. Gastroenterol.* 105, 2195–2201. doi: 10.1038/ajg.2010.192

Johnson, J. A., and Summerfelt, R. C. (2000). Spray-dried blood cells as a partial replacement for fishmeal in diets for rainbow trout *Oncorhynchus mykiss*. *J. World Aquac. Soc.* 31, 96–104. doi: 10.1111/j.1749-7345.2000.tb00703.x

Khansari, A. R., Balasch, J. C., Vallejos-Vidal, E., Parra, D., Reyes-López, F. E., and Tort, L. (2018). Comparative immune- and stress-related transcript response induced by air exposure and *Vibrio anguillarum* bacterin in rainbow trout (*Oncorhynchus mykiss*) and Gilthead Seabream (*Sparus aurata*) mucosal surfaces. *Front. Immunol.* 9:856. doi: 10.3389/fimmu.2018.00856

Khansari, A. R., Balasch, J. C., Vallejos-Vidal, E., Teles, M., Fierro-Castro, C., Tort, L., et al. (2019). Comparative study of stress and immune-related transcript outcomes triggered by *Vibrio anguillarum* bacterin and air exposure stress in liver and spleen of gilthead seabream (*Sparus aurata*), zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*). *Fish Shellfish Immunol.* 86, 436–448. doi: 10.1016/j.fsi.2018.11.063

Kósa, B., Bárdová, K., Lešková, L., Reichel, P., Novotný, J., Link, R., et al. (2019). The effect of a feed additive supplemented with porcine plasma protein on growth performance and selected biochemical indices of nursing piglets. *Acta Vet. Brno* 88, 393–399. doi: 10.2754/avb201988040393

Kostovcikova, K., Coufal, S., Galanova, N., Fajstova, A., Hudcovic, T., Kostovcik, M., et al. (2019). Diet rich in animal protein promotes pro-inflammatory macrophage response and exacerbates colitis in mice. *Front. Immunol.* 10:919. doi: 10.3389/fimmu.2019.00919

Krogdahl, A., Bakke-McKellep, A. M., Roed, K. H., and Baeverdfjord, G. (2000). Feeding Atlantic Salmon *Salmo salar* L. soybean products: effects on disease resistance (furunculosis), and lysozyme and IgM levels in the intestinal mucosa. *Aquac. Nutr.* 6, 77–84. doi: 10.1046/j.1365-2095.2000.00129.x

Lalles, J. P. (2010). Intestinal alkaline phosphatase: multiple biological roles in maintenance of intestinal homeostasis and modulation by diet. *Nutr. Rev.* 68, 323–332. doi: 10.1111/j.1753-4887.2010.00292.x

Lambaerts, K., Van Dyck, S., Mortier, E., Ivarsson, Y., Degeest, G., Luyten, A., et al. (2012). Syntenin, a syndecan adaptor and an Arf6 phosphatidylinositol 4,5-bisphosphate effector, is essential for epiboly and gastrulation cell movements in zebrafish. *J. Cell Sci.* 125, 1129–1140. doi: 10.1242/jcs.089987

Lempiäinen, H., and Shore, D. (2009). Growth control and ribosome biogenesis. *Curr. Opin. Cell Biol.* 21, 855–863. doi: 10.1016/j.ceb.2009.09.002

Li, Y., Huang, Y., Cao, X., Yin, X., Jin, X., Liu, S., et al. (2018). Functional and structural characterization of zebrafish ASC. *FEBS J.* 285, 2691–2707. doi: 10.1111/febs.14514

Liu, Q., Chen, X. W., Che, C. J., Ding, D., and Kang, C. J. (2015). Syntenin is involved in the bacteria clearance response of kuruma shrimp (*Marsupenaeus japonicus*). *Fish Shellfish Immunol.* 44, 453–461. doi: 10.1016/j.fsi.2015.02.016

Masumoto, J., Kobayashi, H., Nakamura, T., Kaneko, Y., Ota, H., Hasegawa, M., et al. (2006). Regulation of the ASC expression in response to LPS stimulation is related to IL-8 secretion in the human intestinal mucosa. *Biochem. Biophys. Res. Commun.* 346, 968–973. doi: 10.1016/j.bbrc.2006.06.002

Oehlers, S. H., Flores, M. V., Chen, T., Hall, C. J., Crosier, K. E., and Crosier, P. S. (2011). Topographical distribution of antimicrobial genes in the zebrafish intestine. *Dev. Comp. Immunol.* 35, 385–391. doi: 10.1016/j.dci.2010.11.008

Parra, D., Reyes-Lopez, F. E., and Tort, L. (2015). Mucosal immunity and B cells in teleosts: effect of vaccination and stress. *Front. Immunol.* 6:354. doi: 10.3389/fimmu.2015.00354

Peace, R. M., Campbell, J., Polo, J., Crenshaw, J., Russell, L., and Moeser, A. (2011). Spray-dried porcine plasma influences intestinal barrier function, inflammation, and diarrhea in weaned pigs. *J. Nutr.* 141, 1312–1317. doi: 10.3945/jn.110.136796

Pérez-Bosque, A., Amat, C., Polo, J., Campbell, J. M., Crenshaw, J., Russell, L., et al. (2006). Spray-Dried animal plasma prevents the effects of staphylococcus aureus enterotoxin b on intestinal barrier function in weaned rats. *J. Nutr.* 136, 2838–2843. doi: 10.1093/jn/136.11.2838

Pérez-Bosque, A., Miró, L., Polo, J., Russell, L., Campbell, J., Weaver, E., et al. (2010). Dietary plasma protein supplements prevent the release of mucosal proinflammatory mediators in intestinal inflammation in rats. *J. Nutr.* 140, 25–30. doi: 10.3945/jn.109.112466

Pérez-Bosque, A., Polo, J., and Torrallardona, D. (2016). Spray dried plasma as an alternative to antibiotics in piglet feeds, mode of action and biosafety. *Porc. Heal. Manag.* 2:16. doi: 10.1186/s40813-016-0034-31

Perisic Nanut, M., Sabotic, J., Jewett, A., and Kos, J. (2014). Cysteine cathepsins as regulators of the cytotoxicity of NK and T cells. *Front. Immunol.* 5:616. doi: 10.3389/fimmu.2014.00616

Petschow, B. W., Blikslager, A. T., Weaver, E. M., Campbell, J. M., Polo, J., Shaw, A. L., et al. (2014). Bovine immunoglobulin protein isolates for the nutritional management of enteropathy. *World J. Gastroenterol.* 20, 11713–11726. doi: 10.3748/wjg.v20.i33.11713

Pierce, J. L., Cromwell, G. L., Lindemann, M. D., Russell, L. E., and Weaver, E. M. (2005). Effects of spray-dried animal plasma and immunoglobulins on performance of early weaned pigs. *J. Anim. Sci.* 83, 2876–2885. doi: 10.2527/2005.83122876x

Piomboni, P., Focarelli, R., Stendardi, A., Ferramosca, A., and Zara, V. (2012). The role of mitochondria in energy production for human sperm motility. *Int. J. Androl.* 35, 109–124. doi: 10.1111/j.1365-2605.2011.01218.x

Reyes-Cerpa, S., Vallejos-Vidal, E., José Gonzalez-Bown, M., Morales-Reyes, J., Pérez-Stuardo, D., Vargas, D., et al. (2018). Effect of yeast (*Xanthophyllomyces dendrorhous*) and plant (Saint John's wort, lemon balm, and rosemary) extract based functional diets on antioxidant and immune status of Atlantic salmon (*Salmo salar*) subjected to crowding stress. *Fish Shellfish Immunol.* 74, 250–259. doi: 10.1016/j.fsi.2017.12.061

Reyes-López, F. E., Ibarz, A., Ordóñez-grande, B., Vallejos-Vidal, E., Sánchez-Nuño, S., Firmino, J. P., et al. (2021). Skin multi-omics-based interactome analysis: integrating the tissue and mucus exuded layer for a comprehensive understanding of the teleost mucosa functionality as model of study. *Front. Immunol.* 11:613824. doi: 10.3389/fimmu.2020.613824

Rodríguez, C., Saborido, N., Ródenas, J., and Polo, J. (2016). Effects of spray-dried animal plasma on food intake and apparent nutrient digestibility by cats when added to a wet pet food recipe. *Anim. Feed Sci. Technol.* 216, 243–250. doi: 10.1016/j.anifeedsci.2016.03.026

Salinas, I., and Parra, D. (2015). *Fish Mucosal Immunity: Intestine*. Amsterdam: Elsevier Inc.

Salomón, R., Reyes-López, F. E., Tort, L., Firmino, J. P., Sarasquete, C., Ortiz-Delgado, J. B., et al. (2021). Medicinal plant leaf extract from sage and lemon verbena promotes intestinal immunity and barrier function in gilthead seabream (*Sparus aurata*). *Front. Immunol.* 12:670279. doi: 10.3389/fimmu.2021.670279

Saurabh, S., and Sahoo, P. K. (2008). Lysozyme: an important defence molecule of fish innate immune system. *Aquac. Res.* 39, 223–239. doi: 10.1111/j.1365-2109.2007.01883.x

Schenk, S., Schoenhals, G. J., de Souza, G., and Mann, M. (2008). A high confidence, manually validated human blood plasma protein reference set. *BMC Med. Genomics* 1:41. doi: 10.1186/1755-8794-1-41

Shiohara, M., Taniguchi, S., Masumoto, J., Yasui, K., Koike, K., Komiyama, A., et al. (2002). ASC, which is composed of a PYD and a CARD, is up-regulated by inflammation and apoptosis in human neutrophils. *Biochem. Biophys. Res. Commun.* 293, 1314–1318. doi: 10.1016/S0006-291X(02)00384-4

Sommer, N., Hüttemann, M., Pak, O., Scheibe, S., Knoepp, F., Sinkler, C., et al. (2017). Mitochondrial complex IV Subunit 4 Isoform 2 is essential for acute pulmonary oxygen sensing. *Circ. Res.* 121, 424–438. doi: 10.1161/CIRCRESAHA.116.310482

Suhane, S., Berel, D., and Ramanujan, V. K. (2011). Biomarker signatures of mitochondrial NDUFS3 in invasive breast carcinoma. *Biochem. Biophys. Res. Commun.* 412, 590–595. doi: 10.1016/j.bbrc.2011.08.003

Tacchi, L., Bickerdike, R., Douglas, A., Secombes, C. J., and Martin, S. A. M. (2011). Transcriptomic responses to functional feeds in Atlantic salmon (*Salmo salar*). *Fish Shellfish Immunol.* 31, 704–715. doi: 10.1016/j.fsi.2011.02.023

Tamura, K., Ikutani, M., Yoshida, T., Tanaka-Hayashi, A., Yanagibashi, T., Inoue, R., et al. (2015). Increased production of intestinal immunoglobulins in

Syntenin-1-deficient mice. *Immunobiology* 220, 597–604. doi: 10.1016/j.imbio.2014.12.003

Tapia-Paniagua, S. T., Balebona, M., del, C., Firmino, J. P., Rodríguez, C., Polo, J., et al. (2020). The effect of spray-dried porcine plasma on gilthead seabream (*Sparus aurata*) intestinal microbiota. *Aquac. Nutr.* 26, 801–811. doi: 10.1111/anu.13039

Terry, S., Nie, M., Matter, K., and Balda, M. S. (2010). Rho signaling and tight junction functions. *Physiology* 25, 16–26. doi: 10.1152/physiol.00034.2009

Tran, H., Bundy, J. W., Li, Y. S., Carney-Hinkle, E. E., Miller, P. S., and Burkey, T. E. (2014). Effects of spray-dried porcine plasma on growth performance, immune response, total antioxidant capacity, and gut morphology of nursery pigs. *J. Anim. Sci.* 92, 4494–4504. doi: 10.2527/jas.2014-7620

Turk, V., Stoka, V., Vasiljeva, O., Renko, M., Sun, T., Turk, B., et al. (2012). Cysteine cathepsins: from structure, function and regulation to new frontiers. *Biochim. Biophys. Acta* 1824, 68–88. doi: 10.1016/j.bbapap.2011.10.002

Uran, P. A., Schrama, J. W., Rombout, J. H., Taverne-Thiele, J. J., Obach, A., Koppe, W., et al. (2009). Time-related changes of the intestinal morphology of Atlantic salmon, *Salmo salar* L., at two different soybean meal inclusion levels. *J. Fish Dis.* 32, 733–744. doi: 10.1111/j.1365-2761.2009.01049.x

Van Dijk, A. J., Enthoven, P. M., Van den Hoven, S. G., Van Laarhoven, M. M., Niewold, T. A., Nabuurs, M. J., et al. (2002). The effect of dietary spray-dried porcine plasma on clinical response in weaned piglets challenged with a pathogenic *Escherichia coli*. *Vet. Microbiol.* 84, 207–218.

van Dijk, A. J., Everts, H., Nabuurs, M. J. A., Margry, R. J. C. F., and Beynen, A. C. (2001). Growth performance of weanling pigs fed spray-dried animal plasma: a review. *Livest. Prod. Sci.* 68, 263–274. doi: 10.1080/10495390600956946

Van Vranken, J. G., Na, U., Winge, D. R., and Rutter, J. (2015). Protein-mediated assembly of succinate dehydrogenase and its cofactors. *Crit. Rev. Biochem. Mol. Biol.* 50, 168–180. doi: 10.3109/10409238.2014.990556

Verweij, F. J., Revenu, C., Arras, G., Dingli, F., Loew, D., Pegtel, D. M., et al. (2019). Live tracking of inter-organ communication by endogenous exosomes in vivo. *Dev. Cell* 48, 573–589.e4. doi: 10.1016/j.devcel.2019.01.004.

Warner, J. R. (1999). The economics of ribosome biosynthesis in yeast. *Trends Biochem. Sci.* 24, 437–440. doi: 10.1016/s0968-0004(99)01460-7

Watson, S. A., and McStay, G. P. (2020). Functions of cytochrome c oxidase assembly factors. *Int. J. Mol. Sci.* 21:7254. doi: 10.3390/ijms21197254

Yu, Y., and Schachner, M. (2013). Syntenin-a promotes spinal cord regeneration following injury in adult zebrafish. *Eur. J. Neurosci.* 38, 2280–2289.

Conflict of Interest: JP was employed by APC Europe SL.

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

Publisher's Note: 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 in Marine Science

ORIGINAL RESEARCH
published: 24 February 2022
doi: 10.3389/fmars.2022.843637

Integrated Analysis of Metabolomics and Transcriptomics for Assessing Effects of Fish Meal and Fish Oil Replacement on the Metabolism of Rainbow Trout (*Oncorhynchus mykiss*)

Yiming Cao[1,2], Qinfeng Gao[1,2], Xueqi Li[1,2], Yangen Zhou[1,2], Shuanglin Dong[1,2], Yinbo Wang[1,2] and Zhongyi Dai[1,2]*

[1] Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao, China, [2] Function Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China

OPEN ACCESS

Edited by:
Kang-le Lu,
Jimei University, China

Reviewed by:
Linghong Miao,
Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences, China
M. Virginia Martín,
Centro Oceanográfico de Canarias, Instituto Español de Oceanografía (IEO), Spain
Zhenyan Cheng,
Tianjin Agricultural University, China
Federico Moroni,
University of Insubria, Italy

***Correspondence:**
Qinfeng Gao
qfgao@ouc.edu.cn

Specialty section:
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

Received: 26 December 2021
Accepted: 02 February 2022
Published: 24 February 2022

Citation:
Cao Y, Gao Q, Li X, Zhou Y, Dong S, Wang Y and Dai Z (2022) Integrated Analysis of Metabolomics and Transcriptomics for Assessing Effects of Fish Meal and Fish Oil Replacement on the Metabolism of Rainbow Trout (Oncorhynchus mykiss). Front. Mar. Sci. 9:843637. doi: 10.3389/fmars.2022.843637

Reducing dependency on dietary fish meal (FM) and fish oil (FO) is extremely important for the sustainable development of the aquaculture industry. However, the metabolic consequences and mechanisms underlying the replacement of dietary FM and FO by terrestrial proteins (TPs) and lipids remain unclear. To reveal the effects of replacing dietary FM and/or FO on the metabolic changes, the integrated analysis of metabolomics and transcriptomics were employed to evaluate the changes in metabolites and genes of rainbow trout (*Oncorhynchus mykiss*) feeding different experimental diets. Four diets were formulated for the 84-day duration of the experiment: control group (FMFO), FM and vegetable oil (FMVO), terrestrial protein and FO (TPFO), and terrestrial protein and vegetable oil (TPVO). Integrated metabolomic and transcriptomic analyses revealed the significant difference in the metabolic pathways of *O. mykiss* among the three replacement schemes, i.e., single replacement of dietary FM by TP, single replacement of dietary FO by VO, and combined replacement of FM by TP and FO by VO. The combined replacement of FM and FO by TP and VO, respectively, disturbed immune function, energy metabolism, cellular protein biosynthesis capacity, and lipid metabolism of *O. mykiss*. The reduction of antioxidant capacity was only observed in individuals feeding diets with replacement of FM by TP. Furthermore, as soon as the dietary FM and/or FO were reduced, cellular protein biosynthesis ability was suppressed and accompanied by higher energy consumption in response to fluctuations of dietary quality, resulting in reduced growth performance. Interestingly, adenylosuccinate and adenosine monophosphate involved in purine metabolism were induced by both individual and combined replacement of FM and FO by TPs and lipids, respectively. It suggested that these two metabolites might be potential biomarkers for *O. mykiss* fed diets with reduction of FM and/or FO. This study constitutes a new

understanding of the molecular and metabolic mechanisms of *O. mykiss* in response to the replacement of dietary FM and/or FO by TP and/or VO, respectively, and built a theoretical basis for further improvement of aquafeed formulation and sustainable development of aquaculture.

Keywords: fish meal, fish oil, replacement, *Oncorhynchus mykiss*, metabolomics, transcriptomics

INTRODUCTION

Fish meal (FM) and fish oil (FO) are known as the most desirable ingredients used in the formulation of aquafeeds (Turchini et al., 2019). Firstly, FM is regarded as the most reliable protein source owing to its relatively high protein level, excellent amino acid (AA) composition, good palatability, and digestibility to fulfill the dietary requirement of aquatic species (Olsen and Hasan, 2012). Moreover, FO constitutes one of the major dietary components in aquafeed to provide energy and essential fatty acids (EFA), especially long-chain ω3 polyunsaturated fatty acids, which are short in vegetable oil (VO) (Geay et al., 2015). Such long-chain unsaturated FAs are crucial for the growth, health, and development of aquatic animals (Tocher, 2010). However, overfishing of wild fish to produce FM and FO for aquaculture use is unsustainable and as such reducing the reliance on aquaculture feeds, composed of FM and FO is essential for the sustainable development aquaculture industry (Klinger and Naylor, 2012).

The possibility of feeding farmed fish with diets based on various alternative protein sources, mainly terrestrial proteins (TPs), has been evaluated by numerous studies (Panserat et al., 2009; Glencross et al., 2011; Messina et al., 2013; Oliva-Teles et al., 2015; Rimoldi et al., 2018; Choi et al., 2019; Randazzo et al., 2021). Plant proteins are considered the desirable alternative source for FM because of their reasonable price, consistent quality, and stable supply (Oliva-Teles et al., 2015). However, the unbalanced AAs profiles and presence of antinutritional factors (ANFs) limit the application of plant-based proteins in aquafeed (Krogdahl et al., 2003; Espe et al., 2006). The depression of growth performance caused by the replacement of about 80% dietary FM with one protein source has been documented in carnivorous fish species (Dias et al., 2005). Animal proteins typically have adequate AA profiles, suitable digestible phosphorus, and the absence of ANFs compared to plant proteins. However, the relatively high ash content in some animal proteins can negatively affect the utilization of dietary macromolecules by aquatic species, which results in repressed growth performance (Bureau et al., 1999; Moreno-Arias et al., 2018). Therefore, one strategy to overcome such shortcomings is to combine multiple alternative dietary protein sources to achieve a complementary effect and to offset the undesirable ingredients present in the alternative sources, thus improving the growth performance of farmed fish (Li S. et al., 2021). In addition to the unfavorable components of alternative protein sources, the energy metabolism of fish needs to be considered. It is known that due to the limitation of carbohydrate utilization in fish, dietary proteins (especially several AAs) are typically utilized preferentially as fuel for energy metabolism in fish (Jia et al., 2017). Therefore, the development of alternative FM diets must ensure a sufficient supply of AAs for protein synthesis and energy metabolism.

As regards FO, VO is widely considered as the potential alternative ingredient that can effectively replace dietary FO at high levels without significant effect on the growth performance of fish (Hixson et al., 2014; Betancor et al., 2015; Oliva-Teles et al., 2015; Grayson and Dabrowski, 2020; Lu et al., 2020). Despite the desired positive results, the FA composition of VO can negatively affect the lipid metabolism of the farmed fish. Additionally, previous studies indicated that unbalanced dietary FA profiles can induce intestinal enteritis and oxidative stress, which is the response of the immune system to external physical or chemical stimuli (Petropoulos et al., 2009). Thus, both the growth performance of the fish and the quality of the final product needs to be taken into account when replacing dietary FO with alternative sources. For this consideration, several solutions have been developed to reduce the negative effects of replacing FO with VO on the metabolism and product quality of aquatic species. For example, the use of new oil sources (Hixson et al., 2014; Grayson and Dabrowski, 2020) and transgenic plants (Betancor et al., 2015) have been tested. As such, further understanding of lipid metabolism in fish feeding diets with replacement of FO by alternative sources is required.

Considering that partial replacement of dietary FM or FO has been successfully achieved, it is feasible to conduct investigations to evaluate the effects of combined replacement of dietary FM and FO. Previous studies have investigated the effects of combined replacement of FM and FO on fish. For instance, Torrecillas et al. (2017) successfully reduced dietary FM and FO levels of European sea bass (*Dicentrarchus labrax*) down to 10 and 3%, respectively. Glencross et al. (2016) achieved the total replacement of dietary FO by ricebran oil and 90% dietary FM by TP in Asian seabass (*Lates calcarifer*). Uncertainty and controversy exist regarding the effects of supplementary TP and lipid in feed on fish metabolism and growth. A high level of dietary FM and FO substitution, for example, resulted in reduced growth performance after 1 year due to the unbalanced AA profiles (Torstensen et al., 2008). Moreover, most current studies about the replacement of dietary FM and FO were focused on the growth performance of fish while reports regarding the effects of such replacement on fish metabolism are scarce so far.

Fish metabolism involves a series of complex biological processes with the regulations of numerous genes and the production of various metabolites. Recently, metabolomics and transcriptomics have been increasingly used to study the physiological and molecular mechanisms of organisms in response to external stimuli (Yan et al., 2018; Kong et al., 2020). Transcriptome analysis can help to determine important

information about gene expression levels and is a valuable tool for understanding biological information. However, transcriptome sequencing cannot reflect the metabolic levels in an organism. Meanwhile, metabolomics has been successfully employed in nutrition research because it can reveal the metabolite profiles of an organism and further clarification on its metabolic response to various dietary ingredients (Wei et al., 2017; Alfaro and Young, 2018). As such, a simultaneous analysis combining transcriptomic and metabolomic information can compensate for the shortage of the application of a single method and improve the systematics and accuracy for understanding the physiological processes and the metabolic and molecular mechanisms of the target organism (Hao et al., 2019; Li Y. et al., 2021).

As the second-largest salmonid species in terms of total production worldwide, rainbow trout (*Oncorhynchus mykiss*) is one of the most commercially important species (FAO, 2020). *O. mykiss* aquaculture consumes considerable FM and FO as the main ingredients of fish diets. As such, it is economically and ecologically significant to minimize the usage of FM and FO by means of replacing such feed ingredients with alternative terrestrial sources. The present investigation is aimed to evaluate the effects of combined replacement of dietary FM and FO on the metabolism of *O. mykiss* and the molecular mechanism based on the integrated information from transcriptomic and metabolomic analyses to provide a scientific base for the development of feed techniques in *O. mykiss* farming.

MATERIALS AND METHODS

Experimental Diets

Four experimental diets with isonitrogenous (461.9 g/kg) and isolipidic (160.4 g/kg) were formulated. The use of alternative sources of FM and FO in the present study was referred to previous studies on *O. mykiss* (Panserat et al., 2009; Rimoldi et al., 2018), and the feed formulation was appropriately modified according to the nutritional requirements of juvenile *O. mykiss* (Guillaume et al., 2001). The specific compositions of the four diets are as follows: (1) FMFO diet only containing FM and FO as the protein and lipid sources, respectively, (2) FMVO diet containing FM and 80% VO plus 20% FO, (3) TPFO diet containing FO and 70% TP plus 30% FM, (4) TPVO diet containing 70% TP plus 30% FM and 80% VO plus 20% FO. The feed formulation and proximate analysis are shown in **Table 1**. All feed ingredients were completely mixed and pelletized, dried in an oven at 55°C for 16 h, and frozen at −20°C for future use.

Experimental Conditions and Sample Collection

The feeding experiment was carried out in Haiyang Yellow Sea Aquatic Product Co., Ltd. (Shandong, China). All *O. mykiss* obtained from Wanzefeng Fishery Company (Rizhao, Shandong, China) were acclimated to the experimental conditions for 14 days. A total of 240 *O. mykiss* with an average weight of 38.09 ± 0.73 g were randomly assigned to 12 aquariums with a volume of 400 L, i.e., 20 fish in each tank. Three tanks of fish were grouped as one of the four feed treatments and fed with one of the four diets three times every day (at 8:00, 15:00, and 21:00) for 84 consecutive days. Feces and residual feeds were collected after one hour of feeding, and the weight of residual feed was recorded. The temperature was kept constant at 16 ± 0.5 °C with automatic regulators. The dissolved oxygen was kept at no less than 6.0 mg L^{-1}. An artificially controlled photoperiod was established as 12 h light: 12 h dark during the feeding experiment. Sufficient aeration ensured homogenous environmental conditions for each aquarium.

All individuals in each replicate were weighed to determine the final body weight (FBW), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER), and lipid efficiency ratio (LER) at the end of the experiment. All individuals were anesthetized before tissue sample collection. Condition factor (K) and hepatosomatic index (HSI) of *O. mykiss* were calculated using the body weight, length, and liver weight of four fish from each tank. And feces samples were collected for apparent digestibility coefficient (ADC) analyses. Three individuals were collected from each group for RNA-seq, and six fish from each treatment were collected for metabolomic analysis. And the liver of each individual was quickly removed and frozen at −80°C ultra-low temperature refrigerator until subsequent multi-omics analysis.

Transcriptomic Analysis

TRIzol® Reagent was applied to extract the total RNA from the liver and genomic DNA was cleared by DNase I (TaKara). RNA quality was detected by 2100 Bioanalyser (Agilent) and ND-2000 (NanoDrop Technologies) was applied for quantification. Isolation of mRNA by oligo(dT) beads following the polyA selection method, and sequencing libraries were constructed by the TruSeqTM RNA sample of preparation Kit from Illumina (San Diego, CA, United States). The Illumina HiSeq X Ten/NovaSeq 6000 sequencer was employed to sequence the Paired-end RNA-seq (2 × 150 bp read length). Finally, the clean reads were obtained from the raw reads being trimmed. All RNA-seq data have been uploaded to the NCBI Sequence Read Archive (SRA) database, accession NO: PRJNA788999. Gene abundances were quantified by RSEM[1] and normalized based on transcripts per million reads value. Essentially, differential expression analysis was performed using the DESeq2 with $P < 0.05$, and $|\log2FC| > 1$. In addition, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was applied to detect which differentially expressed genes (DEGs) were significantly enriched at $P < 0.05$. KOBAS[2] was applied for KEGG pathway analysis (Xie et al., 2011).

Validation of Differentially Expressed Genes by qRT-PCR

Ten DEGs were randomly selected to validate the RNA-seq results by qRT-PCR. Primers for qRT-PCR were designed using Primer Premier 5.0 software, and primers' details were listed in **Supplementary Table 1**. EF1α and β-actin were employed as the internal control for qRT-PCR. The temperature programming conditions were: denaturation at 95°C for 30 s, followed by 35

[1]http://deweylab.biostat.wisc.edu/rsem/

[2]http://kobas.cbi.pku.edu.cn/home.do

TABLE 1 | Ingredient and proximate composition of experimental diets (all values are g/kg).

Ingredient	Diets			
	FMFO	FMVO	TPFO	TPVO
Fish meal[a]	620.0	620.0	186.0	186.0
Soybean meal[a]	–	–	120.0	120.0
Meat and bone meal[a]	–	–	120.0	120.0
Corn gluten meal[a]	–	–	200.0	200.0
Wheat gluten[a]	–	–	100.0	100.0
Fish oil[b]	110.0	22.0	110.0	22.0
Rapeseed oil[b]	–	88.0	–	88.0
Lecithin[b]	–	–	10.0	10.0
Corn starch[b]	247.0	247.0	116.1	116.1
Choline chloride[b]	5.0	5.0	5.0	5.0
Betaine[b]	5.0	5.0	5.0	5.0
Vitamin premix[c]	5.0	5.0	5.0	5.0
Mineral premix[d]	5.0	5.0	5.0	5.0
Mold inhibitor[e]	1.0	1.0	1.0	1.0
Yttrium oxide	2.0	2.0	2.0	2.0
Methionine	–	–	2.4	2.4
Lysine	–	–	12.5	12.5
Total	1,000.0	1,000.0	1,000.0	1,000.0
Proximate analysis				
Crude protein	459.5	459.5	464.4	464.4
Crude lipid	161.5	161.5	159.3	159.3
Ash	98.5	97.3	73.3	72.5
Moisture	100.2	96.3	95.5	93.6

[a]*Fish meal: crude protein 740.8 g/kg, crude lipid 79.0 g/kg; soybean meal: crude protein 524.4 g/kg, crude lipid 22.6 g/kg; meat and bone meal: crude protein 490.9g/kg, crude lipid 118.4 g/kg; corn gluten meal: crude protein 621.5g/kg, crude lipid 24.4 g/kg; wheat gluten: crude protein 803.8 g/kg, crude lipid 16.2 g/kg; All these ingredients were obtained from Qihao Co., Ltd. (Shandong, China).*
[b]*All these ingredients were obtained from Qihao Co., Ltd. (Shandong, China).*
[c]*Vitamin premix (mg/kg diet): vitamin D, 5; vitamin K, 10; vitamin B12, 10; vitamin B6, 20; folic acid, 20; vitamin B1, 25; vitamin A, 32; vitamin B2, 45; pantothenic acid, 60; biotin, 60; niacin acid, 200; α-tocopherol, 240; inositol, 800; and ascorbic acid, 2000.*
[d]*Mineral premix (mg/kg diet): $CuSO_4 \cdot 5H_2O$, 10; Na_2SeO_3 (1%), 25; $ZnSO_4 \cdot H_2O$, 50; $CoC_{12} \cdot 6H_2O$ (1%), 50; $MnSO_4 \cdot H_2O$, 60; $FeSO_4 \cdot H_2O$, 80; $Ca(IO_3)_2$, 180; $MgSO_4 \cdot 7H_2O$, 1,200; and zeolite, 8,345.*
[e]*Mold inhibitor contained fumaric acid and calcium propionate in the ratio 1:1.*
FMFO, fishmeal and fish oil; FMVO, fishmeal and vegetable oil; TPFO, terrestrial protein and fish oil; TPVO, terrestrial protein and vegetable oil.

cycles of 95°C for 10 s, 60°C for 30 s, 95°C for 15 s, 60°C for 60 s, and 95°C for 1 s. Gene expression results were obtained using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Metabolomic Analysis

Metabolites Extraction and Analysis With LC-MS

For metabolomics analysis, the 50 mg solid sample from each group was separated with 400 μL of the solution containing methanol and water 4:1 (v/v). The samples were homogenized by a tissue crusher at 50 Hz for 6 min and then sonicated for 30 min at 40 kHz and 5°C. The samples were placed for 30 min at −20°C to achieve protein precipitation. The mixture was centrifuged at 4°C, 13,000*g* for 15 min, and the supernatant was separated for subsequent analysis.

The LC-MS analysis was conducted using an ExionLCTMAD system (AB Sciex, United States) equipped with an ACQUITY UPLC BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm; Waters, Milford, CT, United States). The UPLC system worked in tandem with a quadrupole time-of-flight mass spectrometer (Triple TOF^{TM} 5600+, AB Sciex, United States) that has an electrospray ionization (ESI) source with both positive and negative ionization modes of operation.

Data Preprocessing and Metabolite Identification

The positive and negative data obtained from the LC-MS analysis were combined and loaded into SIMCA software (V14.1, Umea, Sweden) for orthogonal projections for latent structures-discriminant analysis (OPLS-DA). Afterward, sevenfold cross-validation was performed. Differential metabolites (DMs) were identified according to the variable importance in projection values (VIP > 1) obtained from the OPLS-DA model and $P < 0.05$ from the Student's *t*-test. KEGG[3] was employed for annotating metabolites and searching for metabolite pathways. The scipy stats[4] was employed to confirm the statistically significantly enriched pathway by Fisher's exact test.

Comprehensive Analysis of Metabolomics and Transcriptomics

Kyoto Encyclopedia of Genes and Genomes was applied to annotate and enrich the metabolic pathways mapped by DMs and DEGs of *O. mykiss*, and DMs and DEGs enriched to the shared KEGG pathways were filtered for subsequent analysis. Pearson correlation analysis was utilized to identify the correlation between DMs and DEGs *O. mykiss* using the Cytoscape software. A heatmap was used to show the correlation of the DMs and DEGs, respectively. $P < 0.05$ was considered a statistically significant level of difference.

Calculation and Statistical Analysis

The growth performance of fish was determined by the following equations:

$$\mathrm{SGR} = (\mathrm{Ln}W_f - \mathrm{Ln}W_0) \times 100/t$$

$$\mathrm{K} = (W_f/\mathrm{L}^3) \times 100$$

$$\mathrm{FE}{=}(W_f - W_0)/\text{ingested feed}$$

$$\mathrm{ADC} = [1 - (\text{dietary } Y_2O_3 \text{ content} \times \text{ fecal nutrient content})/ (\textit{feed nutrient content} \times \text{ fecal } Y_2O_3 \text{ content})] \times 100$$

$$\mathrm{PER} = (W_f - W_0)/\text{protein fed}$$

$$\mathrm{LER} = (W_f - W_0)/\text{lipid fed}$$

where W_f and W_0 represent final and initial body weight of *O. mykiss*, *t* represents the duration of experiment. L represents the length of *O. mykiss*.

[3]http://www.genome.jp/kegg/
[4]https://docs.scipy.org/doc/scipy/

All results are expressed as mean ± SD. The growth performance of *O. mykiss* was analyzed using two-way ANOVA with the percentage of dietary FM or FO as fixed factors. $P < 0.05$ indicated that the data has statistically significant differences. All analyses were performed on the SPSS 21.0 software.

RESULTS

Growth Performance

After the 84-d experiment, no interaction term between dietary levels of FM and FO on growth performance or feed utilization were observed in *O. mykiss* (**Table 2**). Significant differences in the growth performance in terms of FBW, SGR, and K and the feed utilization in terms of ADC were observed between the two FM inclusion levels ($P < 0.05$) and HSI showed a significant difference in *O. mykiss* between the two FO levels ($P < 0.05$). In addition, there was no significant difference of FE, PER, and LER in *O. mykiss* fed diets with replacement of dietary FM or FO by TP or VO, respectively ($P > 0.05$).

Transcriptomics Analysis of Replacement of Fish Meal and/or Fish Oil by Terrestrial Protein and/or Vegetable Oil

Replacement of Fish Meal by Terrestrial Protein for Differentially Expressed Genes and Metabolic Pathways

A total of 518 and 93 DEGs were identified between FMFO vs. TPFO and FMVO vs. TPVO, respectively. There were 24 overlapping DEGs between FMFO vs. TPFO and FMVO vs. TPVO (**Figure 1A**). In the comparison of FMFO vs. TPFO, 378 and 140 genes were upregulated and downregulated, respectively. Meanwhile, there were 48 and 45 genes showing up-regulated and down-regulated expression in FMVO vs. TPVO, respectively (**Figure 1B** and **Supplementary Figure 1**).

To further explore the metabolic pathways of the replacement of FM by TP, the DEGs in each group were annotated (**Supplementary Table 2**). Based on significant pathways ($P < 0.05$), KEGG was applied to correlate the DEGs with metabolic pathways. It was determined that most of DEGs in the comparison of FMFO vs. TPFO were enriched in "steroid biosynthesis," "aminoacyl-tRNA biosynthesis," "protein processing in endoplasmic reticulum," "glycine, serine and threonine metabolism," and "PPAR signaling pathway" (**Figure 1C**), while most of DEGs in the comparison of FMVO vs. TPVO were enriched in "steroid biosynthesis," "PPAR signaling pathway," "glutathione metabolism," and "pentose and glucuronate interconversions" (**Figure 1D**).

Replacement of Fish Oil by Vegetable Oil for Differentially Expressed Genes and Metabolic Pathways

There were 140 and 48 DEGs identified in FMFO vs. FMVO and TPFO vs. TPVO, respectively. A total of 3 overlapping DEGs between FMFO vs. TPFO and FMVO vs. TPVO. In the comparison of FMFO vs. FMVO, 104 genes were expressed up-regulated and 36 genes were expressed down-regulated. Meanwhile, 21 and 27 gene expressions were up- and down-regulated in TPFO vs. TPVO, respectively (**Supplementary Figure 1**).

To further explore the metabolic pathways of the replacement of FO by VO, the DEGs in each group were annotated (**Supplementary Table 3**). Based on significant pathways ($P < 0.05$), KEGG was employed to correlate the DEGs with metabolic pathways. It was determined that most of DEGs in FMFO vs. FMVO were enriched in "oxidative phosphorylation," "steroid biosynthesis," and "arachidonic acid metabolism" (**Figure 1E**), while most of DEGs in TPFO vs. TPVO were enriched in "alanine, aspartate and glutamate metabolism," "PPAR signaling pathway," "D-glutamine, and D-glutamate metabolism" and "fatty acid biosynthesis" (**Figure 1F**).

Combined Replacement of Fish Meal and Fish Oil by Terrestrial Protein and Vegetable Oil for Differentially Expressed Genes and Metabolic Pathways

A total of 210 DEGs were identified in FMFO vs. TPVO. Compared with the control group, 121 and 89 genes showed up-regulated and down-regulated expression in the TPVO group, respectively (**Supplementary Figure 1**).

To further explore the metabolic pathways of the combined replacement of FM and FO by TP and VO, respectively, the DEGs in each group were annotated (**Supplementary Table 4**). Based on pathway $P < 0.05$, KEGG was employed to correlate the DEGs with metabolic pathways. It was determined that most of DEGs in FMFO vs. TPVO were enriched in "steroid biosynthesis," "protein processing in endoplasmic reticulum," "glycine, serine and threonine metabolism," "purine metabolism," "phenylalanine metabolism," and "arachidonic acid metabolism" (**Figure 1G**).

Metabolomic Analysis of Replacement of Fish Meal and/or Fish Oil by Terrestrial Protein and/or Vegetable Oil

Overall Changes on the Liver in Metabolites in Response to Replacement of Fish Meal and/or Fish Oil by Terrestrial Protein and/or Vegetable Oil

In this LC-MS analysis, a total of 12,928 valid peaks were extracted. To maximize the discrimination between the replacement of dietary FM and/or FO by TP and/or VO and the control groups, and OPLS-DA was applied to determine the differences in metabolite levels between comparable groups. R^2Y represents the percentage of all sample variables explained by the model. Q^2 represents the percentage of all sample variables predicted by the model. OPLS-DA score plots of FMFO vs. TPFO, FMVO vs. TPVO, FMFO vs. FMVO, TPFO vs. TPVO, and FMFO vs. TPVO had the cumulative values of R^2Y being 0.909, 0.927, 0.967, 0.976, and 0.975, and Q^2 being 0.838, 0837, 0.767, 0.629, and 0.923, respectively, indicating that the model derived by OPLS-DA were highly plausible (**Supplementary Figure 2**) and can be employed in the further analysis.

TABLE 2 | Two-way factorial analysis of variance (ANOVA) for the growth performance and feed utilization of *O. mykiss* fed different diets.

Item	*df*	*P*	Multiple comparisons			
			FM		FO	
			100%	30%	100%	20%
Dependent variable: FBW						
FM	1	<0.001	293.99 ± 12.82	263.99 ± 19.42	282.20 ± 18.73	275.77 ± 25.26
FO	1	0.182				
FM × FO	1	0.632				
Dependent variable: SGR						
FM	1	<0.001	2.43 ± 0.06	2.30 ± 0.08	2.38 ± 0.08	2.35 ± 0.11
FO	1	0.134				
FM × FO	1	0.489				
Dependent variable: K						
FM	1	0.023	1.45 ± 0.03	1.42 ± 0.03	1.44 ± 0.04	1.43 ± 0.03
FO	1	0.852				
FM × FO	1	0.536				
Dependent variable: FE						
FM	1	0.126	0.92 ± 0.04	0.88 ± 0.05	0.90 ± 0.04	0.89 ± 0.06
FO	1	0.658				
FM × FO	1	0.682				
Dependent variable: HSI						
FM	1	0.624	2.17 ± 0.13	2.18 ± 0.13	2.07 ± 0.07	2.27 ± 0.09
FO	1	<0.001				
FM × FO	1	0.628				
Dependent variable: ADC (*protein*)						
FM	1	0.003	71.22 ± 0.99	72.16 ± 1.08	71.93 ± 1.21	71.45 ± 1.01
FO	1	0.114				
FM × FO	1	0.627				
Dependent variable: PER						
FM	1	0.066	2.00 ± 0.99	1.89 ± 0.11	1.95 ± 0.09	1.93 ± 0.14
FO	1	0.659				
FM × FO	1	0.683				
Dependent variable: LER						
FM	1	0.356	5.35 ± 0.25	5.50 ± 0.33	5.46 ± 0.23	5.39 ± 0.35
FO	1	0.649				
FM × FO	1	0.672				

Data are expressed as mean ± SD, (n = 3), df: degree of freedom. FBM, final body weight; SGR, specific growth rate; K, condition factor; FE, feed efficiency; HSI, hepatosomatic index; ADC, apparent digestibility coefficient; PER, protein efficiency ratio; LER, lipid efficiency ratio.

Differential Metabolites and Metabolic Pathways for Replacement of Fish Meal by Terrestrial Protein

A total of 204 and 199 DMs were identified between FMFO vs. TPFO and FMVO vs. TPVO, respectively. In the comparison of FMFO vs. TPFO, there were 70 and 134 DMs upregulated and downregulated, respectively. In the comparison of FMVO vs. TPVO, 42 and 157 DMs were upregulated and downregulated, respectively. There were 127 overlapping metabolites between FMFO vs. TPFO and FMVO vs. TPVO (**Supplementary Figure 3**).

To identify the potential metabolic pathways affected by the replacement of FM by TP, KEGG was applied to correlate the DMs with metabolic pathways. The results of pathway analysis described in detail the metabolic pathway changes related to the replacement of FM by TP(**Figure 2**). The most closely related metabolic pathways of FMFO vs. TPFO and FMVO vs. TPVO were shown in **Supplementary Table 5**.

Differential Metabolites and Metabolic Pathways for Replacement of Fish Oil by Vegetable Oil

There were 167 and 122 DMs identified in FMFO vs. FMVO and TPFO vs. TPVO, respectively. In the comparison of FMFO vs. FMVO, 87 DMs were expressed up-regulated and 80 DMs were expressed down-regulated. In the comparison of TPFO vs. TPVO, 36 and 86 DMs were upregulated and downregulated, respectively. A total of 67 overlapping metabolites between FMFO vs. FMVO and TPFO vs. TPVO (**Supplementary Figure 3**).

To identify the potential metabolic pathways affected by the replacement of FO by VO, KEGG was applied to

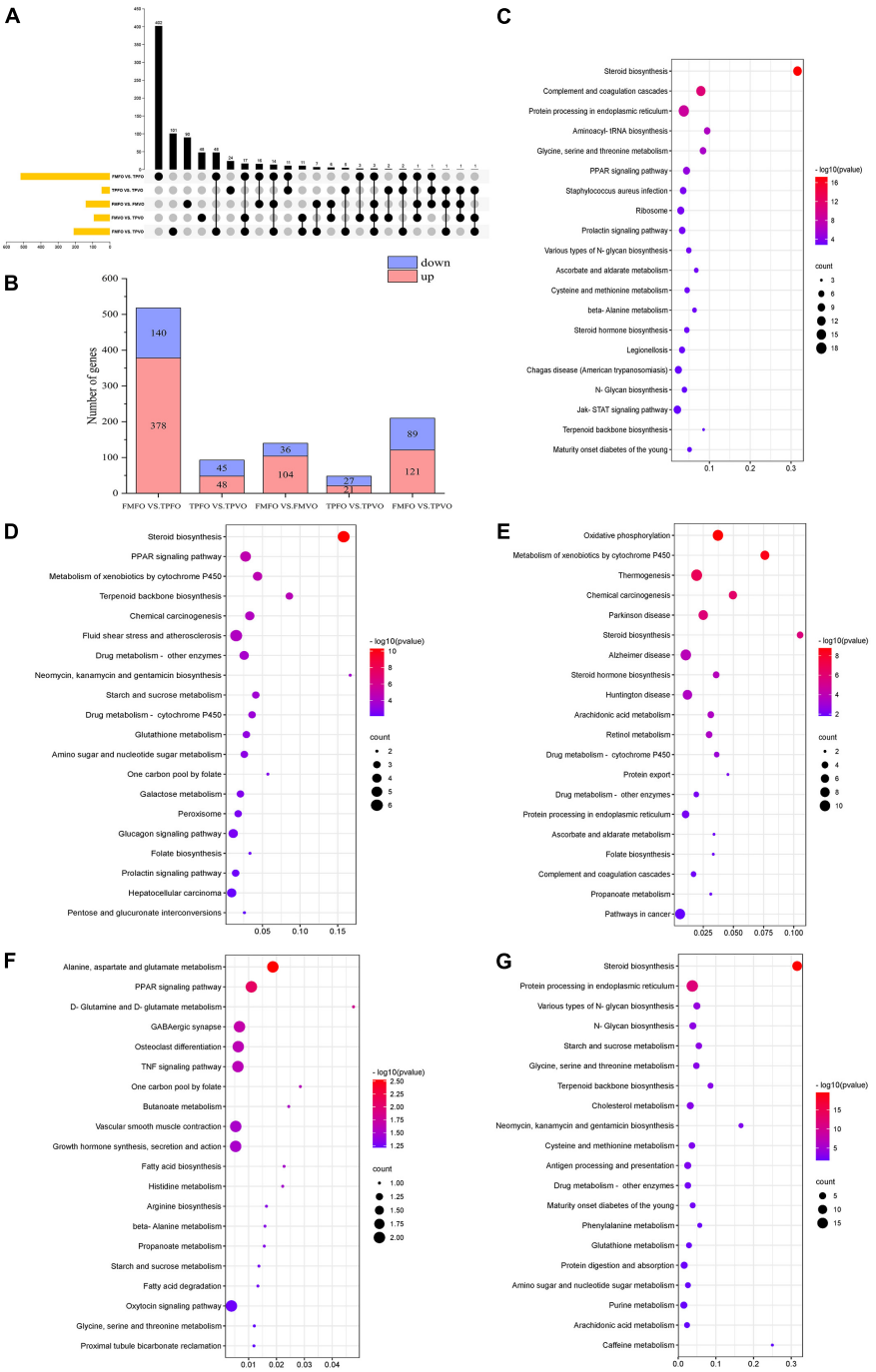


FIGURE 1 | Transcriptomic analysis of *O. mykiss* fed diets with replacement of FM and/or FO. The number of up- and down-regulated DEGs were identified in the FM replacement and FO replacement **(A,B)**. KEGG pathways analysis of DEGs in response to FM replacement **(C,D)**, FO replacement **(E,F)**, and combined replacement of FM and FO **(G)**. Pathway enrichment analysis plots (top 20) of upregulated expressed metabolisms according to $P < 0.05$.

correlate the DMs with metabolic pathways. The results of pathway analysis described in detail the metabolic pathway changes related to the replacement of FO by VO (**Figure 2**). The most closely related metabolic pathways of FMFO vs. FMVO and TPFO vs. TPVO were shown in **Supplementary Table 6**.

Differential Metabolites and Metabolic Pathways for Combined Replacement of Fish Meal and Fish Oil by Terrestrial Protein and Vegetable Oil

A total of 220 DMs were identified between FMFO vs. TPVO. Compared to the control group, 63 and 157 DMs were upregulated and downregulated in the combined

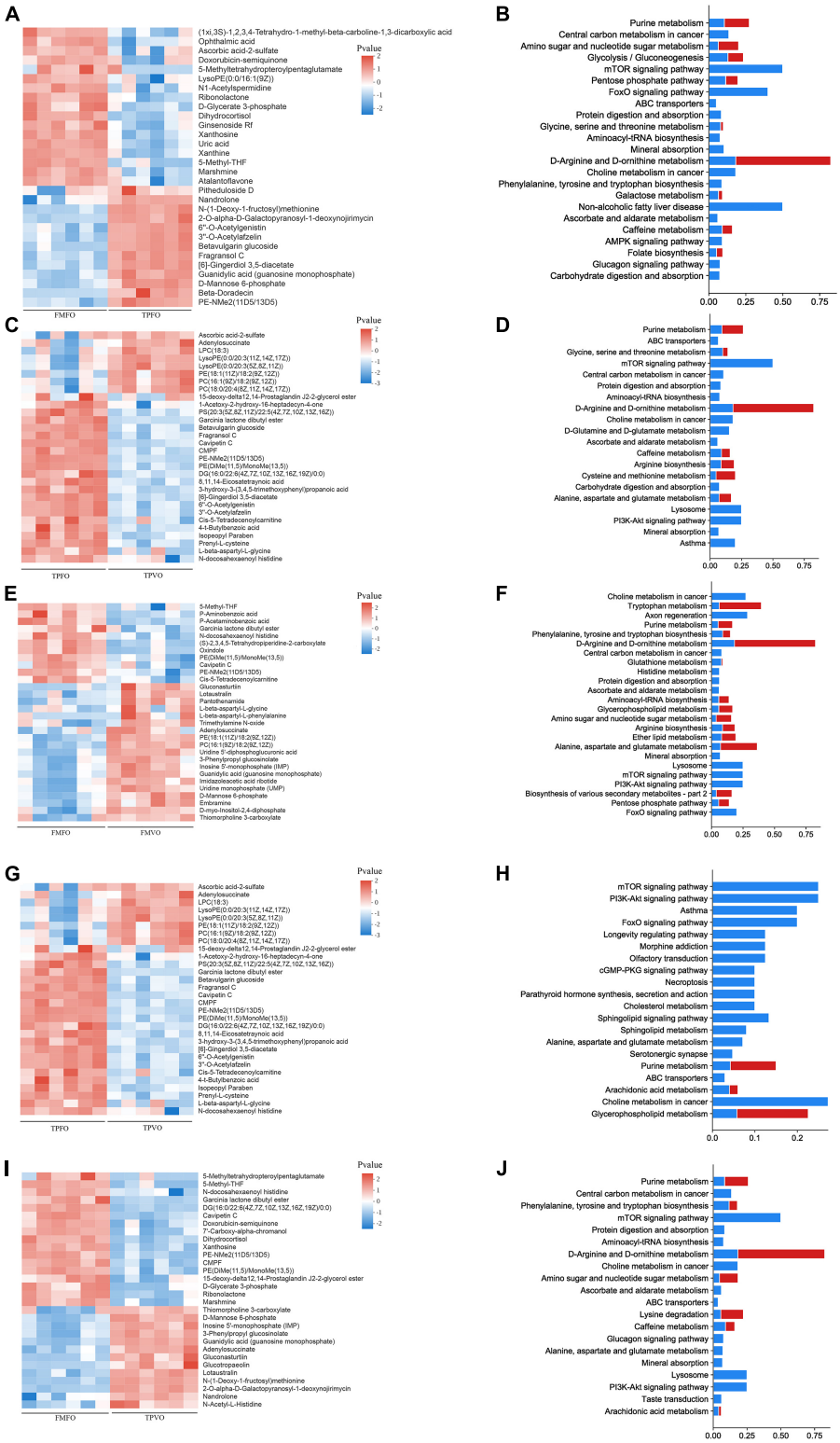


FIGURE 2 | Metabolomic profiles of *O. mykiss* fed diets with replacement of FM and/or FO. Heatmaps and pathway analysis plots of DMs in FM replacement **(A–D)**, FO replacement **(E–H)**, and combined replacement of FM and FO **(I,J)**. The blue bar in pathway analysis plots showed enrichment ratio and the red bar showed pathway impact value calculated from pathway topology analysis.

replacement of FM and FO by TP and VO, respectively (**Supplementary Figure 3**).

To identify the potential metabolic pathways affected by the combined replacement of dietary FM and FO, KEGG was applied to correlate the DMs with metabolic pathways. The results of pathway analysis described in detail the metabolic pathway changes related to the combined replacement of dietary FM and FO by TP and VO, respectively (**Figure 2**). The most closely related metabolic pathways of FMFO vs. TPVO were shown in **Supplementary Table 7**.

Integrated Analysis of Metabolomics and Transcriptomics for the Replacement of Fish Meal and Fish Oil by Terrestrial Protein and Vegetable Oil

The integrative analysis of transcriptome and metabolome revealed the shared KEGG pathways of DEGs and DMs of *O. mykiss* fed with reduction of dietary FM and/or FO in the diets (**Supplementary Table 8**). The shared DEGs and DMs involved in these metabolic pathways were shown in **Supplementary Table 9**. For the replacement of FM by TP, 32 shared KEGG pathways were mapped from 63 and 42 shared KEGG pathways between FMFO vs. TPFO and FMVO vs. TPVO, respectively. Among these pathways, 10 significantly important metabolic pathways were identified via topological pathway analysis ("purine metabolism," "cysteine and methionine metabolism," "lysine degradation," "arginine biosynthesis," "glycolysis/gluconeogenesis," "phenylalanine metabolism," "amino sugar and nucleotide sugar metabolism," "glycine, serine and threonine metabolism," "pentose and glucuronate interconversions," and "glutathione metabolism") (**Figure 3A**). For the replacement of FO by VO, 6 shared KEGG pathways were mapped from 33 and 10 shared KEGG pathways in FMFO vs. FMVO and TPFO vs. TPVO, respectively. Among these pathways, six significantly important metabolic pathways were identified via topological pathway analysis ("purine metabolism," "glycine, serine and threonine metabolism," "aminoacyl-tRNA biosynthesis," "cysteine and methionine metabolism," "sphingolipid metabolism," and "alanine, aspartate and glutamate metabolism") (**Figure 3B**). For the combined replacement of FM and FO by TP and VO, respectively, 52 shared KEGG pathways were mapped from FMFO vs. TPVO. Among these pathways, 11 significantly important metabolic pathways were identified via topological pathway analysis ("tryptophan metabolism," "purine metabolism," "lysine degradation," "amino sugar and nucleotide sugar metabolism," "arginine biosynthesis," "glycolysis/gluconeogenesis," "phenylalanine metabolism," "citrate cycle (TCA cycle)," "glycerophospholipid metabolism," "pentose and glucuronate interconversions," and "cysteine and methionine metabolism") (**Figure 3C**). Correlation analysis calculated by Pearson showed tight correlations between DEGs and DMs, which further emphasized the correlation between each DEG and each DM (**Figure 4**).

Shared DMs and DEGs of individual and combined replacement of FM and FO by TP and VO, respectively, were further aggregated to explore the metabolic process that might occur in the liver of *O. mykiss* (**Table 3**). A comprehensive metabolic network of these shared metabolites and genes involving the replacement of dietary FM and/or FO by TP and/or VO was shown in **Figure 5**. Adenylosuccinate and adenosine monophosphate were all upregulated in replacement of dietary FM and/or FO by TP and/or VO and were involved in purine metabolism. The high concentration of 2-(formamido)-N1-(5′-phosphoribosyl) acetamidine, but low contents of xanthosine, xanthine, and urate which possibly due to the low expression of PNP and XDH were found in purine metabolism. The low concentrations of R-S-glutathione, 5-oxoproline, 5-L-glutamyl-L-alanine, and low expression levels of OPLAH, which are involved in the glutathione metabolism, were identified in fish feeding diets with replacement of FM. Moreover, the concentrations of L-tryptophan and anthranilate were both downregulated, but high expression levels of ACAT2 were identified in the combined replacement of dietary FM and FO by TP and VO, respectively. In glycerophospholipid metabolism, lysine metabolism, and tryptophan metabolism, 1-acyl-SN-glycero-3-phosphocholine, phosphatidylcholine (lecithin), PCYT1, L-lysine, and L-2-aminoadipate were downregulated in combined replacement of dietary FM and FO by TP and VO, respectively. In addition, the low expression levels of D-glycerate 3-phosphate and D-glucuronic acid, but the high expression levels of UDP-D-glucuronate may be mainly controlled by the up-regulation of upstream DEGs, which were found in glycolysis/gluconeogenesis and pentose and glucuronate interconversions. Furthermore, L-arginine, L-phenylalanine, and D-glucuronic acid, which are involved in arginine biosynthesis, phenylalanine metabolism, and amino sugar and nucleotide sugar metabolism were expressed at low levels in individual replacement of dietary FM by TP and combined replacement of dietary FM and FO by TP and VO, respectively.

The RNA Sequencing Data Quality Control and Results of qRT-PCR

In our study, the averages of 44.5, 44.2, 44.8, and 43.3 million clean reads were localized to the *O. mykiss* genome from the FMFO, FMVO, TPFO, and TPVO groups, respectively (**Supplementary Table 10**). The GC contents of the four groups showed the average values with 49.9, 49.88, 49.09, and 49.3%, respectively. And Q30 contents were on the average of 95.24, 94.89, 95.06, and 95.10%, respectively. The average map rates of the four groups were 94.75, 94.82, 93.64, and 94.23%, respectively (**Supplementary Table 10**). These data suggested that the sequencing results were favorable and that the results of the subsequent transcriptome analysis were dependable. To further confirm the accuracy and reliability of transcriptomic data, qRT-PCR was conducted to validate the RNA-seq results of 10 DEGs, and the expression patterns were in accordance with the trend of RNA-seq results (**Supplementary Figure 4**).

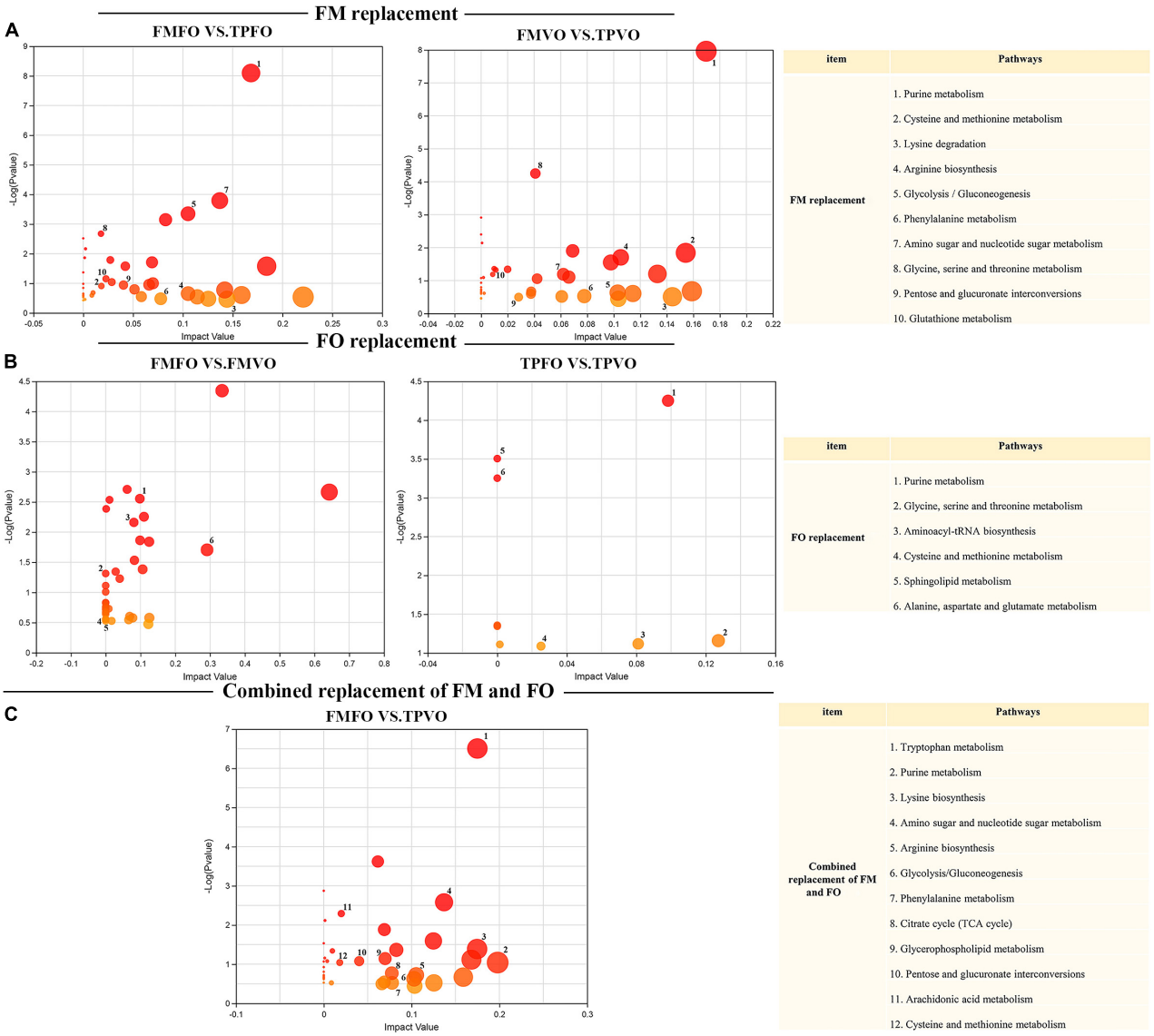


FIGURE 3 | Significant specific pathway identified by multi-omics analysis between FM or FO replacement and combined replacement FM and FO. Pathway analysis of DMs from common pathways shared with DEGs was plotted at the replacement of FM **(A)**, replacement of FO **(B)**, and combined replacement of FM and FO **(C)**. Map00230: purine metabolism; map00310: lysine degradation; map00220: arginine biosynthesis; map00010: glycolysis/gluconeogenesis; map00360: phenylalanine metabolism; map00520: amino sugar and nucleotide sugar metabolism; map00260: glycine, serine, and threonine metabolism; map00040: pentose and glucuronate interconversions; map00480: glutathione metabolism; map00380: tryptophan metabolism; map00020: citrate cycle (TCA cycle); map00564: glycerophospholipid metabolism.

DISCUSSION

Growth Performance

The growth performance results obtained in the present study were consistent with those previously reported in African catfish (*Clarias gariepinus*) (Sourabié et al., 2018), and dietary protein sources were the main limiting factor affecting growth performance. In terms of FO, replacing dietary FO with VO did not compromise the growth performance of *O. mykiss*, which was consistent with the previous studies with partial replacement FO in farmed fish (Glencross et al., 2003; Hardy, 2010). In contrast, individuals feeding diets with reduced dietary FM presented lower feed utilization than fish feeding diets with higher dietary FM content, resulting in poor growth performance regardless of the dietary lipid sources. This result was in agreement with the previous investigations in European sea bass (*D. labrax*) (Torrecillas et al., 2017) and Atlantic cod (*Gadus morhua* L.) (Hansen et al., 2007) that accepted the reduction dietary FM diets. The response of *O. mykiss* to the replacement of FM by TP was mainly associated with the reduction in feed intake and FE (Torstensen et al., 2008). Particularly, some ingredients present in alternative protein sources, such as unbalanced AAs profiles

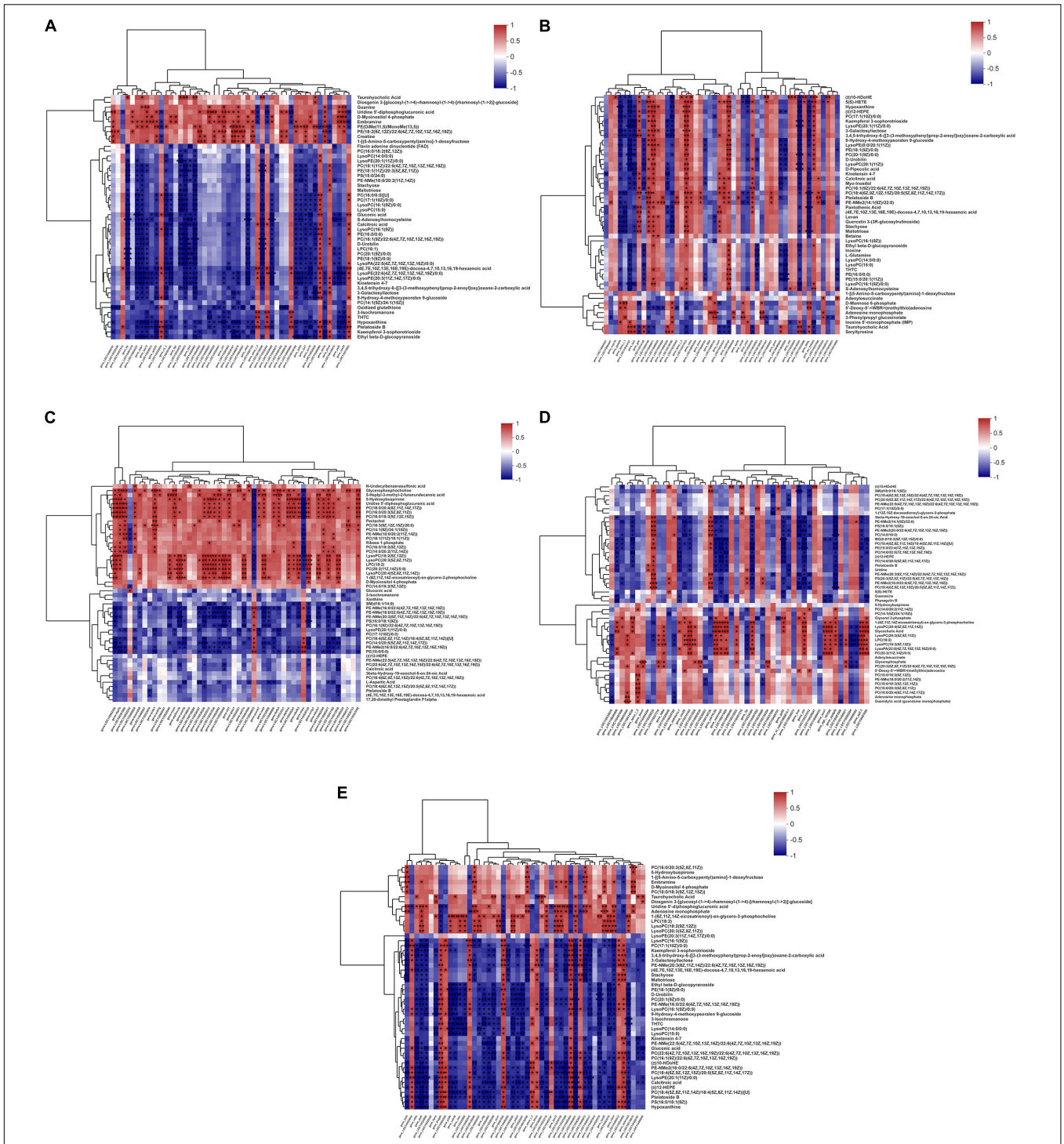


FIGURE 4 | Correlation plots of the correlations between metabolome and transcriptome. The correlation plots with genes in columns and metabolites in rows to reveal the connection between genes and metabolites of *O. mykiss* fed diets with FM replacement **(A,B)**, FO replacement **(C,D)**, and combined replacement FM and FO **(E)**. The red and blue showed the positive and negative correlation between transcriptomics and metabolomics, respectively. The symbol "*" indicates significant correlation between the gene and metabolites ($P < 0.05$).

and anti-nutritional factors repress the growth performance of fish (Li et al., 2014). Additionally, fish feeding diets with reduction of dietary FM and FO appeared to have similar protein and lipid utilization efficiencies to those of individuals in the FMFO group, as no significant differences in PER and LER were observed between individuals in the four experimental groups.

TABLE 3 | Shared DMs and DEGs in the KEGG pathway of *O. mykiss* which fed diets with replacement of FM and/or FO.

Description	Metabolites	Gene
Glutathione metabolism	5-Oxoproline, 5-L-glutamyl-L-alanine, R-S-glutathione	OPLAH
Purine metabolism	2-(Formamido)-N1-(5′-phosphoribosyl)acetamidine, xanthine, xanthosine, adenylosuccinate, adenosine monophosphate, urate	PNP, XDH
Glycerophospholipid metabolism	Phosphatidylcholine (lecithin), 1-acyl-sn-glycero-3-phosphocholine	PCYT1
Glycine, serine, and threonine metabolism	Dimethylglycine	BHMT, DMGDH
Arginine biosynthesis	L-arginine	/
Tryptophan metabolism	L-tryptophan, anthranilate	ACAT2
TCA cycle	Citrate	/
Glycolysis/ gluconeogenesis	D-glycerate 3-phosphate	ALDH3, GCK, HK
Phenylalanine metabolism	L-phenylalanine	TAT, ALDH3
Lysine degradation	L-2-aminoadipate	L-Lysine
Pentose and glucuronate interconversions	UDP-D-glucuronate, D-glucuronic acid	UGP2, UGT
Amino sugar and nucleotide sugar metabolism	D-glucuronic acid, UDP-glucuronate	UGP2

Abbreviations of genes are annotated in ***Figure 5****.*

Similar results have also been documented in Atlantic salmon (Torstensen et al., 2008) and Pacific yellowtail (*Seriola lalandi*) (Nuche-Pascual et al., 2018).

Alteration of Immune Function Pathway Associated With Replacement of Fish Meal and/or Fish Oil

The immune system of fish is the first line of defense against external pathogens and a crucial mechanism in organisms (Saurabh and Sahoo, 2008). In this study, we identified pathways that might be involved in the immune response, such as arginine biosynthesis, amino and nucleotide sugars metabolism, and tryptophan metabolism. Arginine is known to be a multifunctional AA closely related to immune system regulation, mediating immunosuppressive mechanisms while maintaining hepatic urea cycle activity for ammonia detoxification (Wu et al., 2014; Azeredo et al., 2015). Thus, the low concentration of arginine revealed by multi-omics data indicated that the depletion of free arginine and low levels of availability, caused by the individual replacement of FM by TP and combined replacement of dietary FM and FO by TP and VO, respectively, resulted in impaired immunity in the liver of *O. mykiss*. Amino sugar and nucleotide sugars metabolism is also an important immune-related pathway that is associated with external environmental stress (Guo et al., 2014; Xu et al., 2016). In the present investigation, D-glucuronic acid and UGP2 were downregulated in response to the individual replacement of dietary FM by TP and the combined replacement of FM and FO by TP and VO, respectively. Meanwhile, anthranilate is an important intermediate in tryptophan metabolism and involves the synthesis of nonsteroidal anti-inflammatory drugs (NSAIDs) (Sorgdrager et al., 2019). NSAIDs can induce the synthesis of nonsteroidal anti-inflammatory molecules that signal the immune system response and prevent inflammation (Zummo et al., 2012). The low concentrations of L-tryptophan and anthranilate in the present study implied that NSAIDs associated with anthranilate might be inhibited, which might affect the immunity of *O. mykiss* fed diets with combined replacement of FM and FO by TP and VO, respectively. Furthermore, previous reports have demonstrated that numerous energy supplies are crucial for the immune defense processes (Zhang et al., 2019). ACAT2, related to the production of acetyl-CoA, was upregulated and promoted its participation in the TCA cycle, suggesting that fish require additional energy to maintain immune function. Therefore, these results indicated that individuals feeding diets with combined replacement of FM and FO might lead to increasingly higher energy requirements of fish. More attention should be paid to the design of diet formulation to prevent the growth performance of fish from being affected by excessive substitution leading to immune dysfunction and energy requirements that are difficult to meet.

Alteration of Antioxidant Pathway Associated With Replacement Fish Meal and/or Fish Oil

Under physiological homeostasis state, organisms will produce reactive oxygen species and maintain the antioxidant defenses by providing a set of mechanisms to equilibrium. Although antioxidant defense in fish is influenced by nutritional factors, little is known about how the oxidative status in fish responds to the dietary ingredients (Rueda-Jasso et al., 2004). In the present study, the low concentrations of R-S-glutathione, 5-oxoproline, 5-L-glutamyl-L-alanine, and OPLAH in individuals fed a reduction FM diet, indicating that glutathione metabolism was repressed. Furthermore, these metabolites are commonly used as potential biomarkers for hepatic glutathione status assessment (Geenen et al., 2013). Particularly, R-S-glutathione, one of the transformation products of glutathione formation complexes, transports into the specific position, which is a common detoxification mechanism (Liang et al., 2016). Altering the level of dietary FM has been confirmed to affect the antioxidant capacity, thereby repressing the growth and development of Pacific white shrimp (Xie et al., 2020), Barramundi (*L. calcarifer*) (Chaklader et al., 2020), and European sea bass (Guerreiro et al., 2015). Previous study has reported that antioxidant defense is a metabolic process that requires massive energy expenditure in response to external stimuli (Xie et al., 2020). Meanwhile, individuals fed the replacement of FM by TP diet required more energy supply to deal with the oxidative stress caused by the reduction of dietary FM and support the subsequent growth requirements. Given this requirement, the

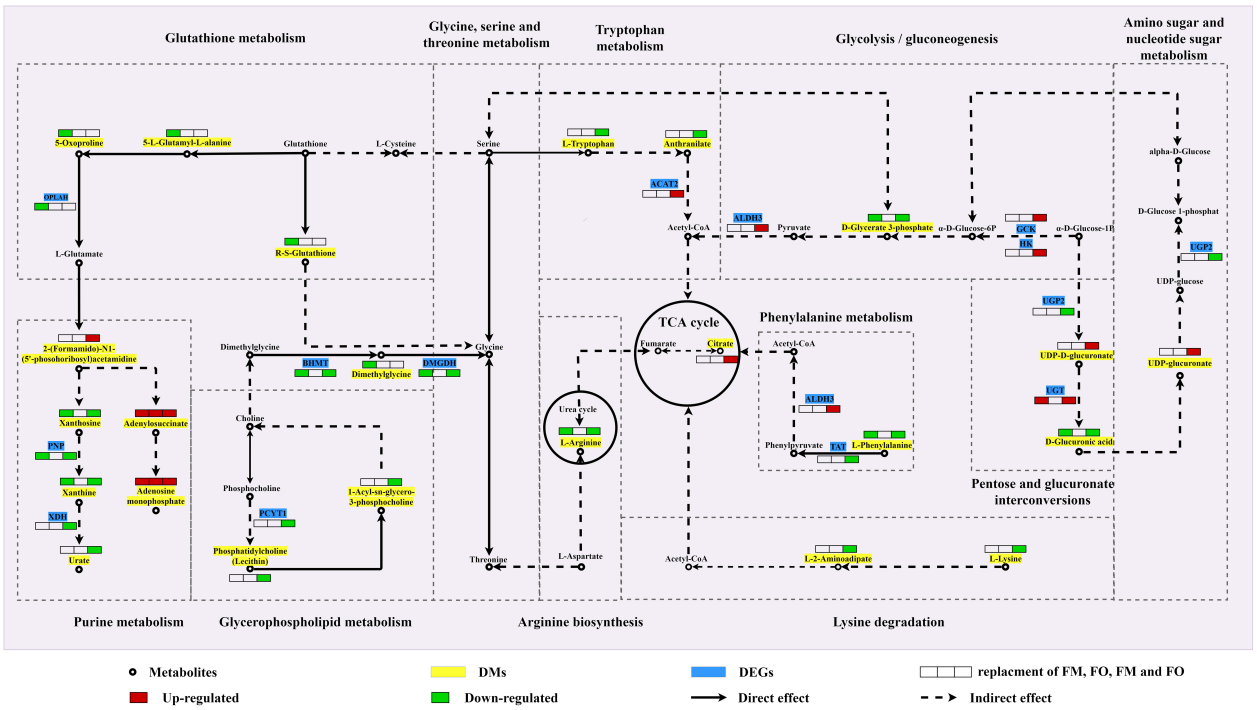


FIGURE 5 | A hypothetical integrated metabolic network of key differential metabolites and genes in significant pathways identified by multi-omics analysis between FM and FO replacement and combined replacement FM and FO based on the KEGG pathway database. Map00230: purine metabolism; map00310: lysine degradation; map00220: arginine biosynthesis; map00010: glycolysis/gluconeogenesis; map00360: phenylalanine metabolism; map00520: amino sugar and nucleotide sugar metabolism; map00260: glycine, serine, and threonine metabolism; map00040: pentose and glucuronate interconversions; map00480: glutathione metabolism; map00380: tryptophan metabolism; map00020: citrate cycle (TCA cycle); map00564: glycerophospholipid metabolism. Metabolites were marked as circles, differential metabolites as green frames, and differential genes as blue frames. The red/green boxes (from left to right, replacement of dietary FM, FO, FM and FO, respectively) show high/low expression of alternative FM and/or FO. *OPLAH*: 5-oxoprolinase; *PNP*: purine-nucleoside phosphorylase; *XDH*: xanthine dehydrogenase/oxidase; *PCYT1*: choline-phosphate cytidylyltransferase; *BHMT*: betaine-homocysteine S-methyltransferase; *DMGDH*: dimethylglycine dehydrogenase; *ACAT2*: acetyl-CoA C-acetyltransferase; *TAT*: tyrosine aminotransferase; *ALDH3*: aldehyde dehydrogenase; *GCK*: glucokinase; *HK*: hexokinase; *UGP2*: UTP-glucose-1-phosphate uridylyltransferase; *UGT*: glucuronosyltransferase.

TCA cycle, pentose and glucuronate interconversions, and glycolysis/gluconeogenesis were activated to provide sufficient energy for the growth and antioxidative requirements of *O. mykiss*. Therefore, our results suggested that the replacement of dietary FM by TP represses the antioxidant system of *O. mykiss*, which could result in lower antioxidant and detoxifying capabilities.

Alteration of Energy Metabolism Pathway Associated With Replacement Fish Meal and/or Fish Oil

The TCA cycle is a central pathway for most metabolic pathways such as AA, lipid, and carbohydrate, and is an important metabolic pathway that provides energy to the organism (Akram, 2014). Comparative multi-omics data in fish between FMFO and TPVO showed several essential intermediates involved in the TCA cycle, including L-tryptophan, L-lysine, and L-phenylalanine (Mishra et al., 2017), all of which were downregulated in TPVO compared with FMFO. The low concentrations of these metabolites might be consumed for the energy metabolism of *O. mykiss*. Meanwhile, adenylosuccinate is the precursor of fumaric acid for further synthesis of citrate, which plays a crucial role in energy metabolism. Citrate is an important intermediate of the TCA cycle and is closely associated with ATP generation. The multi-omics data showed the high concentration of citrate possibly indicated that fish feeding diets with combined replacement of FM and FO by TP and VO, respectively, activated the TCA cycle to supply sufficient energy to accommodate the shift dietary quality. Huang et al. (2017) reported that the decreased AA may be associated with the increased energy requirements to maintain body homeostasis in response to external stimuli. In the present study, the low concentrations of L-tryptophan, L-lysine, and L-phenylalanine were observed in TPVO, indicating that the activated TCA cycle may be a strategy for *O. mykiss* to cope with the combined replacement of FM and FO by TP and VO, respectively. Furthermore, Li et al. (2009) reported that the decrease in phenylalanine could depress growth performance and even negatively affect the survival rate of fish. It was speculated that the poor feeding intake rates and growth performance of *O. mykiss* might result from the low concentration of L-phenylalanine in the liver of *O. mykiss*.

Glycolysis/gluconeogenesis is an important metabolic pathway to maintain glucose homeostasis (Metón et al., 2003). D-glycerate 3-phosphate, a precursor of tryptophan in organisms, is an important intermediate in glycolysis metabolism to produce pyruvate, the final product of glycolysis, which is required to activate the TCA cycle (Tikunov et al., 2014). In our work, the low concentration of D-glycerate 3-phosphate in fish feeding diets with individual replacement of dietary FM and combined replacement of dietary FM and FO by TP and VO, respectively, indicated the consumption of D-glycerate 3-phosphate. Furthermore, the UDP-D-glucuronate, GCK, HK, and ALDH3 are important metabolite and genes involved in the glycolysis/gluconeogenesis, and glucose and pentose interconversions pathways were increased in individuals fed diets with reduction of both FM and FO. The results suggested that the energy metabolism of *O. mykiss* was activated and provided sufficient energy in response to the fluctuation of dietary quality. Thus, once the metabolic energy is consumed in maintaining the adverse reactions caused by the diets, less energy is available for the growth of the fish.

Alteration of Cellular Protein Biosynthesis Pathway Associated With Replacement Fish Meal and/or Fish Oil

Nucleotide metabolism plays an important physiological and biochemical role in organisms, mediating energy metabolism and signal transmission (Cosgrove, 1998). *De novo* synthesis of nucleotides can satisfy the normal growth of organisms, but when nucleotide metabolism is affected, the immune function of organs such as the liver may be impaired (Grimble and Westwood, 2000). The present study showed that nucleotide metabolism, especially purine metabolism in *O. Mykiss*, was significantly affected by the fluctuation of dietary quality. The low concentrations of several purines, including xanthosine, xanthine, and urate may be caused by the downregulated PNP and XDH that could promote the conversion of xanthosine to urate (Baccolini and Witte, 2019). Purines are the main components and metabolites of nucleotides, which can be absorbed by transporters and reused for protein synthesis (Mohlmann et al., 2010). However, the concentrations of purine in fish feeding diets with individual replacement of dietary FM by TP and combined replacement of FM and FO by TP and VO, respectively, were low, suggesting that purine synthesis might be repressed and thus affect the protein synthesis. Moreover, it is worth noting that as soon as the dietary FM and/or FO were reduced, both adenylosuccinate and adenosine monophosphate of *O. mykiss* were upregulated. Most purine nucleotides required for cell replication are derived from the purine biosynthesis pathway, and adenylosuccinate plays a considerable role in *de novo* biosynthesis of purine and replication (Yuan et al., 2011). However, the high concentrations of these metabolites employed for cell replication were detected in the liver, suggesting that the replacement of dietary FM and FO by TP and VO, respectively, repressed the cell protein synthesis ability of *O. mykiss*. Therefore, the results suggested that the impaired growth performance of *O. mykiss* might be associated with the replacement of dietary FM and/or FO by TP and/or VO, especially the inhibition of cellular protein biosynthesis induced by the combined replacement of FM and FO by TP and VO, respectively.

Alteration of Lipid Metabolism Pathway Associated With Replacement Fish Meal and/or Fish Oil

In terms of lipid metabolism, most DEGs and DMs were associated with glycerophospholipid metabolism. For instance, phosphatidylcholine (PC) is one of the main components of biofilms (Zong et al., 2018), and is crucial for the structure and function of these membranes (Gibellini and Smith, 2010). Previous studies reported that PC could bloke the damage of cell membrane that is caused by active free radicals through antioxidant effect, accelerate the lipolysis in the liver, and promote liver fibrosis in collagenase active link (Navder et al., 1997; Mi et al., 2000). In our study, PCYT1 was downregulated in the liver of *O. mykiss* fed reduction of both FM and FO diet, and resulted in low concentration of PC and 1-acyl-sn-glycero-3-phosphocholine, which might reflect significant changes in cell membrane composition. Additionally, previous investigations have demonstrated that the FA profiles of cell membranes were significantly affected by dietary FA profiles (Oxley et al., 2010) which is critical for fluidity and permeability of cell membranes, thereby affecting the function of immune receptors (Arts and Kohler, 2009). Therefore, in our study, the individual reduction of FM and combined replacement of FM and FO by TP and VO, respectively, may impair the immune function and cell membrane fluidity of *O. mykiss* to limit the normal physiological activities and growth performance of fish.

CONCLUSION

Integrated metabolomics and transcriptomics were used to investigate the effects of the replacement of dietary FM and/or FO by TP and/or VO, respectively, on changes of metabolites and genes in *O. mykiss*. The specific metabolic pathways of *O. mykiss* fed diet with combined replacement of FM and FO by TP and VO, respectively, were identified, mainly repressing immune function, cellular protein biosynthesis capacity, and lipid metabolism. The depression of antioxidant capacity was only observed in fish feeding diets with the replacement of dietary FM by TP. Moreover, as soon as the dietary FM and/or FO were reduced, the cellular protein biosynthesis ability of *O. mykiss* was repressed and accompanied by higher energy consumption in response to fluctuations of dietary quality, resulting in reduced growth performance. Furthermore, both adenylosuccinate and adenosine monophosphate related to cellular protein biosynthesis ability were significantly affected by both individual and combined replacement of FM and FO by TP and VO, respectively, suggesting that purine metabolism and its related two metabolites were potential biomarkers for *O. mykiss* fed diets with reduction of dietary FM and/or FO. Overall, our study provides new insights and theoretical basis into the metabolic and molecular mechanism behind the

growth of *O. mykiss* response to the diet with reduction of dietary FM and/or FO.

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/, PRJNA788999.

ETHICS STATEMENT

The animal study was reviewed and approved by the Regulations of the Administration of Affairs Concerning Experimental Animals of China and Regulations of the Administration of Affairs Concerning Experimental Animals of Shandong Province.

AUTHOR CONTRIBUTIONS

YC completed the data analysis and manuscript writing. QG and SD designed the study. QG was the major instructor. XL, YW, and ZD helped to collect the sample. YZ revised the manuscript. All authors contributed to the study and approved the submitted version.

FUNDING

This study was funded by the National Key R&D Program of China (Project No. 2019YFD0901000) and the National Natural Science Foundation of China (Grant No. 31872575).

ACKNOWLEDGMENTS

We acknowledge those who donated the experimental animals and those who helped support this study at the Ocean University of China.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.843637/full#supplementary-material

REFERENCES

Akram, M. (2014). Citric acid cycle and role of its intermediates in metabolism. *Cell Biochem. Biophys.* 68, 475–478. doi: 10.1007/s12013-013-9750-1

Alfaro, A. C., and Young, T. (2018). Showcasing metabolomic applications in aquaculture: a review. *Rev. Aquac.* 10, 135–152. doi: 10.1111/raq.12152

Arts, M. T., and Kohler, C. C. (2009). "Health and condition in fish: the influence of lipids on membrane competency and immune response," in *Lipids in Aquatic Ecosystems*, ed. M. T. Arts (New York, NY: Springer Press), 237–256. doi: 10.1007/978-0-387-89366-2_10

Azeredo, R., Pérez-Sánchez, J., Sitjà-Bobadilla, A., Fouz, B., Tort, L., Aragão, C., et al. (2015). European sea bass (*Dicentrarchus labrax*) immune status and disease resistance are impaired by arginine dietary supplementation. *PLoS One* 10:e0139967. doi: 10.1371/journal.pone.0139967

Baccolini, C., and Witte, C. P. (2019). AMP and GMP catabolism in *Arabidopsis* converge on Xanthosine, which is degraded by a nucleoside hydrolase heterocomplex. *Plant Cell* 31, 734–751. doi: 10.1105/tpc.18.00899

Betancor, M. B., Sprague, M., Sayanova, O., Usher, S., Campbell, P. J., Napier, J. A., et al. (2015). Evaluation of a high-EPA oil from transgenic Camelina sativa in feeds for Atlantic salmon (*Salmo salar* L.): effects on tissue fatty acid composition, histology and gene expression. *Aquaculture* 444, 1–12. doi: 10.1016/j.aquaculture.2015.03.020

Bureau, D. P., Harris, A. M., and Cho, C. Y. (1999). Apparent digestibility of rendered animal protein ingredients for rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 180, 345–358. doi: 10.1016/s0044-8486(99)00210-0

Chaklader, M. R., Siddik, M. A. B., and Fotedar, R. (2020). Total replacement of fishmeal with poultry by-product meal affected the growth, muscle quality, histological structure, antioxidant capacity, and immune response of juvenile barramundi, *Lates calcarifer*. *PLoS One* 15:0242079. doi: 10.1371/journal.pone.0242079

Choi, D. G., He, M., Fang, H., Wang, X., Li, X., and Leng, X. (2019). Replacement of fish meal with two fermented soybean meals in diets for rainbow trout (*Oncorhynchus mykiss*). *Aquac. Nutr.* 26, 37–46. doi: 10.1111/anu.12965

Cosgrove, M. (1998). Nucleotides. *Nutrition* 14, 748–751. doi: 10.1016/S0899-9007(98)00075-6

Dias, J., Alvarez, M. J., Arzel, J., Corraze, G., Diez, A., Bautista, J. M., et al. (2005). Dietary protein source affects lipid metabolism in the European seabass (*Dicentrarchus labrax*). *Comp. Biochem. Physiol. A* 142, 19–31. doi: 10.1016/j.cbpb.2005.07.005

Espe, M., Lemme, A., Petri, A., and El-Mowafi, A. (2006). Can Atlantic salmon grow on diets devoid of fish meal? *Aquaculture* 255, 255–262. doi: 10.1016/j.aquaculture.2005.12.030

FAO (2020). *The State of World Fisheries and Aquaculture, Meeting the Sustainable Development Goals*. Rome: FAO.

Geay, F., Wenon, D., Mellery, J., Tinti, E., Mandiki, S. N. M., Tocher, D. R., et al. (2015). Dietary linseed oil reduces growth while differentially impacting LC-PUFA synthesis and accretion into tissues in Eurasian perch (*Perca fluviatilis*). *Lipids* 50, 1219–1232. doi: 10.1007/s11745-015-4079-8

Geenen, S., Yates, J. W. T., Kenna, J. G., Bois, F. Y., Wilson, I. D., and Westerhoff, H. V. (2013). Multiscale modelling approach combining a kinetic model of glutathione metabolism with PBPK models of paracetamol and the potential glutathione-depletion biomarkers ophthalmic acid and 5-oxoproline in humans and rats. *Integr. Biol.* 5, 877–888. doi: 10.1039/c3ib20245c

Gibellini, F., and Smith, T. K. (2010). The Kennedy pathway-de novo synthesis of phosphatidylethanolamine and phosphatidylcholine. *IUBMB Life* 62, 414–428. doi: 10.1002/iub.337

Glencross, B., Blyth, D., Irvin, S., Bourne, N., Campet, M., Boisot, P., et al. (2016). An evaluation of the complete replacement of both fishmeal and fish oil in diets for juvenile Asian seabass, *Lates calcarifer*. *Aquaculture* 451, 298–309. doi: 10.1016/j.aquaculture.2015.09.012

Glencross, B., Hawkins, W., and Curnow, J. (2003). Evaluation of canola oils as alternative lipid resources in diets for juvenile red seabream, *Pagrus auratus*. *Aquac. Nutr.* 9, 305–315. doi: 10.1046/j.1365-2095.2003.00257.x

Glencross, B., Rutherford, N., and Jones, B. (2011). Evaluating options for fishmeal replacement in diets for juvenile barramundi (*Lates calcarifer*). *Aquac. Nutr.* 17, 722–732. doi: 10.1111/j.1365-2095.2010.00834.x

Grayson, J., and Dabrowski, K. (2020). Partial and total replacement of fish oil with fatty acid ethyl esters in the starter diets of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 522:735018. doi: 10.1016/j.aquaculture.2020.735018

Grimble, G. K., and Westwood, O. M. R. (2000). "Nucleotides," in *Nutrition and Immunology: Principles and Practice*, eds J. B. German and C. L. Keen (Totowa, NJ: Humana Press), 135–144. doi: 10.1007/978-1-59259-709-3_11

Guerreiro, I., Couto, A., Perez-Jimenez, A., Oliva-Teles, A., and Enes, P. (2015). Gut morphology and hepatic oxidative status of European sea

bass (*Dicentrarchus labrax*) juveniles fed plant feedstuffs or fishmeal-based diets supplemented with short-chain fructo-oligosaccharides and xylo-oligosaccharides. *Br. J. Nutr.* 114, 1975–1984. doi: 10.1017/s0007114515003773

Guillaume, J., Kaushik, S. J., Bergot, P., and Métailler, R. (2001). *Nutrition and Feeding of Fish and Crustaceans*. New York, NY: Springer Press. doi: 10.1016/s0044-8486(02)00261-2

Guo, C., Huang, X., Yang, M., Wang, S., Ren, S., Li, H., et al. (2014). GC/MS-based metabolomics approach to identify biomarkers differentiating survivals from death in crucian carps infected by *Edwardsiella tarda*. *Fish Shellfish Immunol.* 39, 215–222. doi: 10.1016/j.fsi.2014.04.017

Hansen, A., Rosenlund, G., Karlsen, Ø., Koppe, W., and Hemre, G. (2007). Total replacement of fish meal with plant proteins in diets for Atlantic cod (*Gadus morhua* L.) I–effects on growth and protein retention. *Aquaculture* 272, 599–611. doi: 10.1016/j.aquaculture.2007.08.034

Hao, R. J., Du, X. D., Yang, C. Y., Deng, Y. W., Zheng, Z., and Wang, Q. H. (2019). Integrated application of transcriptomics and metabolomics provides insights into unsynchronized growth in pearl oyster *Pinctada fucata martensii*. *Sci. Total Environ.* 666, 46–56. doi: 10.1016/j.scitotenv.2019.02.221

Hardy, R. W. (2010). Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. *Aquac. Res.* 41, 770–776. doi: 10.1111/j.1365-2109.2009.02349.x

Hixson, S. M., Parrish, C. C., and Anderson, D. M. (2014). Full substitution of fish oil with camelina (*Camelina sativa*) oil, with partial substitution of fish meal with camelina meal, in diets for farmed Atlantic salmon (*Salmo salar*) and its effect on tissue lipids and sensory quality. *Food Chem.* 157, 51–61. doi: 10.1016/j.foodchem.2014.02.026

Huang, S. S., Benskin, J. P., Veldhoen, N., Chandramouli, B., Butler, H., Helbing, C. C., et al. (2017). A multi-omic approach to elucidate low-dose effects of xenobiotics in zebrafish (*Danio rerio*) larvae. *Aquat. Toxicol.* 182, 102–112. doi: 10.1016/j.aquatox.2016.11.016

Jia, S., Li, X., Zheng, S., and Wu, G. (2017). Amino acids are major energy substrates for tissues of hybrid striped bass and zebrafish. *Amino Acids* 49, 2053–2063. doi: 10.1007/s00726-017-2481-7

Klinger, D., and Naylor, R. (2012). Searching for solutions in aquaculture: charting a sustainable course. *Annu. Rev. Environ. Resour.* 37, 247–276. doi: 10.1146/annurev-environ-021111-161531

Kong, T., Lin, S., Ren, X., Li, S., and Gong, Y. J. (2020). Transcriptome and metabolome integration analysis of mud crab *Scylla paramamosain* challenged to *Vibrio parahaemolyticus* infection. *Fish Shellfish Immunol.* 103, 430–437. doi: 10.1016/j.fsi.2020.05.069

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.). *Aquac. Nutr.* 9, 361–371. doi: 10.1046/j.1365-2095.2003.00264.x

Li, P., Mai, K. S., Trushenski, J., and Wu, G. Y. (2009). New developments in fish amino acid nutrition: towards functional and environmentally oriented aquafeeds. *Amino Acids* 37, 43–53.

Li, S., Dai, M., Qiu, H., and Chen, N. (2021). Effects of fishmeal replacement with composite mixture of shrimp hydrolysate and plant proteins on growth performance, feed utilization, and target of rapamycin pathway in largemouth bass, *Micropterus salmoides*. *Aquaculture* 533:736185. doi: 10.1016/j.aquaculture.2020.736185

Li, Y., Ai, Q. H., Mai, K. S., Xu, W., Deng, J. M., and Cheng, Z. Y. (2014). Comparison of high-protein soybean meal and commercial soybean meal partly replacing fish meal on the activities of digestive enzymes and aminotransferases in juvenile Japanese seabass, *Lateolabrax japonicus* (Cuvier, 1828). *Aquac. Res.* 45, 1051–1060. doi: 10.1111/are.12042

Li, Y., Niu, D. H., Wu, Y. H., Dong, Z. G., and Li, J. L. (2021). Integrated analysis of transcriptomic and metabolomic data to evaluate responses to hypersalinity stress in the gill of the razor clam (*Sinonovacula constricta*). *Comp. Biochem. Phys. D* 38:100793. doi: 10.1016/j.cbd.2021.100793

Liang, T., Ding, H., Wang, G., Kang, J., Pang, H., and Lv, J. (2016). Sulfur decreases cadmium translocation and enhances cadmium tolerance by promoting sulfur assimilation and glutathione metabolism in *Brassica chinensis* L. *Ecotoxicol. Environ. Saf.* 124, 129–137. doi: 10.1016/j.ecoenv.2015.10.011

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C(T)) Method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Lu, J., Tibbetts, S. M., Lall, S. P., and Anderson, D. M. (2020). Use of dietary oil, solvent-extracted meal and protein concentrate from *Camelina sativa* for rainbow trout, *Oncorhynchus mykiss*, at the early fry stage. *Aquaculture* 524:735252. doi: 10.1016/j.aquaculture.2020.735252

Messina, M., Piccolo, G., Tulli, F., Messina, C., Cardinaletti, G., and Tibaldi, E. (2013). Lipid composition and metabolism of European sea bass (*Dicentrarchus labrax* L.) fed diets containing wheat gluten and legume meals as substitutes for fish meal. *Aquaculture* 33, 169–175. doi: 10.1016/j.aquaculture.2012.11.005

Metón, I., Fernandez, F., and Baanante, I. V. (2003). Short- and long-term effects of refeeding on key enzyme activities in glycolysis-gluconeogenesis in the liver of gilthead seabream (*Sparus aurata*). *Aquaculture* 225, 99–107. doi: 10.1016/s0044-8486(03)00281-3

Mi, L. J., Mak, K. M., and Lieber, C. S. (2000). Attenuation of alcohol-induced apoptosis of hepatocytes in rat livers by polyenylphosphatidylcholine (PPC). *Alcohol. Clin. Exp. Res.* 24, 207–212. doi: 10.1111/j.1530-0277.2000.tb04592.x

Mishra, P., Gong, Z. Y., and Kelly, B. C. (2017). Assessing biological effects of fluoxetine in developing zebrafish embryos using gas chromatography-mass spectrometry based metabolomics. *Chemosphere* 188, 157–167. doi: 10.1016/j.chemosphere.2017.08.149

Mohlmann, T., Bernard, C., Hach, S., and Neuhaus, H. E. (2010). Nucleoside transport and associated metabolism. *Plant Biol.* 12, 26–34. doi: 10.1111/j.1438-8677.2010.00351.x

Moreno-Arias, A., López-Elías, J. A., Martínez-Córdova, L. R., Ramírez-Suárez, J. C., Carvallo-Ruiz, M. G., García-Sánchez, G., et al. (2018). Effect of fishmeal replacement with a vegetable protein mixture on the amino acid and fatty acid profiles of diets, biofloc and shrimp cultured in BFT system. *Aquaculture* 483, 53–62. doi: 10.1016/j.aquaculture.2017.10.011

Navder, K. P., Baraona, E., and Lieber, C. S. (1997). Polyenylphosphatidylcholine attenuates alcohol-induced fatty liver and hyperlipemia in rats. *J. Nutr.* 127, 1800–1806. doi: 10.1093/jn/127.9.1800

Nuche-Pascual, M. T., Lazo, J. P., Ruiz-Cooley, R. I., and Herzka, S. Z. (2018). Amino acid-specific δ^{15}N trophic enrichment factors in fish fed with formulated diets varying in protein quantity and quality. *Ecol. Evol.* 8, 9192–9217. doi: 10.1002/ece3.4295

Oliva-Teles, A., Enes, P., and Peres, H. (2015). "Replacing fishmeal and fish oil in industrial aquafeeds for carnivorous fish," in *Feed and Feeding Practices in Aquaculture*, ed. D. A. Davis (Cambridge, MA: Elsevier Press), 203–233. doi: 10.1016/B978-0-08-100506-4.00008-8

Olsen, R. L., and Hasan, M. R. (2012). A limited supply of fishmeal: impact on future increases in global aquaculture production. *Trends Food Sci. Technol.* 27, 120–128. doi: 10.1016/j.tifs.2012.06.003

Oxley, A., Jolly, C., Eide, T., Jordal, A. E. O., Svardal, A., and Olsen, R. E. (2010). The combined impact of plant-derived dietary ingredients and acute stress on the intestinal arachidonic acid cascade in Atlantic salmon (*Salmo salar*). *Br. J Nutr.* 103, 851–861. doi: 10.1017/S0007114509992467

Panserat, S., Hortopan, G., Plagnes-Juan, E., Kolditz, C., Lansard, M., Skiba-Cassy, S., et al. (2009). Differential gene expression after total replacement of dietary fish meal and fish oil by plant products in rainbow trout (*Oncorhynchus mykiss*) liver. *Aquaculture* 294, 123–131. doi: 10.1016/j.aquaculture.2009.05.013

Petropoulos, I. K., Thompson, K. D., Morgan, A., Dick, J. R., Tocher, D. R., and Bell, J. G. (2009). Effects of substitution of dietary fish oil with a blend of vegetable oils on liver and peripheral blood leucocyte fatty acid composition, plasma prostaglandin E_2 and immune parameters in three strains of Atlantic salmon (*Salmo salar*). *Aquac. Nutr.* 15, 596–607. doi: 10.1111/j.1365-2095.2008.00627.x

Randazzo, B., Zarantoniello, M., Gioacchini, G., Cardinaletti, G., Belloni, A., Giorgini, E., et al. (2021). Physiological response of rainbow trout (*Oncorhynchus mykiss*) to graded levels of *Hermetia illucens* or poultry by-product meals as single or combined substitute ingredients to deitary plant proteins. *Aquaculture* 538:73650. doi: 10.1016/j.aquaculture.2021.736550

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. doi: 10.1371/journal.pone.0193652

Rueda-Jasso, R., Conceiçao, L. E., Dias, J., De Coen, W., Gomes, E., Rees, J. F., et al. (2004). Effect of dietary non-protein energy levels on condition and

oxidative status of Senegalese sole (*Solea senegalensis*) juveniles. *Aquaculture* 231, 417–433. doi: 10.1016/s0044-8486(03)00537-4

Saurabh, S., and Sahoo, P. K. (2008). Lysozyme: an important defence molecule of fish innate immune system. *Aquac. Res.* 39, 223–239. doi: 10.1111/j.1365-2109.2007.01883.x

Sorgdrager, F. J., Naudé, P. J., Kema, I. P., Nollen, E. A., and Deyn, P. P. (2019). Tryptophan metabolism in inflammaging: from biomarker to therapeutic target. *Front. Immunol.* 10:2565. doi: 10.3389/fimmu.2019.02565

Sourabié, A., Mandiki, S., Geay, F., Sene, T., Toguyeni, A., and Kestemont, P. (2018). Fish proteins not lipids are the major nutrients limiting the use of vegetable ingredients in catfish nutrition. *Aquac. Nutr.* 24, 1393–1405. doi: 10.1111/anu.12676

Tikunov, A. P., Stoskopf, M. K., and Macdonald, J. M. (2014). Fluxomics of the eastern oyster for environmental stress studies. *Metabolites* 4, 53–70. doi: 10.3390/metabo4010053

Tocher, D. R. (2010). Fatty acid requirements in ontogeny of marine and freshwater fish. *Aquac. Res.* 41, 717–732. doi: 10.1111/j.1365-2109.2008.02150.x

Torrecillas, S., Robaina, L., Caballero, M. J., Montero, D., Calandra, G., Mompel, D., et al. (2017). Combined replacement of fishmeal and fish oil in European sea bass (*Dicentrarchus labrax*): production performance, tissue composition and liver morphology. *Aquaculture* 509, 246–248. doi: 10.1016/j.aquaculture.2017.03.031

Torstensen, B. E., Espe, M., Sanden, M., Stubhaug, I., Waagbo, R., Hemre, G. I., et al. (2008). Novel production of Atlantic salmon (*Salmo salar*) protein based on combined replacement of fish meal and fish oil with plant meal and vegetable oil blends. *Aquaculture* 285, 193–200. doi: 10.1016/j.aquaculture.2008.08.025

Turchini, G. M., Trushenski, J. T., and Glencross, B. D. (2019). Thoughts for the future of aquaculture nutrition: realigning perspectives to reflect contemporary issues related to judicious use of marine resources in aquafeeds. *North Am. J. Aquac.* 81, 13–39. doi: 10.1002/naaq.10067

Wei, Y. L., Liang, M. Q., Mai, K. S., Zheng, K. K., and Xu, H. G. (2017). The effect of ultrafiltered fish protein hydrolysate levels on the liver and muscle metabolic profile of juvenile turbot (*Scophthalmus maximus* L.) by 1H NMR-based metabolomics studies. *Aquac. Res.* 48, 3515–3527. doi: 10.1111/are.13178

Wu, G., Bazer, F. W., Dai, Z., Li, D., Wang, J., and Wu, Z. (2014). Amino acid nutrition in animals: protein synthesis and beyond. *Annu. Rev. Anim. Biosci.* 2, 387–417. doi: 10.1146/annurev-animal-022513-114113

Xie, C., Miao, X., Huang, J., Ding, Y., Wu, J., Dong, S., et al. (2011). KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. *Nucleic Acids Res.* 39, 316–322. doi: 10.1093/nar/gkr483

Xie, S. W., Wei, D., Chen, S. J., Zhuang, Z. X., Yin, P., Liu, Y. J., et al. (2020). Dietary fishmeal levels affect anti-oxidative ability and metabolomics profile of juvenile Pacific white shrimp, *Litopenaeus vannamei*. *Aquac. Nutr.* 26, 978–989. doi: 10.1111/anu.13055

Xu, Z., Li, T., Li, E., Chen, K., Ding, Z., Qin, J. G., et al. (2016). Comparative transcriptome analysis reveals molecular strategies of oriental river prawn *Macrobrachium nipponense* in response to acute and chronic nitrite stress. *Fish Shellfish Immunol.* 48, 254–265. doi: 10.1016/j.fsi.2015.12.005

Yan, J., Risacher, S. L., Shen, L., and Saykin, A. J. (2018). Network approaches to systems biology analysis of complex disease: integrative methods for multi-omics data. *Brief. Bioinf.* 19, 1370–1381. doi: 10.1093/bib/bbx066

Yuan, T., Gu, J. R., Gu, W. B., Wu, J., Ge, S. R., and Xu, H. (2011). Molecular cloning, characterization and expression analysis of adenylosuccinate lyase gene in grass carp (*Ctenopharyngodon idella*). *Mol. Biol. Rep.* 38, 2059–2065. doi: 10.1007/s11033-010-0331-8

Zhang, H., Wang, H., Chen, H., Wang, M. Q., Zhou, Z., Qiu, L. M., et al. (2019). The transcriptional response of the Pacific oyster *Crassostrea gigas* under simultaneous bacterial and heat stresses. *Dev. Comp. Immunol.* 94, 1–10. doi: 10.1016/j.dci.2019.01.006

Zong, L., Xing, J. P., Liu, S., Liu, Z. Q., and Song, F. R. (2018). Cell metabolomics reveals the neurotoxicity mechanism of cadmium in PC12 cells. *Ecotoxicol. Environ. Saf.* 147, 26–33. doi: 10.1016/j.ecoenv.2017.08.028

Zummo, F., Marineo, S., Pace, A., Civiletti, F., Giardina, A., and Puglia, A. M. (2012). Tryptophan catabolism via kynurenine production in *Streptomyces coelicolor*: identification of three genes coding for the enzymes of tryptophan to anthranilate pathway. *Appl. Microbiol. Biotechnol.* 94, 719–728. doi: 10.1007/s00253-011-3833-y

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.

frontiers in Marine Science

ORIGINAL RESEARCH
published: 23 March 2022
doi: 10.3389/fmars.2022.844716

Influence of Soybean Meal on Intestinal Mucosa Metabolome and Effects of Adenosine Monophosphate-Activated Protein Kinase Signaling Pathway in Mirror Carp (*Cyprinus carpio* Songpu)

Jingyi Zhao[1,2,3] *and Qiyou Xu*[1,2,3]*

[1] College of Life Science, Huzhou University, Huzhou, China, [2] Zhejiang Provincial Key Laboratory of Aquatic Bioresource Conservation and Development Technology, Huzhou, China, [3] Nation and Local Joint Engineering Laboratory of Aquatic Animal Genetic Breeding and Nutrition, Huzhou, China

OPEN ACCESS

Edited by:
Kang-le Lu,
Jimei University, China

Reviewed by:
Hongyan Tian,
Yancheng Institute of Technology, China
Xiaodan Wang,
East China Normal University, China
Mahmoud A. O. Dawood,
Kafrelsheikh University, Egypt

***Correspondence:**
Qiyou Xu
02655@zjhu.edu.cn

Specialty section:
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

Received: 28 December 2021
Accepted: 16 February 2022
Published: 23 March 2022

Citation:
Zhao JY and Xu QY (2022) Influence of Soybean Meal on Intestinal Mucosa Metabolome and Effects of Adenosine Monophosphate-Activated Protein Kinase Signaling Pathway in Mirror Carp (Cyprinus carpio Songpu). Front. Mar. Sci. 9:844716. doi: 10.3389/fmars.2022.844716

This study aimed to explore the influence of soybean meal on intestinal mucosa metabolome and signaling pathway of mirror carp (*Cyprinus carpio* Songpu) by integrating liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based metabolomics. Fish meal (Con) was control group, soybean meal (Dou) group, AMPK activator (300 mg kg^{-1} Metformin, Met) and AMPK inhibitor (0.2 mg kg^{-1} Compound-c, c-Com) which added to soybean meal were experimental groups. The metabolome profiles of the intestinal mucosa were determined in fish fed diets Con, Dou, Met, and c-Com on 7th and 59th day. The results were shown: compared with the Con group, the weight gain rate and the specific growth rate of fish experimental groups were significantly decreased ($P < 0.05$), feed conversion ratio (FCR) was significantly increased ($P < 0.05$). Compared with the Con group, sphingosine, glycocholic acid, majorities of sugar metabolites were up-regulated, and phosphatidylcholine (PC) and lysophosphatidylcholine (LysoPC), amino acids were reduced significantly in all experiment groups ($P < 0.05$). Oxidized glutathione was up-regulated in Dou on 7th day, Met on 7th and 59th day ($P < 0.05$). ADP (adenosine diphosphate) and AMP (adenosine monophpsphate) were up-regulated in Dou, Met, c-Com on 59th day ($P < 0.05$). Compared with the Dou group, sphingosine was down-regulated on 7th day, up-regulated on 59th day in Met and on 7th and 59th day in c-Com ($P < 0.05$). Oxidized glutathione and isocitrate on 7th day, L-Valine, L-histidine, and L-isoleucine on 59th day were up-regulated in Met ($P < 0.05$). Nucleoside metabolites and ADP were up-regulated in c-Com on 7th day ($P < 0.05$). In conclusion, soybean meal influenced intestinal mucosa metabolic processes, including lipid, amino acid, sugar, apoptosis, and oxidative injury; and changed energy metabolism in intestinal mucosa, enriched in the AMPK, TOR, FoxO signaling pathway; Metformin could

aggravate oxidative damage, alleciated apoptosis for the short term, and aggravate apoptosis, improve carbohydrate catabolism and amino acid anabolism for the long term; Compound-c exacerbated apoptosis. repaired oxidative damage, and enhanced nucleoside catabolism.

Keywords: intestinal mucosa, metabolome, soybean meal, metformin, compound-c, carp

INTRODUCTION

Currently, the high demand and cost of fish meals entail evaluating the substitution of fish meal with soybean meal in fish diets, but the substitution of fish meal by soybean meal over a certain range could reduce the growth performance, affect the intestinal health of fish (Wang et al., 2016) and decrease the protease activities in both intestine and hepatopancreas (Lin and Luo, 2011). The replacement ratio of fish meal with soybean meal reached 20% can cause intestinal inflammation in common carp (Urn et al., 2008). Soybean meal induces Hsp70 activation by reducing intestinal p38MAPK phosphorylation of part of the intestine and elicits cell protective response (Antonopoulou et al., 2017). One of the main limiting factors in soybean meals is the presence of anti-nutritional factors that cause intestinal injury and antioxidant damage. Among them, protease inhibitors, soybean agglutinin, tannin, phytic acid, soybean antigen have a strong anti-nutritional effect. 3.5% Soya lectin (Buttle et al., 2001) significantly damaged the integrity of posterior intestinal villi in Atlantic salmon. The trypsin inhibitor (Maytorena-Verdugo et al., 2017) may have reacted with the essential group of protease, which inhibits the binding of protease with a substrate, and reduces or even prevents the activity of the protease. Tannins in the SBM diet (Becker and Makkar, 1999) can inhibit gastric enzyme activity after combination with carbohydrates. Evidence suggests that intestinal mucosal barrier dysfunction is a prerequisite for enteropathy (Turner, 2009; Bron et al., 2017). Usually, intestinal inflammation accompanies with abnormal expression of MUC-2 and tight junction proteins such as claudin-4, occluding, and ZO-1 (Hara et al., 2000; Velcich et al., 2002; Jiang et al., 2015). In addition, fish fed a high dose of soybean meal presented gross intestinal inflammation coupled with increased expression of pro-inflammatory cytokines such as IL-1β and TNF-α accompanied by the up-regulation of NF-κB (Jiang et al., 2015; Gu et al., 2016).

The metabolism and absorption of intestinal nutrients play an important role in the growth performance of fish. The integrity and proper functioning of intestinal mucosal cells are essential. Previous experiments have been confirmed, anti-nutritional factors in soybean meal lead to intestinal damage by affecting some signaling pathways. Soybean glycinin (Zhang et al., 2020) increased ROS generation related to the NOX signaling pathway in the mid and distal intestine and decreased ROS elimination capacity related to NrF2 translocation in the whole intestine of juvenile grass carp (*Ctenopharyngodon idella*). Glycinin (Jiang et al., 2015) and β-conglycinin (Zhang et al., 2013) of soybean can affect the gene expression of the intestinal TOR signaling pathway and thus affect protein synthesis, and reduce fish growth performance. Soybean soaking water has a function in ameliorating obesity through inhibiting lipid synthesis as well as stimulating fatty acid oxidation by elevating the levels of phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC) in the experiment in mice (Park et al., 2015). As a cellular energy sensor, AMPK is a serine/threonine-protein kinase that affects fatty acid, cholesterol, glucose metabolism, protein synthesis, and other metabolic pathways by regulating downstream target proteins (Kahn et al., 2005). There is no direct evidence if soybean meal affects fish intestine health and energy metabolism by affecting the AMPK signaling pathway on fish. Metabolomics is the attempt to identify and quantify all endogenous small molecule metabolites in an organism or biofluid sample and is common in experimental studies of aquatic animals (Long et al., 2021; Xiang et al., 2021; Yang et al., 2021). This study is based on metabonomics to investigate the effects of soybean meal on intestinal mucosa metabolome and the influence on AMPK and other signaling pathways.

MATERIALS AND METHODS

Experimental Diets, Fish Feeding, and Sampling

Metformin (97%) and compound-c [98.59%, $C_{24}H_{25}N_5O$; 6-[4-(2-piperidin-1-ylethoxy) phenyl]-3-pyridine-4-ylpyrazolo [1, 5-a] pyrimidine] used in this study were purchased from Sigma-Aldrich. The ingredients and nutrient content of the experimental diets are shown in **Table 1**. The levels of nutrients were designed to meet the requirements of common carp (*Cyprinus carpio*) according to the National Research Council [NRC] (2011). Fish meal (Con) used as the dietary protein source was the control group, 40% soybean meal (Dou), 300 mg kg^{-1} metformin (Met) (Silva et al., 2015), and 0.2 mg kg^{-1} compound-c (c-Com) (Kim et al., 2011), which added to soybean meal were experimental groups. Amino acids were balanced with coated amino acids. All diets were isonitrogenous and isolipidic. All the feed ingredients were stirred evenly in the mixer. After adding lipid and water, the mixture was homogenized and extruded through a pelletizer with a 2 mm mold. The diets were air-dried at room temperature and stored at −20°C until feeding.

The juvenile mirror carps (*Cyprinus carpio* Songpu) used in this study were purchased from Heilongjiang River Fishery Research Institute of the Chinese Academy of Fishery Sciences. Before the trial, the fish were acclimatized to the recirculating aquaculture system for 2 weeks. Then, 348 fish with a mean initial weight of 9.51 (±0.06) g were randomly assigned to 12 aquariums (500 L water) with an initial stocking density of 29

TABLE 1 | Ingredients and nutrient contents of the diets.

Ingredients (g/kg)	Con	Dou	Met	c-Com
Fish meal	308.0	50.0	50.0	50.0
Corn gluten meal	100.0	100.0	100.0	100.0
Peanut meal	71.8	71.8	71.8	71.8
Soybean meal	0.0	400.0	400.0	400.0
Wheat flour	260.0	260.0	260.0	260.0
Cellulose	171.6	3.6	3.3	3.6
Fish oil	5.0	5.0	5.0	5.0
Soya bean oil	13.7	25.6	25.6	25.6
Soyabean lecithin	10.0	10.0	10.0	10.0
Vitamin premix[a]	5.0	5.0	5.0	5.0
Mineral premix[b]	2.0	2.0	2.0	2.0
Choline chloride	5.0	5.0	5.0	5.0
Monopotassium phosphate	12.0	24.5	24.5	24.5
Sodium carboxymethylcellulose	20.0	20.0	20.0	20.0
Lysine	3.3	3.9	3.9	3.9
Methionine	0.0	1.2	1.2	1.2
Threonine	2.6	2.4	2.4	2.4
Glucose	10.0	10.0	10.0	10.0
Metformin	0.0	0.0	0.3	0.0
Compound-c	0.0	0.0	0.0	0.0002
Total	1000.0	1000.0	1000.0	1000.0
Nutrient content (%)				
Crude protein	35.85	35.03	35.17	35.85
Crude lipid	5.86	6.30	6.40	5.86
Ash	6.72	6.61	6.60	6.72

[a] *Vitamin premix provided the following per kg of diets: VA 8 000IU, VB_1 15 mg, VB_2 30 mg, VB_6 15 mg, VB_{12} 0.5 mg, VC 500 mg, VD 3 000IU, VE 60 mg, VK 35 mg;* [b] *Mineral premix provided the following per kg of diets: Fe 190 mg, Zn 15.75 mg, Cu 3.02 mg, Mn 12.88 mg, I 1.05 mg, Co 1.05 mg, Se 0.53 mg.*

fish per aquarium. Each feed was randomly assigned to three aquariums. In the feeding trial, each diet was fed at 5% of the bodyweight of fish twice per day for the 59 days. The water temperature and pH were 26 (±2)°C and 7.0 (±0.3), respectively, dissolved oxygen was higher than 5 mg/l. The water quality was tested using YSI Professional Plus, and one-third of water was changed a week thrice to remove solid substances and reduce ammonia concentration.

At the beginning and end of the trial, fish in each aquarium were counted and weighed. After that, 18 fish per diet were anesthetized by MS-222 (100 mg/L), the intestine was quickly removed on ice, dissected longitudinally, rinsed in cold physiological saline (0.86% NaCl solution, pH = 7.2), and the intestinal mucosa was scraped with a glass slide. The intestinal mucosas of three fishes were pooled into one sample, frozen in liquid nitrogen, and stored at −80°C. Samples were collected on the 7th (short-term) and 59th day (long-term). The following growth parameters were calculated after the feeding trial: weight gain rate (WGR), specific growth rate (SGR), feed conversion ratio (FCR), and survival rate (SR).

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

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

$$\text{Feed conversion ratio (FCR)} = \text{feed intake}/(\text{final fish weight} + \text{dead fish weight} - \text{initial fish weight});$$

$$\text{Survival rate (SR, \%)} = (\text{total fish number} - \text{dead fish number})/\text{total fish number} \times 100.$$

Non-targeted Metabolomic and Statistical Data Analysis

Metabolite extraction and liquid chromatograph-mass spectrometer analysis were performed by Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China). Metabolite data and profilings were obtained and analyzed on the free online platform of Majorbio Cloud Platform.[1] After identification and quantification, metabolites were annotated into HMDB (human metabolome database) and KEGG (Kyoto encyclopedia of genes and genomes), respectively. Differential metabolites in the mucosa of different treatments were identified using univariate analysis and multivariate statistics. Univariate analysis was performed by the methods of *t*-test and folded change (FC) analysis ($P < 0.05$; FC ≥ 1 or ≤ 1). Multivariate statistics were performed by Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) with variable importance for the projection (VIP) values (≥ 1.0). HMDB pie chart showed the superclass of differential metabolites between groups, and KEGG was used to help in understanding the functions of metabolites. Growth performance indicators were analyzed by one-way ANOVA using SPSS IBM Statistics 25. Duncan's analysis was used for multiple comparisons.

RESULTS

Growth Performance

Compared with the Con group, the WGR and SGR of fish fed the soybean meal, metformin, and compound-c diets were significantly decreased ($P < 0.05$). FCR of fish in the experimental groups was significantly increased than those fish in the Con group ($P < 0.05$). There was no significantly difference in SR of fish among groups ($P > 0.05$, in **Table 2**).

Principal Component Analysis

The metabolite peaks extracted from all samples and QC samples were analyzed by principal component analysis (**Figure 1**). It was found that QC samples clustered together, indicating that this experiment had good repeatability, a stable system, and reliable experimental results. Con on 7th day, Dou on 7th day, and Met

[1] www.majorbio.com

TABLE 2 | Growth performance of juvenile Songpu mirror carp fed different diets.

Items	Con	Dou	Met	c-Com
WGR/%	569.86 ± 24.71^{a}	297.92 ± 15.55^{b}	301.97 ± 18.83^{b}	343.01 ± 17.42^{b}
SGR/(%/day)	3.22 ± 0.06^{a}	2.34 ± 0.07^{b}	2.35 ± 0.08^{b}	2.52 ± 0.07^{b}
FCR	1.39 ± 0.03^{b}	2.07 ± 0.07^{a}	2.11 ± 0.05^{a}	2.00 ± 0.03^{a}
SR/%	98.85 ± 1.15^{a}	100.00 ± 0.00^{a}	98.85 ± 1.15^{a}	98.85 ± 1.15^{a}

Data are presented as mean with SD of three replicate tanks (n = 3).
Values in the same line followed by different superscript letters are significantly different (P < 0.05).

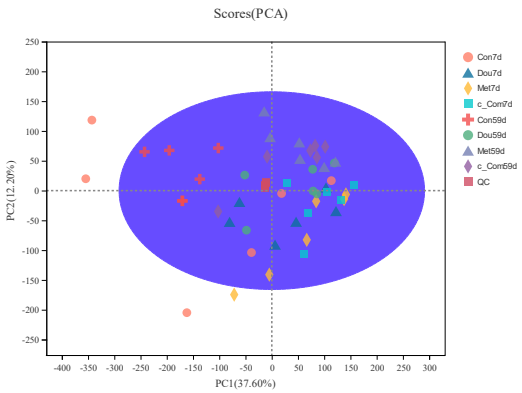


FIGURE 1 | Primcipal component analysis score chart. After dimension reduction analysis, there are relative coordinate points on principal components P1 and P2, and the distance of each coordinate point represents the degree of aggregation and dispersion between samples. The confidence ellipse means that the "real" samples group is distributed in this region with 95% confidence.

on 7th day were dispersed among samples, while c-Com on 7th day, Con on 59th day, Dou on 59th day, Met on 59th day, and c-Com on 59th day groups had more concentrated sample points.

Differential Metabolites and Differential Pathways

The identification and quantification results of metabolites showed that a total of 1,332 metabolites had been obtained, of which 1,157 and 363 were annotated into HMDB (Human Metabolome Database) and KEGG (Kyoto Encyclopedia of Genes and Genomes), respectively. Score plots of the (O) PLS-DA performed to verify the differentiated metabolites between the two groups and multivariate analysis supervision are shown in **Figures 2–6**. R^2X (cum) and R^2Y (cum) represent cumulative interpretation rates. Q^2 indicates the prediction ability of the model. In all comparisons, R^2Y was close to 1, indicating that the model is reliable. In Dou vs. Con, Met vs. Con, c-Com vs. Con, and c-Com on 59th day, vs. Dou on 59th day groups, $Q^2 > 0.5$ indicated the model's prediction ability was good. The pie chart (**Figure 7**) showed the HMDB superclass classification of the different metabolites between every two groups. As shown in the figure, Lipids and lipid-like molecules (purple) accounted for the largest proportion of all groups, respectively. The differential metabolites selected above were enriched into the KEGG database for analysis to obtain differential metabolic pathways. The differential metabolic pathways were sorted according to their significance (*P*-value) (**Figure 8**), and the differential metabolites involved in the differential metabolic pathways were emphatically analyzed (**Supplementary Table 1**).

Dou vs. Con

On the 7th day, compared with the Con group, sphingosine was up-regulated in the Dou group ($P < 0.05$), which was annotated into apoptosis, sphingolipid signaling pathway, sphingolipid metabolism and necroptosis pathway and glycocholic acid in cholesterol metabolism, and most of phosphatidylcholine (PC) and lysophosphatidylcholine (LysoPC) were down-regulated ($P < 0.05$) in choline metabolism in cancer. On the 59th day, compared with the Con group, same with 7th day, sphingosine and glycocholic acid were up-regulated ($P < 0.05$), and most of PC and LysoPC were down-regulated in the Dou group ($P < 0.05$). Furthermore, nucleoside and sugar metabolites in purine metabolism, amino sugar, and nucleotide sugar metabolism pathways were up-regulated ($P < 0.05$), and amino acids enriched in central carbon metabolism in cancer, protein digestion and absorption, aminoacyl-tRNA biosynthesis, mineral absorption, ABC transporters were down-regulated in Dou group ($P < 0.05$). ADP and AMP enriched in the AMPK signaling pathway, mTOR signaling pathway, and FoxO signaling pathway were up-regulated ($P < 0.05$) in the Dou group. And taurine was up-regulated in neuroactive ligand-receptor interaction, primary bile acid biosynthesis, and taurine and hypotaurine metabolism. Taurocholic acid was down-regulated in bile secretion, cholesterol metabolism, primary bile acid biosynthesis in the Dou group on the 59th day.

Met vs. Con

On the 7th day, compared with the Con group, the addition of metformin into soybean meal up-regulated sphingosine in apoptosis, sphingolipid signaling pathway, sphingolipid metabolism, and necroptosis pathways, oxidized glutathione in glutathione metabolism, nucleoside, and sugar metabolites in purine metabolism and amino sugar and nucleotide sugar metabolism pathways ($P < 0.05$), down-regulated PC in choline metabolism in cancer, amino acids in ABC transporters, protein digestion and absorption, phenylalanine, tyrosine and tryptophan biosynthesis, central carbon metabolism in cancer and aminoacyl-tRNA biosynthesis and l-arginine in arginine biosynthesis and mTOR signaling pathway ($P < 0.05$). On the 59th day, compared with the Con group, the Met group up-regulated sphingosine, oxidized glutathione, nucleoside, and sugar metabolites ($P < 0.05$), and down-regulated PC and amino acids ($P < 0.05$), as the same with the 7th day. In addition to that, Met group up-regulated glycocholic acid ($P < 0.05$) and down-regulated taurocholic acid ($P < 0.05$) in bile secretion, cholesterol metabolism, primary bile acid biosynthesis, and up-regulated taurine ($P < 0.05$) in neuroactive ligand-receptor interaction, primary bile acid biosynthesis, and taurine and hypotaurine metabolism at 59th day. Furthermore, up-regulation of ADP and AMP ($P < 0.05$) were enriched in the AMPK signaling pathway, FoxO signaling pathway, and mTOR signaling pathway in the Met group on the 59th day.

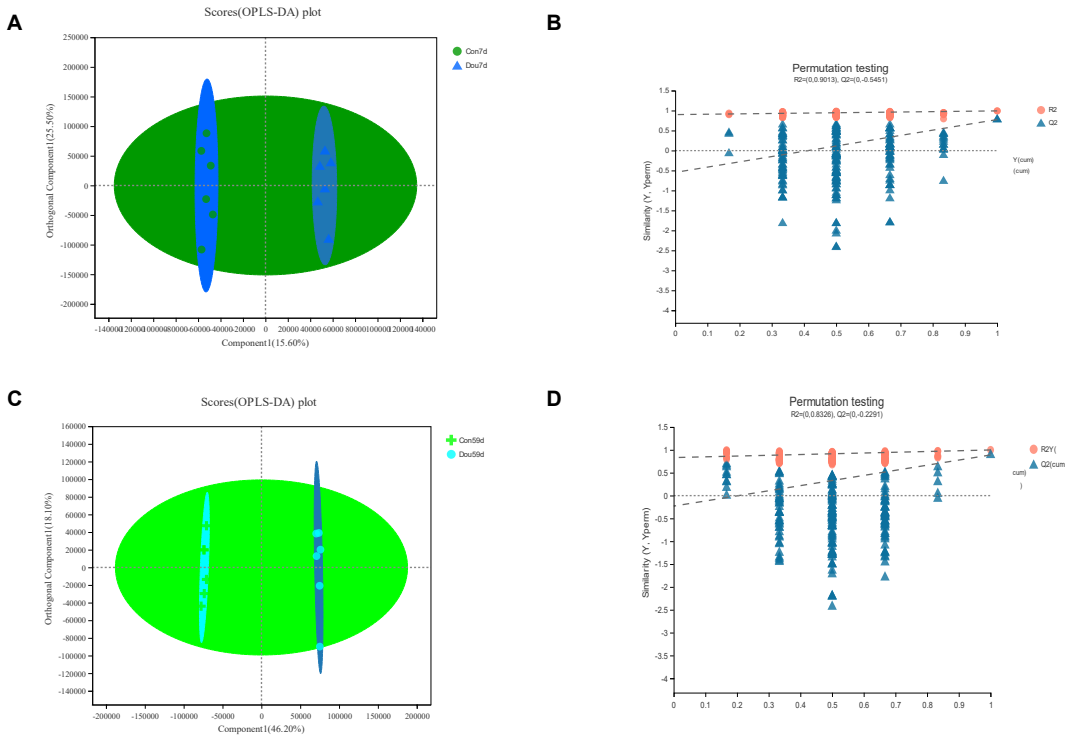


FIGURE 2 | In Dou vs. Con, panels **(A,B)** for 7th day and panels **(C,D)** for 59th day. Panels **(A,C)** are OPLS-DA scores plot. Panels **(B,D)** are OPLA-DA permutation tests. **(B)** R^2X (cum) = 0.571, R^2Y (cum) = 0.994, Q^2 = 0.777. **(D)** R^2X (cum) = 0.705, R^2Y (cum) = 0.999, Q^2 = 0.89.

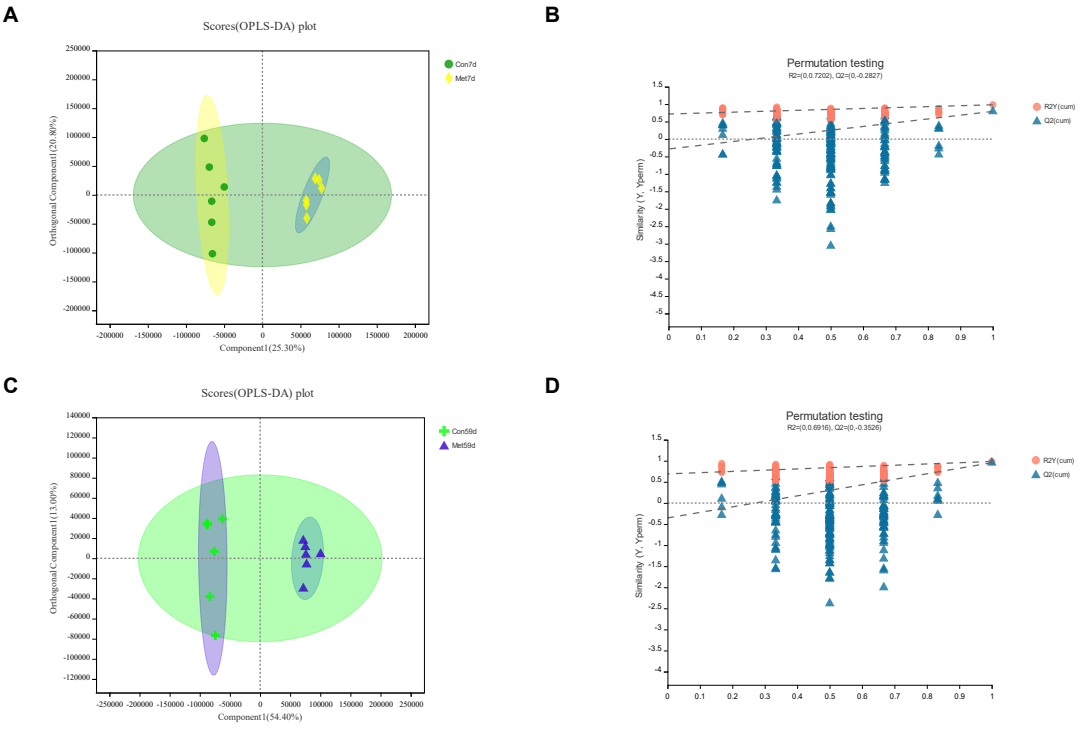


FIGURE 3 | In Met vs. Con, panels **(A,B)** for 7th day and panels **(C,D)** for 59th day. Panels **(A,C)** are OPLS-DA scores plot. Panels **(B,D)** are OPLA-DA permutation tests. **(B)** R^2X (cum) = 0.462, R^2Y (cum) = 0.985, Q^2 = 0.794. **(D)** R^2X (cum) = 0.674, R^2Y (cum) = 0.986, Q^2 = 0.956.

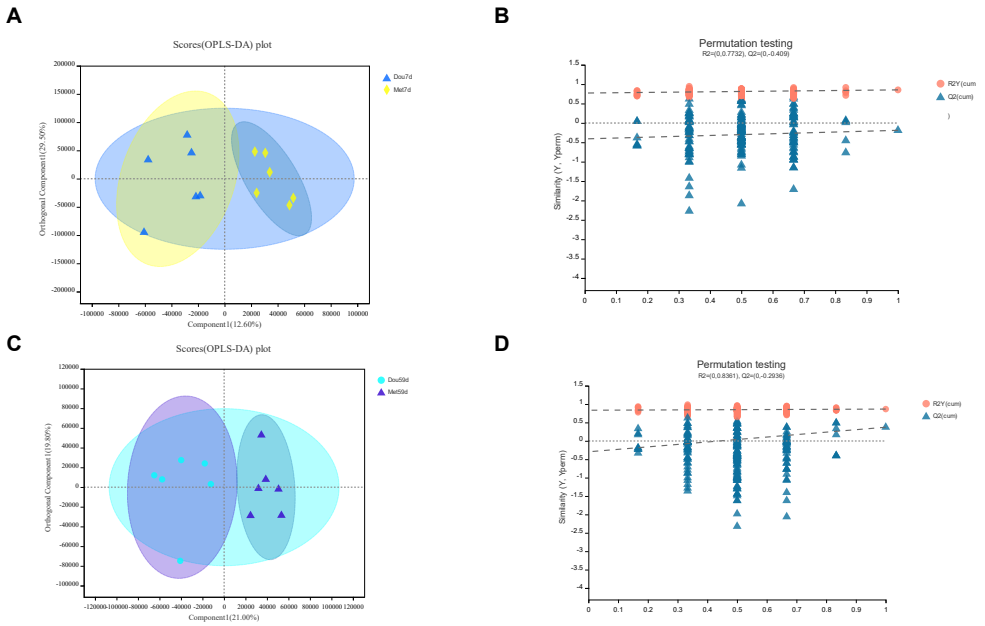


FIGURE 4 | In Met vs. Dou, panels **(A,B)** for 7th day and panels **(C,D)** for 59th day. Panels **(A,C)** are OPLS-DA scores plot. Panels **(B,D)** are OPLA-DA permutation tests. **(B)** R^2X (cum) = 0.421, R^2Y (cum) = 0.855, Q^2 = −0.188. **(D)** R^2X (cum) = 0.408, R^2Y (cum) = 0.867, Q^2 = 0.374.

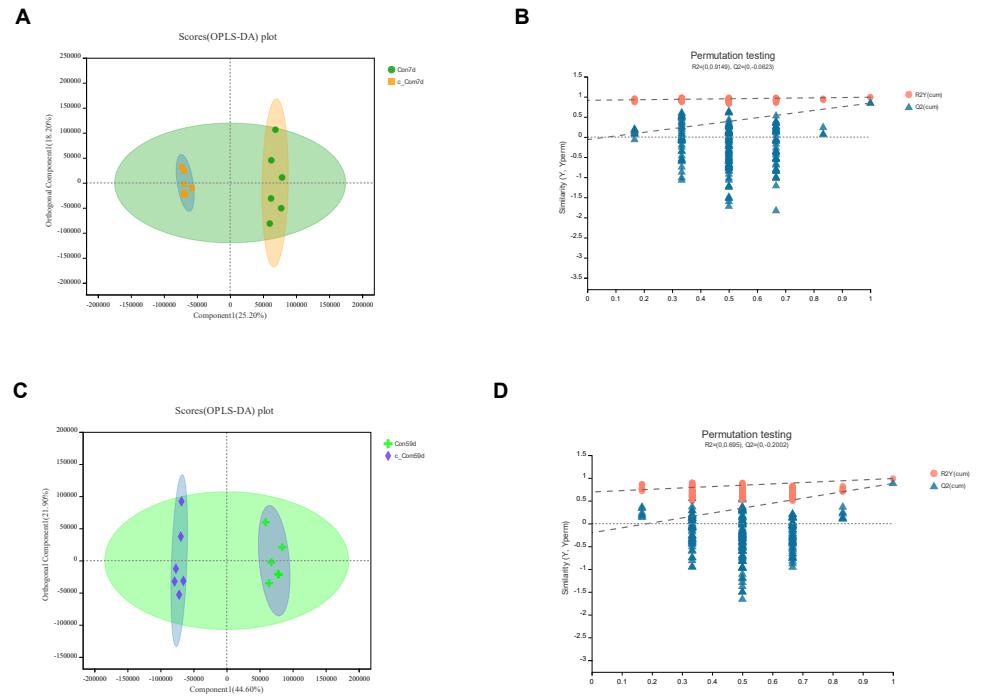


FIGURE 5 | In c-Com vs. Con, panels **(A,B)** for 7th day and panels **(C,D)** for 59th day. Panels **(A,C)** are OPLS-DA scores plot. Panels **(B,D)** are OPLA-DA permutation tests. **(B)** R^2X (cum) = 0.558, R^2Y (cum) = 0.991, Q^2 = 0.844. **(D)** R^2X (cum) = 0.665, R^2Y (cum) = 0.99, Q^2 = 0.881.

Met vs. Dou

On the 7th day, compared with the Dou group, Met down-regulated sphingosine ($P < 0.05$) in apoptosis, necroptosis, and sphingolipid signaling pathway, up-regulated isocitrate ($P < 0.05$) in citrate cycle (TCA cycle), and up-regulated oxidized glutathione ($P < 0.05$) in thyroid hormone synthesis. On the 59th day, differently with 7th day, Met up-regulated sphingosine ($P < 0.05$) in apoptosis and necroptosis,

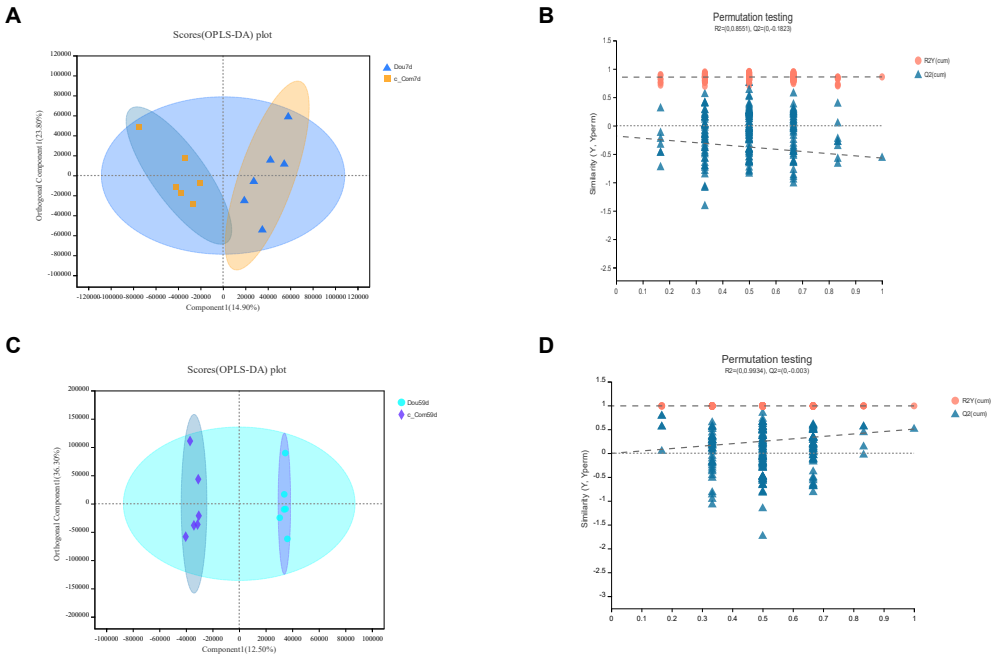


FIGURE 6 | In c-Com vs. Dou, panels **(A,B)** for 7th day and panels **(C,D)** for 59th day. Panels **(A,C)** are OPLS-DA scores plot. Panels **(B,D)** are OPLA-DA permutation tests. **(B)** R^2X (cum) = 0.388, R^2Y (cum) = 0.86, Q^2 = −0.57. D: R^2X (cum) = 0.787, R^2Y (cum) = 0.993, Q^2 = 0.506.

up-regulated amino acids ($P < 0.05$) enriched in protein digestion and absorption, ABC transporters, central carbon metabolism in cancer, aminoacyl-tRNA biosynthesis, valine, leucine, and isoleucine biosynthesis, mineral absorption, and valine, leucine, and isoleucine degradation, increased most of the sugar metabolites ($P < 0.05$) in amino sugar and nucleotide sugar metabolism, and down-regulated taurocholic acid ($P < 0.05$) in cholesterol metabolism.

c-Com vs. Con

On both the 7th and the 59th day, compared with the Con group, the addition of compound-c into soybean meal down-regulated amino acids, l-arginine ($P < 0.05$), and up-regulated nucleoside and sugar metabolites ($P < 0.05$) in purine metabolism, amino sugar, and nucleotide sugar metabolism pathways, and up-regulated sphingosine ($P < 0.05$) in sphingolipid signaling pathway, sphingolipid signaling pathway, necroptosis, and apoptosis, up-regulated ADP ($P < 0.05$) in neuroactive ligand-receptor interaction, thermogenesis, lysosome, down-regulated most of PC and LysoPC ($P < 0.05$) in choline metabolism in cancer. Beyond that, long-term treatment of c-Com up-regulated ADP and AMP ($P < 0.05$) enriched in FoxO signaling pathway, mTOR signaling pathway, PI3K-Akt signaling pathway, and AMPK signaling pathway, and up-regulated taurine ($P < 0.05$) in cholesterol metabolism, primary bile acid biosynthesis, and taurine and hypotaurine metabolism, down-regulated taurocholic acid ($P < 0.05$) and up-regulated glycocholic acid ($P < 0.05$) in bile secretion, cholesterol metabolism, and primary bile acid biosynthesis.

c-Com vs. Dou

On the 7th day, compared with the Dou group, c-Com up-regulated ADP, acetyl adenylate, and UDP-D-galactose ($P < 0.05$), mainly enriched in the AMPK signaling pathway, purine metabolism, FoxO signaling pathway, amino sugar, nucleotide sugar metabolism, and oxidative phosphorylation. On the 59th day, c-Com upregulated sphingosine ($P < 0.05$) in apoptosis and necroptosis and sphingolipid signaling pathway, up-regulated LysoPC in choline metabolism in cancer ($P < 0.05$), increased (R)-(+)-2-Pyrrolidone-5-carboxylic acid ($P < 0.05$) in D-glutamine and D-glutamate metabolism, and up-regulated l-valine and xanthosine ($P < 0.05$) and down-regulated chitobiose and N, N'-diacetylchitobiose ($P < 0.05$) in ABC transporters.

DISCUSSION

Lipid Metabolism

Sphingosine affected intestinal mucosal cells through apoptosis and necroptosis. Sphingolipids and their metabolites are important active molecules, which participate in many important signal transduction processes such as cell growth, differentiation, senescence, and programmed cell death, including many bioactive metabolites ceramide, sphinganine, sphingosine, and sphingosine-1-phosphate [SM(d18:0/14:0)]. Ceramide can be converted to sphingosine. Activated sphingosine kinase catalyzes sphingosine phosphorylation to sphingosine-1-phosphate. Both ceramide and sphingosine can act as second messengers, promoting cell death, cell senescence, and growth stagnation,

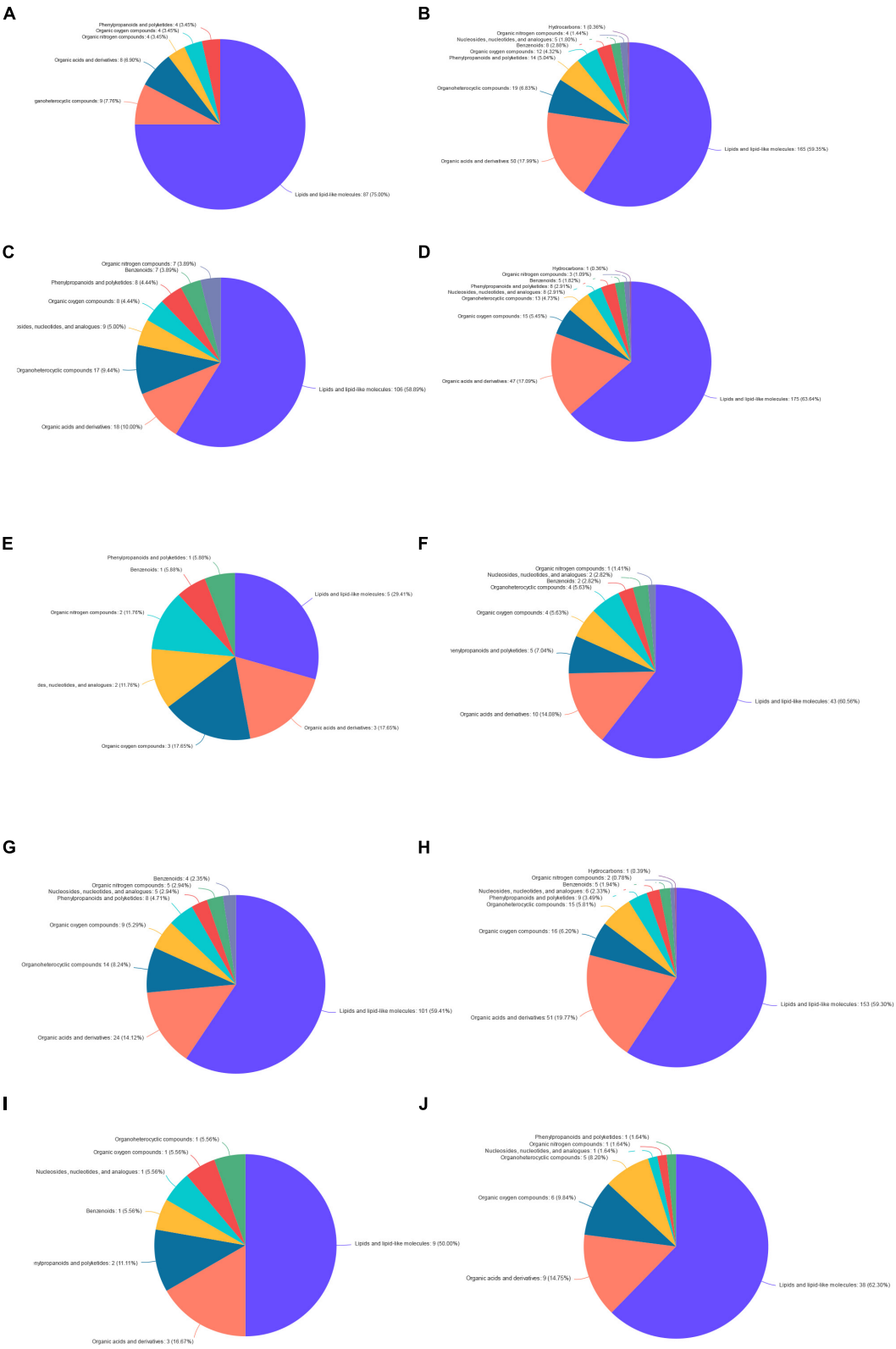


FIGURE 7 | The pie chart. Panel **(A)** for Dou vs. Con on 7th day; panel **(B)** for Dou vs. Con on 59th day; panel **(C)** for Met vs. Con on 7th day; panel **(D)** for Met vs. Con on 59th day; panel **(E)** for Met vs. Dou on 7th day; panel **(F)** for Met vs. Dou on 59th day; panel **(G)** for c-Com vs. Con on 7th day; panel **(H)** for c-Com vs. Con on 59th day; panel **(I)** for c-Com vs. Dou on 7th day; panel **(J)** for c-Com vs. Dou on 59th day.

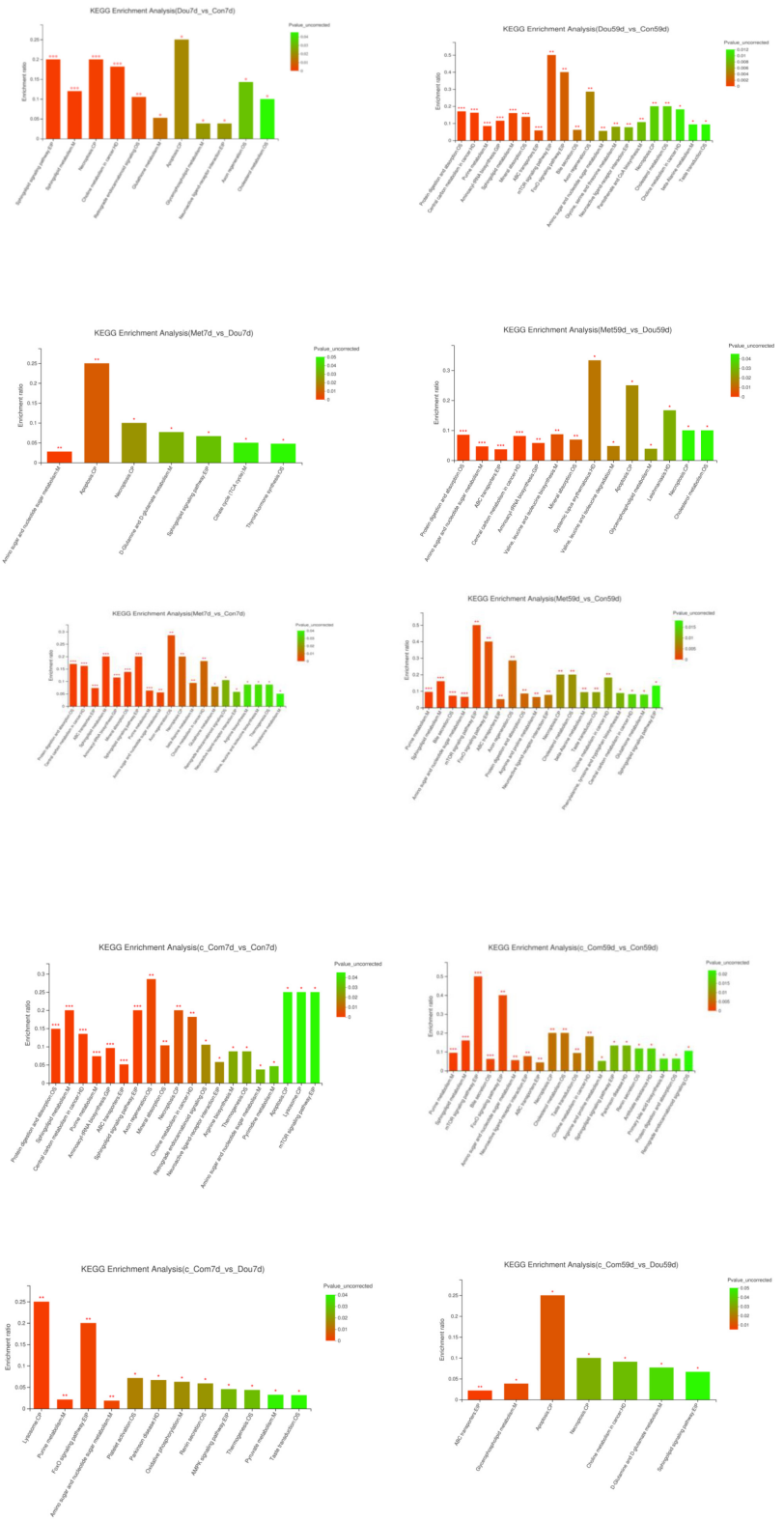


FIGURE 8 | KEGG enrichment analysis. The abscissa represents the pathway name, and the ordinate represents enrichment rate, representing the ratio between the Metabolite number enriched in this pathway, and the Background number annotated. The higher the ratio, the greater the degree of enrichment. The color gradient of the column represents the significance of enrichment. The darker the default color is, the more significant the enrichment of the KEGG term is. In particular, *P*-value < 0.001 is marked as ***, *P*-value < 0.01 is marked as **, and *P*-value < 0.05 is marked as *. The following table is the same.

while sphingosine-1-phosphate regulates signaling pathways that stimulate cell growth and inhibit programmed cell death (Petra et al., 2020). Previous studies have shown that sphingosine induces apoptosis, which depends on the activation of Caspases-3, -7, and -8 (Chang et al., 2003). Several previous studies showed that the pro-apoptotic gene caspases-3, -7, and -8 were up-regulated by high-dose soybean meal (Bakke-McKellep et al., 2007; Chen et al., 2018; Duan et al., 2019). In this study, soybean meal significantly affected the contents of sphingosine and SM(d18:0/14:0) metabolites. Adding metformin was the opposite of that of the soybean meal group, and compound-c was similar to the soybean meal group. Metformin activated AMPK through inhibiting mitochondrial complex I, which led to the inhibition of mTOR signaling, autophagy, and apoptosis activation, decreased ROS generation, DNA damage, and inflammatory response (Lv and Guo, 2020). Compound-c is the primary reagent used as an AMPK inhibitor (Dasgupta and Seibel, 2018). In previous studies, compound C induced apoptosis, but AMPK was not involved in this effect (Jin et al., 2009). Consistent with the studies mentioned above, these results indicate soybean meal-induced intestinal mucosal cell apoptosis with AMPK independence.

Phosphatidylcholine, also known as lecithin, is an important component of the cell membrane. PC and LysoPC were involved in choline metabolism in cancer in this study. This study has shown that PC and LysoPC were down-regulated by soybean meal. However, PC and LysoPC in the Met group did not change significantly compared with the Dou group. These suggested that lipid metabolism, especially PC and LysoPC metabolism in the intestinal mucosa of carp may be affected through AMPK signaling pathway.

Further more, taurine, glycocholic acid, and betaine were changed by soybean meal whether activator of inhibitor was added or not. According to Valenzuela et al. (2021), tolerance to soybean meal is associated with immune and lipid metabolism-related pathways in fish. The results of this experiment are consistent with it. Acted as an activator of AMPK, metformin administration increases the bile acid pool in the intestine, which may affect glucagon-like-peptide-1 secretion and cholesterol levels (Lv and Guo, 2020). Therefore, metformin down-regulated taurocholic acid of cholesterol metabolism with consistent function. The result indicated that soybean meal could affect lipid metabolism of intestinal mucosa of carp unrelated to AMPK signaling pathway.

Protein and Amino Acid Metabolism

Aminoacyl-tRNA synthetases (aaRS) participate in one of the most important steps of life activities–protein biosynthesis. Hence, in the intestinal mucosa, the level of amino acids enriched in the pathways can reflect the availability of amino acids. This experiment found that amino acids were down-regulated in experimental groups, enriched in protein digestion and absorption pathways, ABC transporters, central carbon metabolism in cancer, aminoacyl-tRNA biosynthesis, so soybean meal significantly reduced the amino acids content and the utilization in the carp intestines. This is the same as the results of Zhou in carps (Zhou, 2021), and the difference with the conclusion from Wei et al. (2017) that increased the utilization of amino acids in fish muscles, which the different types of fish should cause a difference. In contrast, metformin up-regulated amino acids in the result. This maybe related with that metformin activates AMPK, which leads to the inhibition of mTOR signaling, and as a result, protein synthesis is disturbed (Lv and Guo, 2020).

In addition to that, arginine plays a vital role in regulating animals' nutritional metabolism, growth, and development (Rhoads and Wu, 2009). Arginine stimulates protein synthesis, cell proliferation, DNA synthesis, cell protection, and migration (Rhoads et al., 2004; Corl et al., 2008; Tan et al., 2010; Tan et al., 2015). Activation of the mTOR signaling pathway is an important mechanism for arginine to alleviate cell stress injury (Tan et al., 2010). Compared with Con, enriched in the mTOR signaling pathway, arginine was significantly down-regulated in experimental groups. Zhang et al. (2013) found that anti-nutritional factors in soybean decreased TOR gene expression in foregut and midgut and increased 4E-BP gene expression in the hindgut of Jian carp. This is consistent with this experiment. These suggested that soybean meal caused intestinal mucosa injury, which may be related to the down-regulation of arginine, which does not activate the mTOR signaling pathway.

Sugar Metabolism and Purine Metabolism

In addition to lipid and protein, sugar is also one of the essential energy materials in fish, which can be directly used to power the body to reduce fat and protein consumption in fish. This experiment suggested that the soybean meal group significantly raised sugar metabolites on the 59th day. This was the same as Zhou's research in carp and the result of Wei in turbot (*Scophthalmus maximus* L) that glucose increased in the liver metabolites. Compared with the soybean meal group, isocitrate was significantly up-regulated and enriched in the citrate cycle in Met on 7th day, indicating regulating glucose metabolism. This result was consistent with the property that metformin can regulate the AMPK signaling pathway and further increase glucose catabolism by activating the AMPK signaling pathway (Lv and Guo, 2020). However, this phenomenon did not last for 59 days. It was speculated that soybean meal had an effect on glucose metabolism in the intestinal mucosa; as a result, there was no significant change in glucose metabolism when Met was added. Therefore, adding an activator could not enhance glucose metabolism on the 59th day. Additionally, metabolites in purine metabolic pathways were significantly increased in Dou on 59th day vs. Con on 59th day and c-Com on 7th day vs. Dou on 7th day, and not changed between Met and Dou, which indicated that both soybean meal and compound-c up-regulated the nucleotide metabolism of intestinal mucosal cells. We speculated that the mechanism of soybean meal on intestinal nucleoside metabolism might not be through the AMPK signaling pathway.

Energy Metabolism

Adenosine triphosphate box transporter (ATP-binding Cassette transporter, ABC transporter) is involved in many important physiological processes, such as nutrient intake, cellular

detoxification, lipid homeostasis, signal transduction, disease virus defense, and antigen presentation (Wilkens, 2015), whose primary main function is to utilize the energy generated by ATP (adenosine triphosphate) hydrolysis to transport the substrate across the membrane against the concentration gradient (Borths et al., 2002; Locher, 2004; Kathawala et al., 2015). This experiment found down-regulated amino acids in experimental groups enriched in ABC transporters. At the same time, compared with the Con group, ADP and AMP of Dou on 59th day, Met on 7th day and 59th day and c-Com on 7th day, and 59th day were significantly increased and enriched in FoxO and AMPK signaling pathways. The content of ADP, AMP, and ATP in intestinal mucosa reflects the level of energy metabolism. Metformin acts as an AMPK activator by inhibiting mitochondrial complex I (Batandier et al., 2006; Viollet et al., 2012). Mitochondrial complex I is critical for electron transport. As a result, the production of ATP was reduced, and the intracellular concentration of ADP increased. Consequently, the cellular levels of AMP increases and ultimately activate AMPK (Viollet et al., 2012; Pryor and Cabreiro, 2015). Members of the class O of forkhead box transcription factors (FoxO) have essential roles in metabolism, cellular proliferation, stress tolerance, and probably lifespan (Weigel et al., 1989). The AKT/PKB-mediated phosphorylation of FoxO inhibits FoxO function by promoting its interaction with 14-3-3 proteins and its relocalization from the nucleus to the cytosol (Van der Horst and Burgering, 2007). Lee et al. (2017) suggested that FoxO transcription factors could feasibly act downstream of AMPK to mediate the induction of antioxidant systems in response to H_2. This study suggested that soybean meal can improve ADP and AMP content then activate the AMPK and FoxO pathway.

Oxidative Damage

The soybean meal can cause intestinal oxidative damage, which has been shown in grass carp (Duan et al., 2019; Zhang et al., 2020), turbot (Chen et al., 2018), and *Scophthalmus maximus* L. (Gu et al., 2021). In this study, enriched in glutathione metabolism, oxidized glutathione was up-regulated in the soybean meal group on the 7th day, and the amount of oxidized glutathione in the Met group was significantly higher than that in the Dou group at 7th day. These suggested that soybean meal might cause oxidative damage to the intestinal mucosa in short-time treatment, and metformin supplementation aggravated the injury. However, in a previous study, metformin suppresses mitochondrial complex I, thereby preventing the generation of reactive oxygen species (ROS) and further decreasing DNA damage (Algire et al., 2012). The results of this experiment are inconsistent with this and require further validation.

CONCLUSION

In conclusion, soybean meal influenced intestinal mucosa metabolic processes, including lipid, amino acid, sugar, apoptosis, and oxidative injury; and changed energy metabolism in intestinal mucosa, enriched in the AMPK, TOR, FoxO signaling pathway; Metformin could aggravate oxidative damage, alleciated apoptosis for the short term, and aggravate apoptosis, improve carbohydrate catabolism, and amino acid anabolism for the long term; Compound-c exacerbated apoptosis. repaired oxidative damage, and enhanced nucleoside catabolism.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ebi.ac.uk/metabolights/, MTBLS4074.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee on the Ethics of Animal Experiments of Huzhou University.

AUTHOR CONTRIBUTIONS

JZ: experimental operation, field sampling, and writing—original draft preparation. QX: conceptualization and writing—review and editing. Both authors read and approved the final manuscript.

FUNDING

This work was financially supported by the Natural Science Foundation of China, NSFC (31972800), and the China Agriculture Research System of MOF and MARA (CARS-45).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.844716/full#supplementary-material

REFERENCES

Algire, C., Moiseeva, O., Deschnes-Simard, X., Amrein, L., Petruccelli, L., Birman, E., et al. (2012). Metformin reduces endogenous reactive oxygen species and associated DNA damage. *Cancer Prev. Res.* 5, 536–543. doi: 10.1158/1940-6207.CAPR-11-0536

Antonopoulou, E., Chouri, E., and Feidantsis, K. (2017). Effects of partial dietary supplementation of fish meal with soymeal on the stress and apoptosis response in the digestive system of common dentex (*Dentex dentex*). *J. Biol. Res.* 24, 1–10. doi: 10.1186/s40709-017-0071-1

Bakke-McKellep, A. M., Penn, M. H., Salas, P. M., Refstie, S., Sperstad, S., Landsverk, T., et al. (2007). Effects of dietary soyabean meal, inulin and oxytetracycline on intestinal microbiota and epithelial cell stress, apoptosis and proliferation in the teleost Atlantic salmon (*Salmo salar* L.). *Br. J. Nutr.* 97, 699–713. doi: 10.1017/S0007114507381397

Batandier, C., Guigas, B., Detaille, D., El-Mir, M., Fontaine, E., Rigoulet, M., et al. (2006). The ROS production induced by a reverse-electron flux at respiratory-chain complex 1 is hampered by metformin. *J. Bioenerg. Biomembr.* 38, 33–42. doi: 10.1007/s10863-006-9003-8

Becker, K., and Makkar, H. P. S. (1999). Effects of dietary tannic acid and quebracho tannin on growth performance and metabolic rates of common carp (*Cyprinus carpio* L.). *Aquaculture* 175, 327–335. doi: 10.1016/S0044-8486(99)00106-4

Borths, E. L., Locher, K. P., Lee, A. T., and Rees, D. C. (2002). The structure of *Escherichia coli* BtuF and binding to its cognate ATP binding cassette transporter. *Proc. Natl. Acad. Sci. U. S. A.* 99, 16642–16647. doi: 10.1073/pnas.262659699

Bron, P. A., Kleerebezem, M., Brummer, R. J., Cani, P. D., Mercenier, A., MacDonald, T. T., et al. (2017). Can probiotics modulate human disease by impacting intestinal barrier function? *Br. J. Nutr.* 117, 93–107. doi: 10.1017/S0007114516004037

Buttle, L. G., Burrells, A. C., and Good, J. E. (2001). The binding of soybean agglutinin (SBA) to the intestinal epithelium of Atlantic salmon, *Salmo salar* and Rainbow trout, *Oncorhynchus mykiss*, fed high levels of soybean meal. *Vet. Immunol. Immunopathol.* 80, 237–244. doi: 10.1016/S0165-2427(01)00269-0

Chang, H. C., Hsu, C., Hsu, H. K., and Yang, R. C. (2003). Functional role of caspases in sphingosine-induced apoptosis in human hepatoma cells. *IUBMB Life* 55, 403–407. doi: 10.1080/15216540310001594184

Chen, Z. C., Zhao, S. F., Liu, Y., Yang, P., Ai, Q. H., Zhang, W. B., et al. (2018). Dietary citric acid supplementation alleviates soybean meal−induced intestinal oxidative damage and micro−ecological imbalance in juvenile turbot, *Scophthalmus maximus* L. *Aquac. Res.* 49, 3804–3816. doi: 10.1111/are.13847

Corl, B. A., Odle, J., Niu, X. M., Moeser, A. J., Gatlin, L. A., Phillips, O. T., et al. (2008). Arginine activates intestinal $p70^{S6k}$ and protein synthesis in piglet rotavirus enteritis. *J. Nutr.* 138, 24–29. doi: 10.1093/jn/138.1.24

Dasgupta, B., and Seibel, W. (2018). Compound C/Dorsomorphin: its Use and Misuse as an AMPK Inhibitor. *Methods Mol. Biol.* 1732, 195–202. doi: 10.1007/978-1-4939-7598-3_12

Duan, X. D., Feng, L., Jiang, W. D., Wu, P., Liu, Y., Kuang, S. Y., et al. (2019). Dietary soybean β-conglycinin suppresses growth performance and inconsistently triggers apoptosis in the intestine of juvenile grass carp (*Ctenopharyngodon idella*) in association with ROS-mediated MAPK signalling. *Aquac. Nutr.* 25, 770–782. doi: 10.1111/anu.12895

Gu, M., Bai, N., Zhang, Y. Q., and Krogdahl, A. (2016). Soybean meal induces enteritis in turbot *Scophthalmus maximus* at high supplementation levels. *Aquaculture* 464, 286–295. doi: 10.1016/j.aquaculture.2016.06.035

Gu, M., Pan, S. H., Li, Q., Qi, Z. Z., Deng, W. Z., and Bai, N. (2021). Protective effects of glutamine against soy saponins-induced enteritis, tight junction disruption, oxidative damage and autophagy in the intestine of *Scophthalmus maximus* L. *Fish Shellfish Immunol.* 114, 49–57. doi: 10.1016/j.fsi.2021.04.013

Hara, A., Saegusa, M., Mitomi, H., Kurihara, M., Ishihara, K., Hotta, K., et al. (2000). Colonic mucin-carbohydrate components in colorectal tumors and their possible relationship to MUC2, p53 and DCC immunoreactivities. *Pathol.-Res. Pract.* 196, 159–166. doi: 10.1016/S0344-0338(00)80096-5

Jiang, W. D., Hu, K., Zhang, J. X., Liu, Y., Jiang, J., Wu, P., et al. (2015). Soyabean glycinin depresses intestinal growth and function in juvenile Jian carp (*Cyprinus carpio* var Jian): protective effects of glutamine. *Br. J. Nutr.* 114, 1569–1583. doi: 10.1017/S0007114515003219

Jin, J. F., Mullen, T. D., Hou, Q., Bielawski, J., Bielawska, A., Zhang, X. M., et al. (2009). AMPK inhibitor Compound C stimulates ceramide production and promotes Bax redistribution and apoptosis in MCF7 breast carcinoma cells. *J. Lipid Res.* 50, 2389–2397. doi: 10.1194/jlr.M900119-JLR200

Kahn, B. B., Alquier, T., Carling, D., and Hardie, D. G. (2005). AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab.* 1, 15–25. doi: 10.1016/j.cmet.2004.12.003

Kathawala, R. J., Gupta, P., Ashby, C. R., and Chen, Z. S. (2015). The modulation of ABC transporter-mediated multidrug resistance in cancer: a review of the past decade. *Drug Resist. Updates* 18, 1–17. doi: 10.1016/j.drup.2014.11.002

Kim, Y. M., Kim, M. Y., Kim, H. J., Roh, G. S., Ko, G. H., Seo, H. G., et al. (2011). Compound-c independent of AMPK inhibits ICAM-1 and VCAM-1 expression in inflammatory stimulants-activated endothelial cells in vitro and in vivo. *Atherosclerosis* 219, 57–64. doi: 10.1016/j.atherosclerosis.2011.06.043

Lee, J. A., Yang, G. B., Kim, Y. J. C., Tran, Q. H. B., Choe, W. B. D., Kang, I. B. D., et al. (2017). Hydrogen-rich medium protects mouse embryonic fibroblasts from oxidative stress by activating LKB1-AMPK-FoxO1 signal pathway. *Biochem. Biophys. Res. Commun.* 491, 733–739. doi: 10.1016/j.bbrc.2017.07.119

Lin, S., and Luo, L. (2011). Effects of different levels of soybean meal inclusion in replacement for fish meal on growth, digestive enzymes and transaminase activities in practical diets for juvenile tilapia, *Oreochromis niloticus* × *O. aureus*. *Anim. Feed Sci. Technol.* 168, 80–87. doi: 10.1016/j.anifeedsci.2011.03.012

Locher, K. P. (2004). Structure and mechanism of ABC transporters. *Curr. Opin. Struct. Biol.* 14, 426–431. doi: 10.1016/j.sbi.2004.06.005

Long, S. S., Dong, X. H., Tan, B. P., Zhang, S., Xie, S. W., Yang, Q. H., et al. (2021). Growth performance, antioxidant ability, biochemical index in serum, liver histology and hepatic metabolomics analysis of juvenile hybrid grouper (♀ *Epinephelus fuscoguttatus* × ♂ *Epinephelus lanceolatus*) fed with oxidized fish oil. *Aquaculture* 545:737261. doi: 10.1016/j.aquaculture.2021.737261

Lv, Z., and Guo, Y. (2020). Metformin and its benefits for various diseases. *Front. Endocrinol.* 11:191. doi: 10.3389/fendo.2020.00191

Maytorena-Verdugo, C. I., Córdova-Murueta, J. H., and García-Carreño, F. L. (2017). Peptidase compensation in the digestive system of whiteleg shrimp Penaeus vannamei against dietary Kunitz−type soybean trypsin inhibitor. *Aquac. Nutr.* 23, 1095–1103. doi: 10.1111/anu.12477

National Research Council [NRC] (2011). *Nutrient Requirements of Fish and Shrimp*. Washington, DC: The National Academies Press.

Park, Y. M., Lim, J. H., and Seo, E. W. (2015). Anti-obesity effect of by-product from soybean on mouse fed a high fat diet. *Korean J. Plant Resour.* 28, 168–177. doi: 10.7732/kjpr.2015.28.2.168

Petra, G., Markova, C. E. P., and Mirela, S. (2020). Targeting ceramide metabolism in hepatocellular carcinoma: new points for therapeutic intervention. *Curr. Med. Chem.* 27, 6611–6627. doi: 10.2174/0929867326666190911115722

Pryor, R., and Cabreiro, F. (2015). Repurposing metformin: an old drug with new tricks in its binding pockets. *Biochem. J.* 471, 307–322. doi: 10.1042/BJ20150497

Rhoads, J. M., Chen, W., Gookin, J., Wu, G. Y., Fu, Q., Bliklslager, A. T., et al. (2004). Arginine stimulates intestinal cell migration through a focal adhesion kinase dependent mechanism. *Gut* 53, 514–522. doi: 10.1136/gut.2003.027540

Rhoads, J. M., and Wu, G. Y. (2009). Glutamine, arginine, and leucine signaling in the intestine. *Amino Acids* 37, 111–122. doi: 10.1007/s00726-008-0225-4

Silva, F. H., Alexandre, E. C., Calmasini, F. B., Calixto, M. C., and Antunes, E. (2015). Treatment with metformin improves erectile dysfunction in a murine model of obesity associated with insulin resistance. *Urology* 86, 423.e1–6. doi: 10.1016/j.urology.2015.04.035

Tan, B., Xiao, H., and Xiong, X. (2015). L-arginine improves DNA synthesis in LPS-challenged enterocytes. *Front. Biosci.* 20, 989–1003. doi: 10.2741/4352

Tan, B., Yin, Y. L., Kong, X. F., Li, P., Li, X. L., Gao, H. J., et al. (2010). L-arginine stimulates proliferation and prevents endotoxin-induced death of intestinal cells. *Amino Acids* 38, 1227–1235. doi: 10.1007/s00726-009-0334-8

Turner, J. R. (2009). Intestinal mucosal barrier function in health and disease. *Nat. Rev. Immunol.* 9, 799–809. doi: 10.1038/nri2653

Urn, P. A., Gonalves, A. A., Taverne-Thiele, J. J., Schrama, J. W., Verreth, J. A. J., and Rombout, J. H. W. M. (2008). Soybean meal induces intestinal inflammation in common carp (*Cyprinus carpio* L.). *Fish Shellfish Immunol.* 25, 751–760. doi: 10.1016/j.fsi.2008.02.013

Valenzuela, L., Pacheco, S., Rincón, G., Pavez, L., Lam, N., Hernández, A. J., et al. (2021). Intestinal transcriptome analysis reveals enrichment of genes associated with immune and lipid mechanisms, favoring soybean meal tolerance in high-growth zebrafish (*danio rerio*). *Genes* 12:700. doi: 10.3390/genes12050700

Van der Horst, A., and Burgering, B. M. T. (2007). Stressing the role of FoxO proteins in lifespan and disease. *Nat. Rev. Mol. Cell Biol.* 8, 440–450. doi: 10.1038/nrm2190

Velcich, A., Yang, W. C., Heyer, J., Fragale, A., Nicholas, C., Viani, S., et al. (2002). Colorectal cancer in mice genetically deficient in the mucin Muc2. *Science* 295, 1726–1729. doi: 10.1126/science.1069094

Viollet, B., Guigas, B., Sanz Garcia, N., Leclerc, J., Foretz, M., and Andreelli, F. (2012). Cellular and molecular mechanisms of metformin: an overview. *Clin. Sci.* 122, 253–270. doi: 10.1042/CS20110386

Wang, Y. Y., Yu, S. L., Wang, Y. Y., Che, J. F., Zhao, L. P., Bu, X. Y., et al. (2016). Effect of replacing fish meal with soybean meal on growth, feed utilization and nitrogen and phosphorus excretion of juvenile *Pseudobagrus ussuriensis*. *Aquac. Res.* 47, 3145–3155. doi: 10.1111/are.12765

Wei, Y., Liang, M., Mai, K., Zheng, K., and Xu, H. (2017). H-1 NMR-based metabolomics studies on the effect of size-fractionated fish protein hydrolysate, fish meal and plant protein in diet for juvenile turbot (*Scophthalmus maximus* L.). *Aquac. Nutr.* 23, 523–536. doi: 10.1111/anu.12420

Weigel, D., Jurgens, G., Kuttner, F., Seifert, E., and Jackle, H. (1989). The homeotic gene fork head encodes a nuclear protein and is expressed in the terminal regions of the *Drosophila embryo*. *Cell* 57, 645–658. doi: 10.1016/0092-8674(89)90133-5

Wilkens, S. (2015). Structure and mechanism of ABC transporters. *F1000prime Rep.* 7:14. doi: 10.12703/P7-14

Xiang, Q. Q., Yan, H., Luo, X. W., Kang, Y. H., Hu, J. M., and Chen, L. Q. (2021). Integration of transcriptomics and metabolomics reveals damage and recovery mechanisms of fish gills in response to nanosilver exposure. *Aquat. Toxicol.* 237:105895. doi: 10.1016/j.aquatox.2021.105895

Yang, B. T., Wen, B., Ji, Y., Wang, Q., Zhang, H. R., Zhang, Y., et al. (2021). Comparative metabolomics analysis of pigmentary and structural coloration in discus fish (*Symphysodon haraldi*). *J. Proteom.* 233:104085. doi: 10.1016/j.jprot.2020.104085

Zhang, J. X., Guo, L. Y., Feng, L., Jiang, W. D., Kuang, S. Y., Liu, Y., et al. (2013). Soybean β-conglycinin induces inflammation and oxidation and causes dysfunction of intestinal digestion and absorption in Fish. *PLoS One* 8:e58115. doi: 10.1371/journal.pone.0058115

Zhang, Y. L., Jiang, W. D., Duan, X. D., Feng, L., Wu, P., Liu, Y., et al. (2020). Soybean glycinin caused NADPH-oxidase-regulated ROS overproduction and decreased ROS elimination capacity in the mid and distal intestine of juvenile grass carp (*Ctenopharyngodon idella*). *Aquaculture* 516:734651. doi: 10.1016/j.aquaculture.2019.734651

Zhou, Z. L. (2021). *Effects of glycinin and β-conglycinin on growth performance, intestinal health and metabolic pathways of Songpu mirror carp*. Ph.D. thesis. Shanghai: Shanghai Ocean University.

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.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership