frontiers | Research Topics

New progresses of fish meal replacement in aquatic animals

Edited by
Jin Niu, Min Gu, Ikram Belghit, Shiwei Xie and Xiang-Jun Leng

Published in
Frontiers in Marine Science
Frontiers in Physiology

ISSN 1664-8714
ISBN 978-2-83250-962-3
DOI 10.3389/978-2-83250-962-3

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

New progresses of fish meal replacement in aquatic animals

Topic editors

Jin Niu — Sun Yat-sen University, China
Min Gu — Shandong University, Weihai, China
Ikram Belghit — Norwegian Institute of Marine Research (IMR), Norway
Shiwei Xie — Guangdong Ocean University, China
Xiang-Jun Leng — Shanghai Ocean University, China

Citation

Niu, J., Gu, M., Belghit, I., Xie, S., Leng, X.-J., eds. (2022). *New progresses of fish meal replacement in aquatic animals.* Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-83250-962-3

Table of contents

frontiers in Marine Science

ORIGINAL RESEARCH
published: 02 July 2021
doi: 10.3389/fmars.2021.688601

The Single-Molecule Long-Read Sequencing of Intestine After Soy Meal-Induced Enteritis in Juvenile Pearl Gentian Grouper, *Epinephelus fuscoguttatus*♀ × *Epinephelus lanceolatus*♂

Wei Zhang[1,2,3], Beiping Tan[1,2,3], Junming Deng[1,2,3], Xiaohui Dong[1,2,3], Qihui Yang[1,2,3], Shuyan Chi[1,2,3], Hongyu Liu[1,2,3], Shuang Zhang[1,2,3], Shiwei Xie[1,2,3] and Haitao Zhang[3]*

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

OPEN ACCESS

Edited by:
Menghong Hu,
Shanghai Ocean University, China

Reviewed by:
Kang-le Lu,
Jimei University, China
Mansour Torfi Mozanzadeh,
South Iran Aquaculture Research Center, Iran
Min Jin,
Ningbo University, China

***Correspondence:**
Beiping Tan
bptan@126.com

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

Received: 31 March 2021
Accepted: 21 May 2021
Published: 02 July 2021

Citation:
Zhang W, Tan B, Deng J, Dong X, Yang Q, Chi S, Liu H, Zhang S, Xie S and Zhang H (2021) The Single-Molecule Long-Read Sequencing of Intestine After Soy Meal-Induced Enteritis in Juvenile Pearl Gentian Grouper, Epinephelus fuscoguttatus♀ × Epinephelus lanceolatus♂. Front. Mar. Sci. 8:688601. doi: 10.3389/fmars.2021.688601

Intestinal inflammatory disease induced by excessive soy protein substitutions for fish meal (FM) protein is a common phenomenon. The pearl gentian grouper, *Epinephelus fuscoguttatus*♀ × *Epinephelus lanceolatus*♂, a marine fish with important economical and nutritional values, exhibited a similar problem. As far as we know, there are no reports on the full-length transcriptome of the pearl gentian grouper. In the present study, seven isonitrogenous and isolipidic (10% lipid) diets were prepared and fed to fish for 10 weeks. The water volume in each barrel was about 1 m^3, using natural light and temperature. The results showed that 40% dietary soy proteins significantly negatively affected the growth performance of the pearl gentian grouper. Compared to the FM control, the content of immunoglobulin M and the enzyme activities of glutathione reductase, glutathione peroxidase, and total superoxide dismutase in the intestine significantly increased; the content of malondialdehyde in the intestine significantly decreased; and the enzyme activities of alanine transaminase and aspartate transaminase in the liver significantly increased. A library composed of seven different treated distal intestine tissues, including the FM control group, 20% soybean meal substitute for FM (SBM20), SBM40, 20% soybean protein concentrate (SPC20), SPC40, 20% fermented soybean meal substitute for FM (FSBM20), and FSBM40, was constructed and sequenced using PacBio single-molecule real-time (SMRT) and the RNA-Seq technology. As a whole, this study obtained 420,006 full-length non-chimeric (FLNC) reads. After error correction, sequence clustering, and redundancy, 82,351 transcripts with high quality were obtained. In addition, a total of 77,815 transcripts were annotated in seven databases (non-redundant protein sequences, non-redundant nucleotide sequences, Protein family, Clusters of Orthologous Groups of proteins, Gene Ontology, Swiss-Prot, and KEGG Orthology). Also, 49,093 long non-coding RNAs (lncRNAs) and 141,702 simple sequence repeats were identified. Based on full-length transcriptome sequencing, the present study found that the Toll-like receptor/nuclear

factor kappa-B signaling pathway plays an important role in the development of SBM- and FSBM-induced enteritis. SPC-induced enteritis is mainly accompanied by a general imbalance of the nutrition absorption-related signaling pathways, which only affects a small part of the immune-related signaling pathways. This study supplies new and valuable reference transcripts, which would better facilitate further research on the pearl gentian grouper.

Keywords: *Epinephelus fuscoguttatus*♀ × *Epinephelus lanceolatus*♂, full-length transcriptome sequencing of groupe, Illumina sequencing, intestinal health, soy protein, third-generation sequencing

INTRODUCTION

Fish meal (FM) is an important source of protein in aquatic feed, especially for carnivorous species. Most of the FM originates from wild oil-rich fishes, and its supply is unsustainable due to the limited abundance of these populations (Booman et al., 2018; Cai et al., 2020). The total amount of global aquaculture continues to increase, while FM production is relatively constant (Guardiola et al., 2016). Therefore, it is very important to find new protein sources with good potential to replace FM (Teves and Ragaza, 2014). At present, soy products are the first choice to replace FM protein, such as soybean meal (SBM), soybean protein concentrate (SPC), and fermented soybean meal (FSBM) (Xiang, 2017). However, intestinal inflammation disease induced by high levels of soy protein, especially SBM as a substitute for FM, is a common phenomenon in aquaculture, which would affect fish growth and feed utilization.

Up to now, the exact reason and mechanism of fish enteritis induced by soy proteins are not very clear, and the molecular mechanism of enteritis formation is still lacking of a systematic research. In order to further understand the effect of aquatic feed on fish physiology, it is necessary to apply different research methods to investigate the relationship between diet and intestinal health from different perspectives. New omics technologies such as transcriptomics can provide great potential for studying the complex relationship between nutrition and the immunity of fish in health and disease (Martin and Król, 2017). Despite the reduced cost of deep sequencing, only a few partially completed genome information are available of the key aquaculture fish species, such as the Atlantic cod (*Gadus morhua*) (Star et al., 2011), common carp (*Cyprinus carpio*) (Xu et al., 2014), European sea bass (*Dicentrarchus labrax*) (Tine et al., 2014), tilapia (*Oreochromis niloticus*) (Brawand et al., 2014), grass carp (*Ctenopharyngodon idellus*) (Wang et al., 2015), and the channel catfish (*Ictalurus punctatus*) (Liu et al., 2016).

The pearl gentian grouper (*Epinephelus fuscoguttatus*♀ × *E. lanceolatus*♂) is a carnivorous fish species that has the advantages of fast growth, high market value, and good disease resistance (Zhou et al., 2020). As far as we know, the pearl gentian grouper is a non-model species that has not been reported in any published genomic library. Previously, our lab carried out grouper transcriptome sequencing using Illumina, such as the liver, blood, and intestine. However, the length of the sequencing reads was short (usually 100–250 bp) and the full-length transcripts obtained by splicing were not complete, which would hinder further study of the molecular mechanism.

The third-generation sequencing technology is also called *de novo* sequencing technology, namely, the single-molecule real-time (SMRT) DNA sequencing. The third-generation sequencing technology is the development trend in the future, which is mainly used in genome sequencing, methylation research, and mutation identification (SNP detection) (Jia et al., 2020). Compared with the next-generation sequencing technologies, the SMRT sequencing technology shows many superiorities, such as (1) obtaining full-length transcripts directly without transcript splicing; (2) a longer sequencing length and an ultrahigh sequencing flux; (3) discovering new functional genes and supplementing genome annotation; and (4) the analysis of alternative splicing (Roberts et al., 2013). Full-length sequencing is crucial for fully characterizing the transcriptomes of lesser studied and non-model organisms (Workman et al., 2018), but up to now, the application of which in aquaculture is scare.

Previously, our lab found that different levels of soybean diets, including 20% SBM protein as a substitute for FM protein (SBM20), SBM40, 20% soybean protein concentrate (SPC20), SPC40, 20% fermented soybean meal as substitute for FM (FSBM20), or FSBM40, can induce enteritis of the pearl gentian grouper. In this study, sequencing was carried out to generate the full-length transcriptome of the pearl gentian grouper intestine using the Pacific Biosciences (PacBio) SMRT sequencing technology (PacBio, Menlo Park, CA, United States) for the first time. Based on the obtained transcriptome data, the present study performed transcript functional annotation, coding sequence prediction, long non-coding RNA (LncRNA) prediction, and simple sequence repeat (SSR) analysis. In addition, the differential mechanisms of enteritis in the pearl gentian grouper induced by three soy proteins were preliminarily investigated. This study would be a valuable genome resource for further research of the pearl gentian grouper and also provides more reference results for the study of soy meal-induced enteritis in fish.

MATERIALS AND METHODS

Experimental Diets

The composition and chemical analysis of the experimental diets are presented in **Supplementary Table 1**. The red FM used in this study containing 72.53% crude protein and 8.82% total lipid was provided by Corporación Pesquera Inca S.A.C., Bayovar Plant,

Peru. The SBM and SPC used in this study contained 48.92 and 70.72% crude protein, respectively, which were provided by Zhanjiang Haibao Feed Co., Ltd. (Zhanjiang, China). The FSBM used in this study that contained 60.75% crude protein was provided by Xijie Foshan Co., Ltd. (Foshan, China). The fermentation strain is *Bacillus subtilis*. Seven isonitrogenous (approximately 50% crude protein) and isolipidic (10% total lipid) experimental diets were formulated to replace 0, 20, and 40% of FM protein with SBM, SPC, and FSBM protein, which were named FM (control), SBM20, SBM40, SPC20, SPC40, FSBM20, and FSBM40, respectively. Lysine and methionine were used to compensate for the amino acid imbalance of the diets (Miao et al., 2018). The detailed preparation process and the storage conditions of the experimental diets are described in our previously published literature (Zhang et al., 2019). Briefly, the raw materials were ground into a fine powder, crushed through a 60-mesh sieve, and weighed accurately according to the formula. The micro-constituents were mixed homogenously using the sequential expansion method. Then, deionized water and lipids were added and stirred evenly to obtain a homogenous mixture. After that, the mixture was passed through a pelletizer with 2.0 and 3.00 mm diameter. The pellets were air-dried to 10% moisture, sealed in plastic bags, and stored at −20°C until use. The essential amino acid contents of the diets are shown in **Supplementary Table 2**.

Feeding Trial and Experimental Condition

The detailed feeding trial and experimental conditions are described in our previously published literature (Zhang et al., 2019). Briefly, when juvenile groupers have adapted to the experimental environment, fish of similar size were randomly distributed into 1,000-L cylindrical fiberglass tanks. The fish had initial weight of about 12.55 g and length of about 7.66 cm. Each tank had 60 fish. Each diet group was fed to four replicates twice daily at 0800 and 1600 h, respectively, until apparent satiation for 10 weeks. The experiment was carried out at the indoor farming systems of the Marine Biological Research Base, Zhanjiang, China. All tanks were provided with continuous aeration by air stones. The light cycle used natural conditions, and the temperature was 29 ± 1°C. Ammonia and nitrate were no more than 0.03 mg L^{-1}, and dissolved oxygen was not less than 7 mg L^{-1}. In the first 2 weeks, 60% of the water in each tank was changed every day; all of the water was changed every day thereafter.

Sampling

Samples were collected at the end of the experiment. Before the experiment, the fish were starved for 24 h and then anesthetized with eugenol (1:10,000) for sampling. After cutting the abdomen along the midline, the intestine was gently pulled out and the mesenteric adipose tissue cleared up, and then the external residue was washed off with deionized water. Subsequently, some distal intestine (DI) and liver samples were quickly put into liquid nitrogen immediately after being placed in a cryopreservation tube. After sampling, the samples were stored at −80°C for transcriptome sequencing and enzyme activity analysis. Some DI samples were cut into pieces and placed into a tube containing RNAlater. After storing overnight at 4°C, the samples were then stored at −80°C for gene expression determination. Some DI tissues were stored in 4% paraformaldehyde general tissue fixative (Wuhan Servieobio Technology Co., Ltd., Wuhan, China) for 24 h for histology observation. In addition, the present study mainly analyzed the physiological changes in the 40% substitution groups. The weight gain rate (WGR), specific growth rate (SGR), feed conversion ratio (FCR), hepatosomatic index (HSI), and the survival rate (SR) were evaluated.

Histology

Intestinal histological observation (hematoxylin and eosin staining) was done according to Zhang et al. (2019). Briefly, the height-and-width ratio of the plica, the width of the lamina propria, and the length of the microvilli were determined. Each index was determined through 10 different scans. The stained sections were observed and photographed with an optical microscope (Olympus CKX41 microscope, Tokyo, Japan).

Analysis of Biochemical Indicators

The enzyme activities of glutathione reductase (GR), glutathione peroxidase (GPx), and total superoxide dismutase (T-SOD) in DI tissues and alanine aminotransferase (ALT) and aspartate transaminase (AST) in liver tissues were detected by fish enzyme-linked immunosorbent assay (ELISA) kits. The immunoglobulin M (IgM) and malondialdehyde (MDA) contents in DI tissues were determined by the fish ELISA kit. All the kits were purchased from Shanghai Jianglai Biotechnology Co., Ltd. (Shanghai, China). The detailed test steps were according to the instruction manual.

RNA Extraction

Total RNA was extracted separately from each group of distal intestinal tissues of the pearl gentian grouper using the RNeasy Plus Mini Kit (QIAGEN, Valencia, CA, United States). The quality of RNA is usually measured by 1% agarose gels and its purity and concentration usually measured by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, United States) with an OD_{260}/OD_{280} reading value. The integrity of RNA was assessed by the RNA Nano 6000 Assay Kit of Agilent Bioanalyzer 2100 system (Agilent, Santa Clara, CA, United States). For PacBio isoform sequencing (Iso-Seq), only the total RNA samples from seven groups with an RNA integrity number (RIN) >7 were mixed together for sequencing. For Illumina RNA sequencing (RNA-Seq), equal amounts of total RNA from three fish were pooled for each group. Indexed complementary DNA (cDNA) libraries were then prepared for each group.

SMRT Library Construction and Sequencing

The Iso-Seq library was prepared according to the isoform sequencing protocol using the (Clontech, Japan) SMARTer PCR cDNA Synthesis Kit and the BluePippin Size Selection System protocol as described by PacBio (PN 100-092-800-03). Briefly, after enrichment by Oligo(dT) magnetic beads, the messenger

RNA (mRNA) was reverse transcribed into cDNA using the SMARTer PCR cDNA Synthesis Kit. PCR was used to amplify and enrich the synthesized cDNA, and the optimal conditions for PCR were determined by cycle optimization. Part of the cDNA was screened by BluePippin, and the >4-kb fragments were enriched; then, the screened fragments were subjected to large-scale PCR to obtain enough cDNA quantity. The full-length cDNA was used for damage repair, end repair, and connection of the SMRT dumbbell-shaped connector. The equimolar library of the non-screened fragments and fragments larger than 4 kb was constructed. Exonuclease digestion was used to remove the sequence of the unconnected junctions at both ends of cDNA. Finally, a complete SMRT bell library was constructed by binding the primers and DNA polymerase. After passing library inspection, the library was sequenced by the PacBio Sequel platform according to the effective concentration of the library and data output requirements.

Illumina Library Construction and Sequencing

After the RNA samples of each individual were mixed equally, the cDNA library was constructed according to Li et al. (2013). Briefly, polyadenylated (polyA) mRNA was enriched using magnetic beads containing Oligo(dT), and the fragment buffer was added to make it into short fragments. The short mRNA was used as a template to synthesize cDNA. Terminal repair, polyA addition, and sequencing adaptor were performed. Then, the target fragments were recovered for PCR amplification to complete the preparation of the whole library. Finally, the libraries were sequenced using the Illumina HiSeqTM 4000 by Gene Denovo Co., Ltd. (Guangzhou, China).

PacBio SMRT Data Processing and Error Correction

After sequence completion, the off-line raw data are despliced and read with low quality. The output was filtered and processed by the software SMRTlink V5.1. The parameters are –minlength = 200 and –minreadscore = 0.65; then, the final data are the valid data. In order to obtain the full-length transcripts, first, the subread sequence was self-corrected to form circular consensus sequencing (CCS; parameters: –minpasses = 2, minpredicted accuracy = 0.8), and a high-quality consistent transcript sequence was obtained. A non-chimeric sequence with a 5′-end primer, a 3′-end primer, and a polyA tail is called a full-length non-chimeric (FLNC) sequence. An iterative isoform clustering (IEC) algorithm was used to cluster the FLNC sequences of the same transcript to obtain CCS, and then non-full-length sequences were used to correct the CCS. Then, the fused consensus sequences (CS) were obtained for subsequent analysis. After that, the Illumina data were used to correct the polished consensus sequence with the LoRDEC software (parameters: –k21 and –s3) to further improve the accuracy of sequencing. Finally, CD-HIT v4.6.7 (–c0.95 –T6 –G0 –aL0.00 –aS0.99) software was used to cluster and compare the protein or nucleic acid sequences by sequence alignment and to remove redundant and similar sequences.

Functional Annotation

Full-length (FL) transcripts were searched against the NCBI non-redundant protein sequences (Nr), NCBI non-redundant nucleotide sequences (Nt), Protein family (Pfam), Clusters of Orthologous Groups of proteins (KOG/COG), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes Orthology database (KEGG Orthology), and Gene Ontology (GO). Diamond BLASTX software was used for functional annotation with an *e*-value of 1e–10 in the Nr, KOG, Swiss-Prot, and KEGG database analysis. BLAST software with the *e*-value of 1e–10 was used in the Nt database analysis. The Hmmscan software was used in the Pfam database analysis. GO annotation was analyzed using the Blast2GO software (Conesa et al., 2005) with the Nr annotation results of transcripts.

Coding Sequencing and LncRNA Prediction

The ANGEL pipeline, a long-read implementation of ANGLE, was used in order to determine the protein coding sequences (CDS) from the cDNAs. We used this species' or the confident protein sequences of closely related species for ANGEL training and then ran the ANGEL prediction for the given sequences (Shimizu et al., 2006).

Long non-coding RNAs (lncRNAs) are RNA molecules with transcripts longer than 200 nt and do not encode proteins. Due to the limitation of the principle of library construction, we can only obtain lncRNAs with a polyA tail. Usually, four software—CNCI (Sun et al., 2013), CPC (Kong et al., 2007), Pfam (Finn et al., 2016), and PLEK (Aimin et al., 2014)—are used to predict the coding potential of genes obtained from CD-HIT de-redundancy.

Simple Sequence Repeats Analysis

Simple sequence repeats (SSRs) are also known as short tandem repeats or microsatellites. They are a class of repeats consisting of several nucleotides (one to six) as repeat units, which are short in length and widely distributed in eukaryotic genomes. In our analysis, MISA software (version 1.0, default parameters) was used. The minimum repetition times of each unit size were 1–10, 2–6, 3–5, 4–5, 5–5, and 6–5 to detect genes by (Simple Sequence Repeats Analysis) SSRs (Thiel, 2003; Gulcher, 2012).

Analysis of the Differentially Expressed Genes

The present study compared the effects of SBM40, SPC40, and FSBM40 on the transcriptome level of the distal intestine in the pearl gentian grouper. Firstly, the differentially expressed genes (DEGs) in the SBM40, SPC40, and FSBM40 groups were screened. The screened thresholds were $|\text{Log2FC}| > 1$ and $P < 0.05$. The genes meeting the above conditions were identified as DEGs. Then, the DEGs in the SBM40, SPC40, and FSBM40 groups were analyzed by a Venn diagram for common and unique DEGs. Finally, GO annotation and KEGG enrichment analyses were conducted for the common and unique DEGs, respectively, and the signaling pathways related to immune diseases/system, infectious diseases, and signal transduction that

were significantly affected in the KEGG enrichment results were analyzed ($P < 0.05$).

Validation of Real-Time Quantitative PCR

In order to test the accuracy of the full-length PacBio SMRT sequencing results, samples stored at −80°C were selected for quantitative reverse transcription PCR (RT-qPCR). In this study, 18 genes related to immune and inflammatory development were selected, which included *TLR1*, *TLR2*, *TLR3*, *TLR5*, *TLR8*, *TLR9*, *TLR13*, *TLR21*, *TLR22*, *IgA*, *pIgR*, *IL4*, *IL5*, *IL10*, *MyD88*, *IκBα*, and *p65*. The primers were designed by the Primer Premier 5.0 software and the primer sequence templates were from the full-length PacBio SMRT transcriptome sequencing database. The primers were synthesized by Shenggong Bioengineering Co., Ltd. (Shanghai, China). The internal reference gene is β-*actin*. The primers are displayed in **Supplementary Table 3**. The PCR reaction conditions were: 95°C for 2 min, 1 cycle; 95°C for 15 s, 60°C for 10 s, 72°C for 20 s, 40 cycles. The expressions of the target genes were determined by $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

Statistics

Analysis of the omics data refers to the above mentioned. The rest of the data were analyzed using SPSS 22.0 software (SPSS Inc., Chicago, IL, United States). The results were presented as the mean ± standard deviation ($x \pm SD$). In order to test differences among groups, one-way ANOVA was used after the homogeneity variance test. The significance threshold was $P < 0.05$. Growth performance was calculated using the following formulas:

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

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

$$\text{Feed conversion ratio (FCR)} = \frac{\text{feed intake}}{(\text{final body weight} - \text{initial weight})}$$

$$\text{Hepatosomatic index (HSI, \%)} = 100 \times \left(\frac{\text{hepatic weight}}{\text{body weight}}\right)$$

$$\text{Survival rate (SR, \%)} = 100 \times \left(\frac{\text{final fish number}}{\text{initial fish number}}\right)$$

RESULTS

Growth Performance

Figure 1 shows that, compared to the FM control group, there was a significant decrease in the WGR and SGR in the experimental groups ($P < 0.05$), and there was no significant difference among the experimental groups ($P > 0.05$). There was a significant increase in the FCR in the experimental groups ($P < 0.05$), which indicated that fish fed diets containing different soy proteins had worse FCR values; there was no significant difference among the experimental groups ($P > 0.05$). The HSI and SR had no significant differences among the groups ($P > 0.05$) (**Supplementary Figure 1**).

Histological Observation of Enteritis

The results illustrated that the plica height/width and the microvilli length significantly decreased in each experimental group compared to the FM control group ($P < 0.05$). On the contrary, the lamina propria width significantly increased in each experimental group ($P < 0.05$) (**Supplementary Figure 2** and **Table 1**).

Biochemical Indicators

Table 2 exhibits that, compared to the FM control group, the enzyme activities of T-SOD, GR, and GPX significantly increased in the experimental groups ($P < 0.05$), and the highest value appeared in SPC40, followed by the SBM40 and FSBM40 groups. The MDA content also significantly increased in the experimental groups ($P < 0.05$), and the highest value appeared in SBM40, followed by the FSBM40 and SPC40 groups. The content of IgM significantly decreased in the experimental groups ($P < 0.05$), and the highest value appeared in SBM40, followed by the FSBM40 and SPC40 groups. The enzyme activities of ALT and AST in the liver were significantly increased in the experiment groups ($P < 0.05$), and the highest value appeared in SPC40, followed by the FSBM40 and SPC40 groups.

SMRT Sequencing of the Intestine

The flow process diagram of the transcriptome of the pearl gentian grouper by SMRT and Illumina sequencing is shown in **Supplementary Figure 3A**. In total, there were 487,152 CCS reads with an average length of 3,013 bp isolated from the PacBio SMRT raw data (30.31 G of subreads) in the mixed library (**Supplementary Table 4**), among which FLNC made up 86.22%, while the ratios of the non-full-length (NFL), full-length chimeric (FLC), and short reads were 11.39, 0.89, and 1.50%, respectively (**Supplementary Figure 3B**).

After ICE correction (iterative correction and eigenvector decomposition), 225,854 CS were obtained. Then, the Illumina sequencing data were used to further correct the CS using LoRDCE software. Thereafter, the sequences were removed by CD-HIT software; 225,854 non-redundant transcripts (2,998 bp on average) and 82,351 unigenes (3,486 bp on average, N50 = 4,131 and N90 = 2,173) in all were obtained. The length distribution of the unigenes and the number of transcripts corresponding to genes are shown in **Supplementary Figure 3C** and **Figure 2D**. Transcripts with lengths ranging from 1,700 to 5,100 bp make up 73.29% of the unigenes.

The unigene length sequenced by third-generation technology in this study is much longer than that sequenced using Illumina, in which the N50 of the non-assembled unigenes by PacBio sequencing is 4,131 bp; however, the N50 values of the unigenes sequenced in our previous unpublished transcriptomes using Illumina for the pearl gentian grouper intestine and liver were 1,886 and 1,921 bp, respectively (**Table 3**).

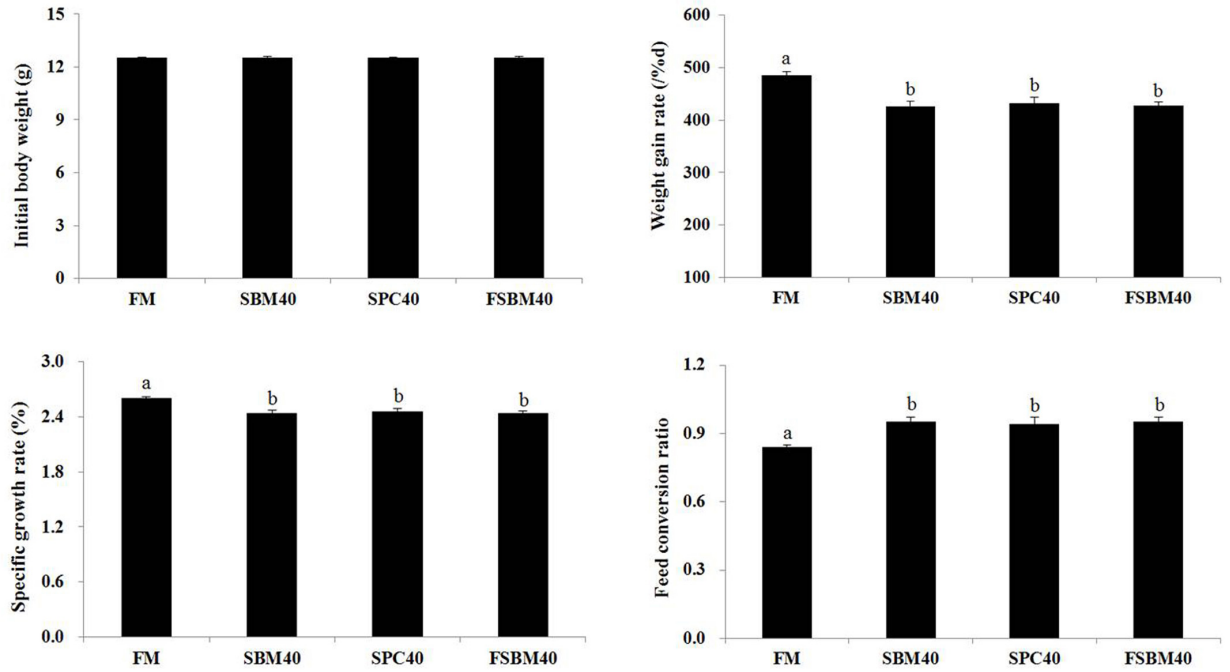


FIGURE 1 | Effect of the different soy proteins at 40% substitution levels for fish meal on the growth of the pearl gentian grouper (n = 3).

TABLE 1 | Effect of different soy protein substitutions for fish meal protein on the distal intestine morphology of the pearl gentian grouper (n = 10).

Parameters	FM	SBM40	SPC40	FSBM40
Plica height/width	7.80 ± 0.63a	3.31 ± 0.60b	4.82 ± 0.53c	4.98 ± 0.80c
Lamina propria width (μm)	16.65 ± 5.78a	63.53 ± 6.76b	35.09 ± 5.49c	29.87 ± 8.08c
Microvilli length (μm)	24.10 ± 2.06a	13.00 ± 1.50b	14.98 ± 3.42b	15.93 ± 1.83b

FM, fish meal control group; SBM40, 40% soy bean meal (SBM) protein substitute for FM protein; SPC40, 40% soybean protein concentrate (SPC) protein substitute for FM protein; FSBM40, 40% fermented soybean meal (FSBM) protein substitute for FM protein. Different letters assigned to the lines represented significantly differences between the groups at $P < 0.05$.

TABLE 2 | Effect of the different soy protein substitutions for fish meal protein on the enzyme activities of the pearl gentian grouper (n = 3).

Parameters	FM	SBM40	SPC40	FSBM40
IgM (μg/mg tissue)	94.33 ± 4.22a	64.70 ± 3.63b	37.77 ± 3.15c	50.84 ± 5.60c
GR (U/g tissue)	32.49 ± 7.07a	52.66 ± 6.81b	71.65 ± 6.31c	62.34 ± 5.67b
GPx (U/mg tissue)	167.20 ± 21.34a	246.58 ± 20.64b	322.51 ± 22.74c	286.12 ± 24.83b
T-SOD (U/mg tissue)	78.23 ± 9.95a	115.50 ± 12.07b	154.04 ± 13.35c	135.55 ± 14.25c
MDA (nmol/mg tissue)	2.49 ± 0.41a	4.25 ± 0.33b	4.01 ± 0.14b	4.15 ± 0.46b
ALT(U/g tissue)	25.53 ± 3.34a	36.64 ± 4.13b	48.55 ± 4.23c	43.03 ± 4.30b
AST (U/g tissue)	26.88 ± 4.02a	38.94 ± 4.34b	50.36 ± 3.72c	44.32 ± 4.04c

GR, glutathione reductase; GPx, glutathione peroxidase; T-SOD, total superoxide dismutase; ALT, alanine aminotransferase; AST, aspartate transaminase; IgM, immunoglobulin M; MDA, malondialdehyde; FM, fish meal control group; SBM40, 40% soy bean meal (SBM) protein substitute for FM protein; SPC40, 40% soybean protein concentrate (SPC) protein substitute for FM protein; FSBM40, 40% fermented soybean meal (FSBM) protein substitute for FM protein. Different letters assigned to the lines represented significantly differences between the groups at $P < 0.05$.

Functional Annotation of the Full-Length Transcripts

All of the full-length transcripts were blasted against seven databases, including the Nr, Swiss-Prot, KEGG, KOG, GO, Nt, and Pfam databases (**Table 4**). Based on the annotation results of the seven databases, five databases were selected to draw the Venn diagram (**Figure 2**). In total, 77,815 (94.5%) FL transcripts were annotated in at least one of the databases. The Nt database annotated the highest gene number (75,956), followed by Nr (61,243), KEGG (59,092), Swiss-Prot (52,750), KOG

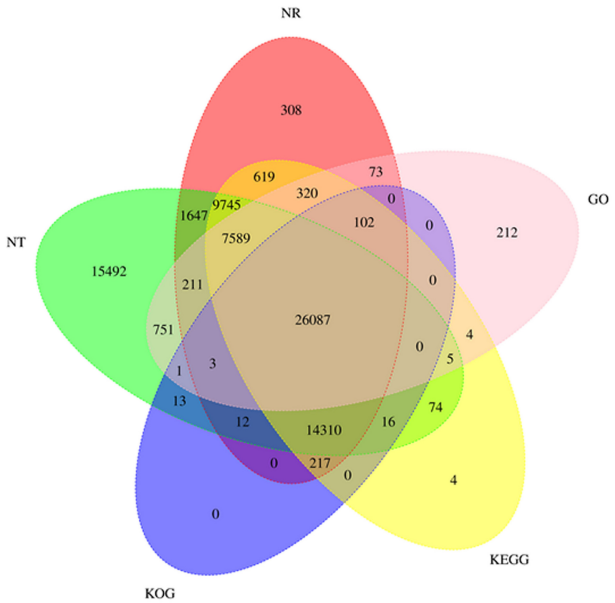


FIGURE 2 | Venn diagram of the functional annotation of the long-read transcriptomes in Nr, Nt, GO, KOG, and KEGG databases. *Nr*, non-redundant protein database; *Nt*, non-redundant nucleotide sequences; *GO*, Gene Ontology; *KOG*, Clusters of Orthologous Eukaryotic Genes; *KEGG*, Kyoto Encyclopedia of Genes and Genomes.

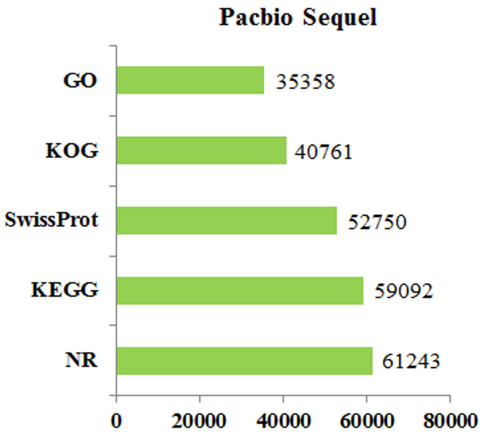


FIGURE 3 | Annotated gene number of single-molecule real-time (SMRT) sequencing.

(40,761), and GO (35,358) (**Figure 3**). The species distribution annotation in the Nr database showed that the top 10 species were *Lates calcarifer* (27.03%), *Larimichthys crocea* (17.12%), *Stegastes partitus* (6.93%), *Notothenia coriiceps* (3.43%), *Paralichthys olivaceus* (2.44%), *O. niloticus* (2.15%), *Epinephelus coioides* (1.77%), *Nothobranchius furzeri* (0.93%), *D. labrax* (0.92%), and *Epinephelus lanceolatus* (0.76%) (**Figure 4**). However, the remaining 36.52% of the matched FL transcripts showed similarities to other species due to limited genome information. This suggested that the FL transcripts of the pearl gentian grouper should be further annotated with updated published fish genes and related gene background information.

In the KOG database, the 77,815 annotated FL transcripts were categorized into 26 KOG classifications (**Figure 5A**). The largest cluster was "general function prediction only (R)" (7,805 isoforms), which indicates that the functions of most genes still need experimental confirmation. This was followed by "signal transduction mechanisms (T)" (7,797 isoforms), "posttranslational modification, protein turnover, chaperones (O)" (4,178 isoforms), and "function unknown (S)" (2,740 isoforms).

Functional annotation using GO exhibits the properties of the gene products by classifying them into the "biological process," "cellular component," and "molecular function" categories. **Figure 5B** showed that there were 77,139, 44,427, and 42,465 transcripts assigned to the "molecular function," "cellular component," and "biological process" categories, respectively. In detail, in the "molecular function" annotation, "binding" (21,517 isoforms) and "catalytic activity" (13,382 isoforms)

TABLE 3 | Comparison of unigenes from the PacBio and Illumina sequencing platforms of the pearl gentian grouper.

Sequencing platforms	Tissues	Transcript number	Mean length (bp)	N50 (bp)	References
PacBio Sequel	Distal intestine	82,351	3,486	4,131	This study
Illumina Hiseq2000	Distal intestine	111,196	874.67	1,886	Unpublished
Illumina Hiseq2000	Liver	120,896	912.36	1,921	Unpublished

TABLE 4 | Annotation results of seven databases.

	Databases							At least one database	All databases
	Nr	Swiss-Prot	KEGG	KOG	GO	Nt	Pfam		
Annotation_num	61,243	52,750	59,092	40,761	35,358	75,956	35,358	77,815	26,087

Nr, non-redundant protein database; KEGG, Kyoto Encyclopedia of Genes and Genomes; KOG, Clusters of Orthologous Eukaryotic Genes; GO, Gene Ontology; Nt, non-redundant nucleotide sequences; Pfam, protein family; FM, fish meal control group; SBM40, 40% soybean meal (SBM) protein substitute for FM protein; SPC40, 40% soybean protein concentrate (SPC) protein substitute for FM protein; FSBM40, 40% fermented soybean meal (FSBM) protein substitute for FM protein.

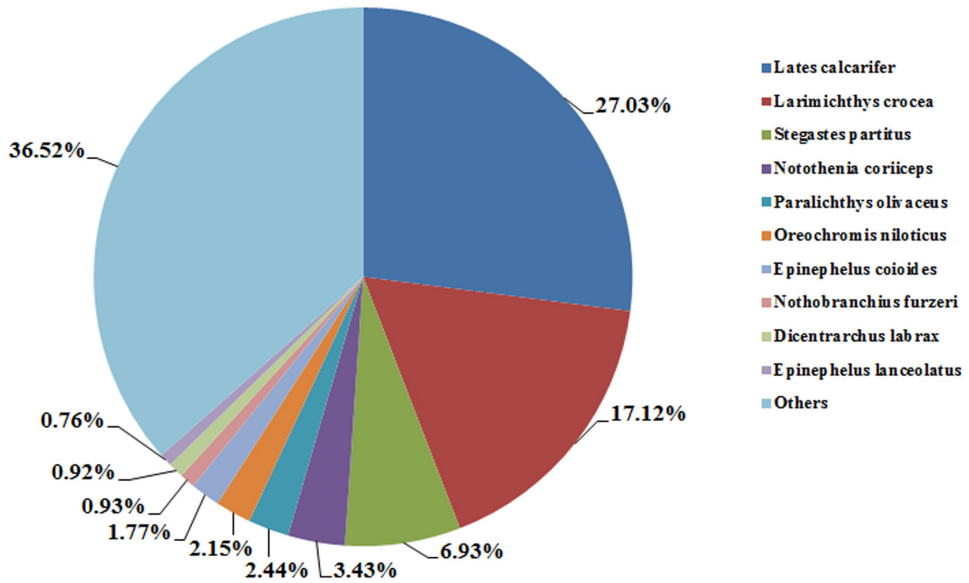


FIGURE 4 | Species distribution of the Nr annotation of the long-read transcripts.

were the top two abundant terms; in the "cellular component" annotation, "cell" (7,847 isoforms), "cell part" (7,847 unigenes), and "membrane" (6,164 isoforms) were the majority of level 2 terms. In the "biological process" annotation, "cellular process" (14,884 isoforms), "metabolic process" (13,824 isoforms), and "single-organism process" (10,951 isoforms) comprised the largest proportions.

KEGG annotation found that 23,082 genes were assigned to five pathways (level 1), including "cellular processes," "environmental information processing," "genetic information processing," "metabolism," and "organismal systems." At level 2 of the KEGG pathways, "signal transduction," (6,250 isoforms), "cancers: overview" (3,975 isoforms), "immune system" (3,851 isoforms), "transport and catabolism" (3,808 isoforms), and "endocrine system" (3,103 isoforms) were the most abundant terms (**Figure 5C**).

CDS and LncRNA Prediction

Coding sequences (CDS) is the sequence that encodes a protein product, which completely corresponds to the codon of the protein. After BLAST comparison of the obtained polished consensus in the protein database, 8,243 CDS were found. The lengths of the sequences ranged from 0 to 5,000 bp, mainly concentrated in 0–2,500 bp (**Figure 6**), indicating that the unigenes had good sequence quality.

The results showed that 38,219 lncRNAs were identified using the CNCI software, followed by 24,640 lncRNAs identified using the CPC software, 16,655 lncRNAs using the PLEK software, and 44,249 lncRNAs using the Pfam software, among which 8,874 common lncRNAs were identified by four different bioinformatics software (**Figure 7A**). From the length distribution of the lncRNAs and mRNAs, it can be seen that the peak value of lncRNA length is about 2,000 bp and that of mRNA length is about 2,500 bp (**Figure 7B**).

Analysis of SSRs

In aggregate, 63,118 SSRs were obtained from 53,759 unigenes, among which had at least one SSR. Most of the SSRs were mononucleotide repeats, accounting for 59.86%, followed by the dinucleotide repeats accounting for 25.97%, trinucleotide repeats accounting for 11.01%, tetranucleotide repeats accounting for 2.53%, pentanucleotide repeats accounting for 0.56%, and the hexanucleotide repeats accounting for 0.08% (**Supplementary Figure 4** and **Supplementary Table 5**).

Statistics of the DEGs

Table 5 shows that the SBM40 group had 2,305 significant DEGs ($P < 0.05$), of which 1,256 were significantly upregulated and 1,049 were significantly downregulated. The SPC40 group had 4,076 significant DEGs ($P < 0.05$), of which 2,328 were significantly upregulated and 1,748 were significantly downregulated. The FSBM40 group had 3,462 significant DEGs ($P < 0.05$), of which 2,005 were significantly upregulated and 1,457 were significantly downregulated.

The Venn diagram of the DEGs displayed that, compared to the FM control group, the common DEGs in SBM40, SPC40, and FSBM40 were 554, named Profile G; 1,003 unique DEGs in the SBM40 group, named profile H; 2,254 unique DEGs in the SPC40 group, name Profile I; and 1,656 unique DEGs in the FSBM40 group, name Profile J (**Figure 8**). Only 7.80% (554/7,101) of the DEGs in the three groups have similar expression patterns, indicating that the three soy proteins have different metabolic strategies.

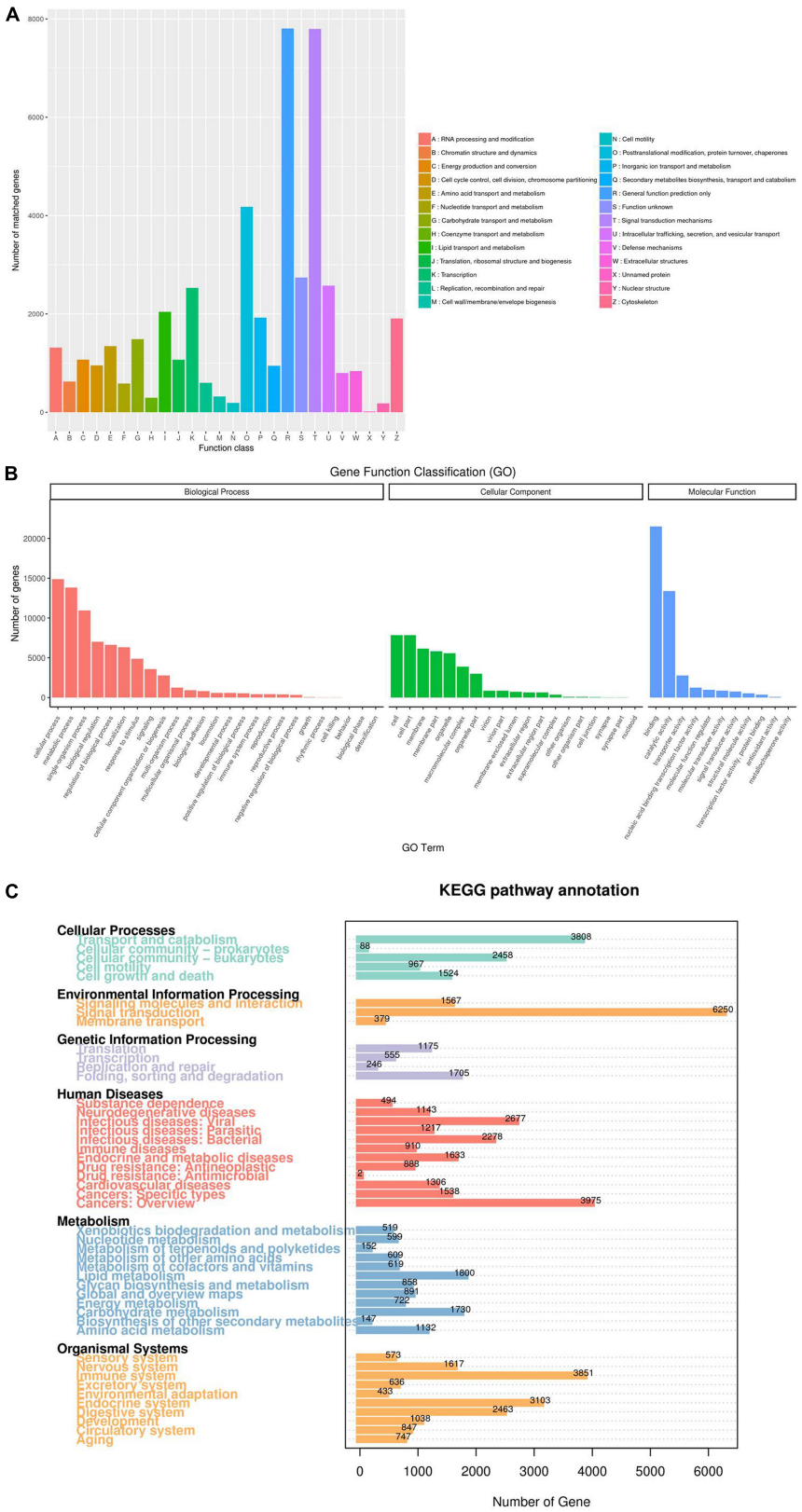


FIGURE 5 | Functional classification of the long-read transcripts. **(A)** Clusters of Orthologous Eukaryotic Genes (KOG) classification. **(B)** Gene Ontology (GO) classification. **(C)** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway classification.

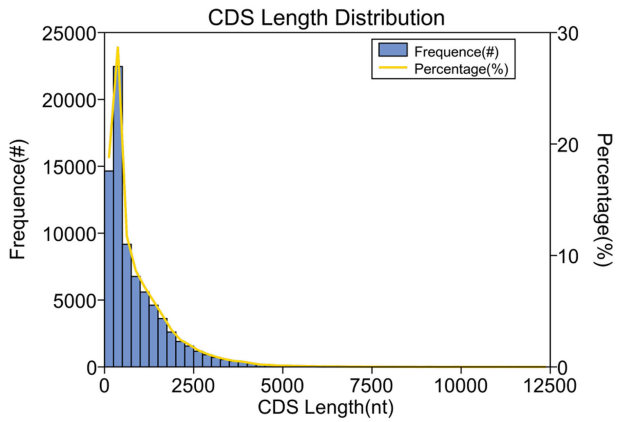


FIGURE 6 | Length distribution of the coding sequences (n = 4). The x-axis represents the coding sequence length and the y-axis represents the number of predicted coding sequences.

TABLE 5 | Comparison of the significantly differential expressed genes of the three soy protein substitutions for fish meal in the distal intestine of the pearl gentian grouper (n = 4).

	Number		
	SBM40 vs. FM	**SPC40 vs. FM**	**FSBM40 vs. FM**
Up	1,256	2,328	2,005
Down	1,049	1,748	1,457
Total	2,305	4,076	3,462

FM, fish meal control group; SBM40, 40% SBM protein substitution for FM protein; SPC40, 40% SPC protein substitution for FM protein; FSBM40, 40% FSBM protein substitution for FM protein.

KEGG Enrichment Analysis of the DEGs

KEGG enrichment analysis was performed on profiles G, H, I, and J. The enrichment of Profile G showed that 238 pathways were enriched and 30 pathways were significant ($P < 0.05$). Among all the pathways, 73 were related to immune disease/system, infectious diseases, and signal transduction, nine of which were significantly enriched ($P < 0.05$). That is to say, 30% (9/30) of all the pathways were related to immune diseases/system, infectious diseases, and signal transduction (**Figure 9A**). Profile H enrichment results found that 297 pathways were enriched and 51 pathways were significant ($P < 0.05$). Among all the pathways, 79 were related to immune disease/system, infectious diseases, and signal transduction, 23 of which were significantly enriched ($P < 0.05$). That is to say, 45.10% (23/51) of all the pathways were related to immune diseases/system, infectious diseases, and signal transduction (**Figure 9B**). Profile I enrichment results found that 320 pathways were enriched and 35 pathways were significant ($P < 0.05$). Among all the pathways, 80 were related to immune disease/system, infectious diseases, and signal transduction, one of which was significantly enriched ($P < 0.05$). That is to say, 2.86% (1/35) of all the pathways were related to immune diseases/system, infectious diseases, and signal transduction. Most of the significant pathways were related to fat digestion and absorption, alpha-linolenic acid metabolism, glycerophospholipid metabolism, fatty acid metabolism, linoleic acid metabolism, biosynthesis of unsaturated fatty acid and protein digestion and absorption, etc., accounting for 85.71% (30/35) (**Figure 9C**). Profile J enrichment results found that 305 pathways were enriched and 38 pathways were significant ($P < 0.05$). Among all the pathways, 81 were related to immune disease/system, infectious diseases, and signal transduction, 23 of which were significantly enriched ($P < 0.05$). That is to say, 60.53% (23/38) of all the pathways were related to immune diseases/system, infectious diseases, and signal transduction (**Figure 9D**).

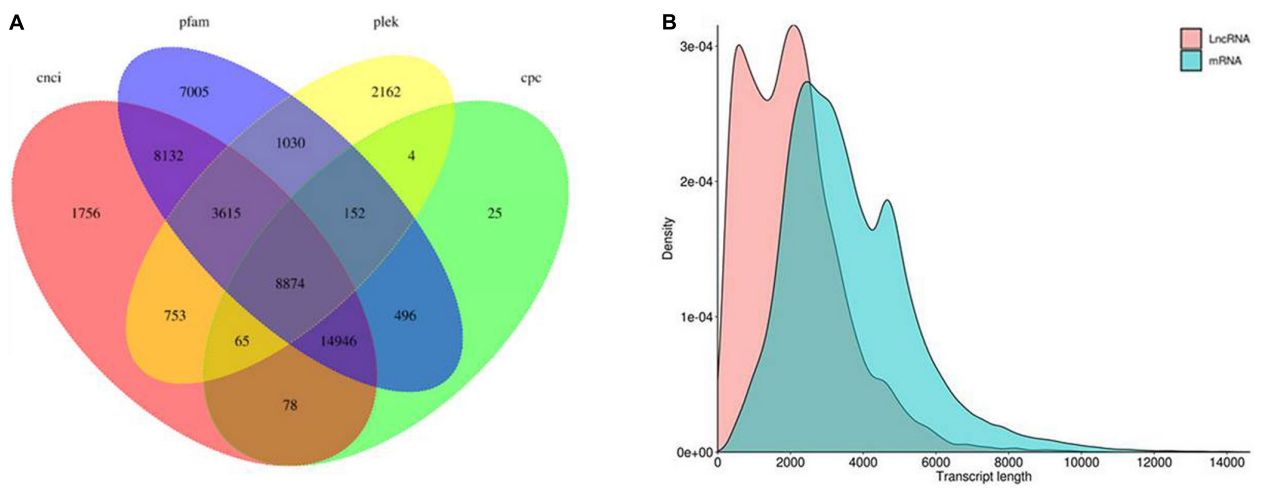


FIGURE 7 | Long non-coding RNA (lncRNA) prediction. **(A)** Venn diagram of the lncRNA prediction results by four software. **(B)** Length distribution of lncRNAs and mRNAs.

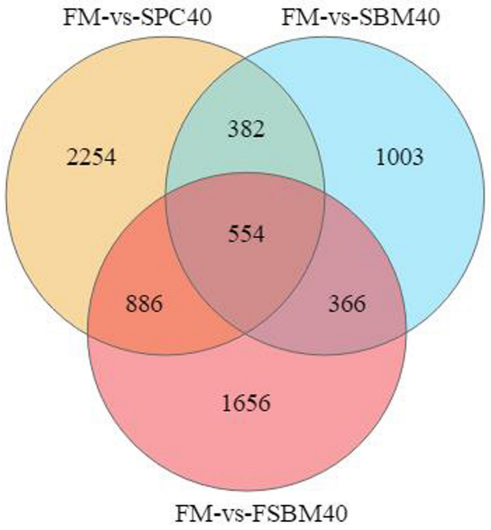


FIGURE 8 | Venn diagram analysis of the significantly differentially expressed genes (DEGs) of the soy protein substitutes for fish meal in the distal intestine of the pearl gentian grouper (n = 4).

Validation of the RNA-Seq Data by RT-qPCR

Generally speaking, the trend of the RT-qPCR results was consistent with that of the transcriptome sequencing data, indicating that the RNA-Seq results were relatively accurate (**Figure 10**). These results further confirmed the reliability of the "3 + 2" transcriptome sequencing strategy.

DISCUSSION

The present study showed that experimental levels of soy proteins from SBM, SPC, and FSBM as substitutes for FM presented significantly negative effects on the growth performance and intestinal health of the pearl gentian grouper. A related study on *E. coioides* (initial weight = 84 ± 2.5 g) found that fish had the best growth performance at the level of 20% SBM substitution for FM (basal *FM* = 60%) (An et al., 2018). The unpublished research in our lab also found that the optimal substitution level of SBM for the pearl gentian grouper (initial weight = 17.01 ± 0.01 g) was 12.05% (basal *FM* = 50%). Research has found that 20–50% SPC substitution for FM (50% basal FM) had no significant effects on growth performance in the brown-marbled grouper (initial weight = 6.1 ± 0.7 g), while the growth performance significantly decreased at 60% SPC substitution level (Faudzi et al., 2018). However, it was found that the replacement of FM with SPC less than 40% had significantly negative effects on the WGR, SGR, and feed efficiency of *Scophthalmus maximus* and *P. olivaceus* (Deng et al., 2006; Liu et al., 2014). There were no significant effects on the final body weight, SGR, and FCR by replacing 30% of FM with FSBM (50% basal FM) in the diet of *Acanthopagrus latus*, while in the diet of *Micropterus salmoides*, fish had better growth, physiology, and apparent digestibility when the FSBM substitution for FM was no more than 10% (Wang, 2009; Ehsani et al., 2014). A previous study on *E. coioides* showed that a 14% dietary FSBM did not significantly affect the WGR and SGR values; however, at higher levels, the WGR and SGR values decreased significantly. The optimal level of FSBM substitution was 10% (52% basal FM) (Luo et al., 2004). In general, the present study obtained consistent results. The difference may be caused by the different varieties of breeding animals.

Previous studies have shown that the characteristics of soy meal-induced enteritis include a reduced mucosal fold height, swelling of the lamina propria and subepithelial mucosa, loss of normal enterocyte supranuclear absorptive vacuolization, and profound infiltration of various inflammatory cells, which decreased the capacity of the DI to digest and absorb nutrients (Gu et al., 2018). The present study found a similar phenomenon. The intestinal structure of fish is very sensitive to oxidative damage. Fish can resist oxidative damage through antioxidant enzymes, such as total GR, GPx, and T-SOD (Zhao et al., 2014). The significant increases of the GR, GPx, and T-SOD enzyme activities in this study indicated that soy proteins induced intestinal stress. IgM is an important component of specific immunity. The present study found that the content of IgM significantly decreased, indicating an impairment of the intestinal immune function of the pearl gentian grouper. MDA is one of the final products of oxidative stress, the concentrations of which indicate the rate or intensity of lipid peroxidation in tissues and cells (Wen et al., 2015). In the present study, the concentration of MDA in the DI tissues significantly increased in the experimental groups, indicating that soy proteins caused intestinal injury in the pearl gentian grouper. ALT and AST are two important and sensitive indicators of hepatocyte injury when liver lesions occur (Kalhoro et al., 2018; Zhou et al., 2018). Previous research also revealed that excessive dietary SBM for the Atlantic salmon and grass carp induced liver lesions (De Santis et al., 2015; Wu et al., 2018). The present study found similar results. Based on the above analysis, the pearl gentian grouper showed the typical characteristics of fish intestinal health issues caused by soy proteins. In order to conduct a systematic and in-depth study, the present study constructed and analyzed the characteristics of the full-length transcriptome database of the pearl gentian grouper by using omics technology.

Genome and transcriptome sequencing is a fundamental work in the field of life science. Due to the lack the genomic data for most non-model organisms, full-length transcriptome sequencing becomes particularly important. Full-length transcripts can greatly improve the basic and applied research on gene function, gene expression regulation, and the evolutionary relationships of these species (Ren et al., 2006). Previously, obtainment of a full-length gene on a large scale is almost impossible, which is also time-consuming and expensive through RACE and Illumina technology in general (Wan et al., 2019). Currently, most of the transcriptome data are obtained based on next-generation sequencing technologies, such as Illumine, Heliscope, Roche 454, Solexa, and SOLID (Lobato et al., 2017). The length of the sequences obtained by second-generation sequencing technologies is short, and the splicing

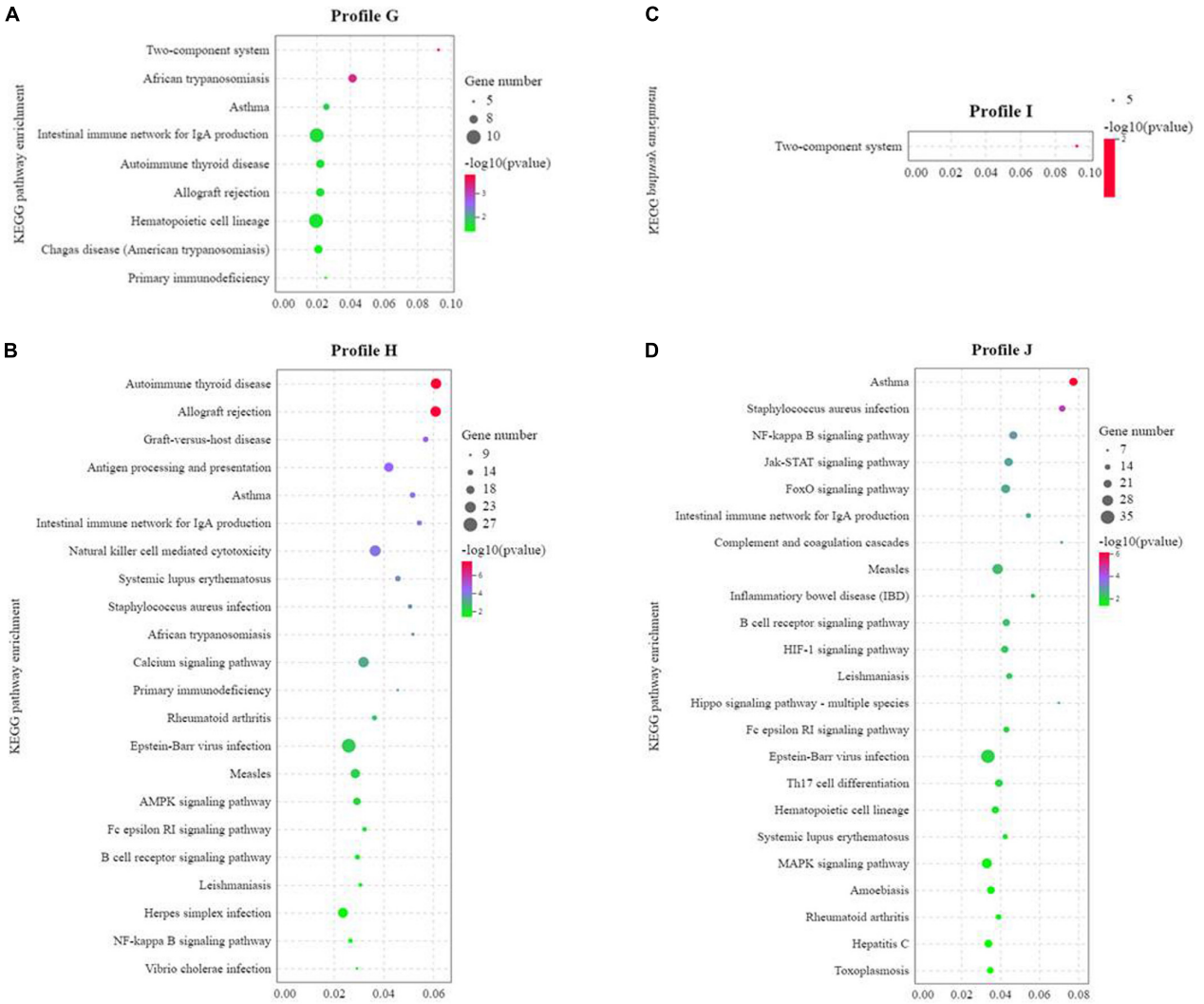


FIGURE 9 | Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the significantly differentially expressed genes (DEGs) of the three soy protein substitutes for fish meal in the distal intestine of the pearl gentian grouper (n = 4). **(A)** The KEGG enrichment analysis of the common differential genes in SBM40, SPC40, and FSBM40 groups. **(B)** The KEGG enrichment analysis of the unique differential genes in SBM40 group. **(C)** The KEGG enrichment analysis of the unique differential genes in SPC40 group. **(D)** The KEGG enrichment analysis of the unique differential genes in FSBM40 group.

of short sequences cannot provide a large number of long transcripts and lose important information, such as alternative splicing (Sun, 2016). Therefore, the third-generation sequencing technology of PacBio SMRT is usually used for transcriptome *de novo* sequencing.

Full-length transcripts are very important for the research of genome assembly and gene function, and the PacBio SMRT sequencing technology can obtain full-length transcripts on a large scale (Wong et al., 2019). This study obtained 82,351 high-quality unique transcripts, and 86.22% were full-length transcripts. This result showed that the third-generation sequencing technology is more efficient than the next-generation sequencing technology (Yang et al., 2017). According to the published literature, only a small number of species had their transcriptomes obtained based on PacBio platform, including the transcriptome data of the hybrid splicing of second- and third-generation sequencing or the corrected third generation through second-generation sequencing technology. Most of the transcriptome data obtained completely based on the PacBio platform are from human beings (Au et al., 2013), and there are also data on HIV virus (Ocwieja et al., 2012), bovine (Larsen and Smith, 2012), *Mus musculus* (Treutlein et al., 2014), *Propithecus coquereli* (Larsen et al., 2014), etc. However, research on full-length transcriptome sequencing based on the PacBio platform was just carried out in recent years, and it was not until 2015 that the sequencing of fungi (Gordon et al., 2015), *Gossypium hirsutum* (van Eijk, 2015), and *Sepia officinalis* (Worley, 2015) was carried out. The full-length transcripts obtained in this research would improve further investigation of the pearl gentian grouper.

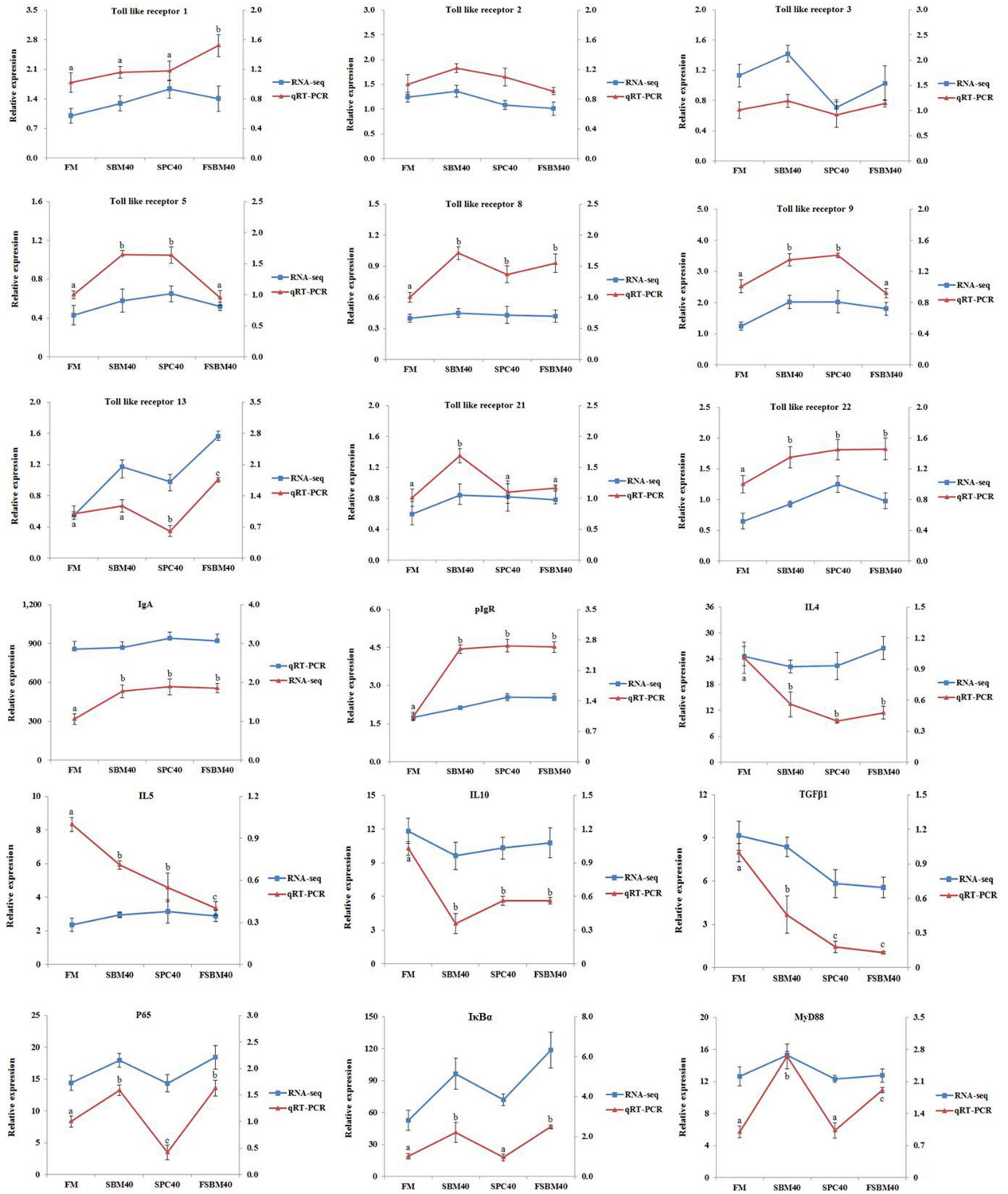


FIGURE 10 | Comparison of the RNA sequencing (RNA-Seq) and the quantitative reverse transcription PCR (RT-qPCR) results. In order to validate the RNA-Seq results, RT-qPCR was used to detect the gene expression levels of the TLR/myD88/NF-κB pathway and the intestinal immune network for IgA production pathway in the distal intestine (DI) tissues of the pearl gentian grouper. The mRNA expression level in RT-qPCR was normalized by β-*actin*. The relative expression level in the RNA-Seq analysis was calculated by the FPKM (fragments per kilobase of transcript per million mapped reads) value. The statistical results were expressed as the mean ± SD. *Different letters assigned to the lines* represent significant differences among the groups at $P < 0.05$. *FM*, fish meal control group; *SBM40*, 40% soybean meal (SBM) protein replacement level for FM protein; *SPC40*, 40% soybean protein concentrate (SPC) protein replacement level for FM protein; *FSBM40*, 40% fermented soybean meal (FSBM) protein replacement level for FM protein.

The longest transcript obtained in this study is 14,637 bp and the N50 is 4,131 bp, which is much longer than that in the pearl gentian grouper used in the Illumina sequencing. For example, our unpublished research found that the N50 values of the assembled unigenes were only 1,886 and 1,921 bp in the intestine and liver transcriptomes of the pearl gentian grouper, respectively. The results indicate that the PacBio SMRT sequencing technology has more advantage in terms of reading the sequence length.

Previous studies have pointed out that the annotation rate of the third-generation sequencing data is higher than that of the second-generation sequencing data (Zeng et al., 2018). In our unpublished research on the pearl gentian grouper transcriptomes of the intestine and liver tissues, the transcript annotation rates were 32.64 and 36.58%, respectively. Also, in our published articles on *M. salmoides* and *Trachinotus ovatus*, in which the transcriptomes were sequenced by the Illumina 2000 platform, the annotation rates of the transcripts were 52.98% (26,886/50,743) (Zhang et al., 2019) and 43.30% (27,366/62,377) (Liu et al., 2019), respectively. Although the raw data obtained from third-generation sequencing had relatively more error, it can be corrected through the data obtained from next-generation sequencing (Hackl et al., 2014). The raw data in this study had been corrected using the transcriptome data sequenced by the Illumina 4000 platform, which would ensure the accuracy of the PacBio SMRT results. Finally, the annotation rate of the transcripts in this study is 94.5%, which is much higher than that previously obtained using the Illumina sequencing technology.

Public databases such as the Nr, Nt, Pfam, KOG, Swiss-Prot, KEEG, and GO have been widely applied for functional annotation of transcriptome sequences. Nr and Nt are the official protein and nucleic acid sequence databases in NCBI (Feng et al., 2019). In this study, 78.70 and 97.61% of the FL transcripts were annotated in Nr and Nt, respectively, which indicated that most of the transcripts were annotated and only contained few non-coding sequences, such as lncRNAs. For the rest of the databases, the highest ratio of the transcripts was in KEGG (75.94%), followed by Swiss-Prot (67.79%), KOG (52.38%), GO (45.44%), and Pfam (45.44%). In our previous second-generation transcriptome sequencing data of pearl gentian grouper intestinal tissues, the annotation rate in Nr was 30.78%, followed by KOG (18.11%), KEGG (17.18%), and Swiss-Prot (15.35%, unpublished). The percentage of the annotated transcripts in this study was higher than those reported by RNA-Seq, which also showed advantages of the third-generation sequencing technology.

lncRNAs are rapidly evolving and are often species-specific, which play vital roles in many physiological processes such as translation, transcription, differentiation, splicing, immune responses, epigenetic regulation, and cell cycle control (Chen and Yan, 2013). Previous research reported that the function and mechanism of lncRNAs are complex and may have competitive relationship with miRNAs when interacting with lncRNAs (Yoon et al., 2014). However, the identification of lncRNAs in the pearl gentian grouper using full-length sequencing technology has not been reported yet. There are 8,874 common lncRNAs that were predicted by the four software in this study, which would be useful for further research on the pearl gentian grouper, including epigenetics, immunology, and phylogenomics (Zeng et al., 2018).

Based on PacBio SMRT full-length transcriptome sequencing, the present study preliminarily investigated the differential mechanisms of enteritis in the pearl gentian grouper induced by different soy proteins. Similar to previous studies on plant protein-induced fish enteritis, some conserved signaling pathways, such as the nuclear factor kappa B (NF-κB) signaling pathway, were found in the intestine transcriptome of pearl gentian fed with the SBM40 and FSBM40 diets. Previous studies indicated that Atlantic salmon fed with SPC did not show changes in the transcriptome levels similar to SBM-induced enteritis (Król et al., 2016). The present study also found that the intestinal transcription profile of pearl gentian grouper fed the SPC40 diet was significantly different from those of the SBM40 and FSBM40 diets. Only 2.86% of the signaling pathways related to immune diseases/system, infectious diseases, and signal transduction were significantly affected, while 85.71% of the signaling pathways related to nutrition digestion and absorption were significantly affected. However, in the common Profile G, some signaling pathways closely related to intestinal immunity were also enriched, such as intestinal immune network for immunoglobulin A (IgA) production.

The intestinal tract is the largest lymphoid tissue in the human body. A remarkable feature of intestinal immunity is that it can produce a large number of IgA antibodies as the first line of defense against microorganisms (Mestecky et al., 1999). There are a few studies on the signaling pathway of the intestinal immune network for IgA production in fish. In mammalian studies, it has been found that IgA production is induced by the interaction of specific antigen and innate immune receptors, such as Toll-like receptor 2 (TLR2), TLR4, and TLR9 (Suzuki and Fagarasan, 2008). Related studies also revealed that the TLR/NF-κB signaling pathway is the main component of inflammation and immune response (Tan et al., 2016). Based on the above analysis, this study focused on the role of the TLR-mediated NF-κB signaling pathway and the intestinal immune network for IgA production pathway in the development of SBM-, SPC-, and FSBM-induced enteritis in the pearl gentian grouper.

A total of nine TLR members were found in the intestinal tissues of the pearl gentian grouper, including TLR1, TLR2, TLR3, TLR5, TLR8, TLR9, TLR13, TLR21, and TLR22. At present, there are 20 TLRs found in fish, at least. Among the TLRs found in this experiment, TLR1, TLR2, TLR3, TLR5, TLR9, TLR21, and TLR22 have been reported as sensors for bacterial ligands in fish (Wei et al., 2011; Yeh et al., 2013; Byadgi et al., 2014). The present study showed that the expression levels of *TLR5*, *TLR8*, *TLR9*, *TLR21*, and *TLR22* were significantly increased with the addition of the SBM40 diet; *TLR5*, *TLR8*, *TLR9*, and *TLR22* were significantly increased with the SPC40 diet addition; and *TLR1*, *TLR8*, *TLR13*, and *TLR22* were significantly increased with the addition of the FSBM40 diet, indicating that the signal transduction of the TLRs was activated by various bacterial components/products after different soy protein substitutions for FM protein.

In addition, the present study found that, compared with the control group, the addition of SBM, SPC, and FSBM resulted

in the significant downregulation of *IL4*, *IL5*, *IL10*, and *TGF-β* expressions, but the expression of IgA increased significantly. Relevant studies pointed out that IL4, IL5, IL6, and IL10 secreted by helper T cells may play important roles in promoting IgA secretion (Barnes et al., 2011). In this experiment, the three soy protein diets all caused the high expression of *IgA*, which may be the manifestation of intestinal immune imbalance in the pearl gentian grouper. The specific reasons need to be further studied.

Taken together, this study analyzed the full-length transcriptome of the pearl gentian grouper intestine using the PacBio SMRT sequencing technology, which represents the first third-generation long-read transcriptome sequencing of the pearl gentian grouper. The obtained transcriptome data may improve further studies on the pearl gentian grouper.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. The Pacbio SMRT sequencing raw reads and Illumina sequencing raw reads are deposited in NCBI Sequence Read Archive (SRA) and the accession numbers are PRJNA664623 and PRJNA664416, respectively.

ETHICS STATEMENT

The animal protocol was approved by the Ethics Review Board of the Guangdong Ocean University. All procedures were performed in accordance with the standards of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023, revised 1978) and relevant Chinese policies.

AUTHOR CONTRIBUTIONS

WZ designed and took part in the whole process of the experiment and wrote the draft of this manuscript. BT and JD co-conceived the experiment and revised the draft critically for important intellectual content. XD and QY participated in the experiments. SC revised the first draft. HL and SZ analyzed the data. SX and HZ approved the final version. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by the National Key R&D Program of China (2019YFD0900200).

ACKNOWLEDGMENTS

We are grateful to the Key Laboratory of Aquatic, Livestock and Poultry Feed Science and Technology in South China, Ministry of Agriculture, for providing technical assistances.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.688601/full#supplementary-material

Supplementary Figure 1 | Effect of different soy proteins at 40% substitution levels for fish meal protein on the hepatosomatic index and survival rate of pearl gentian grouper (n = 3).

Supplementary Figure 2 | Hematoxylin-eosin staining in the distal intestine of pearl gentian grouper. Representative images of increased width and cellular (leucocyte) infiltration (asterisk) of the lamina propria (arrow) in distal intestine depicting the inflammatory changes of grouper fed the FM **(A)**, SBM40 **(B)**, SPC40 **(C)**, and FSBM40 **(D)** diets. FM, fish meal control group; SBM40, 40% SBM protein substitution for FM protein; SPC40, 40% SPC protein substitution for FM protein; FSBM40, 40% FSBM protein substitution for FM protein.

Supplementary Figure 3 | Analysis of long read transcriptome of pearl gentian grouper by SMRT sequencing. **(A)** The flow chart of transcriptome analysis of hybrid grouper in this study; **(B)** classification of total consensus sequence reads; **(C)** length distribution of the unigenes; **(D)** number of transcripts corresponding to unigenes.

Supplementary Figure 4 | Predicted simple sequence repeats (SSRs) of the long read transcripts (n = 4).

REFERENCES

Aimin, L., Zhang, J. Y., and Zhou, Z. Y. (2014). Plek: a tool for predicting long non-coding RNAs and messenger RNAs based on an improved *k*-mer scheme. *BMC Bioinformatics* 15:311. doi: 10.1186/1471-2105-15-311

An, M. L., Fan, Z., Wang, Q. K., Sun, J. H., Xu, D. W., Guo, Y. J., et al. (2018). Effect of replacing fish meal with soybean meal on growth, digestion and antioxidant ability of *Epinephelus coioides*. *Jiangsu Agric. Sci.* 46, 128–132. doi: 10.15889/j.issn.1002-1302.2018.16.032

Au, K. F., Sebastiano, V., Afshar, P. T., Durruthy, J. D., Lee, L., Williams, B. A., et al. (2013). Characterization of the human ESC transcriptome by hybrid sequencing. *Proc. Natl. Acad. Sci. U.S.A.* 110, E4821–E4830. doi: 10.1073/pnas.1320101110

Barnes, S., Prasain, J., D'Alessandro, T., Arabshahi, A., Botting, N., Lila, M., et al. (2011). The metabolism and analysis of isoflavones and other dietary polyphenols in foods and biological systems. *Food Funct.* 2, 235–244. doi: 10.1039/c1fo10025d

Booman, M., Forster, I., Vederas, J. C., Groman, D. B., and Jones, S. R. M. (2018). Soybean meal-induced enteritis in Atlantic salmon (*Salmo salar*) and Chinook salmon (*Oncorhynchus tshawytscha*) but not in pink salmon (*O. gorbuscha*). *Aquaculture* 483, 238–243. doi: 10.1016/j.aquaculture.2017.10.025

Brawand, D., Wagner, C. E., Li, Y. I, Malinsky, M., Keller, I., Fan, S., et al. (2014). The genomic substrate for adaptive radiation in African cichlid fish. *Nature* 513, 375–381. doi: 10.1038/nature13726

Byadgi, O., Puteri, D., Lee, Y. H., Lee, J. W., and Cheng, T. C. (2014). Identification and expression analysis of cobia (*Rachycentron canadum*) Toll-like receptor 9 gene. *Fish Shellfish Immun.* 36, 417–427. doi: 10.1016/j.fsi.2013.12.017

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

Chen, X., and Yan, G. Y. (2013). Novel human lncRNA-disease association inference based on lncRNA expression profles. *Bioinformatics* 29, 2617–2624. doi: 10.1093/bioinformatics/btt426

Conesa, A., Gotz, S., Garcia-Gomez, J. M., Terol, J., Talon, M., and Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21, 3674–3676. doi: 10.1093/bioinformatics/bti610

De Santis, C., Ruohonen, K., Tocher, D. R., Martin, S. A. M., Król, E., Secombes, C. J., et al. (2015). Atlantic salmon (*Salmo salar*) parr as a model to predict the optimum inclusion of air classified faba bean protein concentrate in feeds for seawater salmon. *Aquaculture* 444, 70–78. doi: 10.1016/j.aquaculture.2015.03.024

Deng, J. M., Mai, K. S., Ai, Q. H., Zhang, W. B., Wang, X. J., Xu, W., et al. (2006). Effects of replacing fish meal with soy protein concentrate on feed intake and growth of juvenile Japanese flounder, *Paralichthys olivaceus*. *Aquaculture* 258, 503–513. doi: 10.1016/j.aquaculture.2006.04.004

Ehsani, J., Maniat, M., Azarm, H. M., and Ghabtani, A. (2014). Effects of partial substitution of dietary fish meal by fermented soybean meal on growth performance, body composition and activity of digestive enzymes of juvenile yellowfin sea bream (*Acanthopagrus latus*). *Int. J. Biosci.* 5, 99–107. doi: 10.12692/ijb/5.4.99-107

Faudzi, N. M., Yong, A., Shapawi, R., Senoo, S., and Takii, K. (2018). Soy protein concentrate as an alternative in replacement of fish meal in the feeds of hybrid grouper, brown-marbled grouper (*Epinephelus fuscoguttatus*) × giant grouper (*E. lanceolatus*) juvenile. *Aquac. Res.* 49, 431–441. doi: 10.1111/are.13474

Feng, X., Jia, Y. T., Zhu, R., Chen, K., and Chen, Y. F. (2019). Characterization and analysis of the transcriptome in *Gymnocypris selincuoensis* on the Qinghai-Tibetan Plateau using single-molecule long-read sequencing and RNA-seq. *DNA Res.* 26, 353–363. doi: 10.1093/dnares/dsz014

Finn, R. D., Coggill, P., Eberhardt, R. Y., Eddy, S. R., Mistry, J., Mitchell, A. L., et al. (2016). The Pfam protein families database: towards a more sustainable future. *Nucleic Acids Res.* 44, D279–D285. doi: 10.1093/nar/gkv1344

Gordon, S. P., Tseng, E., Salamov, A., Zhang, J., Meng, X., Zhao, Z., et al. (2015). Widespread polycistronic transcripts in fungi revealed by single-molecule mRNA sequencing. *PLoS One* 10:e0132628. doi: 10.1371/journal.pone.0132628

Gu, M., Jia, Q., Zhang, Z. Y., Bai, N., Xu, X. J., and Xu, B. (2018). Soya-saponins induce intestinal inflammation and barrier dysfunction in juvenile turbot (*Scophthalmus maximus*). *Fish Shellfish Immun.* 77, 264–272. doi: 10.1016/j.fsi.2018.04.004

Guardiola, F. A., Porcino, C., Cerezuela, R., Cuesta, A., Faggio, C., and Esteban, M. A. (2016). Impact of date palm fruits extracts and probiotic enriched diet on antioxidant status, innate immune response and immune-related gene expression of European seabass (*Dicentrarchus labrax*). *Fish Shellfish Immun.* 52, 298–308. doi: 10.1016/j.fsi.2016.03.152

Gulcher, J. (2012). Microsatellite markers for linkage and association studies. *Cold Spring Harb. Protoc.* 4:425. doi: 10.1101/pdb.top068510

Hackl, T., Hedrich, R., Schultz, J., and Forster, F. (2014). Proovread: large-scale high-accuracy PacBio correction through iterative short read consensus. *Bioinformatics* 30, 3004–3011. doi: 10.1093/bioinformatics/btu392

Jia, X., Tang, L., Mei, X., Liu, H., and Su, J. (2020). Single-molecule long-read sequencing of the full-length transcriptome of *Rhododendron lapponicum* L. *Sci. Rep.* 10:6755. doi: 10.1038/s41598-020-63814-x

Kalhoro, H., Zhou, J., Hua, Y., Ng, W. K., Ye, L., Zhang, J., et al. (2018). Soy protein concentrate as a substitute for fish meal in diets for juvenile *Acanthopagrus schlegelii*: effects on growth, phosphorus discharge and digestive enzyme activity. *Aquac. Res.* 49, 1896–1906. doi: 10.1111/are.13645

Kong, L., Zhang, Y., Ye, Z. Q., Liu, X. Q., Zhao, S. Q., Wei, L., et al. (2007). CPC: assess the protein-coding potential of transcripts using sequence features and support vector machine. *Nucleic Acids Res.* 35, W345–W349. doi: 10.1093/nar/gkm391

Król, E., Douglas, A., Tocher, D. R., Crampton, V. O., Speakman, J. R., Secombes, C. J., et al. (2016). Differential responses of the gut transcriptome to plant protein diets in farmed Atlantic salmon. *BMC Genomics* 17:156–172. doi: 10.1186/s12864-016-2473-0

Larsen, P. A., Campbell, C. R., and Yoder, A. D. (2014). Next-generation approaches to advancing eco-immunogenomic research in critically endangered primates. *Mol. Ecol. Resour.* 14, 1198–1209. doi: 10.1111/1755-0998.12274

Larsen, P. A., and Smith, T. P. (2012). Application of circular consensus sequencing and network analysis to characterize the bovine IgG repertoire. *BMC Immunol.* 13:52. doi: 10.1186/1471-2172-13-52

Li, P., Deng, W., Li, T., Song, B., and Shen, Y. (2013). Illumina-based *de novo* transcriptome sequencing and analysis of *Amanita exitialis* basidiocarps. *Gene* 532, 63–71. doi: 10.1016/j.gene.2013.09.014

Liu, K., Tan, B. P., Zhang, W., Liu, H. Y., Dong, X. H., Yang, Q. H., et al. (2019). Transcriptome, enzyme activity and histopathology analysis reveal the effects of high level of dietary carbohydrate on glycometabolism in juvenile golden pompano, *Trachinotus ovatus*. *Aquac. Res.* 1, 1–15. doi: 10.1111/are.14096

Liu, X. W., Ai, Q. H., Mai, K. S., Liu, F. Z. G., and Xu, W. (2014). Effects of replacing fish meal with soy protein concentrate on feed intake and growth of turbot (*Scophthalmus maximus*). *J. Fish. China* 38, 91–98. doi: 10.3724/SP.J.1231.2014.48852

Liu, Z., Liu, S., Yao, J., Bao, L., Zhang, J., Li, Y., et al. (2016). The channel catfish genome sequence provides insights into the evolution of scale formation in teleosts. *Nat. Commun.* 7:11757. doi: 10.1038/ncomms11757

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta}$ method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Lobato, F. M. F., Damasceno, C. D., Leite, D. S., dos Santos, ÂK. R., and De Santana, ÁL. (2017). Data analysis of multiplex sequencing at solid platform: a probabilistic approach to characterization and reliability increase. *Am. J. Resp. Cell .Mol.* 8, 26–38. doi: 10.4236/ajmb.2018.81003

Luo, Z. Y., Liu, Y. J., Mai, K. S., Tian, L. X., Liu, D. H., and Tan, X. Y. (2004). Partial replacement of fish meal by soybean protein in diets for grouper *Epinephelus coioides* juveniles. *J. Fisheries China* 2, 175–181.

Martin, S. A. M., and Król, E. (2017). Nutrigenomics and immune function in fish: new insights from omics technologies. *Dev. Comp. Immunol.* 75, 86–98. doi: 10.1016/j.dci.2017.02.024

Mestecky, J., Moro, I., and Underdown, B. J. (1999). *Mucosal Immunology*, eds P. Ogra, et al. (San Diego, CA: Academic Press), 133–152.

Miao, S., Zhao, C., Zhu, J., Hu, J., Dong, X., and Sun, L. (2018). Dietary soybean meal affects intestinal homoeostasis by altering the microbiota, morphology and inflammatory cytokine gene expression in northern snakehead. *Sci. Rep.* 8, 113–123. doi: 10.1038/s41598-017-18430-7

Ocwieja, K. E., Scott, S. M., Rithun, M., Rebecca, C. A., Patricia, D., Michael, B., et al. (2012). Dynamic regulation of HIV-1 mRNA populations analyzed by single-molecule enrichment and long-read sequencing. *Nucleic Acids Res.* 40, 10345–10355. doi: 10.1093/nar/gks753

Ren, Y. P., Zhang, J. Q., Sun, Y., Wu, Z. F., Ruan, J. S., He, B. J., et al. (2006). Full-length transcriptome sequencing on PacBio platform. *Sci. Bull.* 61, 1250–1254. doi: 10.1360/N972015-01384

Roberts, R. J., Carneiro, M. O., and Schatz, M. C. (2013). The advantages of SMRT sequencing. *Genome Biol.* 14:405. doi: 10.1186/gb-2013-14-7-405

Shimizu, K., Adachi, J., and Muraoka, Y. (2006). ANGLE: a sequencing errors resistant program for predicting protein coding regions in unfinished cDNA. *J. Bioinf. Comput. Biol.* 4, 649–664. doi: 10.1142/S0219720006002260

Star, B., Nederbragt, A. J., Jentoft, S., Grimholt, U., Malmstrøm, M., Gregers, T. F., et al. (2011). The genome sequence of Atlantic cod reveals a unique immune system. *Nature* 477, 207–210. doi: 10.1038/nature10342

Sun, L., Luo, H. T., Bu, D. C., Zhao, G. G., Yu, K. T., Zhang, C. H., et al. (2013). Utilizing sequence intrinsic composition to classify protein-coding and long non-coding transcripts. *Nucleic Acids Res.* 41:e166. doi: 10.1093/nar/gkt646

Sun, L. R. (2016). *Design and Evaluation of Two Hybrid Genome Assembly Approaches Using Illumina, Roche 454, and PacBio Datasets*. Fargo, ND: North Dakota State University.

Suzuki, K., and Fagarasan, S. (2008). How host-bacterial interactions lead to IgA synthesis in the gut. *Trends Immunol.* 29, 523–531. doi: 10.1016/j.it.2008.08.001

Tan, P., Dong, X. J., Mai, K. S., Xu, W., and Ai, Q. H. (2016). Vegetable oil induced inflammatory response by altering TLR-NF-κB signalling, macrophages infiltration and polarization in adipose tissue of large yellow croaker (*Larimichthys crocea*). *Fish Shellfish Immun.* 59, 398–405. doi: 10.1016/j.fsi.2016.11.009

Teves, J. F. C., and Ragaza, J. A. (2014). The quest for indigenous aquafeed ingredients: a review. *Rev. Aquac.* 8, 154–171. doi: 10.1111/raq.12089

Thiel, T. (2003). *MISA-Microsatellite Identifcation Tool*. Available online at: http://pgrc.ipk-gatersleben.de/misa/ (accessed June 17, 2016).

Tine, M., Kuhl, H., Gagnaire, P. A., Louro, B., Desmarais, E., Martins, R. S. T., et al. (2014). European sea bass genome and its variation provide insights into adaptation to euryhalinity and speciation. *Nat. Commun.* 5:5770. doi: 10.1038/ncomms6770

Treutlein, B., Gokce, O., Quake, S. R., and Südhof, T. C. (2014). Cartography of neurexin alternative splicing mapped by single-molecule long-read mRNA sequencing. *Proc. Natl. Acad. Sci. U.S.A.* 111, E1291–E1299. doi: 10.1073/pnas.1403244111

van Eijk, M. (2015). "Genome assembly and Iso-Seq transcriptome sequencing of tetraploid cotton," in *Proceedings of the Plant and Animal Genome XXIII Conference. Plant and Animal Genome*, San Diego, CA.

Wan, H. F., Jia, X. W., Zou, P. F., Zhang, Z. P., and Wang, Y. L. (2019). The Single-molecule long-read sequencing of *Scylla paramamosain*. *Sci. Rep.* 9:12401. doi: 10.1038/s41598-019-48824-8

Wang, X. X. (2009). Study of fermented soybean meal as a substitute for fish meal in California perch feed. *Aquatic. Feed Techn.* 1, 58–61.

Wang, Y. P., Lu, Y., Zhang, Y., Ning, Z. M., Li, Y., Zhao, Q., et al. (2015). The draft genome of the grass carp (*Ctenopharyngodon idellus*) provides insights into its evolution and vegetarian adaptation. *Nat. Genet.* 47, 625–631. doi: 10.1038/ng.3280

Wei, Y. C., Pan, T. S., Ming, X. C., Bei, H., Zhen, X., Luo, T. R., et al. (2011). Cloning and expression of toll-like receptors 1 and 2 from a teleost fish, the orange-spotted grouper epinephelus coioides. *Vet. Immunol. Immunop.* 141, 173–182. doi: 10.1016/j.vetimm.2011.02.016

Wen, L. M., Jiang, W. D., Liu, Y., Wu, P., Zhao, J., Jiang, J., et al. (2015). Evaluation the effect of thiamin deficiency on intestinal immunity of young grass carp (*Ctenopharyngodon idella*). *Fish Shellfish Immun.* 46, 501–515. doi: 10.1016/j.fsi.2015.07.001

Wong, K. C., Zhang, J., Yan, S., Li, X., Lin, Q., Kwong, S., et al. (2019). DNA sequencing technologies: sequencing data protocols and bioinformatics tools. *ACM Comput. Surv.* 52, 1–30. doi: 10.1145/3340286

Workman, R. E., Myrka, A. M., William, W. G., Elizabeth, T., Welch, K. C., and Winston, T. (2018). Single molecule, full-length transcript sequencing provides insight into the extreme metabolism of ruby-throated hummingbird *Archilochus colubris*. *GigaScience* 7, 1–12. doi: 10.1093/gigascience/giy009

Worley, K. C. (2015). "European cuttlefish whole transcriptome sequencing: a single-molecule full length transcript survey with Iso-Seq method," in *Proceedings of the Lant and Animal Genome XXIII Conference. Plant and Animal Genome*, San Diego, CA.

Wu, N., Wang, B., Cui, Z. W., Zhang, X. Y., Cheng, Y. Y., Xu, X., et al. (2018). Integrative transcriptomic and microRNAomic profiling reveals immune mechanism for the resilience to soybean meal stress in fish gut and liver. *Front. Physiol.* 9:1154. doi: 10.3389/fphys.2018.01154

Xiang, F. Q. (2017). *Effects of Sea Bass on Growth, Immune and Intestinal Flora by Soy Protein Concentrate*. Changsha: Hunan Agricultural University.

Xu, P., Zhang, X., Wang, X., Li, J., Liu, G., Kuang, Y., et al. (2014). Genome sequence and genetic diversity of the common carp, *Cyprinus carpio*. *Nat. Genet.* 46, 1212–1219. doi: 10.1038/ng.3098

Yang, X., Ikhwanuddin, M., Li, X., Lin, F., Wu, Q., Zhang, Y., et al. (2017). Comparative transcriptome analysis provides insights into differentially expressed genes and long non-coding RNAs between ovary and testis of the Mud crab (*Scylla paramamosain*). *Mar. Biotechnol.* 20, 20–34. doi: 10.1007/s10126-017-9784-2

Yeh, D. W., Liu, Y. L., Lo, Y. C., Yuh, C. H., Yu, G. Y., Lo, J. F., et al. (2013). Toll-like receptor 9 and 21 have different ligand recognition profiles and cooperatively mediate activity of CpG-oligodeoxynucleotides in zebrafish. *Proc. Natl. Acad. Sci. U.S.A.* 110, 20711–20716. doi: 10.1073/pnas.1305273110

Yoon, J. H., Abdelmohsen, K., and Gorospe, M. (2014). Functional interactions among microRNAs and long noncoding RNAs. *Semin. Cell Dev. Biol.* 34, 9–14. doi: 10.1016/j.semcdb.2014.05.015

Zeng, D. G., Chen, X. L., Peng, J. X., Yang, C. L., Peng, M., Zhu, W. L., et al. (2018). Single-molecule long-read sequencing facilitates shrimp transcriptome research. *Sci. Rep.* 8:16920. doi: 10.1038/s41598-018-35066-3

Zhang, W., Liu, K., Tan, B. P., Liu, H. Y., Dong, X. H., Yang, Q. H., et al. (2019). Transcriptome, enzyme activity and histopathology analysis reveal the effects of dietary carbohydrate on glycometabolism in juvenile largemouth bass, *Micropterus salmoides*. *Aquaculture* 504, 39–51. doi: 10.1016/j.aquaculture.2019.01.030

Zhao, J., Feng, L., Liu, Y., Jiang, W., Wu, P., Jiang, J., et al. (2014). Effect of dietary isoleucine on the immunity, antioxidant status, tight junctions and microflora in the intestine of juvenile Jian carp (*Cyprinus carpio* var. Jian). *Fish Shellfish Immun.* 41, 663–673. doi: 10.1016/j.fsi.2014.10.002

Zhou, W., Samad, R., Lu, K., Wang, L., and Liu, W. (2018). 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, 1–9. doi: 10.1007/s10695-018-0536-7

Zhou, Z. Y., Wu, X. Y., Li, X. J., Dong, Y., Wang, X., Mu, W., et al. (2020). The optimum dietary isoleucine requirement of juvenile hybrid grouper (*Epinephelus fuscoguttatus*♀ × *Epinephelus lance*olatus♂). *Aquacult. Nutr.* 26, 1295–1310. doi: 10.1111/anu.13085

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: 19 July 2021
doi: 10.3389/fmars.2021.705332

Dynamic Alterations of the Distal Intestinal Microbiota, Transcriptome, and Metabolome of Hybrid Grouper by β -Conglycinin With Reconciliations by Sodium Butyrate in Feed

Bin Yin[1,2,3], Hongyu Liu[1,2,3], Beiping Tan[1,2,3]*, Xiaohui Dong[1,2,3], Shuyan Chi[1,2,3], Qihui Yang[1,2,3] and Shuang Zhang[1,2,3]*

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

OPEN ACCESS

Edited by:
Ikram Belghit,
Norwegian Institute of Marine Research (IMR), Norway

Reviewed by:
Fotini Kokou,
Wageningen University and Research, Netherlands
Seyyed Morteza Hoseini,
Iranian Fisheries Science Research Institute (IFSRI), Iran

***Correspondence:**
Hongyu Liu
liuhyu@gdou.edu.cn
Beiping Tan
bptan@126.com

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

Received: 05 May 2021
Accepted: 23 June 2021
Published: 19 July 2021

Citation:
Yin B, Liu H, Tan B, Dong X, Chi S, Yang Q and Zhang S (2021) Dynamic Alterations of the Distal Intestinal Microbiota, Transcriptome, and Metabolome of Hybrid Grouper by β -Conglycinin With Reconciliations by Sodium Butyrate in Feed. Front. Mar. Sci. 8:705332. doi: 10.3389/fmars.2021.705332

Different doses of β-conglycinin produce different regulations on the intestinal health of aquatic animals, affecting the absorption of nutrients, indirectly changing water quality. Sodium butyrate (NaB) can effectively alleviate the negative effects caused by high-dose β-conglycinin. We investigated the positive response to low-dose (1.5%, bL) and negative response to high-dose (6.0%, bH) β-conglycinin and supplementation with NaB (6.0% β-conglycinin + 0.13% NaB, bHNaB) in terms of water pollutants, microbiota, transcriptome, and metabolome in hybrid grouper (*Epinephelus fuscoguttatus*♀ × *E. lanceolatus*♂). The ammonia nitrogen, nitrite, total nitrogen, and total phosphorus contents were significantly higher in the water from bH than from FMb, bL, and bHNaB. Supplementing with NaB significantly reduced the ammonia nitrogen, nitrite, total nitrogen, and total phosphorus contents. Low-dose β-conglycinin increased the relative abundance of *Pelagibacterium*, *Pediococcus*, *Staphylococcus*, and *Lactobacillus* and promoted the "ribosome," "peroxisome proliferator-activated receptor (PPAR) signaling" and "histidine metabolism." High-dose β-conglycinin increased the relative abundance of pathogenic bacteria *Ralstonia* and *Photobacterium* and inhibited the "cell cycle" "PPAR signaling" and "starch and proline metabolism." NaB supplementation at high-dose β-conglycinin reduced the *Ralstonia* and *Photobacterium* abundance and promoted the "cell cycle," "linoleic acid metabolism," and "ABC transporters." Overall, these results reveal differences in the effects of high- and low-dose β-conglycinin, as well as NaB supplementation, on the utilization of proteins, carbohydrates, and lipids and on substance transport and signaling among distal intestinal cells of hybrid grouper. A total of 15 differential metabolite biomarkers were identified: FMb vs. bL contained 10-methylimidazole acetic acid, *N*-acetyl histamine,

urocanic acid, creatinine, glutathione, taurine, nervonic acid, stearic acid, docosanoic acid, and D-serine; FMb vs. bH contained 4-L-fucose, sucrose, α,α-trehalose, and quercetin; and bH vs. bHNaB contained 4-*N*-acetyl histamine, urocanic acid, creatinine, and *S*-adenosylhomocysteine, respectively. Our study provides new insights into the regulation of intestinal health by β-conglycinin in aquatic animals and the protective mechanism of NaB.

Keywords: hybrid grouper (*Epinephelus fuscoguttatus*♀ × *E. lanceolatus*♂), β-conglycinin, sodium butyrate, intestinal microbiota, transcriptome, metabolome

INTRODUCTION

The hybrid grouper (*Epinephelus fuscoguttatus*♀ × *E. lanceolatus*♂) is an economically important fish along the southern coast of China, with a fast growth rate and strong environmental tolerance. In 2017, groupers were officially included in the construction of China's National Marine Fish Industry Technology System. As a typical carnivorous fish, it usually requires up to about 50% of protein in its feed (Jiang et al., 2016). Fishmeal is a high quality protein source and it has been traditionally used as the main protein source in the aquafeed industry. However, due to the high demand for fishmeal leading to a high price and lack of sources, the farming industry had to find dietary alternative ingredients. The high nitrogen and phosphorus content in the fishmeal feeds is not completely absorbed by hybrid grouper and is most likely to be excreted along with the feces, leading to serious contamination of the farmed water. Currently, the use of soybean meal to replace fish meal can relieve the pressure of fish meal shortage and greatly promote the sustainable development of aquaculture industry (Yang et al., 2011; Smith et al., 2017; Miao et al., 2018). However, owing to low tolerance to soybean meal, excessive intake of soybean meal can lead to varying degrees of intestinal injury (Zhang et al., 2019).

The intestines of aquatic animals are responsible for the dual functions of digestion and absorption. The mucosa of the intestinal tract is the main interface between the internal and external environments of the organism. The intestinal tract is the main site of nutrient exchange and infection or initiation of infection by many pathogens (Jiang et al., 2019) and is highly susceptible to the effects of feed ingredients and living environmental conditions (Duan et al., 2017). Thus, it is essential to maintain the health and stability of the intestinal tracts of aquatic animals. The presence of a large number of microbiota in the intestinal tract is the result of long-term evolution, and they are closely related to the immune function and nutritional requirements of the organism (Yang et al., 2018). Intestinal microbiota can participate in the mediation of multiple metabolic pathways in the host, interacting with host metabolism and signal transduction to form a physiologically connected gut-immune-inflammation axis (Matsumoto et al., 2012). Similarly, intestinal health status could also influence the composition of microorganisms. Therefore, the relationship between intestinal flora and metabolite associations and host intestinal health requires further investigation.

It is accepted that β-conglycinin (7S) of soybean meal is a major factor in the induction of intestinal injury. β-Conglycinin, one of the major antigen proteins in soybean, often causes allergic reactions in young animals, causing inflammatory damage to intestinal epithelial cells accompanied by lipid peroxidation of cell membranes, negatively affecting the function of tight junction protein structures and increasing intestinal epithelial permeability (Zhao et al., 2014). The unbalanced amino acid in soybean meal may lead to increased excretion of nitrogen and phosphorus by fish (Tantikitti et al., 2005), which is not conducive to the healthy and sustainable development of the aquaculture industry. As a widely used additive in livestock and poultry animals, sodium butyrate (NaB) can not only act as a food attractant, but also improve the tight junctions of intestinal epithelium (Huang et al., 2015) and alleviate inflammation by inhibiting nuclear factor kappa B (NF-kB) (Albino et al., 2012), and it also has a positive regulatory effect on the host's intestinal microbiota (Zou et al., 2019). The active ingredient in NaB is butyric acid, which is a short-chain, volatile fatty acid (O'Hara et al., 2018). In aquatic animals, NaB can stimulate the growth and proliferation of intestinal mucosa and inhibit the proliferation of intestinal pathogenic microorganisms, thus, promoting the growth of fish and enhancing intestinal digestion and antioxidant capacity (Tian et al., 2017; Jesus et al., 2018; Fu et al., 2019). Nevertheless, the physiological mechanisms of intestinal injury and inflammation in hybrid grouper caused by β-conglycinin and the protective effects of NaB on the intestinal tract are not well understood.

In a previous study, we demonstrated that ingestion of high levels of soybean meal by hybrid grouper resulted in intestinal injury (Zhang et al., 2019). The inhibitory effects of β-conglycinin on the growth of fish is usually thought to be due to intestinal inflammation (Zhang et al., 2013); nevertheless, the reports of whether β-conglycinin can negatively affect other physiological functions in fish are rare. In this study, we investigated the effects of β-conglycinin and the protective mechanism of NaB on transcription, intestinal microbiota, and metabolites in the distal intestine of hybrid grouper. Then, transcriptomic, intestinal microbiota, and metabolomic means were combined to model the associations among host metabolism, intestinal microbiota, and aquaculture water pollutants at the system biology level. This will help us understand the metabolic processes of the microflora in the host and their effects on host transcription, as well as their interactions with each other, and search for potential targets for the treatment of soybean meal enteritis.

MATERIALS AND METHODS

Fish and Rearing Conditions

Healthy juvenile hybrid groupers were purchased from a fish hatchery in East Island (Zhanjiang, China). The juveniles were housed in a concrete pond at the Biological Research Base of Guangdong Ocean University and fed commercial feed for 1 week to acclimatize them to the base environment.

After 1 week, 480 robust and uniformly fit hybrid groupers with an average body weight of 7.70 ± 0.05 g were randomly selected. The experiment was divided into four treatment groups with four replicates of 30 fish each and farmed for 8 weeks. 0.3 m^3 fiberglass tanks were used for each replicate breeding indoors. During the experimental period, apparent satiety feeding was carried out twice daily (08:00 and 16:00) with a daily water change of approximately 70% (water flow: 100 L/min). The water temperature was 30.00 ± 1.30°C, salinity was 32.00 ± 2.00, dissolved oxygen was ≥7.00 mg/L, pH was 7.80–8.10, and ammonia nitrogen was <0.09 mg/L.

Diet Formulations

Four different design approaches to experimental feeds were used in this experiment. Based on the approximate amount of 7S in soybean meal protein (30%) (Ogawa et al., 1991) and the research of soybean meal substitution for fish meal in aquatic animals (Shiu et al., 2015), we set 1.5% 7S as the low dose (equivalent to supplementing 10% dehulled soybean meal to feed) and 6.0% as the high dose group (equivalent to supplementing 40% dehulled soybean meal to feed). Optimum NaB addition level was determined based on the results of studies on aquatic animals (Mirghaed et al., 2019). We used the group with 0.00% 7S and 0.00% NaB as the control group, named FMb group. To the control group, 1.5% of 7S was supplemented as a low-dose 7S addition group, named bL group; to the control group, 6.0% of 7S was supplemented as a high-dose 7S addition group, named bH group; to the control group, both 6.0% of 7S and 0.13% of NaB were supplemented as a repair group, named bHNaB group. Fishmeal, casein, and gelatin were used as the main protein sources for experimental feeds, while fish oil and soy lecithin were used as the main lipid sources. Methionine and lysine were added to the experimental diets at the FM diet level. The diet formulations and amino acid profiles of the four diets are shown in **Supplementary Tables 1, 2**. Purified 7S was purchased from China Agricultural University (Patent No. 200410029589.4, China). After all the ingredients were thoroughly blended, they were passed through a 380 μm sieve. Then, 30% water (dissolved choline chloride) was added and mixed thoroughly, and a pelletizer was used to produce 2.5 mm diameter pellets. The samples were air-dried at room temperature until the moisture content was close to 10% and then stored at −20°C until use.

Sample Collection and Determination

Six experimental fish were randomly selected from each treatment at the end of the 8-week breeding experiment. The fish were dissected in a sterile environment using sterile scissors and forceps, and the distal intestine contents were removed and placed in sterile cryopreservation tubes. Following this, the intestines were immediately cleaned with pre-chilled phosphate buffered saline and divided equally into two portions, one for the transcriptomic sample and the other for the metabolomic sample, which were placed in cryopreservation tubes. Each individual fish served as a biological replicate of each microbiota, transcriptome, and metabolome, and corresponded one to one.

The siphon method was used to obtain water samples approximately 10–20 cm from the bottom of each tank for the determination of ammonia nitrogen (AN), nitrite (NIT), total nitrogen (TN), and total phosphorus (TP). One end of the polyvinylchlorid pipe was placed below the water surface, 10–20 cm from the bottom of the tank, and the other end was connected to the sample bottle. Then the air inside the tube was extracted so that the water enters the sample bottle through the tube due to the air pressure difference. The water samples for determining TN and TP were additionally filtered using a filter extractor with a pore size of 0.45 μm. All water samples were temporarily stored at −20°C. According to the Specification for Marine Monitoring-Part 4: Seawater analysis (GB 17378.4-2007) (China, 2007), the hypobromite oxidation method and *N*-(1-naphthyl)-ethylenediamine dihydrochloride spectrophotometric method were used to determine the contents of NH_4^+-N and NO_2-N, respectively, and the potassium persulfate oxidation method was used to determine the contents of TN and TP.

Trizol (1 mL; TRI Reagent solution, Invitrogen, Carlsbad, CA, United States) was used to extract total RNA from 100–150 mg of the distal intestinal tissue. Electrophoresis of 1% agarose gels and spectrophotometric analysis with a NanoDrop 2000 (260:280 nm) was used to determine the total RNA quality and quantity. Total RNA was reverse-transcribed into cDNA using the PrimerScript^{TM} RT-PCR kit (TaKaRa, Kusatsu, Japan). SYBR® Premix Ex Taq^{TM} kit was used to perform real-time PCR reactions using the Applied Biosystems 7500 Real-Time PCR System (Life Technologies, Carlsbad, CA, United States). Relative mRNA levels were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Distal Intestinal Microbiome Analysis

The hexad ecyltrimethyl ammonium bromide (CTAB) method was used to extract the total genomic DNA from the distal intestine samples (Griffith and Shaw, 1998). 20 uL of lysozyme was added to 1000 uL of CTAB lysis solution (0.1 M Tris HCl, pH 8.0, 1.4 M NaCl, 0.02 M EDTA, 2% CTAB) and the mixed to be used for adequate lysis of the samples. The lysed solution was centrifuged and 950 uL of the supernatant was taken. The supernatant was again taken and added to a mixture equal in volume to the supernatant [V(phenol):V(chloroform):V(isoamyl alcohol) = 25:24:1], mixed well, and centrifuged at 12,000 rpm for 10 min. The supernatant was again taken and added to a mixture equal in volume to the supernatant [V(chloroform):V(isoamyl alcohol) = 24:1], then mixed and centrifuged at 12,000 rpm for 10 min. The supernatant was then taken and three-fourths of the supernatant volume of isopropanol was added and precipitated at −20°C. Centrifuged the above mixture at

12,000 rpm for 10 min. After pouring out the supernatant, the obtained precipitate was washed using 1 mL of 75% ethanol and the washing was repeated twice. The DNA was dried on the ultra clean bench and was dissolved in 51 uL of double distilled water. Finally, 1 uL of RNase A was added and the RNA was removed by placing it at 37°C for 15 min. The quality and quantity of extracted DNA were examined using agarose gel electrophoresis and Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific), respectively. The V3–V4 region of the bacterial 16S rDNA gene was amplified using a pair of barcoded fusion primers, 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′). All PCR reactions were carried out with 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs). PCR products were mixed in equi-density ratios and purified using the Qiagen Gel Extraction Kit (Qiagen, Germany). Library quality was assessed using the Qubit@2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on an Illumina NovaSeq platform using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, United States), and 250 bp paired-end reads were generated. The UCHIME Algorithm[1] (Edgar et al., 2011) was used to compare tags with the reference database (Silva database[2]) to remove chimera sequences (Haas et al., 2011).

The FLASH (v.1.2.7[3]) analysis tool was used to merge paired-end reads (Magoè and Salzberg, 2011). Quality filtering on the raw tags was performed to obtain high-quality clean tags (Bokulich et al., 2013) according to the QIIME (v.1.9.1[4]) (Caporaso et al., 2010) quality controlled process. Uparse software (v7.0.1001[5]) (Edgar, 2013) was used to perform sequence analysis. Sequences with ≥97% similarity were assigned to the same operational taxonomic unit (OTU). Alpha diversity was applied to analyze the complexity of species diversity for a sample through four indices, namely, observed-species, Shannon, Simpson, and Good's coverage, using the Mothur method (Schloss et al., 2011). QIIME (v.1.7.0) and R software (v.2.15.3) were used to calculate and display all indices in the experimental samples, respectively. Shannon and Simpson indices were used to identify the community diversity. Good's coverage was used to characterize the sequencing depth. β-diversity analysis was used to determine differences of samples in species complexity. QIIME software (v. 1.9.1) was used to calculate β-diversity on both unweighted and weighted unifrac. And Principal coordinate analysis (PCoA) was used to perform principal coordinates and visualize from complex multidimensional data. WGCNA package, stat packages and ggplot2 package was used to display the PCoA analysis in R software (v. 2.15.3). High-throughput sequencing data in this study are deposited in the NCBI SRA repository, accession number PRJNA733825.

[1]http://www.drive5.com/usearch/manual/uchime_algo.html

[2]http://www.arb-silva.de/

[3]http://ccb.jhu.edu/software/FLASH/

[4]http://qiime.org/scripts/split_libraries_fastq.html

[5]http://drive5.com/uparse/

Distal Intestinal Transcriptome Analysis

Total RNA from distal intestinal tissues was extracted using the TRIzol Reagent (Life Technologies, United States). Agarose gel electrophoresis, NanoDrop microspectrophotometer, and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, United States) were used to determine the quality and quantity of the extracted total RNA. Total RNA was enriched using oligo (dT). Sequencing libraries were constructed on high-quality RNA samples using the NEB #7530 kit (New England Biolabs, #E7530) on an Illumina HiSeq™ 2500 by Gene *Denovo* Biotechnology Co. (Guangzhou, China). Clean reads were filtered using fastp (Chen et al., 2018) (v. 0.18.0) to obtain high-quality reads. The remaining reads were mapped to the reference genome by TopHat2 (Kim et al., 2013) (v. 2.1.1) after rRNA was removed using the short read alignment tool Bowtie2 (Langmead and Salzberg, 2012) (v. 2.2.8). Differentially expressed genes (DEGs) of FMb vs. bL, FMb vs. bH, and bH vs. bHNaB were identified. Each transcript expression level was calculated using the fragments per kilobase of transcript per million mapped reads (FPKM) method. The edgeR package (v. 3.12.1)[6] was used for identifying DEGs between two groups. DEGs with a fold change (FC) ≥ 2 and a *P*-value <0.05 were considered significant. All DEGs were mapped to gene ontology (GO) terms of the GO database[7]. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed using OmicShare tools[8]. The GO and KEGG enrichment statistical analyses were set at corrected *P*-values <0.05 as the threshold for significance. Transcriptome sequencing data in this study are deposited in the NCBI SRA repository, accession number PRJNA735581.

Distal Intestinal Metabolomics Analysis

Six distal intestinal tissue sample replicates of hybrid grouper from each treatment were used for metabolomic analysis. 100 mg of intestinal tissue samples ground in liquid nitrogen were placed in Eppendorf tubes and 500 μL of 80% formaldehyde aqueous solution was added. The samples were subjected to vortex shaking, allowed to stand in an ice bath for 5 min, and centrifuged at 15,000 × *g* at 4°C for 20 min. Mass spectrometry-grade water (liquid chromatograph-mass spectrometer (LC-MS) Grade, Merck, Germany) was added to dilute the formaldehyde content of the sample to a concentration of 53%. The sample was then centrifuged at 15,000 × *g* at 4°C for another 20 min. The supernatant was collected and detected by LC-MS (Vanquish UHPLC, Thermo Fisher, Germany; Q Exactive™ HF, Thermo Fisher, Germany). The liquid chromatograph was equipped with a Hypersil Gold column (100 × 2.1 mm, 1.9 μm). In the assay, an equal volume of sample from each experimental sample was mixed as a quality control (QC) sample, and an aqueous formaldehyde solution at 53% concentration was used as a blank sample. Mass spectrometry scans ranged from 100 to 1500 (mass to charge ratio, m/z), and the electrospray ionization source was set up

[6]http://www.r-project.org/

[7]http://www.geneontology.org/

[8]http://www.omicshare.com/

as follows: spray voltage, 3.2 kV; sheath gas flow rate, 40 arb; auxiliary gas flow rate, 10 arb; and capillary temperature, 320°C. Polarity: positive, negative; MS/MS secondary scans were data-dependent.

The offline data (raw) files were imported into Compound Discoverer 3.1 (CD3.1, Thermo Fisher) to perform a simple filtering of retention time, mass to charge ratio, etc. The different samples were then peak-aligned according to a retention time deviation of 0.2 min and a mass deviation of 5 ppm for more accurate identification. Peaks were then extracted based on a set mass deviation of 5 ppm, signal strength deviation of 30%, signal to noise ratio of 3, and minimum signal intensity of 100,000. The peak area was also quantified, and molecular formulae were predicted by ion peaks and fragment ions and compared with mzCloud[9], mzVault, and Masslist databases to obtain accurate qualitative and relative quantitative results. The softwares R (R version R-3.4.3), Python (Python 2.7.3 version), and CentOS (CentOS release 6.6) were used for statistical analyses. The transformation was performed using area normalization when the data were not normally distributed. QC and quality assurance were used to determine all data. The KEGG[10], HMDB[11], and LIPIDMaps[12] databases were used to annotate the metabolites. MetaX was used to perform principal component analysis and partial least squares discriminant analysis (PLS-DA). The univariate analysis (*t*-test) was used to calculate statistical significance (*P* value). Metabolites with variable importance in projection (VIP) > 1, *P*-value <0.05, and FC ≥ 2 or ≤ 0.5 were considered to be differential metabolites (DMs). DMs of FMb vs. bL, FMb vs. bH, and bH vs. bHNaB were identified. The metabolites of interest were filtered using Volcano plots based on $\log_2$(FC) and $-\log_{10}$(*P*-value) of metabolites. For clustering heat maps, *z*-scores of the intensity areas of the DMs were used to normalize the data. The Pheatmap package was used to plot the data in R. The functions of these metabolites and metabolic pathways were studied using the KEGG database[13]. Metabolic pathway enrichment of the DMs was performed. The pathways were considered to be enriched when ratios were satisfied by x/n > y/N and significantly enriched when $P < 0.05$.

Correlation Analysis of Intestinal Bacteria With Water Pollutants, DEGs, and DMs

The correlation between distal intestinal bacteria and water pollutants, distal intestinal DEGs, and DMs was revealed by Spearman correlation analysis using the Cytoscape software coNetplug-in. The *P*-value and correlation coefficient were not set; * indicated significant differences ($P < 0.05$), ** indicated very significant differences ($P < 0.01$), and *** indicated extremely significant differences ($P < 0.001$).

[9]http://www.mzcloud.org/

[10]http://www.genome.jp/kegg/pathway.html

[11]http://hmdb.ca/metabolites

[12]http://www.lipidmaps.org/

[13]www.metaboanalyst.ca

Statistical Analysis

Under the premise of variance homogeneity, statistical evaluations of pollutants and RT-PCR data were subjected to one-way analysis of variance followed by Tukey's multiple range tests to determine significant differences among the four groups. SPSS (v. 22, SPSS Inc., Chicago, IL, United States) was used as described previously (Guo et al., 2017). The results are presented as mean ± standard error.

RESULTS

Aquaculture Water Quality Determination

After 24 h of water exchange, water samples were collected to determine the quality (**Figure 1**). bH showed a significantly higher ammonia nitrogen concentration than FMb, while the concentration in bL or bHNaB was not significantly different from that in FMb. As for nitrite, significant increases occurred in bH and bHNaB compared with that in FMb and bL, and no significant difference was found between the FMb and bL. TN and TP concentrations showed the same trend as that of ammonia nitrogen, with the concentrations being significantly higher in bH than in FMb, bL, and bHNaB.

Distal Intestinal Microbiota Changes in Hybrid Grouper

Richness and Diversity

A total of 1,218,101 raw reads were observed in all 16 microbial samples (two samples were excluded from each group). Average numbers of raw reads per treatment were 70,628.50 ± 4793.95, 80,868.00 ± 5197.54, 74,132.00 ± 4256.19, and 78,896.75 ± 5944.74 for FMb, bL, bH, and bHNaB, respectively. After clustering OTUs with 97% consistency, we obtained a total of 11,707 OTUs. The average OTUs in bL (916.75 ± 212.02), bH (561.75 ± 46.00), and bHNaB (352.50 ± 17.59) were lower than those in FMb (1095.75 ± 212.02). In addition, the rarefaction curves indicated that the identification rate of new OTUs gradually decreased as the number of reads per sample increased (**Supplementary Figure 1**), while the Good's coverage of all samples exceeded 99%. This indicated that the sequencing results had good accuracy and reproducibility. Compared with FMb and bL, in bH and bHNaB, the Shannon and Simpson indices showed the same significant differences and were decreased, but no difference was found between bH and bHNaB (**Supplementary Figure 2**). The number of unique OTUs in bL was increased, while that in bH and bHNaB decreased (**Figure 2A**). Principal coordinate analysis plots of unweighted and weighted UniFrac matrix distances showed that FMb was separated from bL, bH, and bHNaB, while bH and bHNaB were very close to each other (**Figures 2B,C**).

Distal Intestinal Microbial Composition

At the phylum level, the top 10 phyla in terms of overall relative abundance included Proteobacteria, Cyanobacteria, Firmicutes, Bacteroidetes, and Actinobacteria. The relative abundance of Proteobacteria decreased in bL and increased

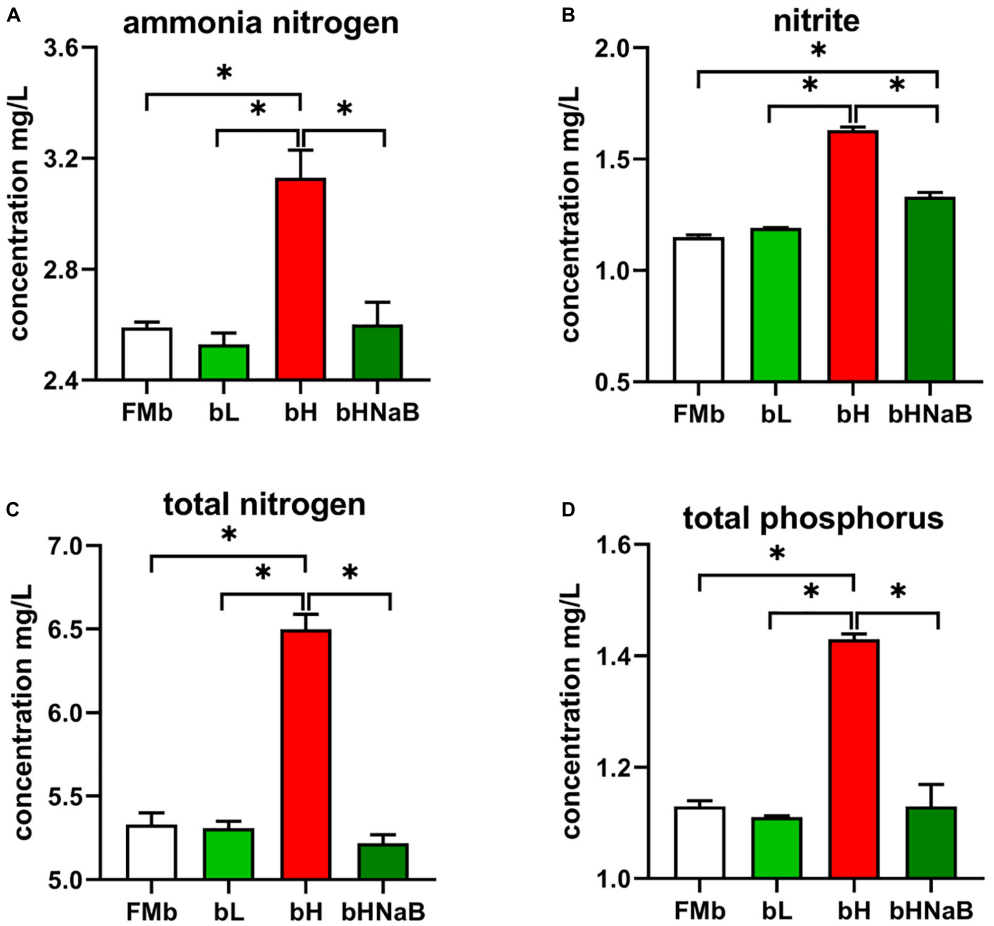


FIGURE 1 | Detection of harmful substances in aquaculture water. The concentrations of **(A)** ammonia nitrogen, **(B)** nitrite, **(C)** total nitrogen concentration, and **(D)** total phosphorus were determined by Specification for Marine Monitoring-Part 4: Seawater analysis (GB 17378.4-2007). *$P < 0.05$.

in bH and bHNaB compared with that in FMb. The relative abundance of Cyanobacteria increased in bL, bH, and bHNaB compared with that in bH. The relative abundance of Firmicutes, Bacteroidetes, and Actinobacteria decreased in all three treatment groups (**Figure 2D**). At the genus level, the dominant bacteria in FMb included *Pseudomonas*, *Prevotellaceae*_UCG-001, *Prevotella*, *Bifidobacterium*, and *Moraxella*. *Pelagibacterium*, *Vibrio*, *Pediococcus*, *Staphylococcus*, and *Lactobacillus* showed a higher abundance in bL than in the other three groups. Classification of bacterial relative abundance showed a higher abundance of *Ralstonia* and *Photobacterium* in bH than in FMb, while the relative abundance of these genera in bL was also lower than that in FMb. The unidentified_*Mitochondria*, unidentified_*Chloroplast*, and *Burkholderia-Caballeronia-Paraburkholderia* showed higher relative abundance in bHNaB than in bH (**Figure 2E**).

Changes in the Distal Intestinal Bacterial Phylotypes

Previous studies have revealed differences in the composition and structure of the bacterial taxa of the distal intestine of hybrid groupers from different treatment groups. To identify the key bacterial taxa with significant differences, we used LEfSe to analyze the differences in the abundance of taxa among the four groups. LEfSe analysis of all the samples revealed that 34 taxa with significantly different taxa information among the four groups were found at the phylum, class, order, family, genus, and species levels. Among them, the relative abundance of 31 bacterial taxa, including Firmicutes, Clostridia, Bacteroidota, Bacteroidia, Bacteroidales, Lachnospiraceae, Lachnospirales, Bacilli, Lactobacillales, and Negativicutes, in FMb was significantly higher than that in the other groups ($P < 0.05$). The relative abundance of one bacterial taxon, Lactobacillaceae, in bL was significantly higher than that in the other groups ($P < 0.05$). The relative abundance of two bacterial taxa, *Ralstonia* and *Ralstonia pickettii*, in the bH group was significantly higher than that in the other groups ($P < 0.05$). No discriminant taxa were found in bHNaB (**Supplementary Figures 3A,B**).

Distal Intestinal Transcriptome Analysis

Identification and Functional Annotation of DEGs

After transcriptome sequencing analysis of 16 distal intestine samples from the four groups (two samples were excluded from each group), a total of 527,803,196 raw reads and 79,170,479,400

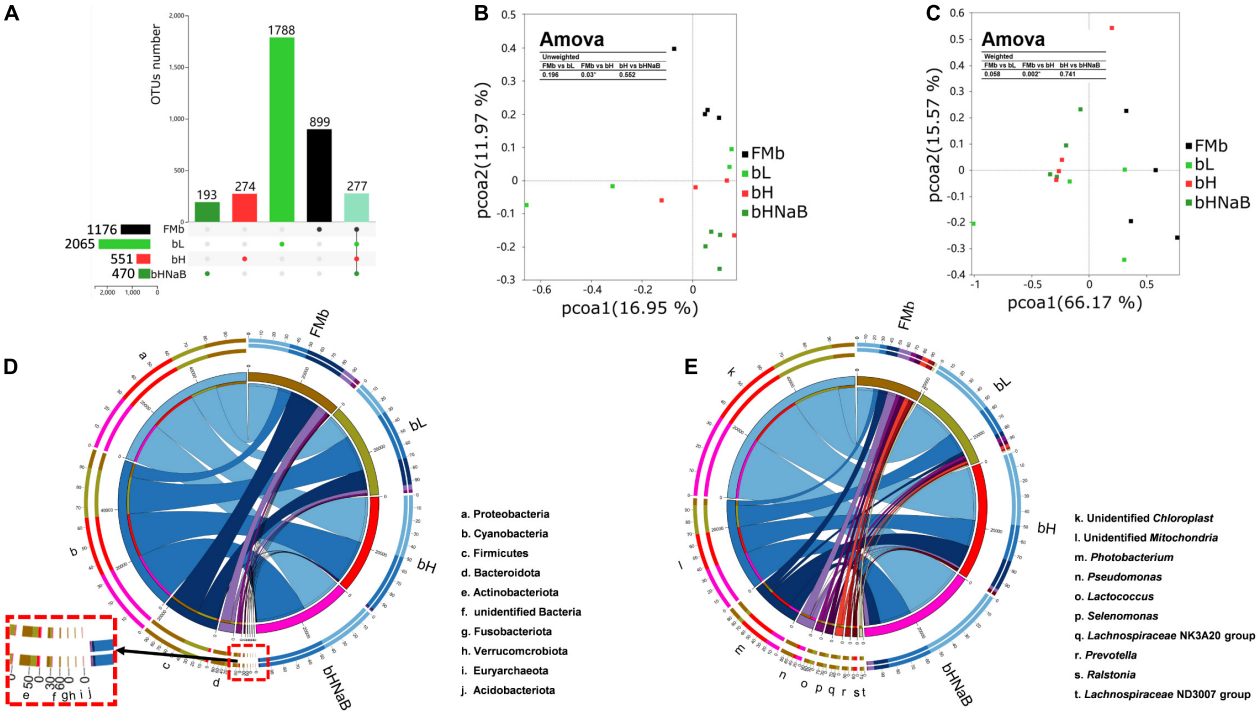


FIGURE 2 | Distal intestinal microbial diversity and composition of hybrid grouper. The numbers of unique and shared OTUs between different treatments indicated by UPSET **(A)**. The horizontal bars indicate the total OTUs for each treatment, the vertical bars indicate the specific OTUs for each treatment, and the rightmost vertical bar indicates the shared OTUs of all treatments. PCoA plots based on unweighted UniFrac metrics **(B)** and weighted UniFrac metrics **(C)**, and Amova analysis was used to determine the significant differences in distance between two groups. Color code, FMb: #000000; bL: #31CB31; bH: #FF3131; bHNaB: #319831; shared OUTs: 91E1BD. Circos graphs of dominant bacterial phyla (top 10) **(D)** and genera (top 10) **(E)** in the distal intestinal. The right semicircle represents the phyla and genera composition of each treatment, and the left semicircle indicates the distribution of each phyla and genera in the different treatments.

raw bases were obtained, from which 526,786,184 clean reads and 78,684,777,954 clean bases were obtained after filtering and QC.

A total of 1878 DEGs (1231 upregulated and 647 downregulated) in FMb vs. bL, 1455 (702 upregulated and 753 downregulated) in FMb vs. bH, and 802 (570 upregulated and 232 downregulated) in bH vs. bHNaB were identified (**Supplementary Figures 4**, **5**).

DEG Trend Analysis

After all the DEGs ($P < 0.05$) were annotated by GO enrichment analysis, the dominant GO terms of the three comparison groups were almost the same. The dominant sub-categories in biological process were "single-organism process," "cellular process," and "metabolic process" (**Figures 3A,D–F**); the dominant sub-categories in molecular function were "binding" and "catalytic activity" (**Figures 3B,G–I**); and the dominant sub-categories in cellular components were "cell," "cell part," and "organelle" (**Figures 3C,J–L**). After significant enrichment analysis, in FMb vs. bL, DEGs were significantly enriched in "cellular component disassembly," "multi-organism process," and "protein complex subunit organization" for biological process; "nucleic acid binding," "protein binding," and "binding" for molecular function; and "organelle part," "organelle," and "intracellular organelle" for cellular component. In FMb vs. bH, DEGs were significantly enriched in "cell cycle," "cell cycle process," and "immune system process" for biological process; "tubulin binding," "cytoskeletal protein binding," and "nucleoside-triphosphatase activity" for molecular function; and "intrinsic component of membrane," "membrane part," and "intracellular non-membrane-bounded organelle" for cellular component. In bH vs. bHNaB, DEGs were significantly enriched in "regulation of protein stability," "organonitrogen compound catabolic process," and "cell cycle" for biological process; "protein binding," "oxidoreductase activity," and "RNA binding" for molecular function; and "organelle," "intracellular organelle," and "intracellular part" for cellular component. According to the KEGG enrichment analysis, DEGs were enriched in six KEGG A classes: "organismal system," "metabolism," "human diseases," "cellular processes," "genetic information processing," and "environmental information processing" (**Figure 4**). Nineteen of 283 pathways were significantly enriched in FMb vs. bL compared with those in FMb; the enriched pathways of bL were "ribosome," "protein digestion and absorption," "*Salmonella* infection," and "peroxisome proliferator-activated receptor (PPAR) signaling" (**Figures 4A,D**). Twenty-six of 261 pathways were significantly enriched in FMb vs. bH compared with those in FMb; the enriched pathways of bH were "cell cycle," "cytokine–cytokine receptor interaction," "PPAR signaling," and "ferroptosis" (**Figures 4B,E**). Twenty of 198 pathways were significantly enriched in bH vs. bHNaB ($P < 0.05$) compared with

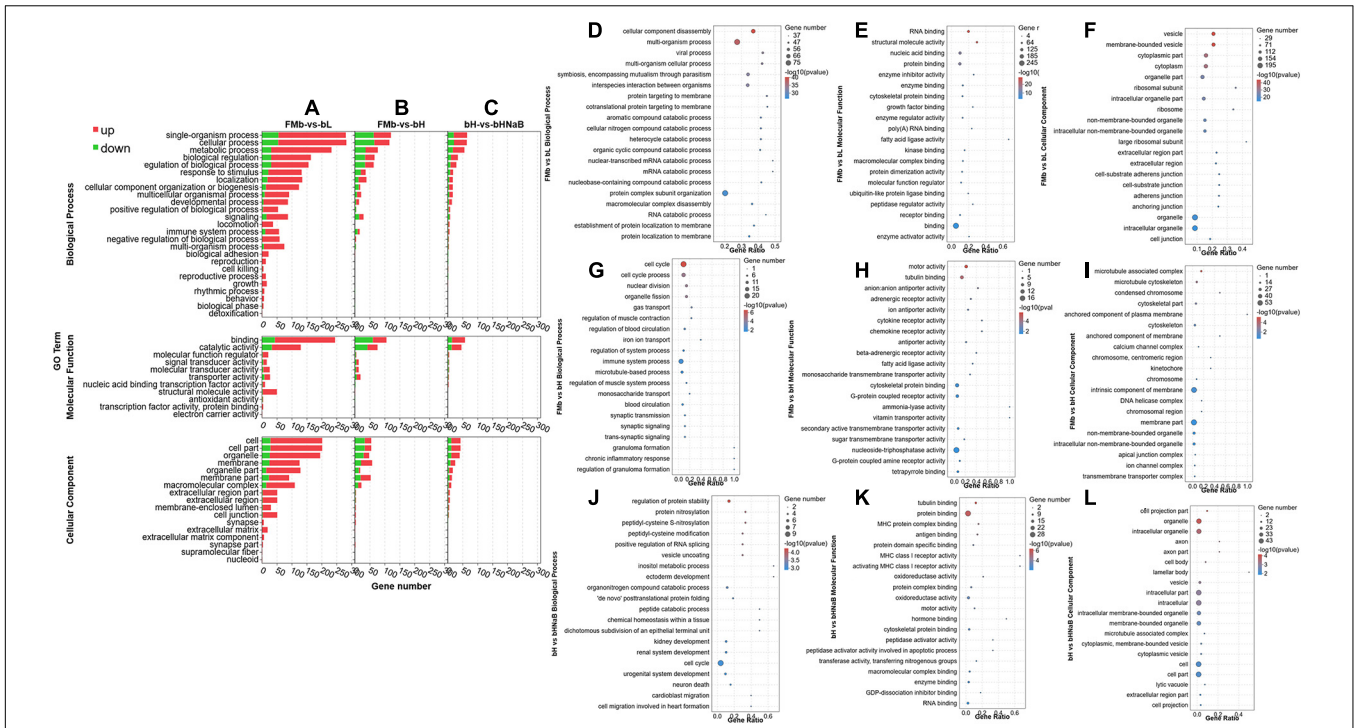


FIGURE 3 | GO classification and enrichment analysis of the DEGs in the distal intestine of hybrid grouper. GO terms of FMb vs. bL, FMb vs. bH and bH vs. bHNaB. Distribution of the top 20 (level-2) GO terms significantly enriched in the categories of biological process, molecular function, and cellular component in FMb vs. bL **(A,D–F)**, FMb vs. bH **(B,G–I)**, and bH vs. bHNaB **(C,J–L)**.

those in bH; the enriched pathways of the bHNaB were "protein processing in endoplasmic reticulum," "cell cycle," and "linoleic acid metabolism" (**Figures 4C,F**).

Identification of Related DEGs

To verify the accuracy of the present transcriptome results, we selected 24 genes related to three aspects, namely, intestinal tight junctions, amino acid transporters, and inflammatory factors, and validated the results using RT-PCR (**Supplementary Figure 6**). The primers used in the experiment (**Supplementary Table 3**) were designed based on the full-length intestinal sequence of hybrid grouper (Zhang, 2020). Overall, the RT-PCR results were generally consistent with the trend of the transcriptome results, indicating that the transcriptome sequencing results were relatively accurate.

Distal Intestinal Metabolome Analysis

Multivariate Analysis of the Metabolite Profiles

We used metabolome analysis to explore the differences in the distal intestinal metabolite profiles of different comparison objects. 24 distal intestine samples were analyzed by LC-MS in both positive and negative ion modes, and the LC-MS spectra are shown in **Supplementary Figure 7** in positive (A) and negative (B) ions. Score plots of the PLS-DA were performed to verify the DMs in different comparison objects, and a multivariate analysis was performed to validate **Supplementary Figure 8**. The samples in each comparison object were clearly separated in both positive and negative ions, and the samples in each group were within the 95% confidence interval (**Supplementary Figures 8A1,A3,B1,B3,C1,C3**), indicating that different doses of 7S and NaB produced significant effects on the distal intestinal metabolic profile of hybrid grouper. Meanwhile, the R^2 value was greater than the Q^2 value, and the intercept of the Q^2 regression line with the Y-axis was less than zero, which indicated that the PLS-DA model in this experiment was not over-fitted and that the model was stable and reliable (**Supplementary Figures 8A2,A4,B2,B4,C2,C4**).

Identification and Functional Annotation of the DMs

Next, we performed DM analysis for the two groups in each comparison object using a VIP threshold of 1 and an FC threshold of 1.5 ($P < 0.05$). Subsequently, hierarchical clustering analysis was performed on the DMs obtained for each group to derive the differences in metabolic expression patterns between and within the two groups of one comparison object. For FMb vs. bL, 161 positively ionized DMs (122 significantly upregulated and 39 significantly downregulated) (**Figures 5A1**, **6A**) and 84 negatively ionized DMs (50 significantly upregulated and 34 significantly downregulated) (**Figures 5A2**, **6B**) were screened. For FMb vs. bH, 154 positively ionized DMs (111 significantly upregulated and 43 significantly downregulated) (**Figures 5B1**, **7A**) and 100 negatively ionized DMs (53 significantly upregulated and 47 significantly downregulated) (**Figures 5B2**, **7B**) were screened. For bH vs. bHNaB, 89 positively ionized DMs (47 significantly upregulated and 42 significantly downregulated) (**Figures 5C1**, **8A**) and 55 negatively

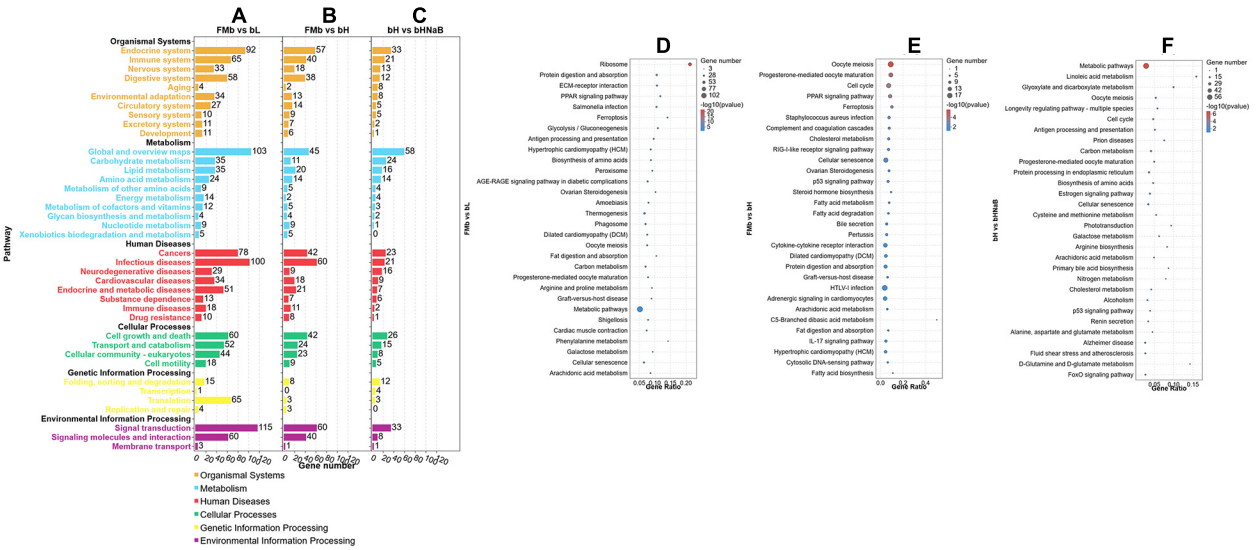


FIGURE 4 | KEGG pathway classification and enrichment analysis of the DEGs in the distal intestine of hybrid grouper. KEGG classification of all DEGs **(A–C)**. Top 30 enriched of KEGG pathways of FMb vs. bL **(D)**, FMb vs. bH **(E)** and bH vs. bHNaB **(F)**.

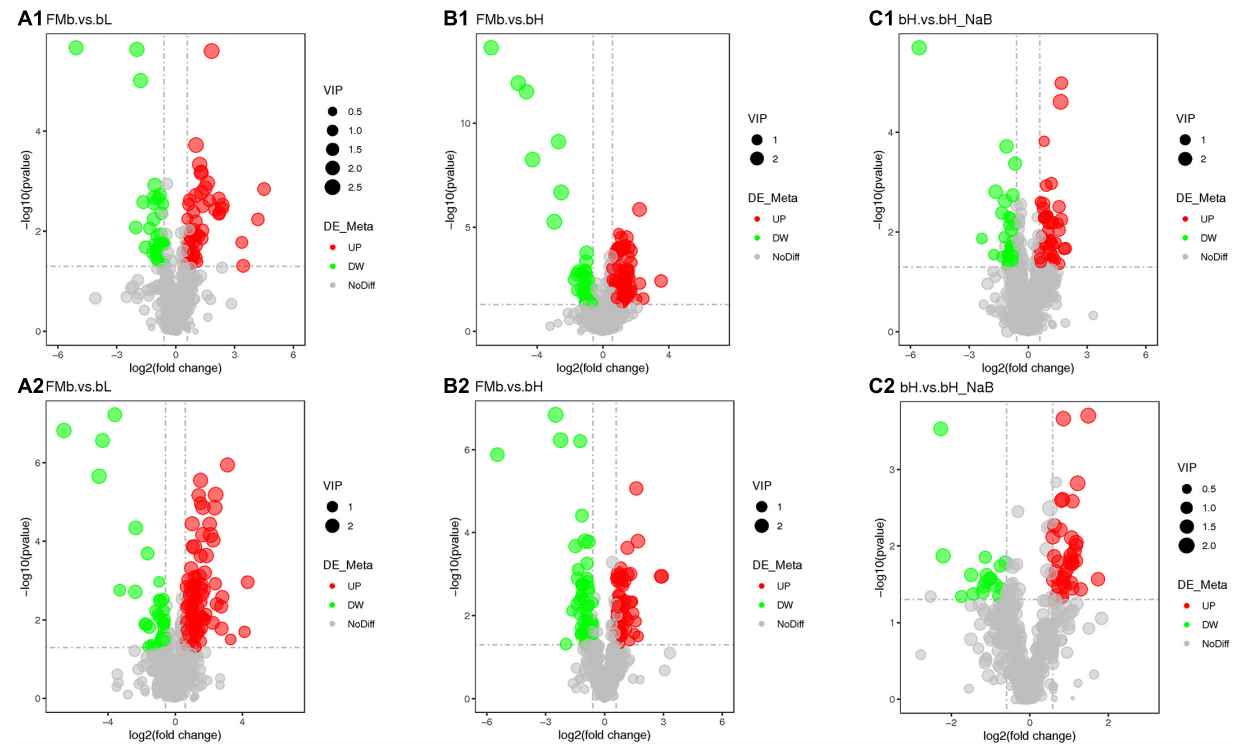


FIGURE 5 | Volcano plots of the DEGs in the distal intestine of hybrid grouper at different comparison objects. FMb vs. bL: positive **(A1)** and negative **(A2)** ions; FMb vs. bH: positive **(B1)** and negative **(B2)** ions; bH vs. bHNaB: positive **(C1)** and negative **(C2)** ions.

ionized DMs (38 significantly upregulated and 17 significantly downregulated) (**Figures 5C2**, **8B**) were screened.

Furthermore, the DMs were analyzed using KEGG annotation. In the FMb vs. bL comparison object, for the positive ionization data, 11 DMs were enriched in 19 pathways, and the "histidine metabolism," "arginine and proline metabolism," "cysteine and methionine metabolism," and "glycine, serine, and threonine metabolism" pathways were enriched (**Figure 9A1**); for the negative ionization data, 46 DMs were enriched in 31 pathways, and the "biosynthesis of unsaturated fatty acids,"

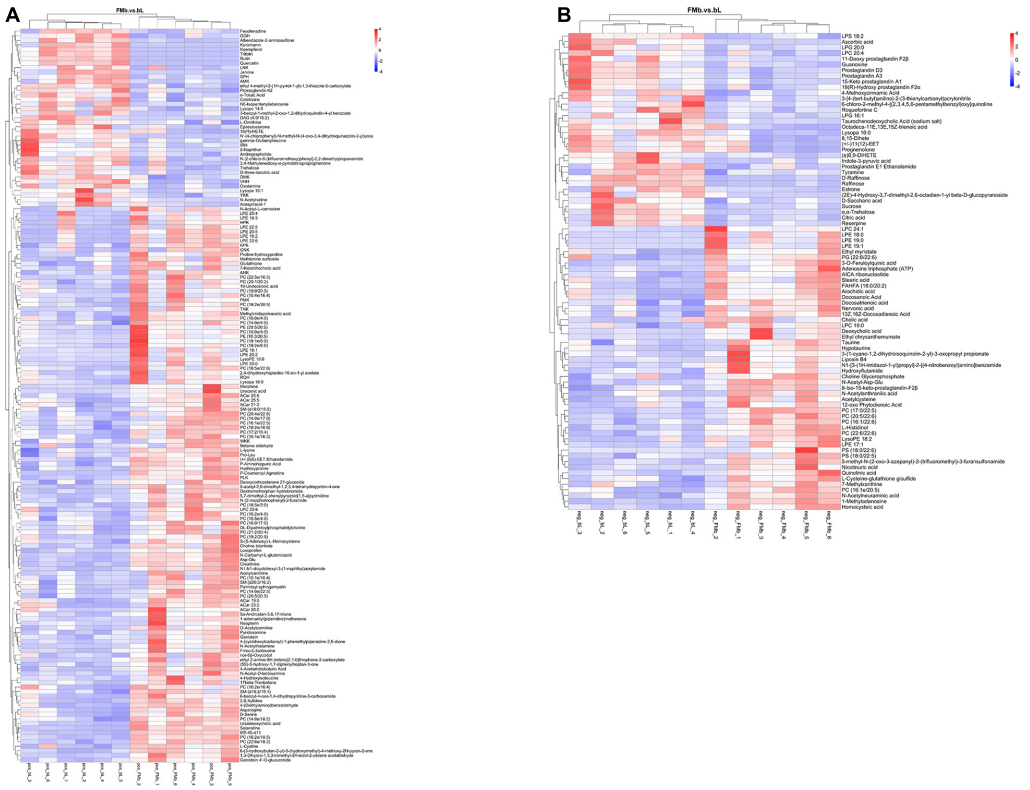


FIGURE 6 | Hierarchical clustering analysis of the DMs in FMb vs. bL: positive **(A)** and negative **(B)** ions. Red and blue indicate that the DMs were upregulated and downregulated, respectively.

"primary bile acid biosynthesis," "fatty acid biosynthesis," and "taurine and hypotaurine metabolism" pathways were enriched (**Figure 9A2**). In the FMb vs. bH comparison object, for the positive ionization data, 70 DMs were enriched in 36 pathways, and the "cysteine and methionine metabolism," "ferroptosis," and "AMP-activated protein kinase (AMPK) signaling" pathways were enriched (**Figure 9B1**); for the negative ionization data, 37 DMs were enriched in 25 pathways, and the "galactose metabolism," "starch and sucrose metabolism," and "amino sugar and nucleotide sugar metabolism" pathways were enriched (**Figure 9B2**). In the bH vs. bHNaB comparison object, for the positive ionization data, 53 DMs were enriched in 33 pathways, and the "histidine metabolism," "glycine, serine and threonine metabolism," "arginine and proline metabolism," and "ATP-binding cassette (ABC) transporters" pathways were enriched (**Figure 9C1**); for the negative ionization data, 35 DMs were enriched in 19 pathways, and the "biosynthesis of amino acids" pathway was enriched (**Figure 9C2**).

Correlations Between the Intestinal Bacteria and Water Pollutants, DEGs, and DMs

We performed Spearman correlation analysis of the top 30 intestinal genera with pollutants (AN, NIT, TN, and TP), DEGs (**Table 1**), and DMs (**Table 2**). In the correlation between intestinal bacteria and pollutants, *Ralstonia* and *Rothia* were positively correlated with changes in ammonia nitrogen and *Lactococcus*, *Pediococcus*, *Pelagibacterium*, *Anaerovibrio*, *Prevotella*, and *Bacillus* were negatively correlated with changes in nitrite (**Figure 10A**). In the correlation between intestinal bacterial and DEGs, *Lactococcus*, *Pediococcus*, and *Pelagibacterium* were positively correlated with changes in *ccnf*, *Numa1*, *pkb*, *spc25*, and *Cks1b* genes (**Figure 10B**). In the correlation between intestinal bacteria and DMs, *Lactococcus* was negatively correlated with changes in sucrose and α,α-trehalose; *Pelagibacterium* was negatively correlated with changes in methylimidazole acetic acid; *Anaerovibrio* was positively correlated with changes in choline, *S*-adenosylhomocysteine, creatinine, urocanic acid, *N*-acetyl histamine, and methylimidazole acetic acid and negatively correlated with changes in quercetin, α,α-trehalose, sucrose, L-fucose, and UDP-*N*-acetylglucosamine; *Prevotella* was positively correlated with changes in *N*-acetyl histamine and methylimidazole acetic acid and negatively correlated with changes in raffinose, quercetin, α,α-trehalose, sucrose, and L-fucose; and *Bacillus* was positively correlated with changes in *S*-adenosylhomocysteine, urocanic acid, *N*-acetyl histamine, and methylimidazole acetic acid and negatively correlated with changes in raffinose, quercetin, and α,α-trehalose (**Figure 10C**). Finally, 10, 4, and 4 potential biomarkers

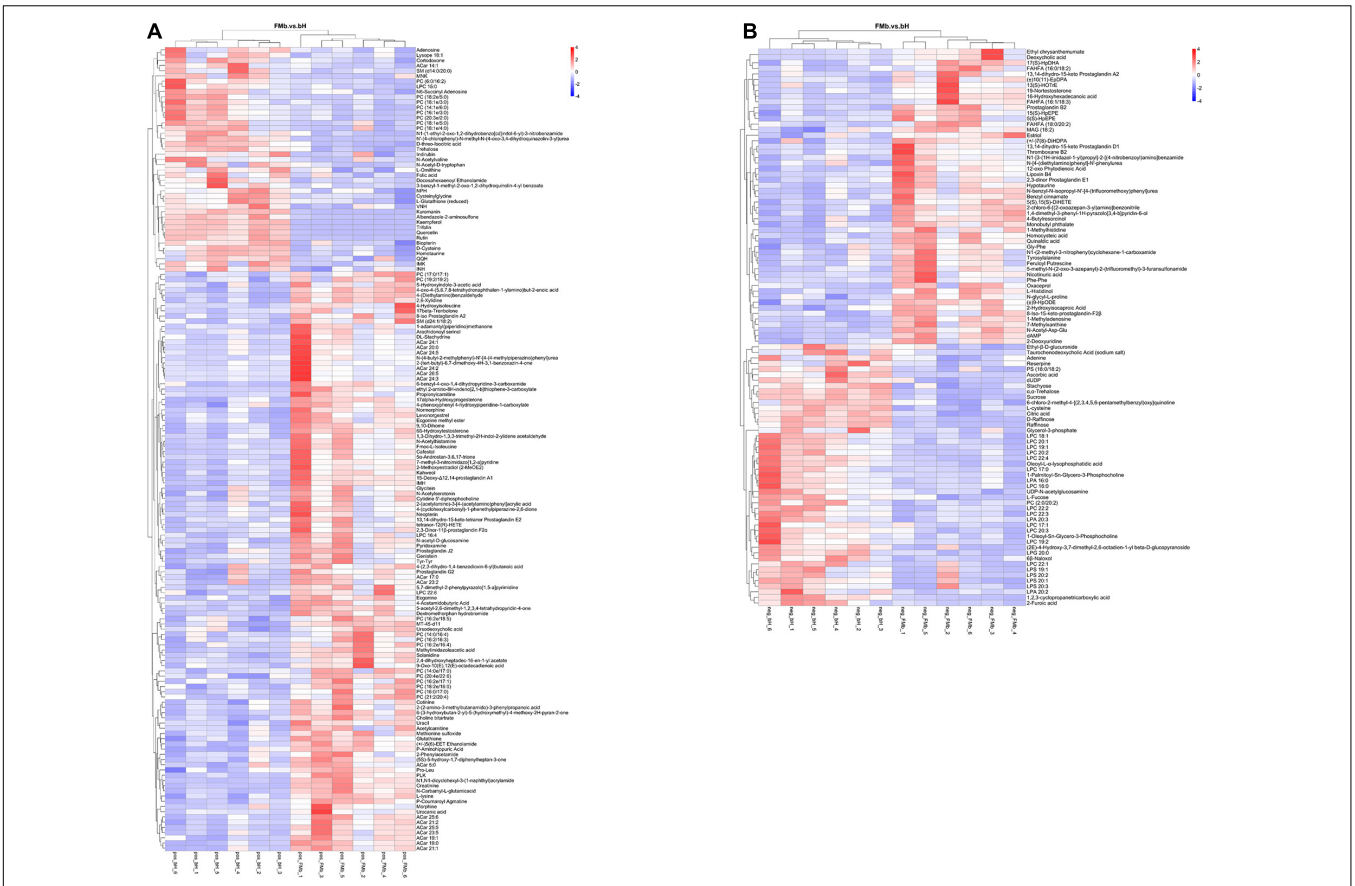


FIGURE 7 | Hierarchical clustering analysis of the DMs in FMb vs. bH: positive **(A)** and negative **(B)** ions. Red and blue indicate that the DMs were upregulated and downregulated, respectively.

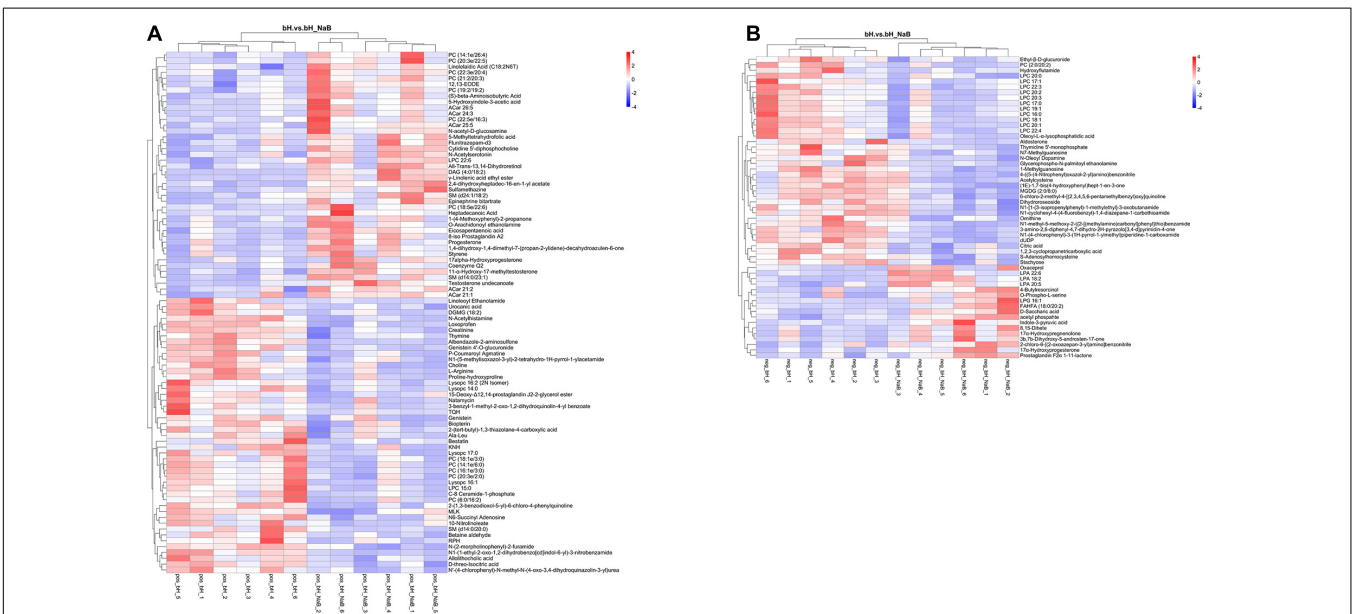


FIGURE 8 | Hierarchical clustering analysis of the DMs in bH vs. bHNaB: positive **(A)** and negative **(B)** ions. Red and blue indicate that the DMs were upregulated and downregulated, respectively.

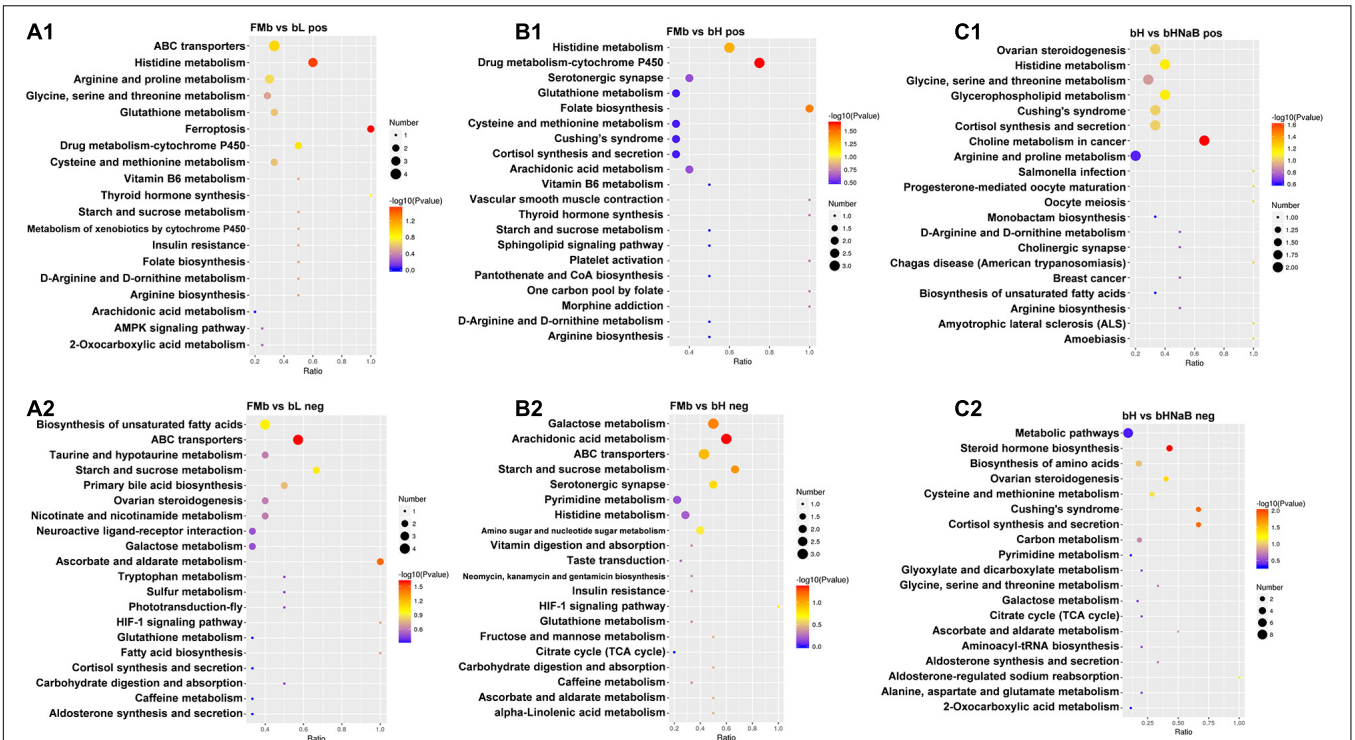


FIGURE 9 | Metabolic pathway enrichment analysis following positive and negative mode ion. DMs were enriched in the different comparison objects. FMb vs. bL: positive **(A1)** and negative **(A2)** ions; FMb vs. bH: positive **(B1)** and negative **(B2)** ions; bH vs. bHNaB: positive **(C1)** and negative **(C2)** ions.

were screened in groups bL, bH, and bHNaB, respectively. ROC analysis showed that the metabolite AUCs all exceeded 0.88 at 95% confidence intervals (**Supplementary Figure 9**), indicating that the potential biomarkers had good predictive power.

DISCUSSION

Distal Intestinal Microbiota in Response to 7S and NaB

The intestinal environment and microbiota interact with food ingested by the host (Wahlström et al., 2016). The intestinal microbiota is quite sensitive to changes in the quality and quantity of food (Wang et al., 2017). In this experiment, we observed that the dominant bacteria were Proteobacteria, Cyanobacteria, Firmicutes, Bacteroidetes, and Actinobacteria, which is consistent with previous results (Li, 2019). The dominant bacteria were influenced by 7S. The process of intestinal microecological dysbiosis is usually accompanied by an increase in Proteobacteria (Shin et al., 2015). This indicates that the intestinal inflammation induced by high-dose 7S in hybrid grouper is closely related to the abundance of Proteobacteria. In addition, the abundance of Proteobacteria and Cyanobacteria were not affected by NaB, and the abundance of Firmicutes, Bacteroidetes, and Actinobacteria decreased further after the addition of NaB compared with that in bH. To further analyze the effects of 7S and NaB on the intestinal microbiota of hybrid grouper, we conducted another assessment at the genus level.

At the genus level, different treatments resulted in different dominant genera in each group. We selected some bacteria that were closely related to the intestinal health of hybrid grouper for analysis. *Pelagibacterium*, *Pediococcus*, *Staphylococcus*, and *Lactobacillus* became the dominant bacteria in the bL. These bacteria played important roles in maintain intestinal resistance to disease and absorption of nutrients (Uymaz et al., 2009; Jiang et al., 2012; Liu and Yu, 2015; Ortiz-Rivera et al., 2017). Therefore, the growth promotion of hybrid grouper by low doses of 7S may be closely related to the enhanced intestinal resistance of these four dominant bacteria (**Supplementary Table 4**). The relative abundance of *Ralstonia* is highly positively correlated with the host inflammatory response (Fu et al., 2017), and a decrease in its relative abundance is effective in alleviating chronic inflammation (Kwon et al., 2018). In addition, increased abundance of *Photobacterium* may disrupt the intestinal morphology of *Litopenaeus vannamei* (Tzuc et al., 2014). The increased abundance of *Ralstonia* and *Photobacterium* might be the cause of intestinal inflammation in hybrid groupers owing to high doses of 7S. After supplementation with NaB, the abundance of *Ralstonia* and *Photobacterium* decreased in bH and became similar to that in FMb and bL. Thus, we speculate that the protective effect of NaB on the distal intestine of hybrid grouper may be achieved by suppressing the abundance of *Ralstonia* and *Photobacterium*, which reduces the risk of intestinal inflammation.

TABLE 1 | Significantly changed DEGs in distal intestine of hybrid grouper.

Gene ID	Function annotation	Log_2(FC)		
		FMb vs. bL	FMb vs. bH	bH vs. bHNaB
Unigene0028315	Lymphokine-activated killer T-cell-originated protein kinase homolog (PKB)	−1.32	−2.30	1.75
Unigene0036995	Protein ECT2 isoform X3 (ECT2)	−1.06	−2.11	1.39
Unigene0057278	LOW QUALITY PROTEIN: G2/M phase-specific E3 ubiquitin-protein ligase (G2E3)	−1.43	−2.02	1.38
Unigene0016769	Reverse transcriptase (pol)	2.24	−0.43	2.35
Unigene0009017	Kinetochore protein Spc25 isoform X1 (spc25)	−0.51	−2.04	1.58
Unigene0019408	G2/mitotic-specific cyclin-B3-like (CCNB3)	−0.78	−2.11	1.54
Unigene0023111	Cyclin-F isoform X1 (ccnf)	−0.96	−2.13	1.43
Unigene0030934	Kinetochore-associated protein 1 isoform X2 (KNTC1)	−0.62	−1.36	1.80
Unigene0059615	Spindle and kinetochore-associated protein 1 (ska1)	−1.17	−2.56	1.89
Unigene0084381	Cyclin-dependent kinases regulatory subunit 1 (Cks1b)	−0.92	−1.51	1.22
Unigene0084467	Mitochondrial fission regulator 2 isoform X2 (mtfr2)	−0.92	−1.55	1.11
Unigene0001450	Forkhead box protein M1 isoform X1 (Foxm1)	−1.85	−4.24	3.09
Unigene0033995	Nuclear mitotic apparatus protein 1 (Numa1)	−1.15	−2.15	1.37
Unigene0033658	Borealin (cdca8)	−1.34	−2.41	1.63
Unigene0049714	Ras GTPase-activating-like protein IQGAP3 (IQGAP3)	−1.86	−2.88	1.76

Distal Intestinal Transcriptome in Response to 7S and NaB

To gain further insight into the regulatory mechanism of 7S and the restorative effects of NaB, the distal intestinal tissue was analyzed using RNA sequencing technology. In this study, based on the GO significant enrichment analysis, 7S and NaB have similarities and differences in the regulation of the distal intestine. We selected the top three GO enrichment sub-categories in each category for our analysis. Ribosomes are the site of intracellular protein synthesis (Dauloudet et al., 2020), and ribosomal protein L31 and L3 contribute to the binding and translation of ribosomal subunits (Petrov et al., 2014; Ueta et al., 2017); furthermore, L7 is an important ribosomal component required for translation process (Pettersson and Kurland, 1980) and plays a critical role in the synthesis of host proteins. A low dose of 7S elevated the expression of associated ribosomal proteins, which may imply that a low dose of 7S effectively promoted the synthesis of distal intestine-associated proteins in hybrid grouper. To test this hypothesis, we further analyzed the expression of genes controlling proteins related to intestinal health and observed that the expression of genes controlling collagen (Claudio et al., 2017), cofilin-1 (Wang et al., 2016), calreticulin (Krzysztof et al., 2017), and tubulin (Saegusa et al., 2014) protein synthesis in the distal intestine was significantly increased at low doses of 7S, suggesting improvement in tight junctions and barrier function in the distal intestine. Fructose-bisphosphate A catalyzes fructose 1,6-diphosphate to produce glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (Katebi and Jernigan, 2015). Therefore, the glycolytic capacity of hybrid grouper was also improved to some extent by promoting the expression of fructose-bisphosphate aldolase A.

When high-dose 7S was administered, the intestinal injury became critical. Mitosis plays an important role in maintaining normal growth and development of individuals (Sanz-Gómez et al., 2020). G2/mitotic-specific cyclin-B1 (Xie et al., 2019), B2 (Waesch and Cross, 2002), and B3 (Garrido et al., 2020) are three proteins essential for mitosis, and their deletion or inhibition of expression usually results in varying degrees of cellular damage, consequently affecting cellular function. We observed that these three cyclins were significantly downregulated, and the expression of genes related to cell cycle regulatory proteins and chromosome structure maintenance, such as cyclin-F (D'Angiolella et al., 2012), double-strand break repair protein (Sinha et al., 2020), and structural maintenance of chromosome protein 2 (Heidelberg, 2011) were inhibited. These results suggest that high doses of 7S inhibit the division of hybrid grouper intestinal cells and disrupt their normal functions. In addition, we observed that the expression of genes associated with ion channels, transient receptor potential cation channel subfamily M, and voltage-dependent L-type calcium channel subunit β-4 in intestinal cells was also negatively affected. The function of ion channels in the cell membrane, in addition to regulating the osmotic pressure inside and outside the cell, is maintaining the cell membrane potential (Page et al., 2005; Wu and Cui, 2014), indicating that high-dose 7S also disrupted distal intestinal cell membrane ion channels, thus, affecting the function of regulating ion transport.

To investigate how NaB protects the distal intestine of hybrid groupers, we supplemented NaB after high-dose 7S treatment. The expression of cyclin B1, B2, and B3, which were repressed at high-dose 7S, was significantly increased after NaB supplementation, and the gene expressions related to transcriptional and translational processes, such as RNA polymerase II transcription, eukaryotic translation initiation factor 1A, and transcription factor GATA-4, were also upregulated. This may indicate that NaB effectively protected the process of mitosis in the intestinal cells of hybrid grouper, allowing the intestinal cells to undergo normal division. NaB is a common and effective inhibitor of cell mitosis, which can inhibit the G1 phase of mitosis in mouse fibroblasts

TABLE 2 | Significantly changed DMs in distal intestine of hybrid grouper.

Metabolites	Log_2 (FC)	Annotated pathways	Classification
FMb vs. bL			
Methylimidazoleacetic acid	1.03	Histidine metabolism	Protein absorption and metabolism
Urocanic acid	1.02		
N-Acetylhistamine	1.55		
Hydroxyproline	1.09	Arginine and proline metabolism	
Creatinine	1.51		
L-Ornithine	−0.72		
L-Cystine	1.16	Cysteine and methionine metabolism	
Glutathione	0.89		
Betaine aldehyde	0.75	Glycine, serine and threonine metabolism	
D-Serine	0.67		
Arachidic acid	1.03	Biosynthesis of unsaturated fatty acids	Lipid utilization and biosynthesis
Stearic acid	1.05		
Docosanoic Acid	0.92		
Nervonic acid	1.36		
Cholic acid	2.24	Primary bile acid biosynthesis	
Taurine	0.92		
Stearic acid	1.05	Fatty acid biosynthesis	
Hypotaurine	0.92	Taurine and hypotaurine metabolism	
Taurine	0.78		
FMb vs. bH			
D-Cysteine	−1.03	Cysteine and methionine metabolism	Protein absorption and metabolism
Glutathione	0.59	Ferroptosis	
Quercetin	−5.13	AMPK signaling pathway	Carbohydrate utilization and metabolism
Sucrose	−1.15	Galactose metabolism	
Raffinose	−2.24		
Stachyose	−1.02		
Sucrose	−1.15	Starch and sucrose metabolism	
α,α-Trehalose	−1.24		
Sucrose	−1.15	ABC transporters	
Raffinose	−2.24		
α,α-Trehalose	−1.24		
UDP-*N*-acetylglucosamine	−1.12	Amino sugar and nucleotide sugar metabolism	
L-Fucose	−0.60		
bH vs. bHNaB			
Urocanic acid	0.63	Histidine metabolism	Protein absorption and metabolism
N-Acetylhistamine	0.81		
Creatinine	1.13	Arginine and proline metabolism	
L-Arginine	1.68		
L-Arginine	1.68	ABC transporters	
Choline	0.88		
Citric acid	0.63	Biosynthesis of amino acids	
O-Phospho-L-serine	−0.73		
S-Adenosylhomocysteine	0.86		

(Wintersberger et al., 2010) and also induce G2 blockade of the human breast cancer cell line MDA-MB-231 (Lallemand et al., 1999). However, the addition of NaB to the post-weaned heifer diet can effectively stimulate the mitosis of small intestinal epithelial cells and inhibit their apoptosis, thus, improving intestinal absorption function and promoting the effective absorption and utilization of nutrients (Rice et al., 2019). We speculate that the reason for this difference in mechanism may be species-related and may also be related to direct addition to cells and to feed; NaB may have the same repair mechanism in the intestine of post-weaned heifer and hybrid grouper. Glyceraldehyde 3-phosphate dehydrogenase can catalyze the oxidation (dehydrogenation) and phosphorylation of glyceraldehyde 3-phosphate to produce 1,3-diphosphoglyceric acid, which is the central link in sugar metabolism and, therefore, plays an important role in glycometabolism (Allonso et al., 2015; Zhang et al., 2016). As a member of the glyceraldehyde 3-phosphate dehydrogenase family, chitinase can catalyze the hydrolysis of chitin to produce *N*-acetylglucosamine (Rosa et al., 2016). Galactosyltransferase can transfer active galactose residues from nucleoside diphosphate galactose to glycosyl receptor molecules (Klohs et al., 2010). The activities of all three enzymes, glyceraldehyde 3-phosphate dehydrogenase, chitinase, and galactosyltransferase, were upregulated after NaB supplementation, suggesting that NaB could also enhance the absorption of glucose in hybrid grouper to some extent by promoting distal intestinal glycometabolism.

Based on the KEGG pathway analysis, the results have similarities and differences with the results of GO annotation. Under low-dose 7S conditions, the formation of ribosome-associated component proteins was promoted, which in turn enabled better protein synthesis by the host. In addition, two signaling pathways, "protein digestion and absorption" and "PPAR signaling," which are closely related to the digestion and absorption of two major nutrients, proteins (Yang et al., 2019) and lipids (Calejman et al., 2020), were also positively affected, suggesting that 7S can significantly promote the absorption and metabolism of proteins and lipids in hybrid groupers. *Salmonella* is a common foodborne pathogen that can enhance bacterial virulence by inhibiting autophagy through the plasmid virulence gene *spvB* (Li et al., 2016). *Salmonella* infection in fish can cause disturbances in the intestinal environment and reduce immunity in fish (Wu et al., 2016). A low dose of 7S improved intestinal immunity in hybrid groupers and might be closely related to suppression of the *Salmonella* infection pathway.

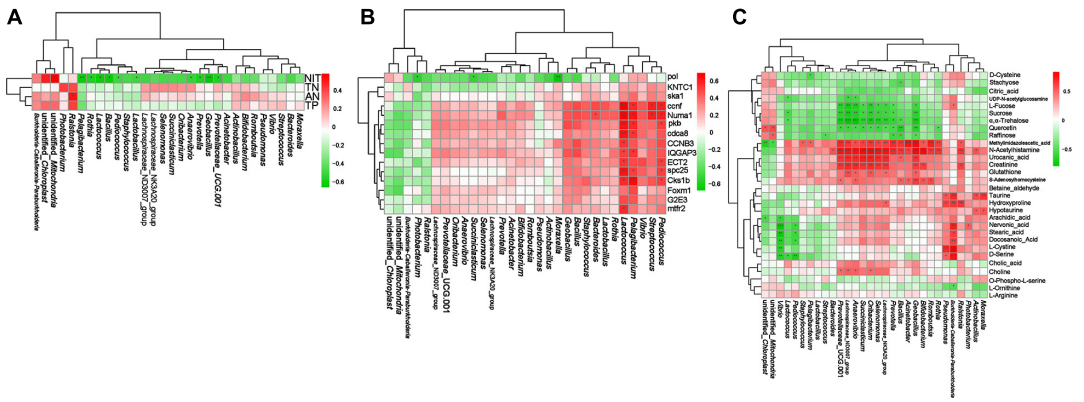


FIGURE 10 | Spearman correlation analysis between distal intestinal bacteria at genus level and water pollutants, DEGs, DMs. The correlation analysis of distal intestinal bacteria and water pollutants **(A)**. The correlation analysis of distal intestinal bacteria and DEGs **(B)**. The correlation analysis of distal intestinal bacteria and DMs **(C)**. Red and green indicate positive and negative correlation, respectively. * significant differences ($P < 0.05$), ** very significant differences ($P < 0.01$), *** extremely significant differences ($P < 0.001$).

Under high-dose 7S conditions, the results were the opposite of the partial results for low-dose 7S. "PPAR signaling pathway" was significantly negatively affected. Ferroptosis is an impairment of intracellular lipid oxide metabolism, with abnormal metabolism catalyzed by iron ions (Yang and Stockwell, 2016). The promotion of "ferroptosis" pathway further illustrates that high-dose 7S leads to disturbances in host lipid metabolism. Similarly, high doses of 7S significantly inhibited "cell cycle" and "cytokine–cytokine receptor interaction," two pathways that are closely related in cell division (Sarraf et al., 2019) and intercellular signaling (Leung, 2004).

After NaB supplementation in the high-dose 7S condition, we observed that the "progesterone-mediated oocyte maturation" pathway was promoted, similar to the results of bL, indicating that NaB enhanced the digestion and absorption of proteins in hybrid grouper. In addition, the "cell cycle" pathway, which was inhibited in bH, was activated after NaB supplementation, indicating that NaB could protect the normal division and maintain the normal function of intestinal cells in hybrid grouper. Linoleic acid is a naturally occurring unsaturated fatty acid with many important physiological functions (Smith et al., 2004). It can promote the secretion of immunoglobulins, and thus, enhance the immunity of fish (Huang et al., 2018), and it can improve the lipid metabolism of fish (Makol et al., 2012). Facilitation of the "linoleic acid metabolism" pathway indicates that NaB can effectively improve lipid utilization by hybrid groupers.

Distal Intestinal Metabolomics in Response to 7S and NaB

To reveal the regulatory mechanism of 7S on the distal intestine of hybrid groupers and the protective effect of NaB in a multidimensional manner, we further utilized the LC-MS technique to analyze the metabolic profile changes in the distal intestine. Low-dose 7S had positive effects on "histidine metabolism," "arginine and proline metabolism," "cysteine and methionine metabolism," and "glycine, serine, and threonine metabolism" in the positive ion mode and positive effects on "biosynthesis of unsaturated fatty acids," "primary bile acid biosynthesis," "fatty acid biosynthesis," and "taurine and hypotaurine metabolism" in the negative ion mode. There are 10 amino acids that fish cannot synthesize in sufficient amount to support maximum growth and must be provided in the diets; these essential amino acids include: methionine, arginine, histidine, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophan and valine (Hua and Bureau, 2019; Nascimento et al., 2020). Proline and glycine are considered as non-essential amino acids, and are involved in key metabolic pathways, they can modulate immune function health, reproduction and growth (Zhao et al., 2015; Li and Wu, 2018). Histidine is involved in the synthesis of hemoglobin in aquatic and terrestrial animals and is important for growth, tissue formation, and repair (Michelato et al., 2017; Khan, 2018). Histidine also inhibits oxidative stress-induced inflammation in human intestinal epithelial cells (Dong et al., 2005). Proline can act as a protective substance for membranes and enzymes and as a free radical scavenger, enhancing the body's antioxidant capacity (Vunnam et al., 2010; Zabirnyk et al., 2010). The immunomodulatory effect of arginine on fish is mainly achieved through the regulation of endocrine hormones by the arginine/nitric oxide pathway, which plays an important role in the regulation of body immunity and protection of intestinal mucosa function (Zhou, 2011). Methionine can improve the antioxidant system by inhibiting apoptosis, and it can also improve immunity by upregulating the tight junction proteins claudin-b, claudin-c, ZO-1, and ZO-2 in the head, kidney, and spleen (Pan et al., 2016). The addition of glycinin to feed can significantly increase the expression of peptide-transporters 1 (PepT1) in the intestines of grass carp (*Cyprinus carpio* L.) and promote the absorption of peptides (Ostaszewska et al., 2010). Serine is an important component of the catalytic active site of serine proteases that can significantly enhance host recognition of pathogens (Liu et al., 2017) and improve the cellular immunity of the organism (Ashton-Rickardt, 2009). Threonine deficiency leads to impaired protein

synthesis and poor proximal intestinal development in grass carp (Gao et al., 2014), upregulates the expression of the pro-inflammatory factors TNF-α and IL-1β, and downregulates the anti-inflammatory factor TGF-β, resulting in increased intestinal inflammation (Dong et al., 2017). These observations combined with the results of this study suggest that 7S could enhance the antioxidant capacity, immunity, and protein hydrolysate absorption efficiency of the distal intestine by promoting the metabolism of essential amino acids.

Highly unsaturated fatty acids (HUFAs) are essential fatty acids for marine fish because they lack the ability to synthesize HUFAs. HUFAs can prevent inflammation caused by high lipid deposition by inhibiting the activity of enzymes related to lipid deposition (Ma, 2008) and also enhance the innate immunity of grass carp by upregulating the expression of TLR22 and MyD88 (Li, 2013). Bile acids (Bertaggia et al., 2017) and taurine (El-Sayed, 2014) can increase the efficiency of intestinal lipid transport and promote lipid absorption. Consistent with these observations, our results suggest that low-dose 7S can promote lipid utilization, inhibit fat production, and avoid inflammation caused by lipid deposition in hybrid groupers.

However, high-dose 7S negatively affected the distal intestine of hybrid grouper from a different perspective. Our results showed that high-dose 7S had negative effects on "cysteine and methionine metabolism," "AMPK signaling pathway," "galactose metabolism," "starch and sucrose metabolism," "ABC transporters," and "amino sugar and nucleotide sugar metabolism" and a positive effect on "ferroptosis." Quercetin could inhibit oxidative stress and inflammatory responses by regulating the AMPK/SIRT1/NF-κB signaling pathway (Zhang et al., 2020), indicating that high-dose 7S might inhibit the absorption and utilization of carbohydrates in the feed of hybrid grouper, resulting in insufficient energy supply. Ferroptosis is caused by the accumulation of reactive oxygen radicals on membrane lipids because of the failure of glutathione peroxidase activity (Doll et al., 2016). Similar to the transcriptome annotation results, in the metabolome, we also found that the "ferroptosis" was activated by high doses of 7S, which further suggests that high-dose 7S could disrupt the normal function of intestinal cells by inducing ferroptosis. Most ABC transporters are extremely energy-dependent and require the transport of various endogenous substrates and xenobiotics across the lipid bilayer via ATP (Popovic et al., 2010). We hypothesize that the negative effect on cell signaling in hybrid grouper is due to 7S inhibition of the uptake and utilization of carbohydrates, such as sucrose, raffinose, and α,α -trehalose.

After supplementation with NaB in the context of high-dose 7S, the DMs closely related to essential amino acid metabolism in hybrid groupers, such as urocanic acid, betaine aldehyde, choline, creatinine, and L-arginine, were all upregulated. Consequently, the uptake and utilization of protein hydrolysis products by hybrid grouper were improved. One of the reasons why NaB can be utilized in livestock animals is that it can supply energy to intestinal epithelial cells (Huang et al., 2015). ATP is required for the proper functioning of ABC transporters, which suggests that the addition of NaB to the feed of hybrid grouper can effectively supply energy to the intestinal cells and, thus, promote the efficiency of ABC transporters and biosynthesis of amino acids.

Correlation Between Distal Intestinal Microbiota and Water Pollutants and Host Health

The intestinal microbiota co-evolved with the host and plays an important role in host nutrient absorption, metabolism, information transfer, and disease infection (Carlos Magno Da Costa et al., 2015; Jones and Guillemin, 2018; Eckel, 2021; Post et al., 2021). Imbalance of intestinal microbiota can disrupt the intestinal internal environment, which is an important site for nutrient absorption and digestion (Liu et al., 2020). This leads to the excretion of unabsorbed nitrogen and phosphorus from the feed, causing pollution of farm water (Wu et al., 2019). In this study, the changes in the abundance of *Ralstonia* were significantly positively correlated with the ammonia nitrogen content, indicating that the increase in *Ralstonia* relative abundance may be the main reason for the high TN content in water. *Lactobacillus* can enhance the uptake of amino acids mediated by PepT1 in in mice with spontaneous colitis (Chen et al., 2010). *Pediococcus* is able to secrete proteases that accelerate protein hydrolysis (Afriani et al., 2018). These results indicate that these bacteria might affect the hydrolysis and absorption processes of nitrogen-containing nutrients, such as proteins, in the diet of hybrid grouper. The abundance of *Pediococcus*, *Pelagibacterium,* and *Lactococcus* was significantly positively correlated with the expression of *ccnf*, *Numa1*, *pkb*, *spc25*, and *Cks1b* genes associated with cell proliferation, suggesting that these bacteria may play important roles in the proliferation of distal intestinal cells in hybrid grouper. The changes in the abundance of *Prevotella* were significantly negatively correlated with nitrite content and significantly positively correlated with methylimidazole acetic acid and *N*-acetyl histamine levels. *Prevotella* has proteolytic activity, a function similar to that of exopeptidases, with positive effects on protein degradation and utilization of hydrolysis products (Griswold and Mackie, 1997), and plays an important role in carbohydrate utilization (Durb'An et al., 2013; Aakko et al., 2020), indicating that the decrease in *Prevotella* abundance caused by high-dose β-conglycinin had a more serious negative effect on the response to nitrogenous nutrients and "carbohydrate utilization and metabolism" in hybrid groupers. The decrease in the abundance of *Anaerovibrio* was significantly negatively correlated with nitrite content and significantly positively correlated with methylimidazole acetic acid, *N*-acetyl histamine, urocanic acid, creatinine, glutathione, and *S*-adenosylhomocysteine levels. *Anaerovibrio* mainly utilizes lipids in the intestine, yet its abundance is highly correlated with metabolites associated with protein absorption and metabolism, which may be related to hindering the utilization of lipids by *Anaerovibrio* to produce unsaturated fatty acids (Castagnino et al., 2015), thereby reducing protein absorption by the host. The increase in the abundance of *Pelagibacterium* was significantly negatively correlated with nitrite level and positively correlated with methylimidazole

acetic acid level, indicating that low-dose 7S may promote protein absorption and metabolism in hybrid grouper and, thus, reduce nitrogen emissions. The increase in the abundance of *Burkholderia-Caballeronia-Paraburkholderia* in bHNaB was significantly positively correlated with taurine, hydroxyproline, nervonic acid, stearic acid, docosanoic acid, L-cystine, and D-serine levels, suggesting that supplementation with NaB significantly improved lipid utilization and biosynthesis in hybrid grouper possibly regulated by *Burkholderia-Caballeronia-Paraburkholderia*. A number of highly relevant bacteria, genes, and metabolites were selected separately in this study, which could serve as potential biomarkers for evaluating the regulation of 7S and NaB in the intestine of hybrid grouper.

CONCLUSION

In this study, altered intestinal flora affected aquaculture water quality, host transcription, and metabolism, thus, affecting host health. Two bacteria, *Ralstonia* and *Rothia*, had negative effects on water quality, and *Lactococcus*, *Pelagibacterium*, *Anaerovibrio*, *Prevotella*, and *Bacillus* had positive effects. Low-dose 7S significantly increased the relative abundance of *Pelagibacterium*, *Pediococcus*, and *Staphylococcus*. High-dose 7S increased the probability of distal intestinal inflammation by increasing the relative abundance of pathogenic bacteria *Ralstonia* and *Photobacterium*, while NaB effectively inhibited the relative abundance of pathogenic bacteria *Ralstonia* and *Photobacterium*. Gene expression also showed significant differences between the treatments. The pathways involved in the regulation of low-dose 7S include "ribosome," "protein digestion and absorption," and "PPAR signaling" to promote protein synthesis and lipid uptake in hybrid grouper; the pathways involved in the regulation of high-dose 7S include "cell cycle," "cytokine–cytokine receptor interaction," and "PPAR signaling," which interfered with the normal nutrient absorption function of intestinal cells in hybrid grouper. The pathways involved in the regulation of NaB supplementation include "protein processing in endoplasmic reticulum," "cell cycle," and "linoleic acid metabolism." Distal intestinal metabolism was observed, and low-dose 7S mainly affected pathways associated with protein absorption, metabolism, and lipid utilization and biosynthesis; high-dose 7S mainly affected pathways associated with carbohydrate utilization and metabolism; and NaB supplementation contributed to protein absorption and metabolism. In addition, 15 DM markers were identified, including UDP-*N*-acetylglucosamine, L-fucose, sucrose, α,α-trehalose, quercetin, raffinose, methylimidazole acetic acid, *N*-acetyl histamine, urocanic acid, creatinine, *S*-adenosylhomocysteine, and choline. Above all, we initially predicted some potential biomarkers associated with water pollutants, host microbiota and genes based on Spearman association analysis, proving that cell proliferation and utilization of three major nutrients (protein, lipid, carbohydrate) might be potential targets for treating the negative effects caused by 7S in hybrid grouper, but further in-depth validation is highly imperative.

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 protocol was approved by the Ethics Review Board of Guangdong Ocean University. All procedures were performed according to the standards of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023, revised 1978) and relevant Chinese policies.

AUTHOR CONTRIBUTIONS

All of the indicated authors have actively contributed to this study. HL and BT designed the study. BY conducted the study and analyzed the data. XD participated in the interpretation of results. BY wrote the manuscript. QY, SC, and SZ purchased the reagents. HL revised the manuscript. All authors have read and approved the final version of the manuscript.

FUNDING

This work was supported by the National Key R&D Program of China (Grant Number: 2019YFD0900200), the National Natural Science Foundation of China (Grant Number: 31772864), and the Natural Science Foundation of Guangdong Province (Grant Numbers: 2018A030313154 and 2020A1515011129).

ACKNOWLEDGMENTS

We would like to thank Shanghai Menon Animal Nutrition Technology Co., Ltd. (Shanghai, China) for kindly providing the sodium butyrate in this experiment.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.705332/full#supplementary-material

Supplementary Figure 1 | Rarefaction curves of the samples.

Supplementary Figure 2 | Differences in hybrid grouper bacterial diversity and richness.

Supplementary Figure 3 | Differences analysis among the groups. **(A)** LefSe cladogram. **(B)** LDA score (3.90) of LEfSe.

Supplementary Figure 4 | The statistics on the number of significantly different genes in different comparison objects.

Supplementary Figure 5 | Volcano plots of the DEGs in the distal intestine of hybrid grouper. **(A)** FMb vs. bL. **(B)** FMb vs. bH. **(C)** bH vs. bHNaB.

Supplementary Figure 6 | Comparison of the RT-PCR and RNA Seq results.

Supplementary Figure 7 | The LC-MS spectra of the distal intestine contents in positive **(A)** and negative **(B)** modes.

Supplementary Figure 8 | Partial least squares discriminant analysis (PLS-DA) plot of distal intestinal of the FMb and bL following positive **(A1,A2)** and negative **(A3,A4)** ions; FMb and bH following positive **(B1,B2)** and negative **(B3,B4)** ions; bH and bHNaB following positive **(C1,C2)** and negative **(C3,C4)** ions.

Supplementary Figure 9 | ROC analysis for discrimination among the groups for the potential biomarker metabolites in positive and negative modes.

Supplementary Table 1 | Composition of the diets.

Supplementary Table 2 | Amino acid profile (%) of the diets used in the experiment.

Supplementary Table 3 | Primers sequences used for real-time quantitative PCR.

Supplementary Table 4 | Growth parameters and feed utilization of juvenile hybrid grouper fed the experimental diets for 8 weeks.

REFERENCES

Aakko, J., Pietil, S., Toivonen, R., Rokka, A., Mokkala, K., Laitinen, K., et al. (2020). A carbohydrate-active enzyme (CAZy) profile links successful metabolic specialization of Prevotella to its abundance in gut microbiota. *Sci. Rep.* 10:12411.

Afriani, Arnim, Marlida, Y., and Yuherman (2018). Isolation and characterization of lactic acid bacteria proteases from Bekasam for use as a beef tenderizer. *Pak. J. Nutr.* 17, 361–367. doi: 10.3923/pjn.2018.361.367

Albino, M. R., Souza, C. L. D., Damiani, T. C., Alberto, R. H., Silva, V. F., Fontana, V. M., et al. (2012). Sodium butyrate decreases the activation of NF-κB reducing inflammation and oxidative damage in the kidney of rats subjected to contrast-induced nephropathy. *Nephrol. Dial. Transplant.* 27, 3136–3140. doi: 10.1093/ndt/gfr807

Allonso, D., Andrade, I. S., Conde, J. N., Coelho, D. R., Rocha, D. C. P., Silva, M. L. D., et al. (2015). Dengue virus NS1 protein modulates cellular energy metabolism by increasing glyceraldehyde-3-phosphate dehydrogenase activity. *J. Virol.* 89, 11871–11883. doi: 10.1128/jvi.01342-15

Ashton-Rickardt, P. G. (2009). Serine protease inhibitors and T lymphocyte immunity. *Curr. Immunol. Rev.* 5, 187–199. doi: 10.2174/157339509788921256

Bertaggia, E., Jensen, K. K., Castro-Perez, J., Xu, Y., Paolo, G. D., Chan, R. B., et al. (2017). Cyp8b1 ablation prevents western diet-induced weight gain and hepatic steatosis due to impaired fat absorption. *Am. J. Physiol. Endocrinol. Metab.* 2, E121–E133.

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

Calejman, C. M., Trefely, S., Entwisle, S. W., Luciano, A. K., and Guertin, D. A. (2020). mTORC2-AKT signaling to ATP-citrate lyase drives brown adipogenesis and de novo lipogenesis. *Nat. Commun.* 11, 1–16.

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

Carlos Magno Da Costa, M., Sandra Bertelli Ribeiro De, C., Gustavo Torres De, S., Cristiano, R., Francisco Carlos Da, G., Maria Anete Santana, V., et al. (2015). Intestinal microbiota as modulators of the immune system and neuroimmune system: impact on the host health and homeostasis. *J. Immunol. Res.* 2015:931574.

Castagnino, P. S., Messana, J. D., Fiorentini, G., Jesus, R. D., Vito, E. S., Carvalho, I., et al. (2015). Glycerol combined with oils did not limit biohydrogenation of unsaturated fatty acid but reduced methane production in vitro. *Anim. Feed Sci. Technol.* 201, 14–24. doi: 10.1016/j.anifeedsci.2014.12.004

Chen, H. Q., Shen, T. Y., Zhou, Y. K., Zhang, M., Chu, Z. X., Hang, X. M., et al. (2010). *Lactobacillus plantarum* consumption increases PepT1-mediated amino acid absorption by enhancing protein kinase C activity in spontaneously colitic mice. *J. Nutr.* 140, 2201–2206. doi: 10.3945/jn.110.123265

Chen, S. F., Zhou, Y. Q., Chen, Y. R., and Gu, J. (2018). Fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34, i884–i890.

China, S. A. O. (2007). *"PRC National Standard", in: The specification for marine monitoring-Part 4: Seawater analysis*. China: State Administration for Market Regulation.

Claudio, F. D., Muglia, C. I., Smaldini, P. L., Delgado, M. L. O., Trejo, F. M., Grigera, J. R., et al. (2017). Use of a collagen membrane to enhance the survival of primary intestinal epithelial cells. *J. Cell. Physiol.* 232, 2489–2496. doi: 10.1002/jcp.25594

D'Angiolella, V., Donato, V., Forrester, F. M., Jeong, Y.-T., Pellacani, C., Kudo, Y., et al. (2012). Cyclin F-mediated degradation of ribonucleotide reductase M2 controls genome integrity and DNA repair. *Cell* 149, 1023–1034. doi: 10.1016/j.cell.2012.03.043

Dauloudet, O., Neri, I., Walter, J. C., Dorignac, J., Geniet, F., and Parmeggiani, A. (2020). Modelling the effect of ribosome mobility on the rate of protein synthesis. *Eur. Phys. J. E* 44, 60–66.

Doll, S., Proneth, B., Tyurina, Y. Y., Panzilius, E., Kobayashi, S., Ingold, I., et al. (2016). ACSL4 dictates ferroptosis sensitivity by shaping cellular lipid composition. *Nat. Chem. Biol.* 13, 91–98. doi: 10.1038/nchembio.2239

Dong, O. S., Satsu, H., and Shimizu, M. (2005). Histidine inhibits oxidative stress- and TNF-α-induced interleukin-8 secretion in intestinal epithelial cells. *FEBS Lett.* 579, 4671–4677. doi: 10.1016/j.febslet.2005.07.038

Dong, Y. W., Jiang, W. D., Liu, Y., Wu, P., Jiang, J., Kuang, S. Y., et al. (2017). Threonine deficiency decreased intestinal immunity and aggravated inflammation associated with NF-kappa B and i signalling pathways in juvenile grass carp (*Ctenopharyngodon idella*) after infection with *Aeromonas hydrophila*. *Br. J. Nutr.* 118, 92–108. doi: 10.1017/s0007114517001830

Duan, Y. F., Zhang, Y., Dong, H. B., Wang, Y., Zheng, X. T., and Zhang, J. S. (2017). Effect of dietary Clostridium butyricum on growth, intestine health status and resistance to ammonia stress in Pacific white shrimp *Litopenaeus vannamei*. *Fish Shellfish Immunol.* 65, 25–33. doi: 10.1016/j.fsi.2017.03.048

Durb'An, A., Abell'An, J., Latorre, A., and Moya, A. (2013). Effect of dietary carbohydrate restriction on an obesity-related Prevotella-dominated human faecal microbiota. *Metagenomics* 2, c1–c4.

Eckel, J. (2021). Intestinal microbiota and host metabolism a complex relationship. *Acta Physiol.* 232:13638.

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

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

El-Sayed, A. F. M. (2014). Is dietary taurine supplementation beneficial for farmed fish and shrimp? a comprehensive review. *Rev. Aquaculture* 6, 241–255. doi: 10.1111/raq.12042

Fu, J. H., Li, G. F., Wu, X. M., and Zang, B. (2019). Sodium butyrate ameliorates intestinal injury and improves survival in a rat model of cecal ligation and puncture-induced sepsis. *Inflammation* 42, 1276–1286. doi: 10.1007/s10753-019-00987-2

Fu, L. L., Song, J. Y., Wang, C., Fu, S. J., and Wang, Y. B. (2017). *Bifidobacterium infantis* potentially alleviates shrimp tropomyosin-induced allergy by tolerogenic dendritic cell-dependent induction of regulatory T cells and alterations in gut microbiota. *Front. Immunol.* 8:1536. doi: 10.3389/fimmu.2017.01536

Gao, Y. J., Yang, H. J., Liu, Y. J., Chen, S. J., Guo, D. Q., Yu, Y. Y., et al. (2014). Effects of graded levels of threonine on growth performance, biochemical parameters and intestine morphology of juvenile grass carp *Ctenopharyngodon idella*. *Aquaculture* 42, 113–119. doi: 10.1016/j.aquaculture.2013.12.043

Garrido, D., Bourouh, M., Bonneil, R., Thibault, P., and Archambault, V. (2020). Cyclin B3 activates the anaphase-promoting complex/cyclosome in meiosis and mitosis. *PLoS Genet.* 16:e1009184. doi: 10.1371/journal.pgen.1009184

Griffith, W. G., and Shaw, D. S. (1998). Polymorphisms in *Phytophthora infestans*: four mitochondrial haplotypes are detected after PCR amplification of DNA from pure cultures or from host lesions. *Appl. Environ. Microbiol.* 64, 4007–4014. doi: 10.1128/aem.64.10.4007-4014.1998

Griswold, K. E., and Mackie, R. I. (1997). Degradation of protein and utilization of the hydrolytic products by a predominant ruminal bacterium, *Prevotella ruminicola* B1(4). *J. Dairy Sci.* 80:167. doi: 10.3168/jds.s0022-0302(97)75924-1

Guo, X. Z., Ran, C., Zhang, Z., He, S. X., Jin, M., and Zhou, Z. G. (2017). The growth-promoting effect of dietary nucleotides in fish is associated with an intestinal microbiota-mediated reduction in energy expenditure. *J. Nutr.* 147, 781–788. doi: 10.3945/jn.116.245506

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

Heidelberg, S. B. (2011). *Structural Maintenance of Chromosomes Protein*. Cambridge, MA: UniProt.

Hua, K., and Bureau, D. P. (2019). Estimating changes in essential amino acid requirements of rainbow trout and Atlantic salmon as a function of body weight or diet composition using a novel factorial requirement model. *Aquaculture* 513, 734440–734440. doi: 10.1016/j.aquaculture.2019.734440

Huang, C., Song, P. X., Fan, P. X., Hou, C. L., Thancker, P., and Ma, X. (2015). Dietary sodium butyrate decreases postweaning diarrhea by modulating intestinal permeability and changing the bacterial communities in weaned piglets. *J. Nutr.* 145, 2774–2780. doi: 10.3945/jn.115.217406

Huang, Y. S., Lin, Z. D., Rong, H., Hao, M. L., Zhu, D. S., Li, S. K., et al. (2018). Effects of conjugated linoleic acid on growth, body composition, antioxidant status, lipid metabolism and immunity parameters of juvenile Chu's croaker, *Nibea coibor*. *Aquac. Res.* 49, 546–556. doi: 10.1111/are.13486

Jesus, G. F. A., Pereira, S. A., Owatari, M. S., Nicholas, S., Silva, B. C., Allan, S., et al. (2018). Protected forms of sodium butyrate improve the growth and health of *Nile tilapia* fingerlings during sexual reversion. *Aquaculture* 499:S0044848618310305.

Jiang, S., Wu, X., Luo, Y., Wu, M., Lu, S., Jin, Z., et al. (2016). Optimal dietary protein level and protein to energy ratio for hybrid grouper (*Epinephelus fuscoguttatus* ♀×*Epinephelus lanceolatus* ♂) juveniles. *Aquaculture* 465, 28–36. doi: 10.1016/j.aquaculture.2016.08.030

Jiang, X. W., Huo, Y. Q., Cheng, H., Zhang, X. Q., and Wu, Z. M. (2012). Cloning, expression and characterization of a halotolerant esterase from a marine bacterium *Pelagibacterium halotolerans* B2T. *Extremophiles* 16, 427–435. doi: 10.1007/s00792-012-0442-3

Jiang, Y., Wang, Y., Zhang, Z., Liao, M., and Chen, G. (2019). Responses of microbial community structure in turbot (*Scophthalmus maximus*) larval intestine to the regulation of probiotic introduced through live feed. *PLoS One* 14:e0216590. doi: 10.1371/journal.pone.0216590

Jones, T. A., and Guillemin, K. (2018). Racing to stay put: how resident microbiota stimulate intestinal epithelial cell proliferation. *Curr. Pathobiol. Rep.* 6, 1–6.

Katebi, A. R., and Jernigan, R. L. (2015). Aldolases utilize different oligomeric states to preserve their functional dynamics. *Biochemistry* 54, 3543–3554. doi: 10.1021/acs.biochem.5b00042

Khan, M. A. (2018). Histidine requirement of cultivable fish species: a review. *Oceanogr. Fish.* 8, 1–7.

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., and Salzberg, S. L. (2013). TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol.* 14, 1–13.

Klohs, W. D., Wilson, J. R., Weiser, M. M., Frankfurt, O., and Bernacki, R. J. (2010). Galactosyltransferase activity and cell growth: uridine diphosphate (UDP) galactose inhibition of murine leukemic. *J. Cell. Physiol.* 119, 23–28. doi: 10.1002/jcp.1041190105

Krzysztof, G., Maciej, U., Jaroslaw, S., Kedzierska, A. E., Rafal, K., Anna, J., et al. (2017). The novel type 1 fimbriae fimh receptor calreticulin plays a role in *Salmonella* host specificity. *Front. Cell. Infect. Microbiol.* 7:326. doi: 10.3389/fcimb.2017.00326

Kwon, M. S., Ki, L. S., Jang, J. Y., Lee, J., Kyeong, P. H., Kim, N. H., et al. (2018). *Lactobacillus sakei* WIKIM30 ameliorates atopic dermatitis-like skin lesions by inducing regulatory t cells and altering gut microbiota structure in mice. *Front. immunol.* 9:1905. doi: 10.3389/fimmu.2018.01905

Lallemand, F., Courilleau, D., Fagot, B., Atfi, A., Montagne, M. N., and Mester, J. (1999). Sodium butyrate induces G2 arrest in the human breast cancer cells MDA-MB-231 and renders them competent for DNA rereplication. *Exp. Cell Res.* 247, 432–440. doi: 10.1006/excr.1998.4370

Langmead, B., and Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9, 357–359. doi: 10.1038/nmeth.1923

Leung, K.-C. (2004). Regulation of cytokine receptor signaling by nuclear hormone receptors: a new paradigm for receptor interaction. *DNA Cell Biol.* 23, 463–474. doi: 10.1089/1044549041562285

Li, C. (2013). *Effects of n-3 HUFA on Growth Performance, Lipid Metabolism, and Health Status in Grass Carp, Ctenopharyngodon Idellus*. Xianyang: Northwest A&F University.

Li, P., and Wu, G. Y. (2018). Roles of dietary glycine, proline, and hydroxyproline in collagen synthesis and animal growth. *Amino Acids* 50, 29–38. doi: 10.1007/s00726-017-2490-6

Li, R. M. (2019). *The Effects of Inositol and Zinc in Low-Fishmeal Dietary on Nutritional Physiological Functions for Juvenile L. vannamei and Epinephelus lanceolatu* ♂ × *Epinephelus Fuscoguttatus* ♀, *Respectively*. Zhanjiang: Guangdong Ocean University.

Li, Y. Y., Wang, T., Gao, S., Xu, G. M., Niu, H., Huang, R., et al. (2016). *Salmonella* plasmid virulence gene spvB enhances bacterial virulence by inhibiting autophagy in a zebrafish infection model. *Fish Shellfish Immunol.* 49, 252–259. doi: 10.1016/j.fsi.2015.12.033

Liu, F. X., and Yu, B. (2015). Efficient production of reuterin from glycerol by magnetically immobilized *Lactobacillus reuteri*. *Appl. Microbiol. Biotechnol.* 99, 4659–4666. doi: 10.1007/s00253-015-6530-4

Liu, H. R., Liu, Y., Song, C. W., and Cui, Z. X. (2017). A chymotrypsin-like serine protease from Portunus trituberculatus involved in pathogen recognition and AMP synthesis but not required for prophenoloxidase activation. *Fish Shellfish Immunol.* 66, 307–316. doi: 10.1016/j.fsi.2017.05.031

Liu, Q., Long, Y., Li, B., Zhao, L., and Yang, S. (2020). Rice-shrimp culture: a better intestinal microbiota, immune enzymatic activities, and muscle relish of crayfish (*Procambarus clarkii*) in sichuan province. *Appl. Microbiol. Biotechnol.* 104, 9413–9420. doi: 10.1007/s00253-020-10797-4

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

Ma, J. J. (2008). *Effect of Dietary n-3 HUFA on Growth Performance and Lipid Metabolism in Juvenile Black, Sparus macrocephalus*. Zhejiang: Zhejiang University.

Magoè, 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

Makol, A., Torrecillas, S., Caballero, M. J., Fernández-Vaquero, A., and Izquierdo, M. S. (2012). Effect of long term feeding with conjugated linoleic acid (CLA) in growth performance and lipid metabolism of European sea bass (*Dicentrarchus labrax*). *Aquaculture* 36, 129–137. doi: 10.1016/j.aquaculture.2012.09.015

Matsumoto, M., Kibe, R., Ooga, T., Aiba, Y., and Benno, Y. (2012). Impact of intestinal microbiota on intestinal luminal metabolome. *Sci. Rep.* 2:233.

Miao, S. Y., Zhao, C. Z., Zhu, J. Y., Hu, J. T., Dong, X. J., and Sun, L. S. (2018). Dietary soybean meal affects intestinal homoeostasis by altering the microbiota, morphology and inflammatory cytokine gene expression in northern snakehead. *Sci. Rep.* 8:113.

Michelato, M., Zaminhan, M., Boscolo, W. R., Nogaroto, V., Vicari, M., Artoni, R. F., et al. (2017). Dietary histidine requirement of Nile tilapia juveniles based on growth performance, expression of muscle-growth-related genes and haematological responses. *Aquaculture* 467, 63–70. doi: 10.1016/j.aquaculture.2016.06.038

Mirghaed, A. T., Yarahmadi, P., Soltani, M., Paknejad, H., and Hoseini, S. M. (2019). Dietary sodium butyrate (Butirex C4) supplementation modulates intestinal transcriptomic responses and augments disease resistance of rainbow trout (*Oncorhynchus mykiss*). *Fish Shellfish Immunl.* 92, 621–628. doi: 10.1016/j.fsi.2019.06.046

Nascimento, T. M. T. D., Mansano, C. F. M., Peres, H., Rodrigues, F. H. F., Khan, K. U., Romaneli, R. S., et al. (2020). Determination of the optimum dietary essential amino acid profile for growing phase of Nile tilapia by deletion method. *Aquaculture* 523:735204. doi: 10.1016/j.aquaculture.2020.735204

Ogawa, T., Bando, N., Tsuji, H., Okajima, H., Nishikawa, K., Sasaoka, K., et al. (1991). Investigation of the IgE-binding proteins in soybeans by immunoblotting with the sera of the soybean-sensitive patients with atopic dermatitis. *J. Nutr. Sci. Vitaminol.* 37, 555–565. doi: 10.3177/jnsv.37.555

O'Hara, E., Kelly, A., Mccabe, M., and Kenny, D. (2018). Effect of a butyrate-fortified milk replacer on gastrointestinal microbiota and products of fermentation in artificially reared dairy calves at weaning. *Sci. Rep.* 8:14901.

Ortiz-Rivera, Y., Sanchez-Vega, R., Gutierrez-Mendez, N., Leon-Felix, J., Acosta-Muniz, C., and Sepulveda, D. R. (2017). Production of reuterin in a fermented milk product by *Lactobacillus reuteri*: inhibition of pathogens, spoilage microorganisms, and lactic acid bacteria. *J. Dairy Sci.* 100, 4258–4268. doi: 10.3168/jds.2016-11534

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

Page, A. J., Brierley, S. M., Martin, C. M., Price, M. P., and Blackshaw, L. A. (2005). Different contributions of ASIC channels 1a, 2, and 3 in gastrointestinal mechanosensory function. *Gut* 54, 1408–1415. doi: 10.1136/gut.2005.071084

Pan, F. Y., Feng, L., Jiang, W. D., Jiang, J., Wu, P., Kuang, S. Y., et al. (2016). Methionine hydroxy analogue enhanced fish immunity via modulation of NF-kappa B, TOR, MLCK, MAPKs and Nrf2 signaling in young grass carp (*Ctenopharyngodon idella*). *Fish Shellfish Immunol.* 56, 208–228. doi: 10.1016/j.fsi.2016.07.020

Petrov, A., Meskauskas, A., and Dinman, J. D. (2014). Ribosomal protein L3: influence on ribosome structure and function. *RNA Biol.* 1, 58–64. doi: 10.4161/rna.1.1.957

Pettersson, I., and Kurland, C. G. (1980). Ribosomal protein L7/L12 is required for optimal translation. *PNAS* 77, 4007–4010. doi: 10.1073/pnas.77.7.4007

Popovic, M., Zaja, R., Loncar, J., and Smital, T. (2010). A novel ABC transporter: the first insight into zebrafish (*Danio rerio*) ABCH1. *Mar. Environ. Res.* 69, S11–S13.

Post, S. V. D., Birchenough, G. M. H., and Held, J. M. (2021). NOX1-dependent redox signaling potentiates colonic stem cell proliferation to adapt to the intestinal microbiota by linking EGFR and TLR activation. *Cell Rep.* 35:108959.

Rice, E. M., Aragona, K., Moreland, S. C., and Erickson, P. S. (2019). Supplementation of sodium butyrate to postweaned heifer diets: effects on growth performance, nutrient digestibility, and health. *J. Dairy Sci.* 102, 3121–3130. doi: 10.3168/jds.2018-15525

Rosa, M. D., Brundo, V. M., and Malaguarnera, L. (2016). New insights on chitinases immunologic activities. *World J. Immunol.* 6:96. doi: 10.5411/wji.v6.i2.96

Saegusa, K., Sato, M., Sato, K., Nakajima-Shimada, J., Harada, A., and Sato, K. (2014). Caenorhabditis elegans chaperonin CCT/TRiC is required for actin and tubulin biogenesis and microvillus formation in intestinal epithelial cells. *Mol. Biol. Cell* 25, 3095–3104. doi: 10.1091/mbc.e13-09-0530

Sanz-Gómez, N., Pedro, I. D., Ortigosa, B., Santamaria, D., and Gandarillas, A. (2020). Squamous differentiation requires G2/mitosis slippage to avoid apoptosis. *Cell Death. Differ.* 27, 2451–2467. doi: 10.1038/s41418-020-0515-2

Sarraf, S. A., Sideris, D. P., Giagtzoglou, N., Ni, L. N., and Pickrell, A. M. (2019). PINK1/Parkin influences cell cycle by sequestering TBK1 at damaged mitochondria, inhibiting mitosis. *Cell Rep.* 29, 225.e5–235.e5.

Schloss, P. D., Gevers, D., and Westcott, S. L. (2011). Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. *PLoS One* 6:e27310. doi: 10.1371/journal.pone.0027310

Shin, N. R., Whon, T. W., and Bae, J. W. (2015). *Proteobacteria*: microbial signature of dysbiosis in gut microbiota. *Trends Biotechnol.* 33, 496–503. doi: 10.1016/j.tibtech.2015.06.011

Shiu, Y. L., Hsieh, S. L., Guei, W. C., Tsai, Y. T., Chiu, C. H., and Liu, C. H. (2015). Using Bacillus subtilis E20-fermented soybean meal as replacement for fish meal in the diet of orange: potted grouper (*Epinephelus coioides*, Hamilton). *Aquac. Res.* 46, 1–14. doi: 10.1016/j.aquaculture.2015.10.020

Sinha, A. K., Possoz, C., and Leach, D. R. F. (2020). The roles of bacterial DNA double-strand break repair proteins in chromosomal DNA replication. *FEMS Microbiol. Rev.* 44, 351–368. doi: 10.1093/femsre/fuaa009

Smith, A. A., Dumas, A., Yossa, R., Overturf, K. E., and Bureau, D. P. (2017). Effects of soybean meal and high-protein sunflower meal on growth performance, feed utilization, gut health and gene expression in Arctic charr (*Salvelinus alpinus*) at the grow-out stage. *Aquac. Nutr.* 481, 191–201. doi: 10.1016/j.aquaculture.2017.08.038

Smith, D. M., Hunter, B. J., Allan, G. L., Roberts, D. C. K., Booth, M. A., and Glencross, B. D. (2004). Essential fatty acids in the diet of silver perch (*Bidyanus bidyanus*): effect of linolenic and linoleic acid on growth and survival. *Aquaculture* 236, 377–390. doi: 10.1016/j.aquaculture.2003.10.021

Tantikitti, C., Sangpong, W., and Chiavareesajja, S. J. A. (2005). Effects of defatted soybean protein levels on growth performance and nitrogen and phosphorus excretion in Asian seabass (*Lates calcarifer*). *Aquaculture* 248, 41–50. doi: 10.1016/j.aquaculture.2005.04.027

Tian, L., Zhou, X. Q., Jiang, W. D., Liu, Y., Wu, P., Jiang, J., et al. (2017). Sodium butyrate improved intestinal immune function associated with NF-kappa B and p38MAPK signalling pathways in young grass carp (*Ctenopharyngodon idella*). *Fish Shellfish Immun.* 66:548. doi: 10.1016/j.fsi.2017.05.049

Tzuc, J. T., Escalante, D. R., Herrera, R. R., Cortés, G. G., and Ortiz, M. L. A. (2014). Microbiota from Litopenaeus vannamei: digestive tract microbial community of Pacific white shrimp (*Litopenaeus vannamei*). *Springer Plus* 3:280. doi: 10.1186/2193-1801-3-280

Ueta, M., Wada, C., Bessho, Y., Maeda, M., and Wada, A. (2017). Ribosomal protein L31 in *Escherichia coli* contributes to ribosome subunit association and translation, whereas short L31 cleaved by protease 7 reduces both activities. *Genes Cells* 22, 452–471. doi: 10.1111/gtc.12488

Uymaz, B., Şimşek, Ö, Akkoç, N., and Akçelik, H. A. (2009). In vitro characterization of probiotic properties ofPediococcus pentosaceus BH105 isolated from human faeces. *Ann. Microbiol.* 59, 485–491. doi: 10.1007/bf03175135

Vunnam, S., Juvvadi, P., and Merrifield, R. B. (2010). Synthesis and antibacterial action of cecropin and proline-arginine-rich peptides from pig intestine. *J. Rept. Res.* 49, 59–66. doi: 10.1111/j.1399-3011.1997.tb01121.x

Waesch, R., and Cross, F. R. (2002). APC-dependent proteolysis of the mitotic cyclin Clb2 is essential for mitotic exit. *Nature* 418, 556–562. doi: 10.1038/nature00856

Wahlström, A., Sayin, Sama, I., Marschall, H. U., and Bäckhed, F. (2016). Intestinal crosstalk between bile acids and microbiota and its impact on host metabolism. *Cell Metab.* 24, 41–50. doi: 10.1016/j.cmet.2016.05.005

Wang, D., Naydenov, N. G., Feygin, A., Baranwal, S., Kuemmerle, J. F., and Ivanov, A. I. (2016). Actin-depolymerizing factor and cofilin-1 have unique and overlapping functions in regulating intestinal epithelial junctions and mucosal inflammation. *Am. J. Pathol.* 26, 844–858. doi: 10.1016/j.ajpath.2015.11.023

Wang, Y. R., Wang, L., Zhang, C. X., and Song, K. (2017). Effects of substituting fishmeal with soybean meal on growth performance and intestinal morphology in orange-spotted grouper (*Epinephelus coioides*). *Aquac. Rep.* 5, 52–57. doi: 10.1016/j.aqrep.2016.12.005

Wintersberger, E., Mudrak, I., and Wintersberger, U. (2010). Butyrate inhibits mouse fibroblasts at a control point in the G1 phase. *J. Cell. Biochem.* 21, 239–247. doi: 10.1002/jcb.240210306

Wu, D., and Cui, J. (2014). Ion channels in the cell membrane: structure, function, and modeling. *J. Physiol.* 554(Pt 2), 245–247.

Wu, P., Huo, P., Wang, Y., Dong, Y., and Wu, X. (2019). Effluent containing *Rubrivivax gelatinosus* improving the yield, digestion system, intestinal microbiota of crucian carp. *Aquaculture* 514:734418. doi: 10.1016/j.aquaculture.2019.734418

Wu, S. Y., Wang, L. D., Li, J. L., Xu, G. M., He, M. L., Li, Y. Y., et al. (2016). *Salmonella* spv locus suppresses host innate immune responses to bacterial infection. *Fish Shellfish Immunol.* 58, 387–396. doi: 10.1016/j.fsi.2016.09.042

Xie, X. H., Lin, W. Z., Zheng, W. L., Chen, T., Yang, H. T., Sun, L. J., et al. (2019). Downregulation of G2/mitotic-specific cyclinB1 triggers autophagy via AMPK-ULK1-dependent signal pathway in nasopharyngeal carcinoma cells. *Cell Death Dis.* 10:94.

Yang, P., Hu, H., Liu, Y., Li, Y., Ai, Q., Xu, W., et al. (2018). Dietary stachyose altered the intestinal microbiota profile and improved the intestinal mucosal barrier function of juvenile turbot, *Scophthalmus maximus* L. *Aquaculture* 486, 98–106. doi: 10.1016/j.aquaculture.2017.12.014

Yang, W. S., and Stockwell, B. R. (2016). Ferroptosis: death by lipid peroxidation. *Trends Cell Biol.* 26, 165–176. doi: 10.1016/j.tcb.2015.10.014

Yang, W. X., Shi, W. Z., Zhou, S. N., Qu, Y. H., and Wang, Z. H. (2019). Research on the changes of water-soluble flavor substances in grass carp during steaming. *J. Food Biochem.* 43:e12993.

Yang, Y. H., Wang, Y. Y., Lu, Y., and Li, Q. Z. (2011). Effect of replacing fish meal with soybean meal on growth, feed utilization and nitrogen and phosphorus

excretion on rainbow trout (*Oncorhynchus mykiss*). *Aquacult. Int.* 19, 405–419. doi: 10.1007/s10499-010-9359-y

Zabirnyk, O., Liu, W., Khalil, S., Sharma, A., and Phang, J. M. (2010). Oxidized low-density lipoproteins upregulate proline oxidase to initiate ROS-dependent autophagy. *Carcinogenesis* 31, 446–454. doi: 10.1093/carcin/bgp299

Zhang, C. X., Wang, Z. Y., Li, Z., Shen, Q. W., Chen, L. J., Gao, L. L., et al. (2016). Phosphoproteomic profiling of myofibrillar and sarcoplasmic proteins of muscle in response to salting. *Food Sci. Biotechnol.* 25, 993–1001. doi: 10.1007/s10068-016-0161-0

Zhang, F. W., Feng, J., Zhang, J. Y., Kang, X., and Qian, D. (2020). Quercetin modulates AMPK/SIRT1/NF-κB signaling to inhibit inflammatory/oxidative stress responses in diabetic high fat diet-induced atherosclerosis in the rat carotid artery. *Exp. Ther. Med.* 20:280.

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, W. (2020). *Study on the Differential Mechanism of Intestinal Mucosal Barrier Damage in Epinephelus fuscoguttatus♀ ×E. lanceolatus ♂ Caused by Three Kinds of Soybean Protein*. Zhanjiang: Guangdong Ocean University.

Zhang, W., Tan, B. P., Ye, G. L., Wang, J. X., and Zhang, H. T. (2019). Identification of potential biomarkers for soybean meal-induced enteritis in juvenile pearl gentian grouper, Epinephelus lanceolatus ♂ × Epinephelus fuscoguttatus ♀. *Aquaculture* 512:734337. doi: 10.1016/j.aquaculture.2019.734337

Zhao, X. L., Han, Y., Ren, S. T., Ma, Y. M., Li, H., and Peng, X. X. (2015). L-proline increases survival of tilapias infected by *Streptococcus agalactiae* in higher water temperature. *Fish Shellfish Immunol.* 44, 33–42. doi: 10.1016/j.fsi.2015.01.025

Zhao, Y., Qin, G. X., Han, R., Wang, J., Zhang, X. D., and Liu, D. D. (2014). β-Conglycinin reduces the tight junction occludin and ZO-1 expression in IPEC-J2. *Int. J. Mol. Sci.* 15, 1915–1926. doi: 10.3390/ijms15021915

Zhou, F. (2011). *Studies on the Effect of Dietary Lysine and Arginine on the Growth of Juvenile Black Seabream (Acanthopagrus schlegelii) and Their Antagonistic Effect Mechanism*. Hangzhou: Zhejiang University.

Zou, X., Ji, J., Qu, H., Wang, J., Shu, D. M., Wang, Y., et al. (2019). Effects of sodium butyrate on intestinal health and gut microbiota composition during intestinal inflammation progression in broilers. *Poult. Sci.* 98, 4449–4456. doi: 10.3382/ps/pez279

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: 17 August 2021
doi: 10.3389/fmars.2021.706179

Effects of Dietary Silica Nanoparticle on Growth Performance, Protein Digestibility, Hematology, Digestive Morphology, and Muscle Composition of Nile Tilapia, *Oreochromis Niloticus*

OPEN ACCESS

Edited by:
Min Gu,
Shandong University, China

Reviewed by:
Mohamed Hassaan,
Benha University, Egypt
Francisco Javier Moyano,
University of Almeria, Spain

***Correspondence:**
Md. Sazzad Hossain
sazzadbau@gmail.com

†These authors have contributed equally to this work and 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: *06 May 2021*
Accepted: *19 July 2021*
Published: *17 August 2021*

Citation:
Bashar A, Hasan NA, Haque MM, Rohani MF and Hossain MS (2021) Effects of Dietary Silica Nanoparticle on Growth Performance, Protein Digestibility, Hematology, Digestive Morphology, and Muscle Composition of Nile Tilapia, Oreochromis Niloticus. Front. Mar. Sci. 8:706179. doi: 10.3389/fmars.2021.706179

Abul Bashar†, Neaz A. Hasan, Mohammad Mahfujul Haque†, Md. Fazle Rohani and Md. Sazzad Hossain*

Department of Aquaculture, Bangladesh Agricultural University, Mymensingh, Bangladesh

The use of nanotechnology in food production systems is being investigated globally, though there is limited research on its effect on fish nutrition. Therefore, this study aimed to identify the effects of silica nanoparticles (NPs) on the nutrition and physiology of tilapia, *Oreochromis niloticus*. Four isonitrogenous diets (300 g/kg crude protein) with NPs (0, 1, 2, and 3 mg/kg diet) were fed to fish (6.52 ± 0.20 g) in a recirculatory aquaculture system for 56 days. Throughout the study period, the effects of silica NP on survival rate, blood cell count, hemoglobin (Hb) level, condition factor (CF), and final product composition (except lipid content) were insignificant. However, growth performance and feed efficiency increased with an increasing level of silica NP, up to 2 mg/kg, and then decreased. This increase was due to the highest apparent protein digestibility and dry matter digestibility when fish were fed silica NP at 2 mg/kg. However, fish at the early stage showed better performance in all dietary groups than in later. Blood glucose (BG) content and histology of the kidney revealed that fish were stressed when a 3 mg/kg silica NP was used and they adapted through excessive excretion *via* expanded glomeruli. Though no significant effect on villi length was observed, silica NP increased the surface area widening the villi of the gut along with the number of goblet cells in the intestine significantly, when supplemented at a level of 2 mg/kg. The bioaccumulation of silica shows that incorporating silica NP in the fish feed will not compromise human health safety upon consumption. Although silica NP at 1 mg/kg and 3 mg/kg yielded some improvements to growth and final product quality, a 2 mg/kg silica NP generated the best results in all measured parameters.

Keywords: silica nanoparticle, tilapia (*Oreochromis Nilotica*), recirculatory aquaculture system, digestibility, nanotechnology in fish nutrition, bioaccumulation

INTRODUCTION

Increased access to multidisciplinary knowledge and the worldwide availability of low-cost compliances have made the global aquaculture industry one of the fastest-growing and irreplaceable animal protein sectors (Belton and Thilsted, 2014; FAO, 2016; Abd El-Naby et al., 2019). This intensified sector has been proved to be a significant contributor to food security, especially for developing Asian and African countries including Bangladesh (Gui et al., 2018; Chan et al., 2019; Hasan et al., 2021c). Inputs for aquaculture include feed, seed, and water, of which feed accounts for nearly 60–70% of the total production costs (Yuan et al., 2017; Yang et al., 2019; Nguyen et al., 2020a). In aquaculture operations, the quality of feed is a fundamental requirement that must be met (Ahmed, 2007; Singha et al., 2020). Besides the quality, digestibility of nutrients also impacts water quality, disease outbreak (Heal et al., 2021), total yield, and, consequently, the business profitability (Guo et al., 2020; Hassaan et al., 2020a; Kong et al., 2020; Nguyen et al., 2020a). While the sustainable use of feed is a challenge to the aquaculture industry, many strategies have been implemented, including the replacement of fishmeal (Perez-Velazquez et al., 2019; Li et al., 2020), the use of byproducts (Irm et al., 2020), selective breeding (Carlberg et al., 2018), minimizing nutrient waste (de Verdal et al., 2018), and, most recently, the use of feed additives including probiotics (Haque et al., 2021b; Hasan et al., 2021a), and nanoparticles (NPs) (Abd El-Naby et al., 2019; Rathore et al., 2020).

Over the last decade, Tilapia (*Oreochromis niloticus*), the global aquatic chicken, has gained its popularity throughout the world (Abdel-Tawwab et al., 2020) for its compatible characteristics, such as easy to produce seed (Barman and Little, 2011), quick response to artificial feeds (Ahmed et al., 2014; Ogello et al., 2014), a wide range of environmental tolerance (Singha et al., 2020), short crop cycle (Francis and Esa, 2016), nutritive values and larger edible portion with no intermuscular bone (Moesch et al., 2016), and high resistance to physical and biological hazards (Al-Deriny et al., 2020; Chaput et al., 2020; Foysal et al., 2020; Naiel et al., 2020). In the context of the increased feed price because of the increasing cost against the limited protein source, such as fish meal (Nguyen et al., 2020a; Pianesso et al., 2020) and a recession in the farmgate price of tilapia due to supply outstripping the national demand and export barriers for developing countries like Bangladesh (Uddin et al., 2019; Bashar et al., 2021; Haque et al., 2021a; Hasan et al., 2021b), recently dealing with this species in semi-intensive, intensive, and super-intensive commercial culture systems have become a great challenge (Kabir et al., 2019). Moreover, due to the lower nutrient digestion capability of tilapia, nutrients, e.g., protein, end up as metabolic waste, like NH_3 (Crab et al., 2007). These waste metabolites not only increase feed costs by increasing the feed conversion ratio (FCR) but also make the fish more susceptible to pathogens (Kent et al., 2009; Hasan and Haque, 2020; Hasan et al., 2020) through deterioration of water quality. Therefore, finding a way of increasing nutrient digestibility and absorption in *O. niloticus* can be a multifaceted solution for a range of issues existing in aquaculture, which could help to make the industry sustainable.

Due to unique physicochemical properties, NPs are being acknowledged by the food production industries for their medical and nutritional uses (Vidya et al., 2016; Khosravi-Katuli et al., 2017; Kumar et al., 2018; Rodriguez et al., 2018; Bashar et al., 2019; Thangapandiyan and Monika, 2020). NPs can benefit aquaculture production by enhancing the bioactivity of molecules, including micronutrients (Xu et al., 2018; Shah and Mraz, 2020), and enabling tissue-specific applications of disease treatments with no consequences to human health (Jennings et al., 2016). The high specific surface area of NPs facilitates the absorption of micronutrients from the intestine to the bloodstream in terrestrial and aquatic animals and makes them suitable for use as feed additives (Huang et al., 2015; Khosravi-Katuli et al., 2017; Pieszka et al., 2019). Along with other NPs, silica (silicon dioxide) NP can be used for its outstanding optical properties, adsorption capacity, low toxicity, biocompatibility (Bitar et al., 2012), thermal stability, and low production cost (Priyadarsini et al., 2018). In the aquaculture industry, silica NP has been reported to favor fish welfare through easing drug administration and reducing the risk of disease outbreak even in case of huge crowding (Khosravi-Katuli et al., 2017). In addition, silica in nanoform is found to be effective in wastewater treatment (Jarvie et al., 2009), controlling microbial load (Huang et al., 2015), and stimulating diatom growth in aquaculture systems.

Despite the huge potential of silica NP in aquaculture, studies on its use as feed additives for finfish, like tilapia, have not been carried out. Therefore, to make the aquaculture production more sustainable from the nutritional consideration without averting human health safety upon consumption, the current experiment was carried out to investigate the effects of different levels of silica NPs on growth performance, feed utilization, blood physiology, histology, and muscle composition of and bioaccumulation in tilapia.

MATERIALS AND METHODS

Ethical Issues

The design and execution of the experiment were approved by the ethical committee of the Bangladesh Agricultural University Research System (Approval No.: 2021/44/BAU). Fish were fed, handled, sampled, harvested, and sacrificed, maintaining proper care and welfare by all the mentioned authors.

Experimental Site and Culture Design

To conduct the 8-week experiment, a Recirculatory Aquaculture System with 12 fiberglass tanks (0.8 × 0.5 × 0.5 m) arranged in a two-tier system was developed in the Faculty of Fisheries, BAU, Mymensingh, Bangladesh. Continuous aeration and a water depth of 0.4 m were maintained throughout the feeding trial. To avoid the circulation of silica NP leaching from the feed and fecal content of different treatments, the inlet and outlet pipes of every biofilter were fitted with a 1-mm thick ceramic filter (0.01 μm pore size) covered by cheesecloth (double layered) of the 1-μm mesh size. Three tanks were designated as control (T0) and every treatment (T1, T2, and T3) had three replications.

TABLE 1 | The inclusion level of main ingredients and proximate composition of the formulated diets.

Ingredients	Diets			
	Control (T0)	Treatment 1 (T1)	Treatment 2 (T2)	Treatment 3 (T3)
Inclusion level (g/kg)				
Fishmeal[a]	263.400	263.399	263.398	263.397
Soyabean meal[b]	263.400	263.400	263.400	263.400
Mustard oil cake[c]	100.000	100.000	100.000	100.000
Wheat bran[d]	151.350	151.350	151.350	151.350
Rice bran[e]	151.350	151.350	151.350	151.350
Molasses[f]	50.000	50.000	50.000	50.000
Vitamin and mineral premix[g]	20.000	20.000	20.000	20.000
Cr_2O_3	0.500	0.500	0.500	0.500
Silica nanoparticle	0.000	0.001	0.002	0.003
Proximate composition of formulated diets (g/kg)				
Ash	114.3	115.4	113.1	114.6
Moisture	105.1	106.6	107.3	109.7
Crude protein	299.9	299.7	299.6	299.6
Crude lipid	122.4	121.2	119.9	121.1
Crude fiber	113.7	113.3	114.3	111.4
Nitrogen-free extract (NFE)[h]	244.1	243.3	245.3	243.1
Cr2O3	0.5	0.5	0.5	0.5

[a]*Crude protein is 55.28%, the crude lipid is 9.90% (Supplied by ACI Godrej Agrovet Pvt. Ltd., Bangladesh).*
[b]*Crude protein is 36.7%, the crude lipid is 19.53%.*
[c]*Crude protein is 25.74%, the crude lipid is 22.11%.*
[d]*Crude protein is 10.65%, the crude lipid is 7.50%.*
[e]*Crude protein is 10.47%, the crude lipid is 8.10%.*
[f]*Sugarcane is derived and collected from a local market.*
[g]*Composition of premix (per kg): Vitamin A: 50,000 IU; vitamin B1: 12 mg; vitamin B2: 25 mg; pantothenate: 200 mg; vitamin B6: 15 mg; biotin: 12 mg; vitamin B12: 0.04 mg; folic acid: 86 mg; vitamin C: 120 mg; vitamin D: 10,000 IU; vitamin E: 0.4 mg; vitamin K3: 10 mg; inositol: 330 mg; zinc: 4.0 g; iron: 80 gm; manganese: 15.3 mg; copper: 427 mg; calcium: 47 gm; iodine: 2 gm; selenium 42 mg; cobalt 1.3 mg; magnesium: 100 mg; sodium chloride: 20 gm. Supplied by the ACI Godrej Agrovet Pvt. Ltd.*
[h]*Calculated as 100–(crude protein + crude lipid + crude fiber + Ash).*

Silica NP

Highly pure, magnetically activated, and 100% natural silica NP, composed of more than 98% of silicon dioxide (SiO_2), 0.08% Al_2O_3, 0.05% Fe_2O_3, and CaO, 0.5% K_2O, and 0.10% TiO_2, was collected from Ceresco Nutrition Ltd., Canada. The size of the ethanol-extracted silica NP ranges from 100 nm to 400 nm with an average active density of 2.03 ± 0.56 g/cm^3 (3.08×10^{16} NP per gram).

Diet Formulation

Four isonitrogenous (300 g/kg crude protein) (Nguyen et al., 2020b) experimental diets containing different levels of silica NPs were formulated according to the square method of Pearson (Wagner and Stanton, 2012), with locally available fish feed ingredients (**Table 1**). Silica NP was incorporated into diets at levels of 0 mg/Kg (T0), 1 mg/Kg (T1), 2 mg/kg (T2), and 3 mg/Kg (T3). As an innate marker, 0.05% Cr_2O_3 was incorporated into diets for the further determination of the digestibility of tilapia according to Austreng (1978). The proximate compositions of the ingredients and diets were analyzed before and after the formulation of diets (**Table 1**), respectively, according to AOAC (2005) (for details, as shown in section Proximate Composition of Fish Muscle).

All feed ingredients were milled (in powder form) and mixed thoroughly using a mixer machine to ensure homogeneous mixing of ingredients and silica NP. Then, double distilled water was added to make the dough, and pellets were prepared through a pelletizer (0.5 mm diameter). After drying for 4 days, pellets were stored in polythene bags at −20°C until feeding tilapia.

Experimental Fish

Male *O. niloticus* (GIFT strain) fry, weighing 6.52 g (± 0.20 g), from the same breeding stock, were procured from Reliance Hatchery Ltd., Mymensingh, Bangladesh. There was no sign of diseases and/or abnormalities. After collection, the fry was acclimatized for 10 days in the experimental system, following the method adopted by Zhang et al. (2019). During the acclimation period, a constant oxygen supply was maintained and fish were fed with a control diet. After 10 days, fish were randomly assigned to the replication tanks, at a density of 188 per cubic meter (30 fish per 160 L tank). To understand how the age of fish affects silica NP utilization, two sampling stages were considered: the first stage (0–28 days) and the second stage (28–56 days). At the second stage, the number of fish in replications of different treatments reduced and varied between 22 and 24 because of different mortality rates and immolation for histological and

hematological studies after the first stage. To ensure evenness among the treatments, the number of fish was adjusted to 21 (132 per cubic meter) in each tank.

Feeding Trials and Data Collection

After final stocking, all groups of fish were fed with their respective diet, at a level close to the apparent satiation, as defined by Simon et al. (2019) twice daily (09:00 and 15:00). In each sampling trip (14 days), at least 50% of fish from each replication tank was sampled randomly and weighed individually with an analytical balance (Model: AS 310.X2 Plus). Fish were fasted for 6 h before and after sampling. Mortality was observed daily, and dead fish were removed to calculate the survival rate.

Growth and Feed Utilization Parameters

Growth indices were calculated according to the following formulae (Aanyu et al., 2018, 2020; Hassaan et al., 2020a):

1. *Weight gain* (g) = *final weight* (g) − *initial weight*(g)
2. *Percent weight gain* (%) = (*weight gain* (*g*))/(*initial weight* (*g*)) × 100
3. *Specific growth rate* (% *per day*) = (*Ln* (*final weight*) − *Ln* (*initial weight*))/(*study period* (*day*)) × 100
4. *Daily growth coefficient* (% *per day*) = (*final weight* $(g)\hat{}.33$ − *initial weight*$(g)\hat{}.33$)/(*study period* (*day*)) × 100
5. *Condition factor* = (*final weight* (*g*))/(*final length* $(cm)\hat{}3$)
6. *Survival rate* (%) = (*final number*)/(*initial number*) × 100

Feed utilization parameters were calculated from the following formulae (Aanyu et al., 2018, 2020):

1. *Food conversion ratio* = (*dry feed fed* (*kg*))/(*live weight gain* (*kg*))
2. *Protein efficiency ratio* = (*total weight gain* (*g*))/(*protein intake* (*g*))
3. *Dry matter digestibility* (% *DMD*) = 100 − 100 ((% *Cr2O3 in diet*)/(% *Cr2O3 in feces*))
4. *Protein digestibility* (% *APD*) = 100 − 100 (% *Cr2O3 in Diet*)/(% *Cr2O3 in feces*) × (% *CP in feces*)/(% *CP in diet*))

For the estimation of %APD and %DMD, five fish from each replication tank were sacrificed after 28 days (first stage) and 56 days (second stage) of feeding. In both stages, to avoid nutrient and marker leaching into the water, the fecal content was collected from the marginal gut, close to the anal region, following the method of Austreng (1978). Feces were extracted from each of the five fish sacrificed, weighed, and dried individually (thereby 15 replications for each treatment) in a hot-air oven (106°C). Fifty milligrams of each dried sample was digested with 5 ml of concentrated nitric acid (20 min) and then 3 ml of perchloric acid (until they turned reddish) in a micro-Kjeldahl flask, keeping in an electrothermal heater at 170°C. After cooling, double distilled water was added to make the volume to be exactly 100 ml and transferred to a spectrophotometer cuvette. The Cr_2O_3 contents in diet and feces were measured by the optical density at 440 nm (Fenton and Fenton, 1979) using a spectrophotometer (T60UV, PG Instrument, UK) according to the following formula:

$$Amount\ of\ Cr2O3\ \ (mg) = (Y - 0.0032)/0.2089$$

where Y = optical density

Cr_2O_3 % was calculated by the following formula:

$$\%\ Cr2O3 = (Amount\ of\ Cr2O3\ (mg))/(Amount\ of\ sample\ (mg)) \times 100$$

The crude protein contents of the feed and feces were estimated according to AOAC (2005) (as shown in section Proximate Composition of Fish Muscle for details).

Blood Physiology

In both stages of the experiment, five fish (later used for the digestibility study) from each replication were anesthetized randomly with MS-222 (15 μg/L) after sampling, and blood samples were collected from the caudal vein to determine the level of hemoglobin (Hb), blood glucose (BG), red blood cells (RBCs), and white blood cells (WBCs). Immediately after blood collection, the Hb level (g/dl) and BG level (mg/dl) were determined by a digital EasyMate® GHB meter (Model ET 232, Bioptic Technology Inc., Taiwan) using Hb and glucose strips, respectively. WBCs and RBCs were counted using a Neubauer hemocytometer (Blaxhall and Daisley, 1973) placed under a light microscope (Olympus IX71) fitted with a Zeiss camera (Axiocam ERc 5s).

Histology

At the end of the feeding trial, five fish from each replication were necropsied to pick out organs of interest: e.g., liver, kidney, and intestine in the Fish Disease Laboratory, Department of Aquaculture, BAU. Intestines were defined and divided into foregut, midgut, and hindgut, according to Giorgini et al. (2018) and only the hindgut was used for the histomorphological study. The organs were fixed in a 10% buffered formalin, and an automatic tissue processor was used for the histological process (Naiel et al., 2020). Gut (5 μm), liver (4 μm), and kidney (4 μm) sections were cut with a microtome machine. Histological slides stained with hematoxylin (CI 75290) and eosin (Y, CI 45380; 0.1% alcoholic solution) and mounted with Canada balsam (C-1795; Sigma-Aldrich) were observed under a light microscope (Olympus IX71) at X100 magnification. The images of the histological slides were prepared with a fixed Zeiss camera and were analyzed with Image J (version 14.0) software.

Proximate Composition of Fish Muscle

At the end of the feeding trial, six fish from each replication were sacrificed to determine the proximate composition of the muscle. Moisture, ash, crude protein, and crude lipid contents were determined according to AOAC (2005). The total nitrogen content was determined using the micro-Kjeldahl analysis (method 945.01) and multiplied by the conversion factor (6.25) to translate it into the total crude protein content. Crude fat, ash, and moisture content were estimated by Soxhlet extraction (method 920.39C), by calcination in a muffle furnace

TABLE 2 | The effects of silica nanoparticle (NP) on growth performance and feed efficiency of experimental tilapia, Oreochromis niloticus, after 28 and 56 days of feeding trial.

Parameters	Days	Control	T1	T2	T3	*p*-value
Growth parameters						
Initial weight (g)	0–28	6.57 ± 0.03	6.57 ± 0.03	6.52 ± 0.33	6.51 ± 0.31	0.976
	28–56	17.96 ± 0.95^{a}	22.73 ± 0.17^{b}	27.30 ± 0.55^{c}	21.33 ± 0.84^{b}	<0.001
Weight gain (g)	0–28	11.39 ± 0.92^{a}	16.16 ± 0.15^{b}	20.78 ± 0.58^{c}	14.82 ± 1.03^{b}	<0.001
	28–56	31.10 ± 1.22^{a}	37.38 ± 2.63^{b}	44.70 ± 1.77^{c}	31.32 ± 2.25^{a}	<0.001
Initial length (cm)	0–28	6.04 ± 0.12	5.98 ± 0.04	6.01 ± 0.05	6.02 ± 0.25	0.954
	28–56	8.14 ± 0.08^{a}	8.76 ± 0.14^{b}	9.40 ± 0.05^{c}	8.57 ± 0.04^{b}	<0.001
Length gain (cm)	0–28	2.10 ± 0.06^{a}	2.78 ± 0.10^{b}	3.38 ± 0.05^{c}	2.55 ± 0.27^{b}	<0.001
	28–56	2.19 ± 0.19^{a}	2.53 ± 0.37^{ab}	2.84 ± 0.10^{b}	2.25 ± 0.10^{a}	0.023
PWG[1]	0–28	173.37 ± 13.17^{a}	245.84 ± 2.00^{b}	319.35 ± 21.99^{c}	228.45 ± 24.27^{b}	<0.001
	28–56	173.31 ± 8.05	164.46 ± 12.05	163.86 ± 9.25	147.24 ± 16.19	0.129
SGR[2]	0–28	3.59 ± 0.18^{a}	4.43 ± 0.02^{b}	5.12 ± 0.19^{c}	4.24 ± 0.27^{b}	<0.001
	28–56	3.59 ± 0.1^{a}	3.47 ± 0.17^{b}	3.46 ± 0.13^{b}	3.23 ± 0.23^{c}	<0.001
DGC[3]	0–28	2.61 ± 0.15^{a}	3.36 ± 0.02^{b}	4.01 ± 0.12^{c}	3.18 ± 0.20^{b}	<0.001
	28–56	3.64 ± 0.09^{ab}	3.79 ± 0.20^{ab}	4.01 ± 0.15^{b}	3.41 ± 0.24^{a}	0.019
CF[4]	0–28	3.33 ± 0.14	3.39 ± 0.16	3.29 ± 0.05	3.39 ± 0.1	0.412
	28–56	4.44 ± 0.14^{c}	4.17 ± 0.09^{b}	3.93 ± 0.09^{a}	4.16 ± 0.05^{b}	<0.001
Survival rate	0–28	92.59 ± 3.70	92.84 ± 3.60	96.30 ± 3.71	92.59 ± 3.70	0.562
	28–56	100	100	100	100	
Feed efficiency parameters						
FCR[5]	0–28	1.47 ± 0.03^{b}	1.42 ± 0.03^{b}	1.27 ± 0.04^{a}	1.43 ± 0.04^{b}	<0.001
	28–56	1.56 ± 0.06^{b}	1.47 ± 0.01^{b}	1.33 ± 0.03^{a}	1.52 ± 0.04^{b}	<0.001
PER[7] (%)	0–28	2.19 ± 0.04^{a}	2.27 ± 0.02^{a}	2.42 ± 0.04^{b}	2.23 ± 0.02^{a}	<0.001
	28–56	2.07 ± 0.02^{a}	2.15 ± 0.03^{b}	2.33 ± 0.03^{c}	2.11 ± 0.03^{ab}	<0.001
APD[8] (%)	0–28	79.21 ± 1.81^{a}	87.44 ± 4.16^{bc}	94.80 ± 1.71^{c}	84.41 ± 2.58^{b}	0.001
	28–56	77.12 ± 1.41^{a}	85.39 ± 2.10^{bc}	92.35 ± 1.71^{c}	82.34 ± 2.50^{b}	<0.001
DMD[9] (%)	0–28	73.56 ± 1.42^{a}	77.94 ± 1.77^{a}	84.86 ± 3.50^{b}	76.37 ± 2.69^{a}	0.003
	28–56	65.50 ± 3.12^{a}	72.09 ± 2.41^{a}	83.41 ± 2.95^{b}	69.86 ± 3.15^{a}	0.001

Results are presented as mean ± SD. Means in the same row with different superscript letters are significant ($p < 0.05$). [a]Percentage weight gain (%); [b]specific growth rate (% per day); [c]daily growth coefficient (% per day); [d]condition factor; [e]feed conversion ratio; [f]feed conversion efficiency; [g]protein efficiency ratio; [h]apparent protein digestibility; [i]dry matter digestibility.

at 550°C for 5 h (method 942.05), and by drying in a hot-air oven at 105°C (method 950.01), respectively. The crude fiber content (only for feed samples) was estimated with Fiber Tech (Tulin equipment, India) following the calcination in a muffle furnace.

Silica Bioaccumulation in Fish Muscle

To quantify the amount of silica persisting in fish muscle, at the end of the feeding trial, six fish from each replication were analyzed in the Bangladesh Council of Scientific and Industrial Research (BCSIR) laboratory by inductively coupled plasma-optical emission spectrophotometry (ICP-OES) determination method, as defined by Hauptkorn et al. (2001). Samples were digested with 25% tetramethylammonium hydroxide and distilled water for 30 min at 120°C under an 800-W microwave power. ICP-OES measurements of silicon contents were conducted at a 251.66-nm wavelength. Silica in muscle was determined by multiplying the silicon content with a conversion factor of 2.142 [as silica contains 46.69% (Merry, 2017)]. The bioaccumulation factor of silica, from the feed to muscle, was calculated according to the following formula [adapted from Gobas (2001)]:

$$Bioaccumulation\ factor\ (BAF) = \frac{Concentration\ of\ silica\ in\ muscle\ (mg/kg)}{Concentration\ of\ silica\ in\ feed\ fed\ (mg/kg)}$$

Statistical Analysis

The data were analyzed using SPSS (Version 23.0, IBM, Armonk, NY) and presented as mean ± SD. A one-way ANOVA was used to determine the significance of different levels of silica NPs on the measured responses. To specify the differences among the treatments, a multiple range test of Tukey, at a 5% significance level, was performed when a significant influence

TABLE 3 | Hematological parameters of tilapia, O. niloticus, fed with control and experimental diets after 28 or 56 days of feeding trial.

Parameters	Days	Control	T1	T2	T3	*p*-value
Hb[1]	0–28	4.10 ± 0.26	4.27 ± 0.15	4.17 ± 0.31	4.23 ± 0.40	0.902
	28–56	3.87 ± 0.25	4.17 ± 0.32	3.97 ± 0.15	3.93 ± 0.29	0.562
BG[2]	0–28	68.40 ± 3.05[a]	73.60 ± 8.84[a]	76.63 ± 6.65[a]	114.80 ± 14.48[b]	0.001
	28–56	65.23 ± 7.11[a]	72.47 ± 7.49[a]	73.57 ± 9.71[a]	96.93 ± 7.18[b]	0.006
RBC[3] ($\times 10^6$)	0–28	1.97 ± 0.02	1.99 ± 0.04	1.95 ± 0.06	2.00 ± 0.05	0.555
	28–56	1.99 ± 0.03	2.03 ± 0.05	2.01 ± 0.07	2.00 ± 0.01	0.755
WBC[4] ($\times 10^4$)	0–28	8.26 ± 0.32	8.30 ± 0.43	8.52 ± 0.54	7.80 ± 0.63	0.395
	28–56	8.13 ± 0.23	7.99 ± 0.64	8.00 ± 0.32	7.62 ± 0.85	0.732

Results are presented as mean ± SD. Means in the same row indicated by different superscript letters are significant (P < 0.05). [1]Haemoglobin (g/dl); [2]blood glucose (mg/dl); [3]red blood cell count; [4]white blood cell count.

TABLE 4 | Histomorphological data of hindgut collected from tilapia, *O. niloticus*, after 56 days of feeding trial with silica NPs.

Observed factors	Control	T1	T2	T3	*p*-value
Villi width (μm)	45.8 ± 7.01[a]	55 ± 8.09[a]	77.8 ± 8.64[b]	50.8 ± 6.5[a]	<0.001
Villi length (μm)	169.6±16.5	161.6 ± 5.73	167 ± 13.69	163 ± 9.35	0.711
Villi surface area (μm^2)	7793.6 ± 1517.001[a]	8874.8 ± 1245.09[a]	12960 ± 1504.21[b]	8283.2 ± 1193.05[a]	< 0.001
Goblet cells (/10,000 μm^2)	22.8 ± 7.79[a]	28.8 ± 8.17[ab]	44.2 ± 8.41[b]	26.6 ± 10.5[a]	0.007

Results are presented as mean ± SD. Means in the same row indicated by different superscript letters are significantly different (p < 0.05).

of silica NP was observed. A $p < 0.05$ was considered to be statistically significant.

RESULTS

Growth Performance and Feed Efficiency

The growth performance of fish fed with experimental and control diets is presented in **Table 2**. The results show that silica had a significant effect ($P < 0.05$) on all growth parameters measured, except percent weight gain (PWG) in the second stage and CF in the first stage. T2 had the most significant effect ($p < 0.001$) on weight gain (WG), length gain (LG), and daily growth coefficient (DGC) in both stages, while T2 had the most significant effect on PWG and specific growth rate (SGR) in only the first stage. The influence of the silica NP supplementation at 3 mg/kg on WG, SGR, PWG, and DGC was highly significant ($P < 0.001$) for the first 28 days, but no significant influence was found after 56 days. The survival rate throughout the feeding trial, in all the treatments, was above 90% with no significant differences between treatments ($P > 0.05$).

The lowest FCR was found in fish from T2 ($P < 0.001$) in both stages (**Table 2**). PER in T2 was the highest in both stages ($P < 0.001$), while in T3, a significant effect on PER was observed only in the second stage. Though the effects of silica NP on DMD on both sampling days were not significant, except T2 ($P = 0.003, 0.001$), a significant difference was found between all the treatments for APD ($P < 0.001$), with T2 causing the most significant increase in both stages.

Blood Physiology

Silica NP, at all treatment levels, had no significant effect on Hb, RBC, and WBC on either sampling date. Silica NP supplementation at 3 mg/kg (T3) showed a significant effect on the BG level of tilapia in both sampling stages ($P = 0.001$ and 0.006, respectively) (**Table 3**). The BG level was found to increase with the increasing dietary concentration of silica NP.

Histomorphology of Midgut, Liver, and Kidney

Data generated from the histological observations of the hindgut revealed that the villi width and villi surface area increased significantly ($P < 0.001$) by silica NP at 2 mg/kg (**Table 4**). Dietary silica NP had no significant effect on villi length ($P > 0.05$); however, the longest villi were observed in the tilapia from control. Silica NP at 2 mg/kg significantly increased the number of goblet cells ($P = 0.007$), ∼2-folds compared to the control (**Table 4**).

The normal kidney structure was observed in tilapia fed with silica NP at 0 mg/kg (control) and 1 mg/kg (T1), while slightly widened and extremely widened glomeruli were found in tilapia fed with silica NP at 2 mg/kg (T2) and 3 mg/kg (T3), respectively (**Figures 1A–D**). No structural aberration was found in liver histology in all treatments. However, with the increase of dietary silica NP, a substantial amount of fat deposition was observed in liver sections (**Figures 1F–H**), with the highest amount found in T3.

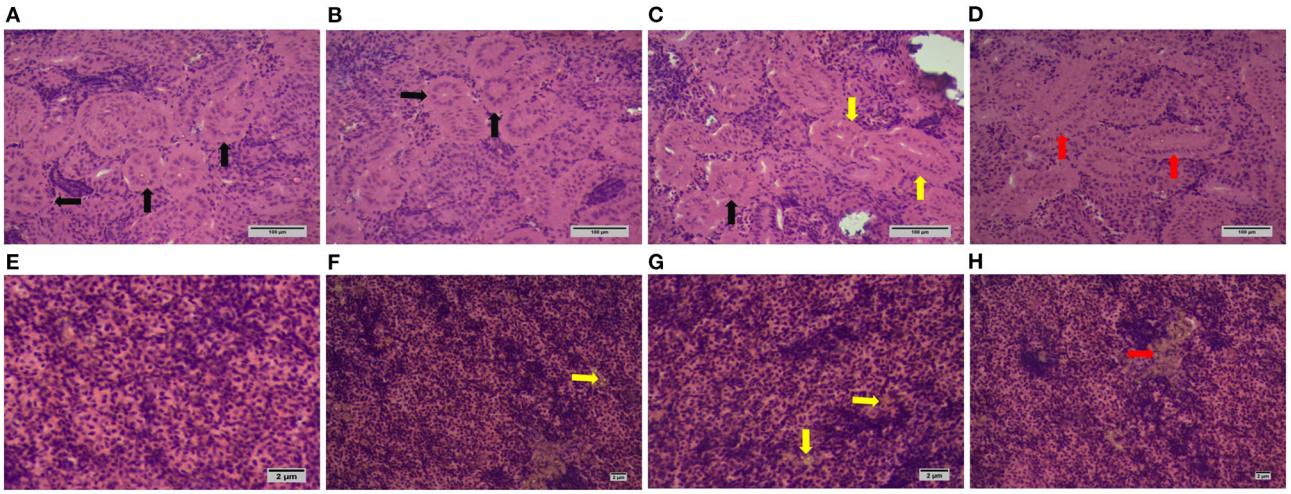


FIGURE 1 | Histoarchitecture of kidney and liver of experimental *Oreochromis niloticus* (H & E; X100 magnification; scale bar: 100 μm for kidney and 1 μm for liver samples). The normal glomerular structure is indicated by black arrows observed in control **(A)** and T1 **(B)**; both normal (black arrow) and slight widened (yellow arrow) structures are found in T2 **(C)**; extremely widened glomerulus is indicated by red arrows observed in T3 **(D)**. **(E)** indicates normal liver histology from control fish with no fat deposition; liver sections from T1 **(F)** and T2 **(G)** showed a slight fat deposition (yellow arrows), while T3 **(H)** experienced a higher deposition (red arrow).

TABLE 5 | Final muscle composition of experimental tilapia in the recirculatory aquaculture system (RAS) and bioaccumulation of silica NPs.

Parameters	Control	T1	T2	T3	*p*-value
Muscle composition (g/kg)					
Moisture	751.1 ± 28.4	725.4 ± 34.8	730.9 ± 27.7	722.1 ± 20.8	0.617
Ash	15.6 ± 1.0	17.3 ± 0.4	16.5 ± 1.3	15.7 ± 1.3	0.228
Crude protein	155.5 ± 14.4	158.1 ± 3.5	148.0 ± 12.5	147.1 ± 11.3	0.573
Crude lipid	25.6 ± 1.4^{a}	28.6 ± 0.9^{b}	28.7 ± 1.0^{b}	28.9 ± 2.2^{b}	0.008
Silica content (mg/kg)					
Silica in muscle	0^{a}	12.16 ± 1.84^{b}	29.27 ± 4.22^{c}	43.76 ± 5.70^{d}	< 0.001
BAF[1]	0^{a}	0.12 ± 0.02^{b}	0.12 ± 0.02^{b}	0.15 ± 0.02^{b}	< 0.001

Results are presented as mean ± SD. Means in the same row indicated by different superscript letters are significant ($P < 0.05$). [a]Bioaccumulation factor.

Proximate Composition of Tilapia Muscle and Silica NP Bioaccumulation

Moisture, ash, and crude protein contents of experimental tilapia at the final harvest showed no significant differences between the dietary groups (**Table 5**). However, the crude lipid content increased with increasing levels of silica NP supplementation. The effect of silica NP on the crude lipid content was significant, irrespective of the incorporation rate ($P < 0.05$), and the highest content was observed in T3 (**Table 5**). The highest silica levels and bioaccumulation of silica NP were found in T3, where the experimental diet was supplemented with 3 mg/kg silica NP.

DISCUSSION

To the best of the knowledge, this is the first study to investigate the effects of silica NP on feed utilization, growth performance, blood physiology, and digestive morphology of *O. niloticus*. The notable outcomes have been achieved in terms of growth performance and feed utilization without compromising human health safety.

Control fish reared with a low proteinous basal diet at a higher stocking density (almost 6-folds than the traditional pond farming system practiced in Bangladesh) showed better growth performance compared to other culture systems like pond-based intensive and semi-intensive cultures (Kabir et al., 2019; Dawood et al., 2020f), raceway culture, tank and aquaria cultures (El-Naby et al., 2019; Abd El-Naby et al., 2020), and even biofloc culture (da Silva et al., 2018; Martins et al., 2019). Controlled environmental conditions and nitrogenous waste maintenance in RAS might ensure optimum welfare for fish and ensure larger spillover advantages of surpassed yield. The growth performances favored from silica NP experienced in this study are far superior to the available findings in the growing literature (Aanyu et al., 2020; Amer et al., 2020; Dawood et al., 2020a,f; Jiang et al., 2020; Wardani et al., 2020) on tilapia, irrespective of culture systems and diets. The supplementation of NPs like nanochitosan (Abd El-Naby et al., 2019, 2020), nanoselenium (Dawood et al.,

2020f; Rathore et al., 2020), nanoselenium with Vit-C (Dawood et al., 2020e), and nanozeolite (Hassaan et al., 2020b) in tilapia have resulted in poorer growth performances than the findings presented in this study. The improved growth performance as seen in this study occurred because silica in nanoform may function as the nutrient carrier (especially for amino acids). These hauled nutrients could have further contributed to increasing the digestion and absorption of nutrient molecules through the controlled encapsulation and release of nutrients from the gastrointestinal tract to the bloodstream (Bahabadi et al., 2017). This increase could also be correlated with the amelioration of DNA and RNA syntheses and an improvement in gut microorganisms, which are supported by NPs (Onuegbu et al., 2018); however, this warrants a further investigation with silica NPs. The survival rate corresponds to those shown in tilapia fed with chitosan NP (Abd El-Naby et al., 2019) and nanoselenium (Dawood et al., 2020a) for the first 28 days. In the later stage, no mortality in any of the treatments, including the control, is in agreement with Abd El-Naby et al. (2019) and Rathore et al. (2020).

The FCR found in the present study is far lower than the results obtained from feeding tilapia with nanoselenium and vitamin E (Dawood et al., 2020e), nanoselenium (Abd El-Kader et al., 2020; Rathore et al., 2020), and nanozeolite (Hassaan et al., 2020b). Similar results were obtained in tilapia fed with chitosan NP supplemented with vitamin C (Naiel et al., 2020) and with only chitosan NP (Abd El-Naby et al., 2019). In the case of T1 and T3, although the growth parameters are significantly different, the FCR was not significant compared to the control. This indicates a high level of feed intake by fish in the T1 and T3 treatments but poorer growth outcomes following the ingestion. The highest PER in this study is similar to those seen in chitosan NP-treated tilapia (Abd El-Naby et al., 2019, 2020); however, it is greater than the findings of PER documented by Rathore et al. (2020) from nanoselenium in tilapia. Fish fed with silica NP demonstrated improved growth performance in tilapia, compared to the control of the current experiment and to other previously mentioned studies. This supports the recommendation of silica NP as one of the best feed additives for *O. niloticus*.

Digestibility data also demonstrate the effect of silica NP on improving growth performance. Digestive enzymes hydrolyze proteins, carbohydrates, and lipids into smaller parts for absorption through the microvilli of the fish intestine (García-Meilán et al., 2016). Tilapia, an omnivorous fish, comparatively possesses an inactive stomach for protein digestion. Digestion

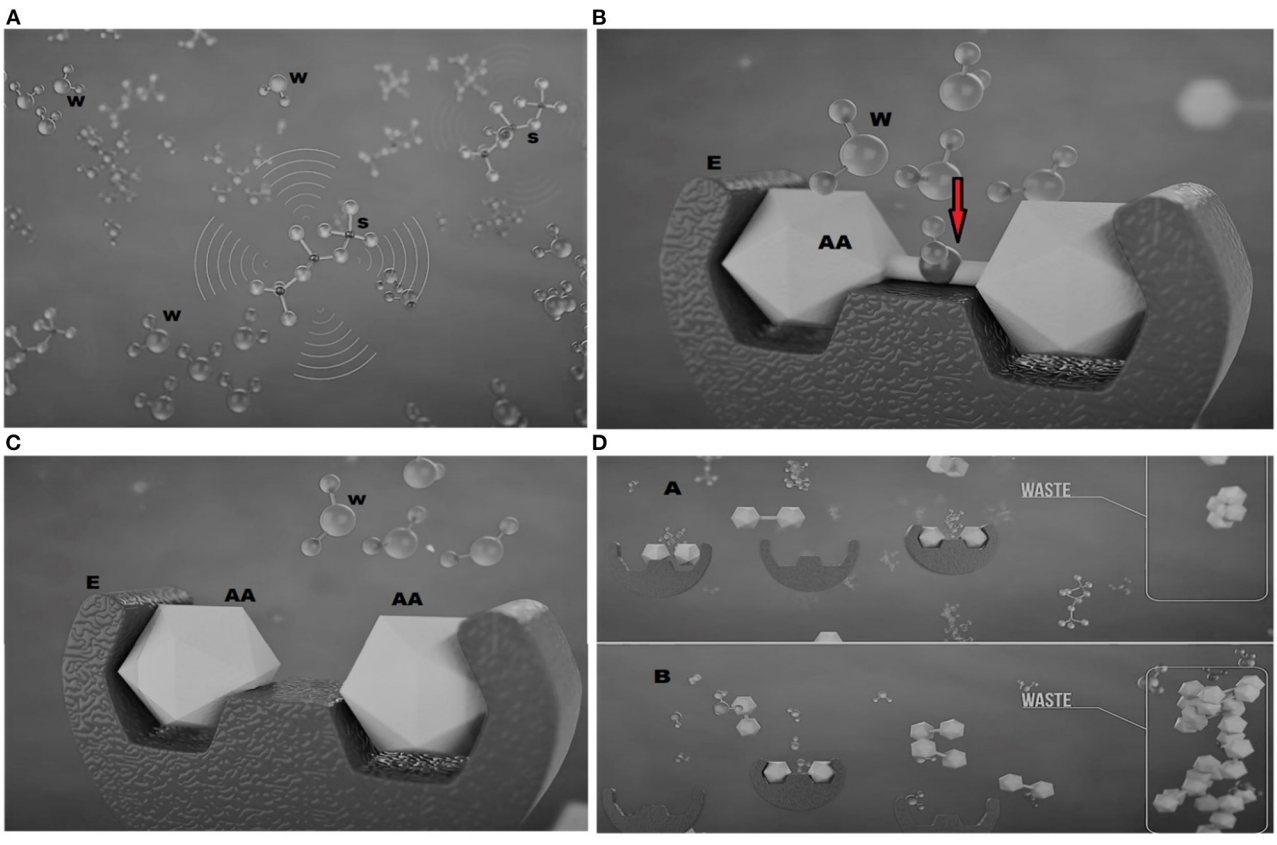


FIGURE 2 | Mechanisms of silica nanoparticles (NPs) responsible for the increased digestibility in tilapia fish (source: Ceresco Nutrition Ltd.) showing **(A)** silica NP(S) activating water molecules (W) inside the gut of fish; **(B)** activated water molecules (W) splitting (red arrow) the amino acid (AA) when it binds to a specific enzyme (E); **(C)** cleaved amino acids (AAs) suitable for absorption through the gut; and **(D)** the reduction in waste in the presence of silica NP **(A)** compared to that without **(B)**.

of protein eminently takes place in the gut just after the stomach, with the action of hydrochloric acid, and due to size limitations, only smaller peptides and amino acids are permitted to access through the gut wall to be absorbed and become available for growth (Wu et al., 2009). The water inside the gut remains inactive and, in general, has no direct role, except facilitating gut microbiota and maintaining homeostasis (Laforenza, 2012; Giatsis et al., 2015); however, in the presence of silica NP, water molecules within the gut may become activated (**Figure 2A**) with the influence of a higher infrared emissivity of silica NP (Faisal et al., 2021). A similar indication with Tourmaline (Borosilicate minerals) NP concluded that radiation emissivity from NP can modulate the structure of water clusters into smaller molecules through breaking hydrogen bonds (Sun et al., 2010). Furthermore, the magnetic treatment of silica NP may have a role in the activation of water molecules through creating magnetic resonance. These activated molecules are reported to facilitate a multitude of reactions, including protein metabolism and immune response (Sun et al., 2010). In the digestion process, ionized water molecules as nucleophiles act on the peptide carbonyl group of the ingested protein (Berg et al., 2002), resulting in smaller peptides and free amino acids, suitable for absorption through the gut epithelium into the blood (**Figures 2B,C**). Enzymatic hydrolysis of protein is largely influenced by the availability of water required for hydration (Butré et al., 2014). Furthermore, the presence of catalytic water is assumed to pledge the enzyme-substrate inhibition through cleaving the peptide bonds of the substrate tying to outside the active sites (Butré et al., 2014). Activated water molecules may also regenerate the enzymes, enabling them to work again within the shortest possible time, as clued by Berg et al. (2002). These evidences clearly disclose that silica NP might accelerate the digestibility of feed and, hence, the growth performance of fish, while the subsequent decrease in nutrient loss results in lower FCR (**Figure 2D**).

Blood physiology data of the experimental fish explain why the growth performance of experimental *O. niloticus* decreased while we incorporated silica at a level of 3 mg/Kg diet. BG is an indicator of stress syndrome, with increased levels providing a biomarker of stress levels in fish (Dawood et al., 2020b,c,d). The BG level of fish fed with 3 mg/kg silica was significantly high, indicating physiological stress due to the high level of silica and rendering that they are not able to use the nutrients properly to acquire growth. Generally, in this state of stress, fish continuously try to respond physiologically, be resistant to the stress, and/or restore homeostasis. These stress responses are energetically costly (Rodnick and Planas, 2016; Schreck and Tort, 2016): consequently, energy might be diverted to maintenance, rather than being available for growth. This explains the lower growth performance in T3, even though the digestibility of protein was still significantly greater than the control. As a defensive agent, WBCs circulate in the bloodstream searching for foreign particles and proliferating when an exogenous particle is identified. There were no significant changes in the Hb level and WBC count throughout the study, indicating that silica NP was not present in the blood and, therefore, no residual effect.

A larger surface area of intestinal villi facilitates nutrient absorption in fish, providing a greater surface area for enzymes for reactions to occur (Dawood et al., 2020a). Though silica NP did not enlarge the villi significantly, it did widen the villi, therefore significantly increasing the surface area. The enhanced surface area enabled the increased absorption of nutrients. Furthermore, the mucus-producing goblet cells have numerous roles in the digestive system, including prevention of gut wall damage and antibacterial action (Pirarat et al., 2015) and maintaining intestinal homeostasis (Junqueira and Carneiro, 2013). Increasing numbers of goblet cells with silica NP treatment may confirm the advantages of silica NP to intestinal health and the gut microbiota of fish that promotes the activities of mucus-secreting goblet cells. The kidney of fish plays an important role in RBC production, osmoregulation, and excretion of waste metabolites. The widened glomerular structure in freshwater fish indicates a high filtration rate for maintaining osmoregulation and excreting detrimental and ionic substances (Oguz, 2015). This reveals the adaptation measures of experimental tilapia against the stress when challenged with silica NP at 3 mg/kg. This adaptation might be accomplished through the excretion of silica NP *via* the enlarged kidney tubules, and this broadening of glomeruli was associated with the increasing level of silica NP in feed. This is also in line with the expansion of glomeruli observed by Hussain et al. (2019) in freshwater fishes coping against different environmental pollutants. Similar adaptation measures against arsenic in *Channa punctata* (Roy and Bhattacharya, 2006) also corroborate the reasoning for enlarged kidney tubules. However, the normal kidney structure in the control and T1 and the nearly normal and slight widening in T2 indicate that there was no such extreme pressure on the fish excretory system below 2 mg/kg dietary silica NP.

Lipids in fish play a crucial role as a source of energy and for the provision of essential fatty acids, necessary for fish growth and development (Kim et al., 2012). However, fish prefer to consume energy from protein, more specifically from the amino acids (Walton and Cowey, 1982; Wu et al., 2020), while lipids are known to be stored in the liver and muscle by fish and to spare the role of proteins (Kim et al., 2012; Zhang et al., 2019) when they are sourced inadequately or fish need to adapt physiologically through energetic cost (McCue, 2013). Silica NP undoubtedly increased digestibility and absorption of nutrients including proteins and lipids. However, due to proper feeding regimes, very little sparing might be required by lipids throughout the study period, and the higher growth performance could have resulted mainly from the absorption of proteins (amino acids). This allowed a greater portion of lipids to remain unused and deposited in the muscle.

Though crystalline silica is considered as a class-1 carcinogenic, amorphous silica in nanoform was non-carcinogenic in rats and mice up to 2,500 mg/kg and 7,500 mg/kg body weights, respectively (Younes et al., 2018). When it is available in food, drugs, and beverages, more than 50% silica in the form of silicon is filtered by the kidney in humans (Kelsay et al., 1979) and the remaining residue disperses through the skin, aorta, bone, and other parts of the body (Carlisle, 1981).

Moreover, if it exists in the blood, as silicic acid, it has no adverse effect on human physiology as it does not bind to proteins (De Araújo et al., 2016). In fact, in favorable concentrations, silica plays an important biological role in bone, brain, nerve, skin, and memory health. It also benefits patients with diabetes by stimulating insulin secretion from the pancreas (Jugdaohsingh, 2007). However, the US Food and Drug Administration has defined 2% by the weight of food as the maximum limit for human consumption, when silica is used as additives (FDA, 2019). The results presented in this study show that only a low level of silica NP accumulated in fish muscle, and therefore, the BAF data show that incorporating silica NP into fish diets will not adversely impact human health upon consumption of the fish.

CONCLUSION

These results have verified the clear advantages of silica NP on growth performance, feed utilization, and the final product quality in the experimental fish. Bioaccumulation study strongly approved a much higher amount of silica NP to be incorporated into feed without averting human health safety; however, growth performance and hematological and histological findings apprehend the limit to 2 mg/Kg for obtaining the highest possible payback from tilapia. On the other hand, the question of maintaining proper dose during manufacturing and feeding is apposite because of the narrow effective range as suggested in this study. The industrial bulk production of feed using appropriate binders to minimize the leaching of silica NP, paradoxically, could knock down the contradiction of accommodating the required dose. Conclusively, silica NP as an input of nanotechnology can be applied as novel feed additives to improve the rate of digestion, as well as improve absorption in the production of *O. niloticus* without making human health safety questioned. Much progress has been made through this experiment, and hopefully, it will make a big sense to widen the gateway of future investigation. It still possesses some limitations on which basis, this publication warrants furthermore studies delving the insights from molecular and physiological prospects.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the first author (AB), without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Bangladesh Agricultural University Research System.

AUTHOR CONTRIBUTIONS

AB: conceptualization, methodology, investigation, writing—original draft, visualization, and fund acquisition. NH: formal analysis, data curation, writing—original draft, and visualization. MHa: conceptualization, validation, writing—review, and editing. MR: methodology, investigation, and visualization. MHo: methodology, validation, resources, and supervision. All authors contributed to the article and approved the submitted version.

FUNDING

As part of the MSc dissertation of first author (AB), this research work was funded by Bangladesh Agricultural University (Grant No. 2020/44/BAU) and National Science and Technology (NST) Fellowship grant (Grant No. MOST/MS/572).

ACKNOWLEDGMENTS

The authors express their gratitude to Professor Dr. Tanvir Rahman, Department of Aquaculture, BAU, for his laboratory assistance during histology and hematology. The authors are also grateful to Ceresco Nutrition Ltd. for permitting to use of their artwork in developing figures for this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.706179/full#supplementary-material

Figure 1 | Kidney of control fish (T0).

Figure 2 | Kidney of fish treated with 1 mg/kg SiNP (T1).

Figure 3 | Kidney of fish treated with 2 mg/kg SiNP (T2).

Figure 4 | Kidney of fish treated with 3 mg/kg SiNP (T4).

Figure 5 | Liver of control fish (T0).

Figure 6 | Liver of fish treated with 1 mg/kg SiNP (T1).

Figure 7 | Liver of fish treated with 2 mg/kg SiNP (T2).

Figure 8 | Liver of fish treated with 3 mg/kg SiNP (T3).

REFERENCES

Aanyu, M., Betancor, M. B., and Monroig, O. (2018). Effects of dietary limonene and thymol on the growth and nutritional physiology of Nile tilapia (*Oreochromis niloticus*). *Aquaculture* 488, 217–226. doi: 10.1016/j.aquaculture.2018.01.036

Aanyu, M., Betancor, M. B., and Monroig, Ó. (2020). The effects of combined phytogenics on growth and nutritional physiology of Nile tilapia *Oreochromis niloticus*. *Aquaculture* 519:734867. doi: 10.1016/j.aquaculture.2019.734867

Abd El-Kader, M. F., Fath El-Bab, A. F., Abd-Elghany, M. F., Abdel-Warith, A.-W. A., Younis, E. M., and Dawood, M. A. O. (2020). Selenium nanoparticles act potentially on the growth performance, hemato-biochemical indices, antioxidative, and immune-related genes of European seabass (Dicentrarchus labrax). *Biol. Trace Elem. Res.* 199, 3126–3313. doi: 10.1007/s12011-020-02431-1

Abd El-Naby, A. S., Al-Sagheer, A. A., Negm, S. S., and Naiel, M. A. E. (2020). Dietary combination of chitosan nanoparticle and thymol affects feed utilization, digestive enzymes, antioxidant status, and intestinal morphology of *Oreochromis niloticus*. *Aquaculture* 515:734577. doi: 10.1016/j.aquaculture.2019.734577

Abd El-Naby, A. S., Khattaby, A. E. R. A., Samir, F., Awad, S. M. M., and Abdel-Tawwab, M. (2019). Stimulatory effect of dietary butyrate on

growth, immune response, and resistance of Nile tilapia, *Oreochromis niloticus* against Aeromonas hydrophila infection. *Anim. Feed Sci. Technol.* 254:114212. doi: 10.1016/j.anifeedsci.2019.114212

Abdel-Tawwab, M., Adeshina, I., and Issa, Z. A. (2020). Antioxidants and immune responses, resistance to Aspergilus flavus infection, and growth performance of Nile tilapia, *Oreochromis niloticus*, fed diets supplemented with yeast, Saccharomyces serevisiae. *Anim. Feed Sci. Technol.* 263:114484. doi: 10.1016/j.anifeedsci.2020.114484

Ahmed, G. U., Upala, S. R., Hasan, M. T., and Hasan, N. (2014). Comparative study on growth performance between Vietnam koi and Thai koi in mini ponds. *J. Bangladesh Agric. Univ.* 12, 405–409. doi: 10.22004/ag.econ.211262

Ahmed, N. (2007). "Economics of aquaculture feeding practices: Bangladesh," in *Economics of Aquaculture Feeding Practices in Selected Asian Countries*, ed M. R. Hasan (Rome: FAO Fisheries Technical Paper. No. 505).

Al-Deriny, S. H., Dawood, M. A. O., Elbialy, Z. I., El-Tras, W. F., and Mohamed, R. A. (2020). Selenium nanoparticles and spirulina alleviate growth performance, hemato-biochemical, immune-related genes, and heat shock protein in Nile Tilapia (*Oreochromis niloticus*). *Biol. Trace Elem. Res.* 198, 661–668. doi: 10.1007/s12011-020-02096-w

Amer, S. A., Ahmed, S. A. A., Ibrahim, R. E., Al-Gabri, N. A., Osman, A., and Sitohy, M. (2020). Impact of partial substitution of fish meal by methylated soy protein isolates on the nutritional, immunological, and health aspects of Nile tilapia, *Oreochromis niloticus* fingerlings. *Aquaculture* 518:734871. doi: 10.1016/j.aquaculture.2019.734871

AOAC (2005). *Official Methods of Analysis*. eds W. Horwitz and G. W. Latimer (Maryland: Association of Official Analytical Chemists (AOAC) International).

Austreng, E. (1978). Digestibility determination in fish using chromic oxide marking and analysis of contents from different segments of the gastrointestinal tract. *Aquaculture* 13, 265–272. doi: 10.1016/0044-8486(78)90008-X

Bahabadi, M. N., Delavar, F. H., Mirbakhsh, M., Niknam, K., and Johari, S. A. (2017). Assessment of antibacterial activity of two different sizes of colloidal silver nanoparticle (cAgNPs) against Vibrio harveyi isolated from shrimp Litopenaeus vannamei. *Aquac. Int.* 25, 463–472. doi: 10.1007/s10499-016-0043-8

Barman, B. K., and Little, D. C. (2011). Use of hapas to produce Nile tilapia (*Oreochromis niloticus* L.) seed in household foodfish ponds: A participatory trial with small-scale farming households in Northwest Bangladesh. *Aquaculture* 317, 214–222. doi: 10.1016/j.aquaculture.2011.04.005

Bashar, A., Heal, R. D., Hasan, N. A., and Haque, M. M. (2021). Effect of COVID-19 on shrimp aquaculture in Bangladesh. *SSRN Electron. J.* 31:7896. doi: 10.2139/ssrn.3867896

Bashar, A., Islam, M. R., Zarin, I., Rohani, M. F., Haque, M. M., and Hossain, M. S. (2019). "Nutritional Intervention of *Oreochromis niloticus* the application of Silica nano-particles in Recirculatory Aquaculture System (RAS)," in *Abstract Book (Biennial International Conference-2019) (*Mymensingh: Fisheries Society of Bangladesh).

Belton, B., and Thilsted, S. H. (2014). Fisheries in transition: Food and nutrition security implications for the global South. *Glob. Food Sec.* 3, 59–66. doi: 10.1016/j.gfs.2013.10.001

Berg, J. M., Tymoczko, J. L., and Stryer, L. (2002). *Biochemistry. 5th edition. Proteases: Facilitating a Difficult Reaction*. New York, NY: W H Freeman.

Bitar, A., Ahmad, N. M., Fessi, H., and Elaissari, A. (2012). Silica-based nanoparticles for biomedical applications. *Drug Discov. Today* 17, 1147–1154. doi: 10.1016/j.drudis.2012.06.014

Blaxhall, P. C., and Daisley, K. W. (1973). Routine haematological methods for use with fish blood. *J. Fish Biol.* 5, 771–781. doi: 10.1111/j.1095-8649.1973.tb04510.x

Butré, C. I., Wierenga, P. A., and Gruppen, H. (2014). Influence of water availability on the enzymatic hydrolysis of proteins. *Process Biochem.* 49, 1903–1912. doi: 10.1016/j.procbio.2014.08.009

Carlberg, H., Nilsson, J., Brännäs, E., and Alanär,ä, A. (2018). An evaluation of 30 years of selective breeding in the Arctic charr (*Salvelinus alpinus* L.) and its implications for feeding management. *Aquaculture* 495, 428–434. doi: 10.1016/j.aquaculture.2018.06.008

Carlisle, E. M. (1981). Silicon: A requirement in bone formation independent of vitamin D1. *Calcif. Tissue Int.* 33, 27–34. doi: 10.1007/BF02409409

Chan, C. Y., Tran, N., Pethiyagoda, S., Crissman, C. C., Sulser, T. B., and Phillips, M. J. (2019). Prospects and challenges of fish for food security in Africa. *Glob. Food Sec.* 20, 17–25. doi: 10.1016/j.gfs.2018.12.002

Chaput, D. L., Bass, D., Alam, M. M., Hasan, N., Al Stentiford, G. D., van Aerle, R., et al. (2020). The Segment Matters: Probable Reassortment of Tilapia Lake Virus (TiLV) Complicates Phylogenetic Analysis and Inference of Geographical Origin of New Isolate from Bangladesh. *Viruses* 12:258. doi: 10.3390/v12030258

Crab, R., Avnimelech, Y., Defoirdt, T., Bossier, P., and Verstraete, W. (2007). Nitrogen removal techniques in aquaculture for a sustainable production. *Aquaculture* 270, 1–14. doi: 10.1016/j.aquaculture.2007.05.006

da Silva, M. A., de Alvarenga, É. R., Alves, G. F., Manduca, L. G., Turra, E. M. (2018). Crude protein levels in diets for two growth stages of Nile tilapia (*Oreochromis niloticus*) in a biofloc system. *Aquac. Res.* 49, 2693–2703. doi: 10.1111/are.13730

Dawood, M. A. O., Eweedah, N. M., Khalafalla, M. M., and Khalid, A. (2020a). Evaluation of fermented date palm seed meal with Aspergillus oryzae on the growth, digestion capacity and immune response of Nile tilapia (*Oreochromis niloticus*). *Aquac. Nutr.* 26, 828–841. doi: 10.1111/anu.13042

Dawood, M. A. O., Magouz, F. I., Mansour, M., Saleh, A. A., Asely, A. M., and El, Fadl, S. E., et al. (2020b). Evaluation of yeast fermented poultry by-product meal in Nile Tilapia (*Oreochromis niloticus*) feed: effects on growth performance, digestive enzymes activity, innate immunity, and antioxidant capacity. *Front. Vet. Sci.* 6:516. doi: 10.3389/fvets.2019.00516

Dawood, M. A. O., Metwally, A. E. S., El-Sharawy, M. E., Ghozlan, A. M., Abdel-Latif, H. M. R., Van Doan, H., et al. (2020c). The influences of ferulic acid on the growth performance, haemato-immunological responses, and immune-related genes of Nile tilapia (*Oreochromis niloticus*) exposed to heat stress. *Aquaculture* 525:735320. doi: 10.1016/j.aquaculture.2020.735320

Dawood, M. A. O., Moustafa, E. M., Gewaily, M. S., Abdo, S. E., AbdEl-kader, M. F., SaadAllah, M. S., et al. (2020d). Ameliorative effects of Lactobacillus plantarum L-137 on Nile tilapia (*Oreochromis niloticus*) exposed to deltamethrin toxicity in rearing water. *Aquat. Toxicol.* 219:105377. doi: 10.1016/j.aquatox.2019.105377

Dawood, M. A. O., Zommara, M., Eweedah, N. M., and Helal, A. I. (2020e). Synergistic effects of selenium nanoparticles and vitamin e on growth, immune-related gene expression, and regulation of antioxidant status of Nile Tilapia (*Oreochromis niloticus*). *Biol. Trace Elem. Res.* 195, 624–635. doi: 10.1007/s12011-019-01857-6

Dawood, M. A. O., Zommara, M., Eweedah, N. M., and Helal, A. I. (2020f). The evaluation of growth performance, blood health, oxidative status and immune-related gene expression in Nile tilapia (*Oreochromis niloticus*) fed dietary nanoselenium spheres produced by lactic acid bacteria. *Aquaculture* 515:734571. doi: 10.1016/j.aquaculture.2019.734571

De Araújo, L. A., Addor, F., and Campos, P. M. B. G. M. (2016). Use of silicon for skin and hair care: An approach of chemical forms available and efficacy. *An. Bras. Dermatol.* 91, 331–335. doi: 10.1590/abd1806-4841.20163986

de Verdal, H., Vandeputte, M., Mekkawy, W., Chatain, B., and Benzie, J. A. H. (2018). Quantifying the genetic parameters of feed efficiency in juvenile Nile tilapia *Oreochromis niloticus*. *BMC Genet.* 19:105. doi: 10.1186/s12863-018-0691-y

El-Naby, F. S. A., Naiel, M. A. E., Al-Sagheer, A. A., and Negm, S. S. (2019). Dietary chitosan nanoparticles enhance the growth, production performance, and immunity in *Oreochromis niloticus*. *Aquaculture* 501, 82–89. doi: 10.1016/j.aquaculture.2018.11.014

Faisal, A. M., Salaün, F., Giraud, S., Ferri, A., Chen, Y., and Wang, L. (2021). Far-infrared emission properties and thermogravimetric analysis of ceramic-embedded polyurethane films. *Polymers.* 13, 1–9. doi: 10.3390/polym13050686

FAO (2016). *The State of World Fisheries and Aquaculture 2016 (SOFIA): Contributing to Food Security and Nutrition for All*. Rome: Food and Agriculture Organization

FDA (2019). *CFR - Code of Federal Regulations Title 21. Department of Health and Human Services, USFDA, Maryland, USA*. Available online at: https://bit.ly/30JvCRh (sccessed August 10, 2020).

Fenton, T. W., and Fenton, M. (1979). An improved procedure for the determination of chromic oxide in feed and feces. *Can. J. Anim. Sci.* 59, 631-−634. doi: 10.4141/cjas79-081

Foysal, M. J., Alam, M., Kawser, A. Q. M. R., Hasan, F., Rahman, M. M., Tay, C. Y., et al. (2020). Meta-omics technologies reveals beneficiary effects of *Lactobacillus plantarum* as dietary supplements on gut microbiota, immune response and disease resistance of Nile tilapia (*Oreochromis niloticus*). *Aquaculture* 520:734974. doi: 10.1016/j.aquaculture.2020.734974

Francis, N. O., and Esa, Y. (2016). A review of production protocols used in producing economically viable monosex tilapia. *J. Fish. Aquat. Sci.* 11, 1–11. doi: 10.3923/jfas.2016.1.11

García-Meilán, I., Ordóñez-Grande, B., Machahua, C., Buenestado, S., Fontanillas, R., and Gallardo, M. A. (2016). Effects of dietary protein-to-lipid ratio on digestive and absorptive processes in sea bass fingerlings. *Aquaculture* 463, 163–173. doi: 10.1016/j.aquaculture.2016.05.039

Giatsis, C., Sipkema, D., Smidt, H., Heilig, H., Benvenuti, G., Verreth, J., et al. (2015). The impact of rearing environment on the development of gut microbiota in tilapia larvae. *Sci. Rep.* 5, 1–15. doi: 10.1038/srep18206

Giorgini, E., Randazzo, B., Gioacchini, G., Cardinaletti, G., Vaccari, L., Tibaldi, E., et al. (2018). New insights on the macromolecular building of rainbow trout (*O.* mykiss) intestine: FTIR Imaging and histological correlative study. *Aquaculture* 497, 1–9. doi: 10.1016/j.aquaculture.2018.07.032

Gobas, F. A. P. C. (2001). "Assessing bioaccumulation factors of persistent organic pollutants in aquatic food-chains," in *Persistent Organic Pollutants*, ed S. Harrad (Boston, MA: Springer). doi: 10.1007/978-1-4615-1571-5_6

Gui, J. F., Tang, Q., Li, Z., Liu, J., and De Silva, S. S. (2018). *Aquaculture in China: Success Stories and Modern Trends*. Chichester: John Wiley & Sons Ltd.). doi: 10.1002/9781119120759

Guo, J., Huang, Y., Salze, G., Roy, L. A., and Davis, D. A. (2020). Use of plant-based protein concentrates as replacement for fishmeal in practical diets for the Pacific white shrimp (*Litopenaeus vannamei*) reared under high stocking density and low salinity conditions. *Aquac. Nutr.* 26, 225–232. doi: 10.1111/anu.12982

Haque, M. M., Alam, M. M., Hoque, M. S., Hasan, N. A., Nielsen, M., Hossain, M. I., et al. (2021a). Can Bangladeshi pangasius farmers comply with the requirements of aquaculture certification? *Aquac. Rep*. 21:100811. doi: 10.1016/j.aqrep.2021.100811

Haque, M. M., Hasan, N. A., Eltholth, M. M., Saha, P., Mely, S. S., Rahman, T., et al. (2021b). Assessing the impacts of in-feed probiotic on the growth performance and health condition of pangasius (Pangasianodon hypophthalmus) in a farm trial. *Aquac. Rep.* 20:100699. doi: 10.1016/j.aqrep.2021.100699

Hasan, N. A., and Haque, M. M. (2020). Dataset of white spot disease affected shrimp farmers disaggregated by the variables of farm site, environment, disease history, operational practices, and saline zones. *Data Br.* 31:105936. doi: 10.1016/j.dib.2020.105936

Hasan, N. A., Haque, M. M., Bashar, A., Hasan, M. T., Faruk, M. A. R., and Ahmed, G. U. (2021a). Effects of dietary Papaveraceae extract on growth, feeding response, nutritional quality and serum biochemical indices of striped catfish (Pangasianodon hypophthalmus). *Aquac. Rep*. 21:100793. doi: 10.1016/J.AQREP.2021.100793

Hasan, N. A., Haque, M. M., Hinchliffe, S. J., and Guilder, J. (2020). A sequential assessment of WSD risk factors of shrimp farming in Bangladesh: looking for a sustainable farming system. *Aquaculture* 526:735348. doi: 10.1016/j.aquaculture.2020.735348

Hasan, N. A., Heal, R. D., Bashar, A., Bablee, A. L., and Haque, M. M. (2021b). Impacts of COVID-19 on the finfish aquaculture industry of Bangladesh: A case study. *Mar. Policy* 130:104577. doi: 10.1016/j.marpol.2021.104577

Hasan, N. A., Heal, R. D., Bashar, A., and Haque, M. M. (2021c). Face masks: protecting the wearer but neglecting the aquatic environment? - A perspective from Bangladesh. *Environ. Challenges* 4:100126. doi: 10.1016/j.envc.2021.100126

Hassaan, M. S., Mohammady, E. Y., Soaudy, M. R., Palma, J., Shawer, E. E., and El-Haroun, E. (2020a). The effect of dietary sericite on growth performance, digestive enzymes activity, gut microbiota and haematological parameters of Nile tilapia, *Oreochromis niloticus* (L.) fingerlings. *Anim. Feed Sci. Technol.* 262:114400. doi: 10.1016/j.anifeedsci.2020.114400

Hassaan, M. S., Nssar, K. M., Mohammady, E. Y., Amin, A., Tayel, S. I., and El-Haroun, E. R. (2020b). Nano-zeolite efficiency to mitigate the aflatoxin B1 (AFB1) toxicity: Effects on growth, digestive enzymes, antioxidant, DNA damage and bioaccumulation of AFB1 residues in Nile tilapia (*Oreochromis niloticus*). *Aquaculture* 523:735123. doi: 10.1016/j.aquaculture.2020.735123

Hauptkorn, S., Pavel, J., and Seltner, H. (2001). Determination of silicon in biological samples by ICP-OES after non-oxidative decomposition under alkaline conditions. *Anal. Bioanal. Chem.* 370, 246–250. doi: 10.1007/s002160100759

Heal, R. D., Hasan, N. A., and Haque, M. M. (2021). Increasing disease burden and use of drugs and chemicals in Bangladesh shrimp aquaculture: a potential menace to human health. *Mar. Pollut. Bull.* 171:112796. doi: 10.1016/j.marpolbul.2021.112796

Huang, S., Wang, L., Liu, L., Hou, Y., and Li, L. (2015). Nanotechnology in agriculture, livestock, and aquaculture in China. *A review. Agron. Sustain. Dev.* 35, 369–400. doi: 10.1007/s13593-014-0274-x

Hussain, B., Fatima, M., Al-Ghanim, K. A., and Mahboob, S. (2019). Environmentally induced nephrotoxicity and histopathological alternations in Wallago attu and Cirrhinus mrigla. *Saudi J. Biol. Sci.* 26, 752–757. doi: 10.1016/j.sjbs.2019.02.003

Irm, M., Taj, S., Jin, M., Luo, J., Andriamialinirina, H. J. T., and Zhou, Q. (2020). Effects of replacement of fish meal by poultry by-product meal on growth performance and gene expression involved in protein metabolism for juvenile black sea bream (*Acanthoparus schlegelii*). *Aquaculture* 528:735544. doi: 10.1016/j.aquaculture.2020.735544

Jarvie, H. P., Al-Obaidi, H., King, S. M., Bowes, M. J., Lawrence, M. J., Drake, A. F., et al. (2009). Fate of silica nanoparticles in simulated primary wastewater treatment. *Environ. Sci. Technol.* 43, 8622–8628. doi: 10.1021/es901399q

Jennings, S., Stentiford, G. D., Leocadio, A. M., Jeffery, K. R., Metcalfe, J. D., Katsiadaki, I., et al. (2016). Aquatic food security: insights into challenges and solutions from an analysis of interactions between fisheries, aquaculture, food safety, human health, fish and human welfare, economy and environment. *Fish Fish.* 17, 893–938. doi: 10.1111/faf.12152

Jiang, M., Ma, L., Shao, H., Wu, F., Liu, W., Tian, J., et al. (2020). Dietary vitamin E requirement of sub-adult genetically improved farmed tilapia strain of Nile tilapia (*Oreochromis niloticus*) reared in freshwater. *Aquac. Nutr.* 26, 233–241. doi: 10.1111/anu.12983

Jugdaohsingh, R. (2007). *Silicon and Bone Health in Journal of Nutrition, Health and Aging (Europe PMC Funders)*. Available online at: /pmc/articles/PMC2658806/?report=abstract (accessed August 10, 2020).

Junqueira, L. C., and Carneiro, J. (2013). *Histologia Básica*. 12th ed. Rio de Janeiro: Guanabara Koogan.

Kabir, K. A., Verdegem, M. C. J., Verreth, J. A. J., Phillips, M. J., and Schrama, J. W. (2019). Effect of dietary protein to energy ratio, stocking density and feeding level on performance of Nile tilapia in pond aquaculture. *Aquaculture* 511:634200. doi: 10.1016/j.aquaculture.2019.06.014

Kelsay, J. L., Behall, K. M., and Prather, E. S. (1979). Effect of fiber from fruits and vegetables on metabolic responses of human subjects. II. Calcium, magnesium, iron, and silicon balances. *Am. J. Clin. Nutr.* 32, 1876–1880. doi: 10.1093/ajcn/32.9.1876

Kent, M. L., Feist, S. W., Harper, C., Hoogstraten-Miller, S., Law, J., Mac, Sánchez-Morgado, J. M., et al. (2009). Recommendations for control of pathogens and infectious diseases in fish research facilities. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 149, 240–248. doi: 10.1016/j.cbpc.2008.08.001

Khosravi-Katuli, K., Prato, E., Lofrano, G., Guida, M., Vale, G., and Libralato, G. (2017). Effects of nanoparticles in species of aquaculture interest. *Environ. Sci. Pollut. Res.* 24, 17326–17346. doi: 10.1007/s11356-017-9360-3

Kim, K. D., Lim, S. G., Kang, Y. J., Kim, K. W., and Son, M. H. (2012). Effects of dietary protein and lipid levels on growth and body composition of juvenile far eastern catfish Silurus asotus. *Asian-Austr. J. Anim. Sci.* 25, 369–374. doi: 10.5713/ajas.2011.11089

Kong, W., Huang, S., Yang, Z., Shi, F., Feng, Y., and Khatoon, Z. (2020). Fish feed quality is a key factor in impacting aquaculture water environment: evidence from incubator experiments. *Sci. Rep.* 10:187. doi: 10.1038/s41598-019-57063-w

Kumar, N., Krishnani, K. K., and Singh, N. P. (2018). Effect of dietary zinc-nanoparticles on growth performance, anti-oxidative and immunological status of fish reared under multiple stressors. *Biol. Trace Elem. Res.* 186, 267–278. doi: 10.1007/s12011-018-1285-2

Laforenza, U. (2012). Water channel proteins in the gastrointestinal tract. *Mol. Aspects Med.* 33, 642–650. doi: 10.1016/j.mam.2012.03.001

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

Martins, G. B., da Rosa, C. E., Tarouco, F., M., and Robaldo, R. B. (2019). Growth, water quality and oxidative stress of Nile tilapia *Oreochromis niloticus* (L.) in biofloc technology system at different pH. *Aquac. Res.* 50, 1030–1039. doi: 10.1111/are.13975

McCue, M. D. (2013). *Comparative Physiology of Fasting, Starvation, and Food Limitation*. Berlin: Springer-Verlag. doi: 10.1007/978-3-642-29056-5

Merry, M. (2017). *How to Calculate Silicon From Silica. Sciencing*. Available online at: https://sciencing.com/calculate-silicon-silica-7548918.html (accessed December 8, 2020).

Moesch, A., Meurer, F., Zadinelo, I. V., Carneiro, W. F., da Silva, L. C. R., and dos Santos, L. D. (2016). Growth, body composition and hepatopancreas morphology of Nile tilapia fingerlings fed crude glycerol as a replacement for maize in diets. *Anim. Feed Sci. Technol.* 219, 122–131. doi: 10.1016/j.anifeedsci.2016.05.009

Naiel, M. A. E., Ismael, N. E. M., Abd El-hameed, S. A. A., and Amer, M. S. (2020). The antioxidative and immunity roles of chitosan nanoparticle and vitamin C-supplemented diets against imidacloprid toxicity on *Oreochromis niloticus*. *Aquaculture* 523:735219. doi: 10.1016/j.aquaculture.2020.735219

Nguyen, L., Dinh, H., and Davis, D. A. (2020a). Efficacy of reduced protein diets and the effects of indispensable amino acid supplements for Nile tilapia *Oreochromis niloticus*. *Anim. Feed Sci. Technol.* 268:114593. doi: 10.1016/j.anifeedsci.2020.114593

Nguyen, L., Salem, S. M. R., Salze, G. P., Dinh, H., and Davis, D. A. (2020b). Optimizing amino acid balance in diets for Nile tilapia *Oreochromis niloticus*. *Aquaculture* 515:734566. doi: 10.1016/j.aquaculture.2019.734566

Ogello, E. O., Munguti, Jonathan Sakakura, Y., and Hagiwara, A. (2014). Complete replacement of fish meal in the diet of Nile tilapia (*Oreochromis niloticus* L.) grow-out with alternative protein sources. *Int. J. Adv. Res.* 2, 962–978.

Oguz, A. R. (2015). A histological study of the kidney structure of Van fish (*Alburnus tarichi*) acclimated to highly alkaline water and freshwater. *Mar. Freshw. Behav. Physiol.* 48, 135–144. doi: 10.1080/10236244.2015.1004838

Onuegbu, C. U., Aggarwal, A., and Singh, N. B. (2018). ZnO nanoparticles as feed supplement on growth performance of cultured African catfish fingerlings. *J. Sci. Ind. Res.* 77, 213–218. Retrieved from: http://nopr.niscair.res.in/handle/123456789/44151

Perez-Velazquez, M., Gatlin, D. M., González-Félix, M. L., García-Ortega, A., de Cruz, C. R., Juárez-Gómez, M. L., et al. (2019). Effect of fishmeal and fish oil replacement by algal meals on biological performance and fatty acid profile of hybrid striped bass (Morone crhysops ♀ × M. *saxatilis* ♂). *Aquaculture* 507, 83–90. doi: 10.1016/j.aquaculture.2019.04.011

Pianesso, D., Adorian, T. J., Mombach, P. I., Dalcin, M. O., Loebens, L., Telles, Y. B., et al. (2020). Nutritional assessment of linseed meal (*Linum usitatissimum* L.) protein concentrate in feed of silver catfish. *Anim. Feed Sci. Technol.* 265:114517. doi: 10.1016/j.anifeedsci.2020.114517

Pieszka, M., Bederska-Łojewska, D., Szczurek, P., and Pieszka, M. (2019). The membrane interactions of nano-silica and its potential application in animal nutrition. *Animals* 9:1041. doi: 10.3390/ani9121041

Pirarat, N., Boonananthanasarn, S., Laddawan Katagiri, T., and Maita, M. (2015). Effect of activated charcoal-supplemented diet on growth performance and intestinal morphology of Nile Tilapia (*Oreochromis niloticus*). *Thai J. Vet. Med.* 45, 113–119. Retrieved from: https://he01.tci-thaijo.org/index.php/tjvm/article/view/32140

Priyadarsini, S., Mukherjee, S., and Mishra, M. (2018). Nanoparticles used in dentistry: a review. *J. Oral Biol. Craniofacial Res.* 8, 58–67. doi: 10.1016/j.jobcr.2017.12.004

Rathore, S. S., Murthy, H. S., Mamun, M. A., Al Nasren, S., Rakesh, K., Kumar, B. T. N., et al. (2020). Nano-selenium supplementation to ameliorate nutrition physiology, immune response, antioxidant system and disease resistance against aeromonas hydrophila in monosex Nile Tilapia (*Oreochromis niloticus*). *Biol. Trace Elem. Res.* 199, 3073–3088. doi: 10.1007/s12011-020-02416-0

Rodnick, K. J., and Planas, J. V. (2016). "The stress and stress mitigation effects of exercise: cardiovascular, metabolic, and skeletal muscle adjustments" in *Fish Physiology*, eds C. B. Schreck, L. Tort, A. P. Farrell, and C. J. Brauner (Cambridge: Academic Press-Elsevier). doi: 10.1016/B978-0-12-802728-8.00007-2

Rodriguez, Y. E., Laitano, M. V., Pereira, N. A., López-Zavala, A. A., Haran, N. S., and Fernández-Gimenez, A. V. (2018). Exogenous enzymes in aquaculture: Alginate and alginate-bentonite microcapsules for the intestinal delivery of shrimp proteases to Nile tilapia. *Aquaculture* 490, 35–43. doi: 10.1016/j.aquaculture.2018.02.022

Roy, S., and Bhattacharya, S. (2006). Arsenic-induced histopathology and synthesis of stress proteins in liver and kidney of Channa punctatus. *Ecotoxicol. Environ. Saf.* 65, 218–229. doi: 10.1016/j.ecoenv.2005.07.005

Schreck, C. B., and Tort, L. (2016). "The concept of stress in fish" in *Fish Physiology*, eds C. B. Schreck, L. Tort, A. P. Farrell, and C. J. Brauner (Cambridge: Academic Press-Elsevier) doi: 10.1016/B978-0-12-802728-8.00001-1

Shah, B. R., and Mraz, J. (2020). Advances in nanotechnology for sustainable aquaculture and fisheries. *Rev. Aquac.* 12, 925–942. doi: 10.1111/raq.12356

Simon, C. J., Blyth, D., Ahmad Fatan, N., and Suri, S. (2019). Microbial biomass (Novacq^{TM}) stimulates feeding and improves the growth performance on extruded low to zero-fishmeal diets in tilapia (GIFT strain). *Aquaculture* 501, 319–324. doi: 10.1016/j.aquaculture.2018.11.052

Singha, K. P., Shamna, N., Sahu, N. P., Sardar, P., Harikrishna, V., Thirunavukkarasar, R., et al. (2020). Optimum dietary crude protein for culture of genetically improved farmed tilapia (GIFT), *Oreochromis niloticus* (Linnaeus, 1758) juveniles in low inland saline water: effects on growth, metabolism and gene expression. *Anim. Feed Sci. Technol.* 271:114713. doi: 10.1016/j.anifeedsci.2020.114713

Sun, S., Wei, C. D., and Liu, Y. X. (2010). Characterization and water activation behavior of tourmaline nanoparticles. *J. Nanosci. Nanotechnol.* 10, 2119–2124. doi: 10.1166/jnn.2010.2087

Thangapandiyan, S., and Monika, S. (2020). Green synthesized zinc oxide nanoparticles as feed additives to improve growth, biochemical, and hematological parameters in freshwater fish labeo rohita. *Biol. Trace Elem. Res.* 195, 636–647. doi: 10.1007/s12011-019-01873-6

Uddin, M. T., Goswami, A., Rahman, M. S., and Dhar, A. R. (2019). How can governance improve efficiency and effectiveness of value chains? An analysis of pangas and tilapia stakeholders in Bangladesh. *Aquaculture* 510, 206–215. doi: 10.1016/j.aquaculture.2019.05.055

Vidya, H., Kumara Swamy, B. E., and Schell, M. (2016). One step facile synthesis of silver nanoparticles for the simultaneous electrochemical determination of dopamine and ascorbic acid. *J. Mol. Liq.* 214, 298–305. doi: 10.1016/j.molliq.2015.12.025

Wagner, J., and Stanton, T. L. (2012). *Formulating Rations With the Pearson Square*. Colorado, CO: Colorado State University.

Walton, M. J., and Cowey, C. B. (1982). Aspects of intermediary metabolism in salmonid fish. *Comp. Biochem. Physiol. – Part B Biochem.* 73, 59–79. doi: 10.1016/0305-0491(82)90201-2

Wardani, W. W., Alimuddin, A., Zairin, M., Setiawati, M., Nuryati, S., and Suprayudi, M. A. (2020). Evaluation of cysteamine supplementation in red tilapia (Oreochromis sp.) diet: Serum insulin and somatostatin, IGF-1 and GLUT4 genes expression, growth performance, and robustness against stress. *Aquaculture* 528:735514. doi: 10.1016/j.aquaculture.2020.735514

Wu, T., Sun, L. C., Du, C. H., Cai, Q. F., Zhang, Q. B., Su, W. J., et al. (2009). Identification of pepsinogens and pepsins from the stomach of European eel (Anguilla anguilla). *Food Chem.* 115, 137–142. doi: 10.1016/j.foodchem.2008.11.077

Wu, W., Ji, H., Yu, H., Sun, J., and Zhou, J. (2020). Effect of refeeding dietary containing different protein and lipid levels on growth performance, body composition, digestive enzyme activities and metabolic related gene expression of grass carp (Ctenopharyngodon idellus) after overwinter starvation. *Aquaculture* 523:735196. doi: 10.1016/j.aquaculture.2020.735196

Xu, W., Huang, K., Jin, W., Luo, D., Liu, H., Li, Y., et al. (2018). Catalytic and antibacterial properties of biosynthesized silver nanoparticles using native inulin. *RSC Adv.* 8, 28746–28752. doi: 10.1039/c8ra03386b

Yang, P., Lai, D. Y. F., Yang, H., Tong, C., Lebel, L., Huang, J., et al. (2019). Methane dynamics of aquaculture shrimp ponds in two subtropical estuaries, Southeast China: dissolved concentration, net sediment release, and water oxidation. *J. Geophys. Res. Biogeosciences* 124, 1430–1445. doi: 10.1029/2018JG004794

Younes, M., Aggett, P., Aguilar, F., Crebelli, R., Dusemund, B., Filipič, M., et al. (2018). Re-evaluation of silicon dioxide (E 551) as a food additive. *EFSA J.* 16:5088. doi: 10.2903/j.efsa.2018.5088

Yuan, Y., Yuan, Y., Dai, Y., and Gong, Y. (2017). Economic profitability of tilapia farming in China. *Aquac. Int.* 25, 1253–1264. doi: 10.1007/s10499-017-0111-8

Zhang, H., Luo, Y., Lu, D. L., Jiao, J. G., Li, L. Y., Qin, J. G., et al. (2019). Diacylglycerol oil reduces fat accumulation and increases protein content by inducing lipid catabolism and protein metabolism in Nile tilapia (*Oreochromis niloticus*). *Aquaculture* 510, 90–99. doi: 10.1016/j.aquaculture.2019.05.035

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 August 2021
doi: 10.3389/fmars.2021.707627

Supplementing Chitosan Oligosaccharide Positively Affects Hybrid Grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂) Fed Dietary Fish Meal Replacement With Cottonseed Protein Concentrate: Effects on Growth, Gut Microbiota, Antioxidant Function and Immune Response

Guofeng Chen[1,2,3], *Bin Yin*[1,2,3], *Hongyu Liu*[1,2,3]*, *Beiping Tan*[1,2,3]*, *Xiaohui Dong*[1,2,3], *Qihui Yang*[1,2,3], *Shuyan Chi*[1,2,3] and *Shuang Zhang*[1,2,3]

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

Oligosaccharides have recently received much attention from researchers owing to their multiple biological activities. This study was conducted to investigate the effects of a diet with reduced fish meal and chitosan oligosaccharide (COS) supplementation on a hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂). Seven isonitrogenous and isolipidic types of diet were formulated to feed the groupers for 56 days. To prepare the feed, a reference diet (FM group) containing 55% fish meal was used. Concentrated cottonseed protein (CPC) was used to replace 45% of the fish meal protein, and different COS supplementation levels (0, 0.2%, 0.4, 0.6, 0.8, and 1%) were added. After an 8-week breeding trial, *Vibrio harveyi* bacteria were injected into the groupers for a 7-day challenge test. The results showed that the FM and COS0.4 groups showed the best growth performance among the groups ($p < 0.05$); however, there was no significant difference in the survival rate ($p > 0.05$). Unlike in the FM group, adhesion and breakage of the intestinal plica occurred in the COS0 group. The height and width of the gut fold reached maximum values in the COS0.4 group ($p < 0.05$). Microbiome sequencing suggested that there was a stable microbiota core in the gut of the groupers. With increasing COS levels, the abundance of both beneficial bacteria and conditional pathogens increased; the activities of serum glucose oxidase, catalase, and total superoxide dismutase also increased ($p < 0.05$). In the gut tissue, the activities of glutathione peroxidase, glutathione reductase, and glutamine increased first but then decreased ($p < 0.05$); the contents of lysozyme, acid phosphatase,

Edited by:
Jin Niu,
Sun Yat-Sen University, China

Reviewed by:
Yun-Zhang Sun,
Jimei University, China
Deiene Rodriguez Barreto,
University of La Laguna, Spain

***Correspondence:**
Hongyu Liu
liuhyu@gdou.edu.cn
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: *10 May 2021*
Accepted: *21 July 2021*
Published: *18 August 2021*

Citation:
Chen G, Yin B, Liu H, Tan B, Dong X, Yang Q, Chi S and Zhang S (2021) Supplementing Chitosan Oligosaccharide Positively Affects Hybrid Grouper (Epinephelus fuscoguttatus ♀ × E. lanceolatus ♂) Fed Dietary Fish Meal Replacement With Cottonseed Protein Concentrate: Effects on Growth, Gut Microbiota, Antioxidant Function and Immune Response. Front. Mar. Sci. 8:707627. doi: 10.3389/fmars.2021.707627

complements C3 and C4, and IgM showed upward trends ($p < 0.05$). Compared with that in the FM group, the expression of *IL-1β* and *TNF-α* in the COS0 group was upregulated. Gene expression levels of *TLR22*, *TGF-β*, and *Nrf2* increased first but then decreased with COS supplementation levels ($p < 0.05$). COS supplementation reduced the cumulative mortality of the groupers in the challenge test ($p < 0.05$). In general, the results of this study demonstrated that dietary COS supplementation enhanced growth performance, intestinal health, and antioxidant and immune responses of groupers fed with a low-fish meal diet. The optimal and acceptable levels of COS supplement were 0.45 and 0.4–0.6%, respectively; these values can provide a reference for developing aquatic prebiotics.

Keywords: chitosan oligosaccharide (cos), prebiotics, fishmeal replacement, intestinal microbiota, immune response

INTRODUCTION

The grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂) is a new hybrid fish species that has a fast growth rate, enhanced nutritional value, and powerful stress resistance (Zhou et al., 2019). This species is widely cultivated in Southeast Asia and China, and it has maintained bright commercial prospects (Li et al., 2018b; Ye et al., 2020).

In recent years, aquaculture has become a rapidly growing industry that is essential in terms of global food security, because it provides high-quality protein to the public (Zeng et al., 2017). However, fish meal production cannot meet the demands of the aquatic industry (Hardy and Tacon, 2002; Hardy, 2006). In this context, replacing fish meal with plant protein is a feasible approach for sustainable aquaculture (Carter and Hauler, 2000; Daniel, 2018). As a new type of plant protein, cottonseed protein concentrate (CPC) has recently received much attention from researchers (Wan et al., 2018; Ye et al., 2020). Accordingly, the experimental replacement of fish meal with CPC has been conducted for certain aquaculture species, such as hybrid grouper (Chen et al., 2020), juvenile golden pompano (*Trachinotus ovatus*) (Shen et al., 2020), and pacific white shrimp (*Litopenaeus vannamei*) (Wan et al., 2018). However, researchers have found that replacing fish meal with high levels of CPC can decrease growth performance and cause intestinal inflammation issues in hybrid groupers (Yin et al., 2018).

In the past, antibiotics have been used as feed supplements to treat fish gut inflammation. More recently, prebiotics have become an environmentally friendly alternative, as they do not cause environmental pollution or public health hazards (Song et al., 2014; Carbone and Faggio, 2016). For example, chitosan oligosaccharide (COS), a novel prebiotic, is prepared by enzymatic degradation, chemical derivatization, and chromatographic separation to obtain chitin from marine shells (Wei et al., 2020). Studies have found that COS supplementation can have multiple physiological benefits, such as antioxidant and anti-inflammatory effects, as well as immune stimulation (Liaqat and Eltem, 2018; Naveed et al., 2019). Other benefits of COS supplementation have been observed for terrestrial animals, such as improvement of fetal survival and reproductive performance in sows (*Sus scrofa domesticus*) (Wan et al., 2016), adjustment of gut microbiota and metabolites in weaned piglets (Kong et al., 2014), and enhancement of growth performance and serum immunity in chickens (*Gus gallus*) (Li et al., 2007). COS is a preferred feed additive for livestock, because it acts as an immunostimulant (Swiatkiewicz et al., 2015). In aquaculture, studies have found that dietary COS supplementation can strengthen growth performance, innate immunity, and disease resistance in turbot (*Scopthalmus maximus*) (Cui et al., 2013), koi (*Cyprinus carpio koi*) (Lin et al., 2012), and juvenile rainbow trout (*Oncorhynchus mykiss*) (Luo et al., 2009).

In this context, COS could be provided as an oral immunostimulant to more fish species. However, the role of COS is affected by many factors, such as fish species, body size, and breeding environment (Wei et al., 2020). Therefore, it is worth exploring the effects of prebiotic supplementation after fish meal substitution (Bai et al., 2017; Torrecillas et al., 2018). Currently, COS application is still under development in aquaculture (Zhang, 2019).

To investigate the improvements induced by COS supplementation in hybrid groupers subjected to fish meal replacement with CPC, this study focused on growth, gut microbiota, antioxidant function, and immune response. Additionally, this study evaluated a suitable level of dietary COS supplementation, which will be useful for providing insight into grouper feed development.

MATERIALS AND METHODS

Experimental Diets

Chitosan oligosaccharide with a purity of >90% was obtained from Bozhi Huili Biotechnology Co., Ltd. (Qingdao, China). Fish meal, CPC, starch, wheat gluten, casein, and gelatin were used as protein sources. Lipids were provided in the form of fish oil and phospholipids. Seven isonitrogenous and isolipidic feeds were prepared to feed the grouper. Based on a previous study (Yin et al., 2018), feed with 55% fish meal was designated as the reference diet (FM). For the test diets, 45% of the fish meal protein was replaced with CPC under uniform conditions. COS

TABLE 1 | Ingredients and proximate nutrient composition of test diets for hybrid grouper.

Ingredients (%)	Diets						
	FM	COS0	COS0.2	COS0.4	COS0.6	COS0.8	COS1
Fish meal	55.00	30.25	30.25	30.25	30.25	30.25	30.25
[a]Concentrate cottonseed protein	0	24	24	24	24	24	24
Starch	18	18	18	18	18	18	18
Wheat gluten	5	5	5	5	5	5	5
Casein	7.6	7.6	7.6	7.6	7.6	7.6	7.6
Gelatin	1.9	1.9	1.9	1.9	1.9	1.9	1.9
Fish oil	3.8	5.62	5.62	5.62	5.62	5.62	5.62
Phospholipid	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Calcium monophosphate	1	1	1	1	1	1	1
[b]Vitamin mixture	0.2	0.2	0.2	0.2	0.2	0.2	0.2
[c]Mineral mixture	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Antioxidant	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Choline chloride	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Cellulose microcrystalline	4.9	3.06	2.86	2.66	2.46	2.26	2.06
Vitamin C	0.05	0.05	0.05	0.05	0.05	0.05	0.05
[d] Methionine	0	0.25	0.25	0.25	0.25	0.25	0.25
[d]Lysine	0	0.52	0.52	0.52	0.52	0.52	0.52
Chitosan Oligosaccharide	0	0	0.2	0.4	0.6	0.8	1
Proximate nutrient composition (%, air dry matter)							
[e]Moisture	6.86	7.65	7.98	7.80	7.36	7.41	7.83
[e]Crude protein	50.71	50.73	50.21	51.27	51.26	49.51	49.50
[e]Crude lipid	12.89	13.21	12.06	11.88	12.71	13.08	11.82

[a]Cottonseed protein concentrate was prepared by Hunan Xinrui Biotechnology Co., Ltd. (Hunan, China).

[b]Vitamin mixture (g.kg^{-1} mixture): vitamin B1, 17.00 g; vitamin B2, 16.67 g; vitamin B6, 33.33 g; vitamin B12, 0.07 g; vitamin K, 3.33 g; vitamin E, 66.00 g; retinyl acetate, 6.67 g; VD, 33.33 g; nicotinic acid, 67.33 g; D-calcium pantothenate, 40.67 g; biotin, 16.67g; folic acid, 4.17 g; inositol, 102.04 g; cellulose, 592.72 g. All ingredients were diluted with corn starch to 1 kg.

[c]Mineral mixture (mg g^{-1} mixture):$CaCO_3$, 350 g; $NaH_2PO_4{\cdot}H_2O$, 200 g; KH_2PO_4, 200 g; NaCl, 12 g; $MgSO_4{\cdot}7H_2O$, 10g; $FeSO_4{\cdot}7H_2O$, 2 g; $MnSO_4{\cdot}7H_2O$, 2 g; $AlCl_3{\cdot}6H_2O$, 1 g; $CuCl_2{\cdot}2H_2O$, 1 g; KF, 1 g; $NaMoO_4{\cdot}2H_2O$, 0.5 g; $NaSeO_3$, 0.4 g; $CoCl_2{\cdot}6H_2O$, 0.1 g; KI, 0.1g; zeolite powder, 219.9 g. All ingredients were diluted with corn starch to 1 kg. (Obtained from Yuehai Feed Co. Ltd., China).

[d]Adding methionine and lysine was to avoid amino acid imbalance after fishmeal replacement.

[e]Moisture, Crude protein and crude lipid were measured values.

supplementation was conducted at different concentrations (0, 0.2, 0.4, 0.6, 0.8, and 1%).

The raw materials were fully mixed and extruded into a hard pellet with a diameter of approximately 2.5 mm. The semi-finished feed was dried at room temperature (25°C) to a 10% moisture content. The finished product was stored at −20°C for later use. The ingredients and approximate nutrient compositions of the test diets are shown in **Table 1**. The essential amino acid profiles of the test diets are shown in **Table 2**.

Feeding Management and Challenge Test

This experiment was approved by the Animal Care and Ethics Committee of Guangdong Ocean University. Experimental fish were obtained from East Island, Zhanjiang. A feeding experiment was conducted at the Marine Science and Technology Park of Zhanjiang (Guangdong, China). Seven treatment groups were used in this study, and each treatment group was established in triplicate. The volume of each breeding tank was 0.3 m^3, where 30 hybrid groupers (initial weight = 13.19 ± 0.02 g) were cultured. The groupers were acclimated to the laboratory environment for 1 week prior to the experiment. The experimental breeding time was 8 weeks. Feeding was performed at 8:00 and 17:00 daily, and the fish were fully satiated. Each day, 90% of the water in each tank was changed. During breeding, the water temperature ranged from 28.4 to 31.6°C, and salinity was 30 ± 2‰. The average dissolved oxygen content exceeded 7 mg L^{-1}, the pH ranged from 7.8 to 8.1, and the ammonia nitrogen content was below 0.03 mg L^{-1}.

After the 8-week breeding experiment, 30 fish were randomly selected from each treatment group (10 fish from each tank) for a challenge test. *Vibrio harveyi* bacteria were obtained from the Key Laboratory of Aquatic Economic Animal Disease Control of Guangdong Provincial Colleges and Universities (Zhanjiang, China). According to the method of Liu et al. (2013), 300 μl of a 5 × 10^8 live *V. harveyi* bacteria suspension was injected into the base of the pectoral fin of each grouper using a 1-ml syringe. The cumulative mortality of the groupers was obtained for 7 days after injection.

TABLE 2 | Essential amino acid (EAA) profile (%) of the test diets used in the experiment.

Amino acids	Test diets						
	FM	COS0	COS0.2	COS0.4	COS0.6	COS0.8	COS1
Methionine	1.24	1.12	1.08	1.11	1.12	1.01	1.03
Lysine	3.47	3.25	3.21	3.24	3.35	3.17	3.16
Threonine	2.06	1.83	1.84	1.84	1.82	1.76	1.77
Isoleucine	1.99	1.78	1.74	1.81	1.78	1.80	1.83
Histidine	1.55	1.47	1.49	1.54	1.57	1.52	1.54
Valine	2.35	2.24	2.18	2.28	2.27	2.21	2.21
Leucine	3.72	3.42	3.33	3.42	3.43	3.39	3.38
Arginine	3.65	3.61	3.63	3.64	3.68	3.58	3.60
Phenylalanine	2.12	2.28	2.25	2.37	2.36	2.33	2.33
Tyrosine	1.63	1.59	1.55	1.63	1.63	1.60	1.62

Sample Collection

Before sampling, the groupers were fasted for 24 h counted, and weighed, and their body lengths were measured. Ten fish from each tank were randomly selected and sacrificed. First, blood was drawn from the fish and then centrifuged at 4,000 rpm for 10 min. The serum was stored at −20°C for later use. The visceral mass was separated, and then the visceral mass, liver, and intestine were weighed separately. Intestinal tissue was washed with physiological saline (0.65%) on a sterile operating table, and then it was stored at −80°C for later enzyme activity analysis, microbiome sequencing, and mRNA expression analysis.

Additionally, the intestines of three other fish per tank were separated, washed with sterile saline (0.65%), and preserved in a 4% formaldehyde solution. Intestinal AB–PAS (alcian blue-periodic acid sthiff) sectioning slices were prepared by Seville Biological Technology Co., Ltd. (Guangzhou, China). Histological observations were conducted using a Leica DM6000 optical microscope (Leica, Wetzlar, Germany), and the LAS 3.8 (Leica, Wetzlar, Germany) software (in Windows) was used for taking photographs. Ten visions from each slice were randomly selected for observation.

Growth Parameters and Biochemical Analysis

The parameters of growth performance were calculated as follows:

$$Weight\ gain\ (WG,\ \%) = \frac{final\ body\ weight - initial\ body\ weight}{initial\ body\ weight} \times 100;$$

$$Specific\ growth\ rate\ \left(SGR,\ \%\ day^{-1}\right) = \frac{\ln\left(final\ weight\right) - \ln(initial\ weight)}{days\ of\ feeding\ experiment} \times 100;$$

$$Feed\ conversion\ ratio\ (FCR) = \frac{feed\ consumed}{weight\ gain};$$

$$Survival\ rate\ (SR,\ \%) = \frac{final\ number\ of\ fish}{initial\ number\ of\ fish} \times 100;$$

$$Hepatosomatic\ index\ (HSI,\ \%) = \frac{hepatopancreas\ weight}{body\ weight} \times 100;$$

$$Vicerosomatic\ index\ (VSI,\ \%) = \frac{viscera\ weight}{body\ weight} \times 100;$$

$$Condition\ factor\ (CF,\ \%) = \frac{fish\ weight}{(body\ length)^3} \times 100.$$

The standard procedures of the Association of Official Analytical Chemists were adopted to detect the approximate composition of feed (Chemists, 2005).

Determination of Enzyme Activity and Biochemical Indices

According to the methods of Ayiku et al. (2020), glucose oxidase (GLU), alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride (TG), catalase (CAT), total superoxide dismutase (T-SOD), and malondialdehyde (MDA) in serum were detected using a microplate photometer (Multiskan™ Go, Thermo Fisher Scientific, Waltham, MA, United States), using commercial kits obtained from Jiancheng Bioengineering Institute (Nanjing, China).

The intestinal tissue was washed with pre-chilled phosphate-buffered saline (PBS) (0.01 M, pH = 7.4), and then a PBS solution (1:9) was added. The gut tissue was then ground on ice. After homogenization, the homogenate was centrifuged at 5,000 rpm for 5 min. The supernatant was extracted for further testing. The methods for measuring the intestinal immune indices (such as lysozyme, acid phosphatase, complement proteins 3 and 4, and immunoglobulin M) were based on Ye et al. (2020), using enzyme-linked immunosorbent assay (ELISA) detection kits from Meilian Biotechnology

Co., Ltd. (Shanghai, China) according to the instructions of the manufacturer. The activities of glutathione peroxidase (GSHPx), glutathione reductase (GR), and glutamine (Gln) were determined using the methods described by Chen et al. (2009). Nitric oxide (NO) content analysis was carried out using the method of Wink et al. (1993). Each sample was tested in triplicate.

Intestinal Microbiome Analysis

According to the methods of Chen et al. (2020), microbiome sequencing technology was used to detect the hybrid grouper intestinal microbiota, with the assistance of Biomarker Technologies Co., Ltd. (Beijing, China). The microbiome analyses of the samples of each treatment were performed in triplicate. Microbiota DNA was extracted using the Soil DNA Kit (Magen Co. Ltd., Guangzhou, China) according to the instructions of the manufacturer. The 16S rRNA V3 + V4 amplified areas of the ribosomal RNA gene were amplified by PCR. PCR was performed as follows: 95°C for 2 min, followed by 27 cycles of 98°C for 10 s, 62°C for 30 s, and 68°C for 30 s, and the final step at 68°C for 10 min. The primer sequences were 5′-ACTCCTACGGGAGGCAGCA-3′ (*338F*) and 5′-GGACTACHVGGGTWTCTAAT-3′ (*806R*). The amplicons were separated on a 2% agarose gel and then purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States). The amplicons were quantified using the ABI StepOnePlus real-time PCR system (Life Technologies, Carlsbad< CA, United States). Later, the purified amplicons were pooled in equimolar amounts, and paired-end sequencing (2 × 250) was carried out on the Illumina platform (Hiseq 2,500, Illumina, San Diego, CA, United States) based on standard protocols. The raw reads generated by the microbiome were deposited into the Sequence Read Archive of the National Center of Biotechnology Information (NCBI) database with the accession number PRJNA728963.

After filtering for noisy sequences and checking for the presence of chimeras, the data were clustered at the 97% similarity threshold (VSEARCH, version 10.0) (Edgar, 2013). The data were assigned to the same operational taxonomic units (OTUs) (Bokulich et al., 2013). To evaluate the sequencing depth, sparse curves were drafted by plotting the number of OTUs against the total number of sequences. Between-group Venn analysis was performed to identify unique and shared OTUs using the microbial indicative analysis function of the BMKCloud platform (http://www.biocloud.net/). The alpha indices (such as the coverage and the ACE, Chao1, Shannon, and Simpson indices) were calculated using the Mothur software (version v.1.30, http://www.mothur.org/) (Schloss et al., 2009; Aßhauer et al., 2015). Additionally, a comparison of the overall bacterial composition (beta analysis) was performed by partial least squares discrimination analysis in the BMKCloud platform, to establish a model of the relationship between sample categories and reduce the dimensionality of the data. Microbial species annotation was conducted using RDP Classifier (version 2.2, http://sourceforge.net/projects/rdpclassifier/) (Wang et al., 2007) based on the SILVA database (https://www.arb-silva.de/).

Real-Time Quantitative PCR Analysis

The TRI Reagent solution (Invitrogen, Carlsbad, CA, United States) was used to extract total RNA from three groupers per experimental group. The quality and quantity of RNA were analyzed by 1% agarose gel electrophoresis and spectrophotometry (A260: 280 nm). First-strand cDNA synthesis was conducted using PrimeScript™ RT-PCR kits obtained from Takara (Kusatsu, Japan), following the instructions of the manufacturer. Real-time quantitative PCR was conducted using quantitative thermal LightCycler480 (Roche Diagnostics, Basel, Switzerland). Amplification was performed in a mixture of 10 μl total volume, which included 5 μl TB Green™ Premix Ex Taq™ II (Takara, Kusatsu, Japan), 0.4 μl of each primer, 3.2 μl RNase-free water, and 1 μl cDNA mix. The steps for the real-time PCR program were as follows: one cycle of 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and then 60°C for 30 s, followed by a cycle of 95°C for 5 s, 60°C for 60 s, and then 95°C for 5 s, and finally a cycle of 50°C for 30 s. The specific primers used in this study are listed in **Table 3**. Based on previous results, *β-actin* was used as the reference gene for the groupers (Yin et al., 2020).

All real-time PCRs were conducted using an Applied Biosystems 7,500 real-time PCR system (Life Technologies, Carlsbad, CA, United States). Melting curve analysis of the amplification products was conducted at the end of each PCR to verify that a single PCR product was generated. Standard curves were obtained using four different dilutions of the cDNA samples in triplicate, and amplification efficiency was calculated using the formula:

$$E = 10^{(-1/slope)} - 1.$$

The $2^{-\Delta\Delta CT}$ method was used to calculate the gene expression results using the method described by Dvinge and Bertone (2009).

Statistical Analysis

Statistical analysis was performed using SPSS Statistics 19.0 (IBM, Armonk, NY, United States). All the data were expressed as the mean ± standard error of the mean (*SEM*). The Shapiro–Wilk and Levene's tests were performed for data normality and homogeneity of variance, respectively. Two analysis methods were used in this experiment: (1) the FM group and each COS-containing group (namely, COS0, COS0.2, COS0.4, COS0.6, COS0.8, and COS1) were analyzed by *t*-test; (2) one-way ANOVA was performed to analyze the COS-containing groups, and the Tukey method was used for multiple comparisons between groups. Statistical significance was set at $p < 0.05$.

Ethics Statement

The experimental procedures were approved by the Animal Care and Ethics Committee of Guangdong Ocean University.

TABLE 3 | Sequence of primers used in this study.

Genes	GenBank no.	Forward (5'–3')	Reverse (5'–3')
IL-1β	EF582837.1	ATCATCGCCACACAGAGGTTT	TGCCTCACAACCGAACACAT
IL-10	KJ741852.1	ACACAGCGCTGCTAGACGAG	GGGCAGCACCGTGTTCAGAT
MyD88	JF271883.1	TCCTCTCGTCGCCCTGAA	CGCTTTGGTGGGGTTTACA
TLR22	JQ965995.1	CAAAACTGGAAGGGGAGCAA	GCTCATCAAACAGGCGGAAG
TGF-β	GQ205390.1	CGATGTCACTGACGCCCTGC	AGCCGCGGTCATCACTTATC
TNF-α	HQ011925.1	CTGGTGATGTGGAGATGGGTC	CGTCGTGATGTCTGGCTTTC
Nrf2	KU892416.1	TATGGAGATGGGTCCTTTGGTG	GCTTCTTTTCCTGCGTCTGTTG
β-Actin	KU200949.2	AAGATGAAATCGCCGCAC	GCTCCTCAGGGGCAACTC

β-Actin was reference gene.

TABLE 4 | Growth performance and morphological indices of hybrid grouper fed with experimental diets for 8 weeks.

Parameters	FM	COS0	COS0.2	COS0.4	COS0.6	COS0.8	COS1
Growth performance							
IW (g)	13.20 ± 0.01	13.20 ± 0.01	13.20 ± 0.01	13.17 ± 0.05	13.19 ± 0.02	13.20 ± 0.01	13.19 ± 0.02
FW (g)	64.92 ± 1.04	59.60 ± 1.45^{a*}	64.61 ± 0.33^{bc}	65.97 ± 0.25^{c}	60.58 ± 0.75^{ab}	62.00 ± 0.65^{ab}	59.04 ± 1.82^{a*}
WG (%)	391.79 ± 8.01	351.52 ± 11.02^{a}	389.31 ± 12.33^{ab}	401.42 ± 10.01^{b}	368.04 ± 10.48^{a}	375.96 ± 7.81^{ab}	358.11 ± 17.19^{a}
SGR (%. day^{-1})	2.87 ± 0.25	2.69 ± 0.08^{a*}	2.84 ± 0.02^{bc}	2.88 ± 0.01^{c}	2.79 ± 0.03^{b*}	2.76 ± 0.13^{ab*}	2.68 ± 0.10^{a*}
FCR	0.74 ± 0.02	0.81 ± 0.02^{b*}	0.78 ± 0.07^{ab}	0.75 ± 0.03^{a}	0.79 ± 0.04^{ab}	0.80 ± 0.12^{b*}	0.82 ± 0.17^{b*}
SR (%)	100.00 ± 0.00	100.00 ± 0.00	97.78 ± 2.22	94.44 ± 2.94	98.89 ± 1.11	9.78 ± 2.22	98.89 ± 1.11
Morphological indices							
HSI (%)	1.54 ± 0.05	1.32 ± 0.04^{a}	1.35 ± 0.12^{a}	1.81 ± 0.05^{c*}	1.62 ± 0.07^{bc}	1.48 ± 0.26^{ab}	1.43 ± 0.13^{ab}
VSI (%)	6.87 ± 0.47	6.66 ± 0.14^{a}	7.08 ± 0.31^{ab}	7.32 ± 0.35^{b}	6.97 ± 0.51^{ab}	6.91 ± 0.33^{ab}	6.89 ± 0.70^{ab}
CF (%)	3.06 ± 0.08	3.06 ± 0.06^{a}	3.03 ± 0.32^{a}	3.13 ± 0.27^{a}	3.28 ± 0.13^{b*}	3.12 ± 0.26^{a}	3.17 ± 0.04^{ab}

*Values are means ± SEM (n = 3). Values in the same row with different superscript letters mean significantly different among COS-containing groups (P < 0.05). The superscript * indicates that this COS-containing group is significantly different from FM group (P < 0.05). IW, initial weight; FW, final weight.*

RESULTS

Growth Performance and Morphological Indices

As shown in **Table 4**, there is no significant difference in IW (initial weight) and SR among all the treatments ($p > 0.05$). FW (final weight), WG, and SGR reached maximum values in the COS0.4 group, and the values are significantly greater than those of the COS0 and COS1 groups ($p < 0.05$). However, there were no significant differences in FW, WG, and SGR between the COS0.4 and FM groups ($p > 0.05$). Among the COS-containing groups, FCR was lowest in the COS0.4 group ($p < 0.05$). However, there was no significant difference in FCR between the COS0.4 and FM groups ($p > 0.05$). Regarding morphological indices, HSI was highest in the COS0.4 group out of all the treatments ($p < 0.05$). VSI in the COS0.4 group was higher compared with that in the other COS-containing groups ($p < 0.05$), but it was not significantly different from that in the FM group ($p > 0.05$). CF first increased and then decreased in all the different groups. It was highest in the COS0.6 group out of all the groups ($p < 0.05$). Based on the polynomial curve analysis of SGR for the COS-containing groups, **Figure 1** shows that the optimal level of COS supplementation for groupers was 0.45%.

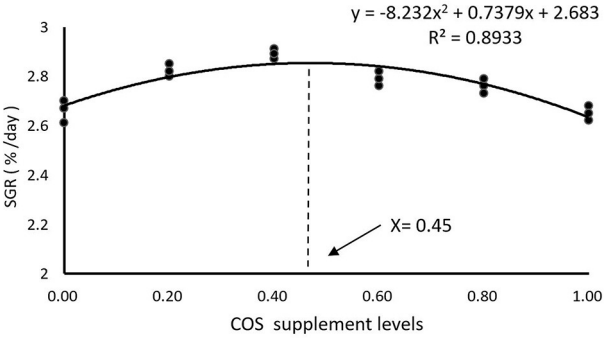


FIGURE 1 | Polynomial curve model analysis of SGR in hybrid grouper fed with experimental diets for 8 weeks.

Intestinal Morphology

Figure 2 shows the intestinal morphology of the hybrid grouper. In the COS0, COS0.8, and COS1 groups, plica adhesion was observed with increase in the level of COS supplementation. In addition, plica breakage appeared in the COS0 and COS1 groups. More vacuolation of epithelial cells was observed in the COS0.6 group than in the other groups. The plica was most uniform in

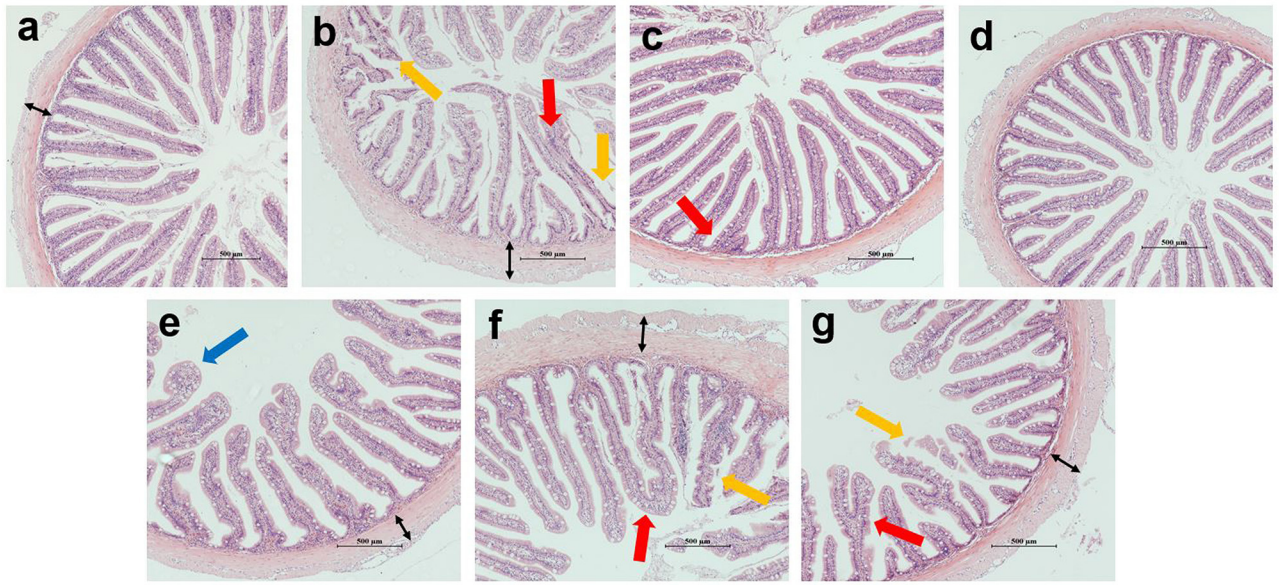


FIGURE 2 | Intestinal morphology of grouper (magnification × 100). **(a–g)** correspond to FM, COS0, COS0.2, COS0.4, COS0.6, COS0.8 and COS1 groups, respectively. Red arrow: plica adhesion; yellow arrow: plica breakage; blue arrow: vacuolation of epithelial cells; black arrow: thickness of lamina propria. Scale size is 500 μm.

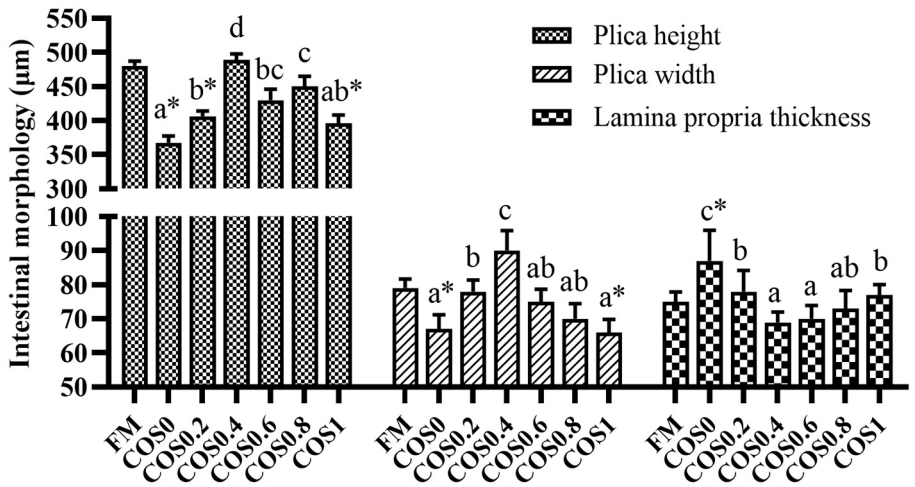


FIGURE 3 | Intestinal morphology measurement of grouper(μm). Randomly selected 10 folds and lamina propria in each slice for measurement. Values are means ± *SEM* ($n = 3$). Values in the same row with different superscript letters mean significantly different among COS-containing groups ($P < 0.05$). The superscript * indicates that this COS-containing group is significantly different from FM group ($P < 0.05$).

the FM and COS0.4 groups, whereas it had a poor uniformity and decreased density in the COS0 group.

As shown in **Figure 3**, there are significant differences in the measurements of intestinal morphology. The height and width of the plica first increased and then decreased with increasing COS supplementation, both reaching the maximum in the COS0.4 group and the minimum in the COS0 group ($p < 0.05$). The plica height and width of the FM group were significantly higher than those of the COS0 and COS1 groups ($p < 0.05$). In contrast, the trend of lamina propria thickness was opposite to that of plica height and width, as it was at a minimum in the COS0.4 group ($p < 0.05$). Lamina propria thickness was greatest in the COS0 group out of all the groups ($p < 0.05$).

Intestinal Microbiota Analysis

Richness, Diversity, and Composition of Microbiota

As shown in **Figure 4**, there are 1,161–1,380 OTUs in each group, and there are 1,445 core OTUs. The proportion of shared

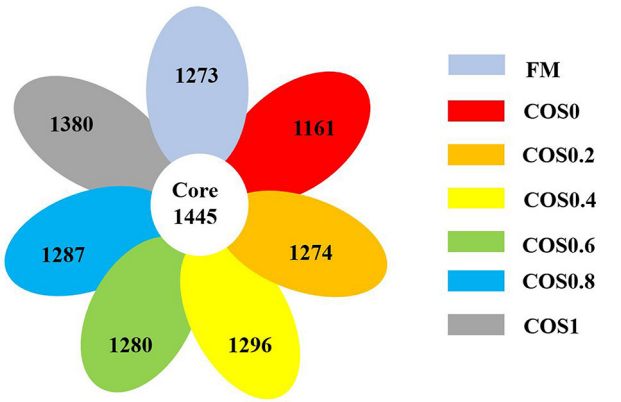


FIGURE 4 | Shared OTUs analysis of intestinal microbiota in hybrid grouper fed with experimental diets for 8 weeks.

OTUs within each group (COS0, COS0.2, COS0.4, COS0.6, COS0.8, and COS1) was 53.16, 55.45, 53.14, 52.72, 53.03, 52.89, and 51.15%, respectively. As these results suggested that the intestinal microbiota of each group was similar, it was concluded that there was a stable microbiota core in the intestines of all tested groupers.

Figure 5 shows the alpha diversity indices of the intestinal microbiota. The sample saturation curves of each treatment group tended to be stable, indicating that the amount of sequencing had reached saturation. Additionally, the coverage was >99% for each treatment group, which suggested that the microbiome sequencing results reflected the gut microbiota composition of the hybrid groupers.

Among the alpha diversity indices, the ACE and Chao1 indices of the different groups both rose and then fell with increasing COS levels ($p < 0.05$), suggesting that microbiota richness was highest in the FM, COS0.4, and COS0.6 groups. There were no significant differences between the Shannon index values of the different groups ($p > 0.05$). However, the Simpson index was highest in the COS0.4 group, where the lowest diversity of microbiota was observed ($p < 0.05$).

As shown in **Figure 6**, there are clear similarities of the overall bacterial composition between treatments. The aggregations of the different groups of each treatment were relatively concentrated, suggesting that the microbiome had good reproducibility. The processing distance of each COS-containing group was similar (blue frame), indicating the bacterial composition of COS-containing groups. In addition, the layouts of the FM group (green frame) and the COS0 group (red frame) were clearly separated from the COS-containing groups. This phenomenon reflected the structural difference in bacterial composition between the groups that included COS supplementation and those that did not (FM and COS0).

Community Analysis of Microbiota

Figure 7A displays the composition and relative abundance of the major bacterial phyla in the grouper intestinal microbiome. Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, Acidobacteria, and Verrucomicrobia were the dominant bacteria at the phylum level. The relative abundance of Firmicutes, Bacteroidetes, and cyanobacteria in the COS-containing groups was higher than of the FM group, and it had an upward trend with increasing COS supplementation. The abundance of Chloroflexi and Rokubacteria showed a downward trend, with the high-COS groups (including COS0.6, COS0.8, and COS1) having significantly lower abundance than the low-COS and FM groups ($p < 0.05$). The abundance of Proteobacteria, Acidobacteria, and Gemmatimonadetes showed a relatively decreasing trend with increasing COS supplementation ($p > 0.05$).

The relative abundance of the major bacterial families comprising the grouper intestinal microbiome is shown in **Figure 7B**. The dominant taxa at the family level were relatively similar across the treatment groups. There were no significant differences in the abundance of Ruminococcaceae, Lachnospiraceae, and Akkermansiaceae across the treatments ($p > 0.05$) but tended to increase first and then decrease. The abundance of Muribaculaceae in the COS0 and COS1 groups was significantly higher than that in the other groups, such as the FM group ($p < 0.05$). The abundance of Erysipelotrichaceae and Bifidobacteriaceae is highest in the COS0.6 group ($p < 0.05$), and it tended to increase first and then decrease in all the groups. The abundance of Chthoniobacteraceae showed a decreasing trend with increasing COS supplementation ($p > 0.05$). The abundance of Chroococcidiopsaceae and Rhodobacteraceae in the COS0.4 and COS0.8 groups is significantly higher than that in the other treatment groups ($p < 0.05$), and the abundance increased first and then decreased.

Serum and Intestinal Antioxidant Indices

Table 5 shows the serum and intestinal antioxidant indices of the hybrid grouper. In all the groups, the amount of GLU in the serum increased first and then decreased, and that in the COS0.6 group was significantly higher than that in the other groups ($p < 0.05$). The ALT level of the COS0.6 group was the lowest among all the groups, including such as the FM group ($p < 0.05$). AST was the lowest in the COS0.2 and COS0.8 groups ($p < 0.05$). There were no significant differences in TG among the groups ($p > 0.05$). CAT was significantly higher in the COS0.4 group than in the other groups, including such as the FM group ($p < 0.05$), whereas it was the lowest in the COS0 group ($p < 0.05$). The T-SOD level of the COS0.4 and COS0.6 groups was significantly higher than that of the other treatment groups ($p < 0.05$). MDA level was the highest in the COS0 group ($p < 0.05$), whereas the other groups showed no significant differences in MDA levels ($p > 0.05$).

In the intestinal tissue, GSHPx was lowest in the COS0 group when compared with that in all the other groups such as the FM group ($p < 0.05$); however, GSHPx was significantly higher in the COS0.4 and COS0.6 groups than in the other groups ($p < 0.05$). GR activity increased first and then decreased; the maximum value appeared in the COS0.6 group, and the lowest value appeared in the COS0 group ($p < 0.05$). Gln was significantly higher in the COS0.4 group than in all the other groups, such as the FM group ($p < 0.05$), whereas it was significantly lowest in

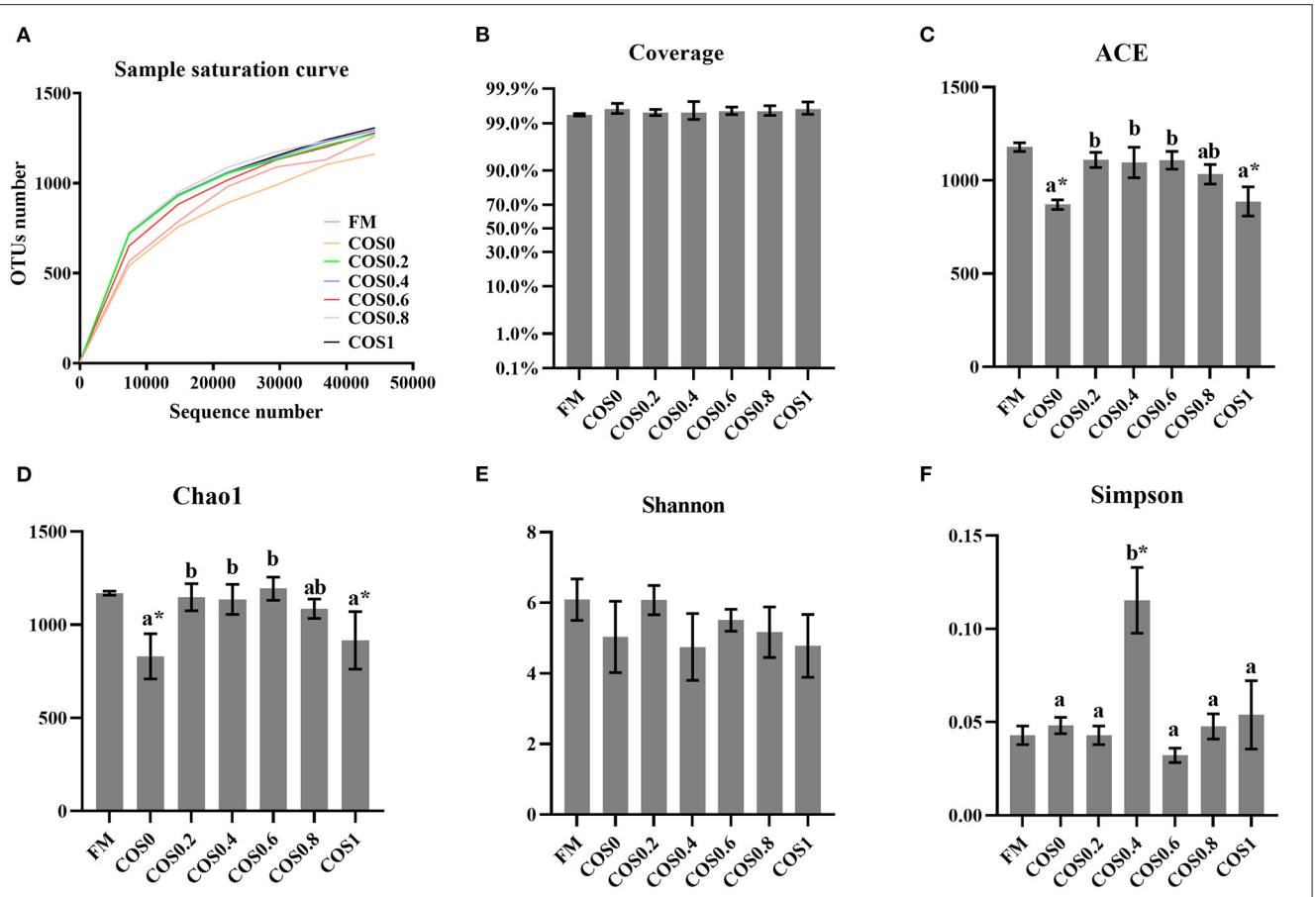


FIGURE 5 | (A) Sample saturation curve analysis of intestinal microbiota in hybrid grouper fed with experimental diets for 8 weeks. **(B–F)** Alpha diversity indices of intestinal microbiota in hybrid grouper fed with experimental diets for 8 weeks. Values are means ± *SEM* ($n = 3$). Values in the same row with different superscript letters mean significantly different among COS-containing groups ($P < 0.05$). The superscript * indicates that this COS-containing group is significantly different from FM group ($P < 0.05$).

the COS0 group ($p < 0.05$). There were no significant differences in NO across the COS-containing groups ($p > 0.05$), but the COS0.2 group had a significantly lower NO level than the FM group ($p < 0.05$).

Intestinal Non-specific Immunity Indices

As shown in **Table 6**, there are significant differences in the intestinal non-specific immunity indices between the hybrid grouper groups. The LYS (lysozyme) of the COS0.6 group was significantly higher than that of all other groups ($p < 0.05$), whereas the COS0 group had the least significant value ($p < 0.05$). The ACP (acid phosphatase) was highest in the COS0.4 group compared with that in all the other groups ($p < 0.05$), and it was least in the COS0 group ($p < 0.05$). C3 (complement protein 3) was lower in the FM group than in the COS-containing groups ($p < 0.05$), whereas it was highest in the COS0.4 group ($p < 0.05$). C4 (complement protein 4) was lowest in the FM group compared with that in all the other treatments, and it was significantly highest in the COS0.4 group compared with that in the other groups ($p < 0.05$). IgM (immunoglobulin M) increased first and then decreased, with the COS0.6 group having a significantly higher value than the other groups and the COS0 group having the least IgM ($p < 0.05$).

Intestinal Immunity Gene Expression

Intestinal immune gene expression is shown in **Figure 8**. The *IL-1β* mRNA level reached the highest significant value in the COS0 group ($p < 0.05$), whereas there was no significant difference in the other groups, such as the FM group ($p > 0.05$). The *IL-10* mRNA level was significantly lower in the FM group than in the COS-containing groups ($p < 0.05$); however, there was no significant difference between the COS-containing groups ($p > 0.05$). There was no significant difference in *MyD88* mRNA levels among the treatments ($p > 0.05$). The *TLR22* mRNA level was upregulated in the COS0 group, and it was significantly higher than that in the other groups ($p < 0.05$); however, there was no significant difference in the FM group ($p > 0.05$). *TGF-β* mRNA levels tended to increase first and then decrease, with levels being significantly higher in the COS0.2 and COS0.4 groups than in the FM, COS0, and COS0.8 groups ($p < 0.05$). The mRNA expression of *TNF-α* showed a downward trend among the COS-containing groups. *TNF-α* mRNA was upregulated in the COS0 group, and it

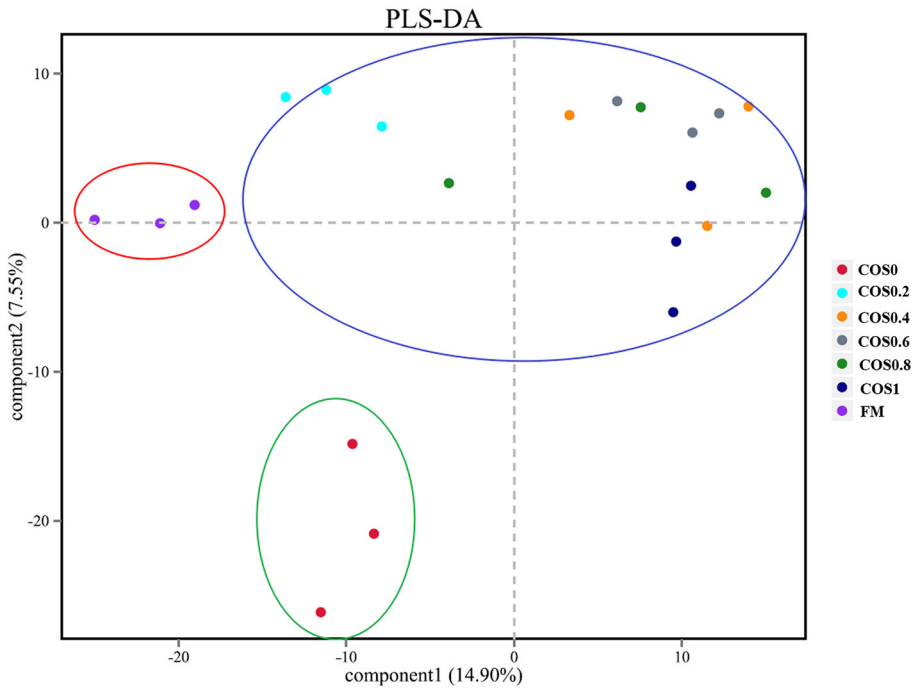


FIGURE 6 | Comparison of overall bacterial composition between treatments by partial least squares discrimination analysis (PLS-DA) in grouper intestinal microbiota.

was significantly higher than that in the other treatment groups, such as the FM group ($p < 0.05$). The *Nrf2* mRNA level was lowest in the COS0 group, whereas the *Nrf2* mRNA expression in the COS0.4 group was upregulated and reached the highest value ($p < 0.05$).

Challenge Test

Figure 9 shows the cumulative mortality of the groupers that were challenged with *V. harveyi* for 7 days. The grouper disease resistance was significantly different between the treatment groups post *V. harveyi* challenge. However, there were no significant differences in disease resistance between the FM and COS-containing groups ($p > 0.05$). Among the COS-containing groups, the cumulative mortality decreased first and then increased, with the highest mortality occurring in the COS0 group and the lowest occurring in the COS0.4 group ($p < 0.05$).

DISCUSSION

Generally, replacing fish meal with plant protein can adversely affect aquatic animals (Wu et al., 1999). For example, the substitution of fish meal with at least 34% CPC can hinder grouper growth and cause intestinal inflammation (Yin et al., 2018). The results of this study showed that FW, WG, and SGR had small values in the COS0 group, which was fed with an experimental diet, with CPC replacing fish meal. This suggests that plant protein replacement has an adverse effect on grouper growth, which may be caused by amino acid imbalance (Belghit et al., 2014; Skiba-Cassy et al., 2016). This is consistent with the result of this study, as the threonine and isoleucine contents of the COS-containing groups were lower than those of the FM group. Thus, the lack of dietary essential amino acids can adversely affect grouper growth. However, COS can have a growth-promoting effect on aquatic animals, such as hybrid tilapia (*Oreochromis niloticus* ♀ × *Oreochromis aureus* ♂) (Chubin et al., 2014), koi (*Cyprinus carpio koi*) (Lin et al., 2012), and *Penaeus monodon* (Jin et al., 2013). In this study, groupers in the COS0.4 group had higher FW, WG, and SGR values than those in the FM group. This illustrates that COS supplementation positively affected hybrid grouper growth, with an optimum effect occurring with COS supplementation of 0.45%. COS may have a growth-promoting effect, because it is fermented in the fish gut (Cha et al., 2008; Ring et al., 2010). The acidic substances produced from COS fermentation can lower the pH value in the gut and enhance digestive enzyme activity. This effect has been observed with mannose oligosaccharides, whose fermentation improved protease and lipase activities in crayfish (*Cherax destructor Clark*) (Sang et al., 2015). Therefore, oligosaccharides fermented in the intestine improve digestive ability and ultimately lead to better growth performance.

The intestine plays an important role in digestion and nutrient absorption in fish (Baker et al., 2014). Normal gut morphology, particularly the fold shape, is the basis for sufficient nutrient absorption and intestinal homeostasis. Optimizing the intestinal morphology can increase the gut absorption area, which is conducive to adequate nutrient absorption (Refstie et al., 2006; Geda et al., 2012). In this study, some undesirable changes occurred to the grouper gut morphology in the CPC-containing

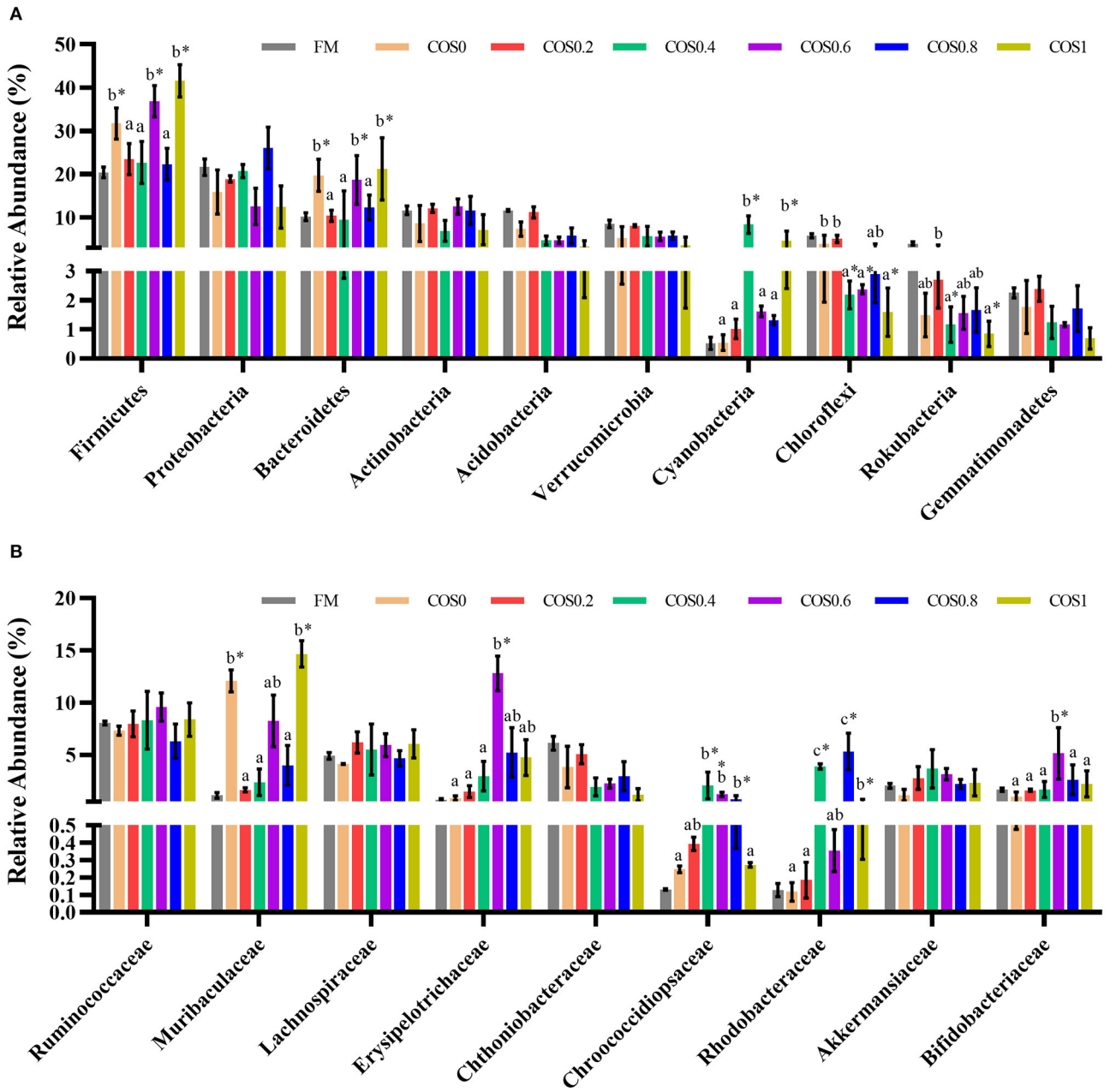


FIGURE 7 | (A) Relative abundance of major bacterial phylum in grouper intestinal microbiota. **(B)** Relative abundance of major bacterial family in grouper intestinal microbiota. Values are means ± *SEM* ($n = 3$). Values in the same row with different superscript letters mean significantly different among COS-containing groups ($P < 0.05$). The superscript * indicates that this COS-containing group is significantly different from FM group ($P < 0.05$).

groups, such as fold breakage and morphological disorders. This may be explained by an imbalance in dietary amino acids caused by the replacement of fish meal with plant protein (Wanga et al., 2018). In carnivorous marine fish, larvae are sensitive to amino acid composition during the growth stage (Aragão et al., 2004). Dietary amino acids are mainly used to meet growth requirements and regulate fish body tissue construction. Thus, malnutrition caused by amino acid imbalance may lead to dysplasia in fish intestinal morphology (Yamamoto et al., 2012; Adriana et al., 2020). In this experiment, the fold height and width, and the lamina propria thickness were improved by the COS-containing treatments, especially in the COS0.2 and COS0.4 groups. These results were similar to those of an experiment conducted on turbot (*Scophthalmus maximus*) supplemented with mannan oligosaccharides (Bai et al., 2017). COS protects the gut by optimizing intestinal morphology and

TABLE 5 | Serum and intestinal antioxidant indices of hybrid grouper fed with experimental diets for 8 weeks.

Parameters	FM	COS0	COS0.2	COS0.4	COS0.6	COS0.8	COS1
Serum							
GLU (mmol/L)	3.99 ± 0.37	2.43 ± 0.11^{a}	4.07 ± 0.11^{ab}	4.55 ± 0.12^{b}	6.67 ± 1.33^{c*}	4.92 ± 0.54^{b}	4.47 ± 0.08^{b}
ALT(U/L)	89.64 ± 2.72	93.24 ± 5.54^{b}	85.03 ± 12.39^{b}	88.63 ± 8.44^{b}	75.49 ± 3.06^{a*}	87.78 ± 2.22^{b}	79.62 ± 6.65^{ab}
AST(U/L)	58.86 ± 6.86	61.39 ± 7.82^{b}	46.1 ± 4.55^{a*}	57.11 ± 12.24^{b}	57.61 ± 6.26^{b}	47.65 ± 1.13^{a*}	58.14 ± 3.88^{b}
TG (mmol/L)	0.38 ± 0.06	0.47 ± 0.05	0.43 ± 0.03	0.38 ± 0.01	0.45 ± 0.04	0.42 ± 0.04	0.43 ± 0.02
CAT(U/ml)	33.36 ± 6.91	28.22 ± 3.97^{a}	48.18 ± 5.15^{bc}	59.81 ± 2.29^{c*}	37.94 ± 6.09^{b}	37.10 ± 2.82^{b}	36.92 ± 8.04^{b}
T-SOD (U/mL)	83.87 ± 6.57	81.11 ± 7.51^{a}	82.66 ± 5.34^{a}	87.97 ± 2.98^{b}	89.63 ± 7.79^{b}	85.66 ± 4.86^{ab}	82.99 ± 7.19^{a}
MDA (nmol/mL)	9.66 ± 1.26	13.37 ± 2.77^{b}	8.75 ± 1.60^{a}	9.23 ± 0.72^{a}	11.01 ± 3.32^{ab}	11.25 ± 2.41^{ab}	10.87 ± 1.31^{ab}
Intestine							
GSHPx (U/L)	94.57 ± 11.30	67.74 ± 9.80^{a*}	83.16 ± 3.52^{ab}	107.5 ± 6.61^{c}	109.58 ± 4.26^{c}	93.99 ± 5.76^{b}	92.79 ± 10.03^{b}
GR (U/L)	72.02 ± 7.63	67.38 ± 1.77^{a}	87.37 ± 7.75^{b}	94.34 ± 4.85^{c*}	111.68 ± 2.09^{d*}	85.68 ± 5.64^{b}	79.12 ± 3.89^{ab}
Gln (umol/L)	1237.84 ± 66.62	1008.09 ± 75.82^{a*}	1571.91 ± 39.27^{c*}	1872.09 ± 68.29^{d*}	1844.74 ± 95.91^{d*}	1440.85 ± 14.47^{c*}	1246.43 ± 48.51^{b}
NO (umol/L)	82.91 ± 3.36	88.51 ± 2.74	66.88 ± 4.42*	76.6 ± 2.65	83.44 ± 7.28	85.55 ± 6.54	82.91 ± 3.36

*Values are means ± SEM (n = 3). Values in the same row with different superscript letters mean significantly different among COS-containing groups (P < 0.05). The superscript * indicates that this COS-containing group is significantly different from FM group (P < 0.05). GLU, glucose oxidase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TG, triglyceride; CAT, catalase; T-SOD, total superoxide dismutase; MDA, malondialdehyde; GSHPx, glutathione peroxidase; GR, glutathione reductase; Gln, glutamine; NO, nitric oxide.*

TABLE 6 | Intestinal non-specific immunity indices of hybrid grouper fed with experimental diets for 8 weeks.

Parameters	FM	COS0	COS0.2	COS0.4	COS0.6	COS0.8	COS1
LYS (U/L)	5.21 ± 0.38	4.78 ± 0.55^{a}	6.49 ± 0.60^{b}	6.99 ± 1.25^{b}	7.72 ± 0.39^{c*}	6.67 ± 0.16^{b}	5.77 ± 0.23^{ab}
ACP (U/L)	7.73 ± 0.23	6.32 ± 0.59^{a*}	8.72 ± 0.75^{c*}	10.46 ± 1.42^{d*}	9.26 ± 0.16^{c*}	7.54 ± 0.27^{b}	8.74 ± 0.36^{c*}
C3 (ug/mL)	61.62 ± 8.15	78.03 ± 6.02^{a*}	83.33 ± 12.98^{a*}	94.17 ± 6.31^{b*}	90.41 ± 15.74^{b*}	83.61 ± 14.59^{a*}	81.86± 15.64^{a*}
C4 (ug/mL)	131.07 ± 12.94	138.82 ± 11.65^{a}	168.23 ± 13.79^{bc*}	179.28 ± 14.97^{c*}	167.35 ± 17.81^{bc*}	160.28 ± 13.05^{b*}	157.49 ± 5.26^{b*}
IgM (ug/mL)	16.29 ± 0.94	16.28 ± 1.64^{a}	20.47 ± 1.05^{a}	25.37 ± 4.94^{b*}	31.68 ± 1.01^{c*}	27.9 ± 3.86^{bc*}	26.11 ± 2.88^{b*}

*Values are means ± SEM (n = 3). Values in the same row with different superscript letters mean significantly different among COS-containing groups (P < 0.05). The superscript * indicates that this COS-containing group is significantly different from FM group (P < 0.05). LYS, lysozyme; ACP, acid phosphatase; C3, complement protein 3; C4, complement protein 4; IgM, immunoglobulin M.*

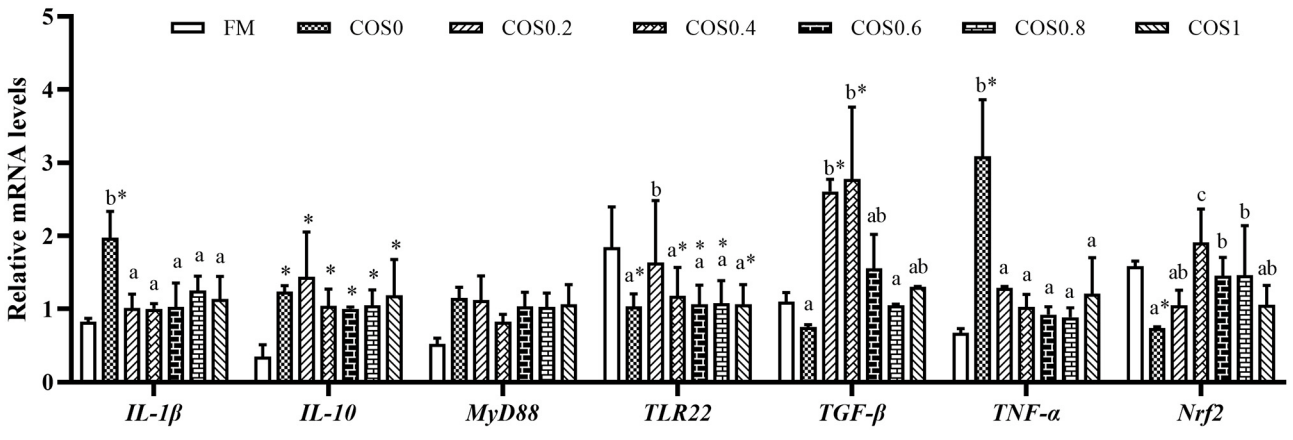


FIGURE 8 | Intestinal immunity gene expression of hybrid grouper fed with experimental diets for 8 weeks. Values are means ± *SEM* (*n* = 3). Values in the same row with different superscript letters mean significantly different among COS-containing groups ($P < 0.05$). The superscript * indicates that this COS-containing group is significantly different from FM group ($P < 0.05$).

acting as a prebiotic. Feeding prebiotic supplements to fish can have a positive effect on enhancing intestinal morphology, as has been shown in *Sparus aurata* (Dimitroglou et al., 2010), juvenile pacu (*Piaractus mesopotamicus*) (Ricardo et al., 2014), *Oncorhynchus mykiss* (Dimitroglou et al., 2009), and larval cobia (*Rachycentron canadum*) (Salze et al., 2008). High-affinity

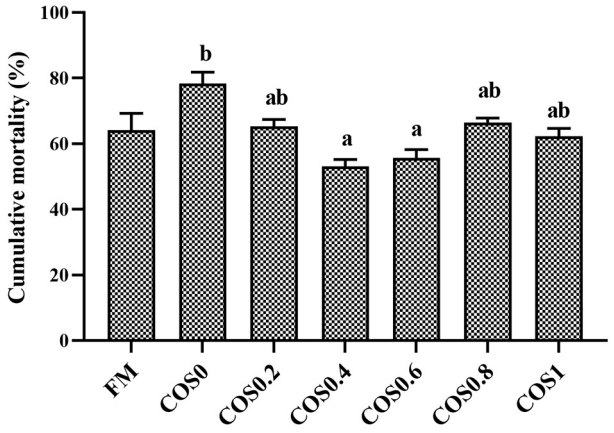


FIGURE 9 | Cumulative mortality of experimental hybrid grouper challenged with *Vibrio harveyi* for 7 days. Values are means ± *SEM* ($n = 3$). Values in the same row with different superscript letters mean significantly different among COS-containing groups ($P < 0.05$).

ligands, prebiotics such as oligosaccharides, can provide binding sites that specifically match bacterial lectins, which could block the binding between pathogens and intestinal mucosal epithelial cells. In addition, prebiotics can promote the proliferation of beneficial bacteria, allowing them to attach more widely to the surface of the intestinal wall, thereby reducing the contact of conditional pathogens with the intestinal wall and protecting the intestines (Ofek et al., 1977; Dimitroglou et al., 2009; Rajendran et al., 2017).

The gut ameliorating effect of COS is closely related to its influence on the intestinal microbiota. The intestinal microbiota plays an indispensable role in maintaining fish intestinal homeostasis. There is lower microbiota diversity in the gut of fish than in that of terrestrial animals. Major bacterial species that are stable after colonization differ depending on fish feeding habits (Coscas et al., 2010; Tarnecki et al., 2017). Furthermore, the gut microbiota community structure is affected by the fish cultivation environment and available dietary nutrients (Li et al., 2018a). Related studies have reported that replacing fish meal with plant protein changes the fish intestinal microbiota community, and a high proportion of fish meal replacement may have adverse effects on the fish microbiota (Gy et al., 2020; Shen et al., 2020). However, oligosaccharide supplementation can adjust the balance between probiotics and conditional pathogens, thereby balancing the intestinal microflora (Mussatto and Mancilha, 2007). In this study, the ACE index and Chao1 index increased first and then decreased with an increase in COS supplementation. Therefore, it can be inferred that COS can enhance the abundance of intestinal microbiota in this grouper species. The comparison of overall bacterial composition revealed that the microbiota in the COS-containing groups were analogous or closely related. In addition, there were clear differences in the microbiota community between the treatments without COS supplementation (FM and COS0) and the COS-containing groups. It is worth noting that oligosaccharide supplementation is only beneficial when a moderate amount is provided. Excess oligosaccharides are excessively fermented by intestinal microorganisms, causing over-proliferation and intestinal microenvironment imbalance. This phenomenon causes the digestive capacity of fish intestines to decrease. In contrast, functional oligosaccharides have suboptimal physical and chemical properties, such as high viscosity and strong hygroscopicity (Mussatto and Mancilha, 2007). Therefore, high oligosaccharide supplementation lowers the palatability of feed, which is not conducive to fish growth; however, within a reasonable range of supplementation, oligosaccharides play a positive role in intestinal functioning. This may explain why 0.4–0.6% COS supplementation was optimal in this experiment.

The results indicated that the relative abundance of beneficial bacteria species, such as Firmicutes and Bacteroides, at the phylum level, and Lactobacteriaceae and Bifidobacterium, at the family level, increased among the COS-containing groups. Related studies have shown that Firmicutes and Bacteroides play important roles in the healthy physiological and metabolic activities of the host. For example, they can produce short-chain fatty acids by fermenting indigestible carbohydrates to resist pathogenic bacteria and further promote intestinal health of the host (Lara et al., 2017; Waite et al., 2017). In addition, beneficial bacteria that colonize the gut, such as Lactobacteriaceae and Bifidobacterium, can secrete enzymes that open β-1,4 glycosidic bonds and then use COS to produce succinic acid and lactic acid, further lowering the pH and maintaining gut stability (Liu et al., 2008). Interestingly, the abundance of conditional pathogens, such as Proteobacteria (phylum level) and Erysipelotrichaceae (family level), also increased with increasing COS supplementation. Proteobacteria is the largest phylum containing many conditional pathogens, such as *Escherichia coli*, *Salmonella*, *Vibriocholerae*, and *Helicobacterpylori* (Waite et al., 2017), and Erysipelotrichaceae is closely related to inflammation-related enteropathy and metabolic disorders (Kaakoush, 2015). The simultaneous increase in the abundance of probiotics and conditional pathogens in this study may have been related to the indigenous bacterial composition of the water environment (Cornejo-Granados et al., 2018). Despite this simultaneous increase, the physiology of the groupers, such as their growth and gut morphology, was not adversely affected. This suggests that COS supplementation enhances grouper intestinal homeostasis and promotes stability of the microbiota habitat, as even the increase in conditional pathogen abundance did not cause intestinal disorders. Mahious et al. (2006) found that 2% oligofructose supplementation promoted intestinal health in turbot (*Psetta maxima*), similar to prebiotics. Furthermore, Qingsen et al. (2016) explored dietary fucoidan supplementation as a prebiotic that can promote intestinal health. The findings suggested that dietary prebiotic supplementation reduced host antigen load and inflammatory response by maintaining a balanced composition of intestinal microbiota, which may be achieved by reducing serum lipopolysaccharide-binding protein levels.

Antioxidant function is an important means of maintaining homeostasis in fish (Pérez-Jiménez et al., 2014). Previous studies have found that dietary fish meal replacement with plant proteins hinders the antioxidant functions in marine fish, such as grouper

(Yin et al., 2018), golden pompano (*Trachinotus ovatus*) (Shen et al., 2020), and totoaba juveniles (*Totoaba macdonaldi*). The liver is the main organ involved in TG metabolism. When abnormal hepatic cells cause TG metabolism disorder, serum TG content increases and further affects energy metabolism (Wu et al., 2011). Lipid peroxidation causes cell damage and produces MDA as the final oxidation product. Thus, the MDA content in the serum can indirectly reflect the degree of cell damage (Box et al., 2020). When the FM group was compared with the COS0 group, it was found that the COS0 group contained higher serum TG and MDA levels. Therefore, the COS0 group had a certain degree of oxidation disorder. Glucose is the main source of energy in fish, and increased GLU activity facilitates energy utilization and physiological efficiency (Li et al., 2020). As one of the key enzymes in the biodefense system, CAT breaks down hydrogen peroxide into water and oxygen, preventing cells from being damaged by hydrogen peroxide (Cai et al., 2017). T-SOD is an important antioxidant enzyme in fish that effectively scavenges free radicals and reduces the production of oxidative stress (Yu et al., 2011). ALT and AST are two important transaminases that play vital roles in the metabolism of amino acids and the interconversion of proteins, lipids, and glucose; serum ALT and AST levels can reflect liver function impairment (Wu et al., 2020). Glutathione is an important antioxidant, and Gln plays a crucial role in glutathione synthesis. GSHPx and GR are two important peroxidases that catalyze glutathione conversion and reduce toxic peroxides to non-toxic hydroxyl compounds, thus protecting the cell membrane from interference and damage by peroxides (Bilzer et al., 2002; Yan and Xiao, 2006; Yin et al., 2018). In this experiment, serum GLU, CAT, and T-SOD activities increased in the COS-containing groups with increasing COS supplementation levels. Conversely, ALT and AST activities decreased. In the gut, the activities of GSHPx, GR, and Gln tended to increase first and then decrease, reaching an optimum in the COS0.4 or the COS0.6 group. These results suggest that COS supplementation can alleviate the antioxidant burden induced by plant protein substitution, thereby refining the antioxidant capacity of this grouper species. Previous studies have suggested that, in addition to its antioxidant properties, COS has a variety of biological activities such as scavenging free radicals and inducing the expression of antioxidant enzymes and genes (Joodi et al., 2011; Sun et al., 2011; Vieira et al., 2020). The amino, primary, and secondary hydroxyl groups in the COS molecule are the structural bases for its antioxidant function (Tao et al., 2007). The molecular structure of COS contains reducing end-groups, whose reducing properties can scavenge or inhibit free radicals, thus maintaining the relative antioxidant stability of the internal environment (Xie et al., 2001). Furthermore, the antioxidant potency of prebiotics is related to their molecular weight and solubility, which may be influenced by differences in the species and habitat. Therefore, the antioxidant effects of prebiotics in aquaculture deserve further research attention (Palframan et al., 2002; Gomez et al., 2010; Mei et al., 2011).

As vertebrates, fish have a relatively well-developed immune system. The non-specific immune defense mechanism is the first barrier and the main protector of fish against pathogens (Alonso and Leong, 2002). The main mechanisms of non-specific immune response include phagocytic activity, respiratory bursts, complement system functioning, and production of lysozyme and cytokines (Little et al., 2005). ACP, a major indicator of fish growth and immune function, plays an important role in cellular regulation and nutrient transport. In addition, ACP is closely related to lysozyme, a cationic protein synthesized in the liver. It is responsible for bacteriolytic, regulatory, and immune responses, as well as antimicrobial activity (Saurabh and Sahoo, 2010; Gudmundsdottir et al., 2014). C3 and C4 are important components of the immune complement system. They can kill pathogens by making small holes in the pathogen cell membrane, thus supplementing the role of antibodies (Cuesta et al., 2016). IgM is an important immune protein that has powerful effects of bactericidal activity, complement activation, immune regulation, and agglutination (Krogdahl et al., 2015). In this experiment, the activities of LYS and ACP showed an upward trend with increased COS supplementation levels. In addition, the C3, C4, and IgM contents in the gut tissue increased first and then decreased. This result is similar to that obtained for *Sparus aurata* and *Dicentrarchus labrax* (Carbone and Faggio, 2016), suggesting that COS supplementation improves grouper health by enhancing lysozyme activity and the complement system.

Previous studies have shown that replacing fish meal with plant protein can induce intestinal inflammatory factor expression, activate inflammatory pathways, and ultimately cause intestinal inflammation in marine fish. This may be related to the unreasonable composition of amino acids and the presence of anti-nutritional factors in plant proteins (Yin et al., 2018, 2020). *IL-1β* is an important mediator of inflammation in infection response (Holland et al., 2002). *TNF-α* is also one of the main mediators of inflammation (Hideaki et al., 2005). The results of this study showed that the expression of *IL-1β* and *TNF-α* was upregulated in the COS0 group, which suggests that dietary fish meal replacement with CPC triggered an inflammatory response in the grouper gut. As an important anti-inflammatory cytokine, *IL-10* participates in inflammatory response and relieves inflammation. It has immunomodulatory effects on teleost fish (Wei et al., 2013). Upregulated *IL-10* expression helps to alleviate inflammation and inhibits T cells from producing excessive *IL-6*, *IL-1β*, and other inflammatory cytokines, thereby preventing excessive immune responses from harming the body (Xiaoyi et al., 2008). *TLRs* play vital roles in the immune system and are widely present in marine fish. *TLR22* is an important inflammatory regulator because of its recognition of dsRNA, recruitment of *TRIF* signaling molecules, and activation of the I-interferon response (Sahoo et al., 2015). *TGF-β* is an important regulatory hub involved in signal transduction (at the stage of transcription phosphorylation) in cellular inflammatory pathways (Liu et al., 2017). *Nrf2* is an important transcription factor that regulates the oxidative stress response of cells; it is also a central regulator that maintains cell redox homeostasis (Hui and Yang, 2018). In this experiment, the expression of *TLR22* and *TGF-β* tended to be upregulated first and then be downregulated, and they had a high expression level in the COS0.2 and COS0.4 groups. This indicates that the intestinal inflammatory response of the groupers was regulated and activated by COS supplementation. Moreover, *IL-10* expression level was higher in

the COS-containing groups than in the FM group, suggesting that COS supplementation alleviates intestinal inflammation in the groupers. This aligns with the observed improvement in intestinal morphology. In addition, *Nrf2* expression was upregulated as COS supplementation was increased. This shows that COS supplementation reduced oxidative damage to the grouper intestinal cells, maintaining a steady state of the gut environment. The conclusions of this study are consistent with those of a study carried out on carp (*Cyprinus carpio L.*), which found that COS supplementation can increase their antioxidant and immune responses (Dautremepuits et al., 2004). The results of this study showed that the resistance of the groupers against *V. harveyi* was enhanced with increased dietary COS supplementation. This may be because oligosaccharides are positively charged polymer substances. The structure of their positively charged and polymerized molecules is the basis for their antibacterial and bactericidal abilities (Anderson and Siwicki, 1994; Talpur et al., 2014). In summary, oligosaccharide prebiotics are immunogenic and stimulate immune responses of the body (Ghosh and Mehla, 2012; Si et al., 2017), thereby improving non-specific immunity (Maqsood et al., 2010; Huo et al., 2015), anti-inflammatory activity (Azuma et al., 2015), and disease resistance (Zhang et al., 2012) in fish species.

CONCLUSION

The results of this study demonstrated that chitosan oligosaccharide supplementation can improve the growth, intestinal morphology, microbiota composition, antioxidant capacity, and immune response of hybrid groupers under the adverse effects of fishmeal replacement with CPC. Hence, COS can be used as a potential enhancer to fortify grouper fed a low-fishmeal diet.

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: NCBI (accession: PRJNA728963).

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

HL, BT, and XD were involved in conceptualization. GC, BY, QY, SC, and SZ designed the experiments. BY provided the necessary assistance. GC were involved in data analysis and writing of the original draft. GC and HL reviewed and edited the manuscript. All the authors approved the final version of the manuscript for submission.

FUNDING

This work was supported by the National Key RD Program of China (2019YFD0900200) and the National Natural Science Foundation of China (No.31772864), Natural Science Foundation of Guangdong Province (2018A030313154; 2020A1515011129).

ACKNOWLEDGMENTS

We thank Bozhi Huili Biotechnology Co., Ltd. for providing chitosan oligosaccharide.

REFERENCES

Adriana, M., Tania, G., Villalobos, Y. D., Hugo, B., Htoo, J. K., González-Vega, J. C., et al. (2020). Dietary protein-bound or free amino acids differently affect intestinal morphology, gene expression of amino acid transporters, and serum amino acids of pigs exposed to heat stress. *J. Anim. Ence* 98:skaa056. doi: 10.1093/jas/skaa056

Alonso, M., and Leong, J. A. (2002). Suppressive subtraction libraries to identify interferon-inducible genes in fish. *Marine Biotech.* 4, 74–80. doi: 10.1007/s10126-001-0001-9

Anderson, D. P., and Siwicki, A. K. (1994). Duration of protection against aeromonas salmonicida in brook trout immunostimulated with glucan or chitosan by injection or immersion. *Progr. Fish-Culturist* 56, 258–261. doi: 10.1577/1548-8640(1994)056<0258:DOPAAS>2.3.CO;2

Aragão, C., Concei, O., L., Dinis, M. T., and Fyhn, H. J. (2004). Amino acid pools of rotifers and Artemia under different conditions: nutritional implications for fish larvae. *Aquaculture* 234, 429–445. doi: 10.1016/j.aquaculture.2004.01.025

Aßhauer, K. P., Wemheuer, B., Daniel, R., and Meinicke, P. (2015). Tax4Fun: predicting functional profiles from metagenomic 16S rRNA data. *Bioinformatics* 31, 2882–2884. doi: 10.1093/bioinformatics/btv287

Ayiku, S., Jianfei, S., Tan, B.-P., Dong, X.-H., and Liu, H.-Y. (2020). Effects of dietary yeast culture on shrimp growth, immune response, intestinal health and disease resistance against Vibrio harveyi. *Fish Shellfish Immunol.* 102, 286–295. doi: 10.1016/j.fsi.2020.04.036

Azuma, K., Osaki, T., Minami, S., and Okamoto, Y. (2015). Anticancer and anti-inflammatory properties of chitin and chitosan oligosaccharides. *J. Funct. Biomater.* 6, 33–49. doi: 10.3390/jfb,6010033

Bai, N., Gu, M., Xu, X., Xu, B., and Krogdahl, A. (2017). Protective effects of mannan oligosaccharides on turbot Scophthalmus maximus suffering from soy enteropathy. *Aquaculture* 476, 141–151. doi: 10.1016/j.aquaculture.2017.04.005

Baker, R., Buckland, A., and Sheaves, M. (2014). Fish gut content analysis: robust measures of diet. *Fish Fish.* 15, 170–177. doi: 10.1111/faf.12026

Belghit, I., Skiba-Cassy, S., Geurden, I., Dias, K., Surget, A., Kaushik, S., et al. (2014). Dietary methionine availability affects the main factors involved in muscle protein turnover in rainbow trout (Oncorhynchus mykiss). *Br. J. Nutr.* 112, 493–503. doi: 10.1017/S0007114514001226

Bilzer, M., Baron, A., Schauer, R., Steib, C., and Ebensberger, S. (2002). Glutathione treatment protects the rat liver against injury after warm ischemia and kupffer cell activation. *Digest. –Basel London* 66, 49–57. doi: 10.1159/000064415

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

Box, A., Capó, X., Tejada, S., Sureda, A., Mejías, L., and Valencia, J. M. (2020). Perkinsus mediterraneus infection induces oxidative stress in the mollusc Mimachlamys varia. *J. Fish Dis.* 43, 1–7. doi: 10.1111/jfd.13085

Cai, H. R., Yang, Q. H., Tan, B. P., Dong, X. H., Chi, S. Y., Liu, H. Y., et al. (2017). Effects of dietary manganese source and supplemental levels on growth performance, antioxidant enzymes activities, tissue Mn

concentrations and cytosolic manganese superoxide dismutase (cMnSOD) mRNA expression level of juvenile *Litopenaeus vannamei*. *Aquacult. Nutri.* 23, 603–610. doi: 10.1111/anu.12427

Carbone, D., and Faggio, C. (2016). Importance of prebiotics in aquaculture as immunostimulants. effects on immune system of *Sparus aurata and Dicentrarchus labrax*. *Fish Shellfish Immunol.* 54, 172–178. doi: 10.1016/j.fsi.2016.04.011

Carter, C., and Hauler, R. (2000). Fish meal replacement by plant meals in extruded feeds for Atlantic salmon, *Salmo salar L*. *Aquaculture* 185, 299–311. doi: 10.1016/S0044-8486(99)00353-1

Cha, S. H., Lee, J. S., Song, C., Lee, K. J., and Jeon, Y. J. (2008). Effects of chitosan-coated diet on improving water quality and innate immunity in the olive flounder, *Paralichthys olivaceus*. *Aquaculture* 278, 110–118. doi: 10.1016/j.aquaculture.2008.01.025

Chemists, A. O. A. (2005). *Official Methods of Analysis*. MD: AOAC International Gaitherburg.

Chen, G., Yin, B., Liu, H., Tan, B., Dong, X., Yang, Q., et al. (2020). Effects of fishmeal replacement with cottonseed protein concentrate on growth, digestive proteinase, intestinal morphology and microflora in pearl gentian grouper (♀ *Epinephelus fuscoguttatus*×♂ *Epinepheluslanceolatu*). *Aquac. Res.* 51, 2870–2884. doi: 10.1111/are.14626

Chen, J., Zhou, X.-Q., Feng, L., Liu, Y., and Jiang, J. (2009). Effects of glutamine on hydrogen peroxide-induced oxidative damage in intestinal epithelial cells of Jian carp (*Cyprinus carpio var. Jian)*. *Aquaculture* 288, 285–289. doi: 10.1016/j.aquaculture.2008.10.053

Chubin, Q., Yuting, Z., Wenshu, L., and Li, X., and Yang, Y. (2014). Effects of chito-oligosaccharides supplementation on growth performance, intestinal cytokine expression, autochthonous gut bacteria and disease resistance in hybrid tilapia *Oreochromis niloticus* ♀ × *Oreochromis aureus* ♂. *Fish Shellfish Immunol.* 40, 267–274. doi: 10.1016/j.fsi.2014.07.010

Cornejo-Granados, F., Gallardo-Becerra, L., Leonardo-Reza, M., Ochoa-Romo, J. P., and Ochoa-Leyva, A. (2018). A meta-analysis reveals the environmental and host factors shaping the structure and function of the shrimp microbiota. *PeerJ.* 6:e5382. doi: 10.7717/peerj.5382

Coscas, G., Quentel, G., Pinon, F., and Soubrane, G. (2010). The ultrastructure of the intestinal epithelium in fishes with different types of feeding. *J. Fish Biol.* 44, 181–193. doi: 10.1111/j.1095-8649.1994.tb01197.x

Cuesta, A., Brinchmann, M., Morcillo, F., Patricia, C., Hector, E., and Maria, A. (2016). Differential proteome profile of skin mucus of gilthead seabream (*Sparus aurata*) after probiotic intake and/or overcrowding stress. *J. Proteomics* 132, 41–50. doi: 10.1016/j.jprot.2015.11.017

Cui, L., Xu, W., Ai, Q., Wang, D., and Mai, K. (2013). Effects of dietary chitosan oligosaccharide complex with rare earth on growth performance and innate immune response of turbot, S cophthalmus maximus L. *Aquac. Res.* 44, 683–690. doi: 10.1111/j.1365-2109.2011.03072.x

Daniel, N. (2018). A review on replacing fish meal in aqua feeds using plant protein sources. *Int. J. Fish. Aquat. Stud* 6, 164–179. Available online at: https://www.researchgate.net/publication/324006059

Dautremepuits, C., Betoulle, S., Paris-Palacios, S., and Vernet, G. (2004). Humoral immune factors modulated by copper and chitosan in healthy or parasitised carp (*Cyprinus carpio L.*) by Ptychobothrium sp. *(Cestoda)*. *Aqua. Toxicol.* 68, 325–338. doi: 10.1016/j.aquatox.2004.04.003

Dimitroglou, A., Merrifield, D. L., Moate, R., Davies, S. J., Spring, P., Sweetman, J., et al. (2009). Dietary mannan oligosaccharide supplementation modulates intestinal microbial ecology and improves gut morphology of rainbow trout, *Oncorhynchus mykiss* (*Walbaum*). *J. Anim. ence* 87, 3226–3234. doi: 10.2527/jas.2008-1428

Dimitroglou, A., Merrifield, D. L., Spring, P., Sweetman, J., Moate, R., and Davies, S. J. (2010). Effects of mannan oligosaccharide (MOS) supplementation on growth performance, feed utilisation, intestinal histology and gut microbiota of gilthead sea bream (*Sparus aurata*). *Aquaculture* 300, 182–188. doi: 10.1016/j.aquaculture.2010.01.015

Dvinge, H., and Bertone, P. (2009). HTqPCR: High-throughput analysis and visualization of quantitative real-time PCR data in R. *Bioinformatics* 25, 3325–3326. doi: 10.1093/bioinformatics/btp578

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

Geda, F., Rekecki, A., Decostere, A., Bossier, P., Wuyts, B., Kalmar, I. D., et al. (2012). Changes in intestinal morphology and amino acid catabolism in common carp at mildly elevated temperature as affected by dietary mannanoligosaccharides. *Anim. Feed Sci. Technol.* 178, 95–102. doi: 10.1016/j.anifeedsci.2012.09.008

Ghosh, S., and Mehla, R. K. (2012). Influence of dietary supplementation of prebiotics (mannanoligosaccharide) on the performance of crossbred calves. *Trop. Anim. Health Prod.* 44, 617–622. doi: 10.1007/s11250-011-9944-8

Gomez, E., Tuohy, K. M., Gibson, G. R., Klinder, A., and Costabile, A. (2010). In vitro evaluation of the fermentation properties and potential prebiotic activity of Agave fructans. *J. Appl. Microbiol.* 108, 2114–2121. doi: 10.1111/j.1365-2672.2009.04617.x

Gudmundsdottir, B. K., Gudmundsdottir, S., and Magnadottir, B. (2014). Yersiniosis in Atlantic cod, *Gadus morhua* (*L.*), characterization of the infective strain and host reactions. *J. Fish Dis.* 37, 511–519. doi: 10.1111/jfd.12139

Gy, A., Xdab, C., Qyab, C., Scab, C., Hlab, C., Hzb, D., et al. (2020). Dietary replacement of fish meal with peanut meal in juvenile hybrid grouper (*Epinephelus fuscoguttatus* ♀ ×*Epinephelus lanceolatus* ♂): Growth performance, immune response and intestinal microbiota. *Aquac. Rep.* 17:100327. doi: 10.1016/j.aqrep.2020.100327

Hardy, R. W., and Tacon, A. G. (2002). Fish meal: historical uses, production trends and future outlook for sustainable supplies. *Respon. Marine Aquacult.* 1, 311–325. doi: 10.1079/9780851996042.0311

Hardy, R. W. (2006). Worldwide fish meal production outlook and the use of alternative protein meals for aquaculture. *Avances en Nutrición Acuicola* 15–17.

Hideaki, K., Shi-Ichi, H., Shin, M., and Lufen, M. (2005). Reactive oxygen species promote TNFα-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell* 5, 649–661. doi: 10.1016/j.cell.2004.12.041

Holland, J. W., Pottinger, T. G., and Secombes, C. J. (2002). Recombinant interleukin-1beta activates the hypothalamic-pituitary-interrenal axis in rainbow trout, Oncorhynchus mykiss. *J. Endocrinol.* 175, 261–267. doi: 10.1677/joe.0.1750261

Hui, L. I., and Yang, L. (2018). Molecular regulatory mechanism of Nrf2 antioxidant. *Chin. J. Bioinform.* 16, 1–6. doi: 10.3969/j.issn.1672-5565.201708001

Huo, P., Pan, J., Han, Y., Su, P., and Jiang, Z. (2015). Effects of alginic acid oligosaccharides on growth performance, hematological indexes and non-specific immunity of juvenile turbot (*Scophthalmus maximus*). *J. Guangdong Ocean Univ*. 35, 10–16. doi: 10.3969/j.issn.1673-9159.2015.04.002

Jin, N., Hei-Zhao, L., Shi-Gui, J., Xu, C., and Kai-Chang, W. (2013). Comparison of effect of chitin, chitosan, chitosan oligosaccharide and N-acetyl-d-glucosamine on growth performance, antioxidant defenses and oxidative stress status of *Penaeus monodon*. *Aquaculture* 372, 1–8. doi: 10.1016/j.aquaculture.2012.10.021

Joodi, G., Ansari, N., and Khodagholi, F. (2011). Chitooligosaccharide-mediated neuroprotection is associated with modulation of Hsps expression and reduction of MAPK phosphorylation. *Int. J. Biol. Macromol.* 48, 726–735. doi: 10.1016/j.ijbiomac.2011.02.011

Kaakoush, N. O. (2015). Insights into the role of erysipelotrichaceae in the human host. *Front. Cell. Infect. Microbiol.* 5:84. doi: 10.3389/fcimb.2015.00084

Kong, X., Zhou, X., Lian, G., Blachier, F., Liu, G., Tan, B., et al. (2014). Dietary supplementation with chitooligosaccharides alters gut microbiota and modifies intestinal luminal metabolites in weaned Huanjiang mini-piglets. *Livest. Sci.* 160, 97–101. doi: 10.1016/j.livsci.2013.11.023

Krogdahl, A., Bakke-McKellep, A. M., Roed, K. H., and Baeverfjord, G. (2015). Feeding Atlantic salmon Salmo salar L. *soybean* products: effects on disease resistance (furunculosis), and lysozyme and IgM levels in the intestinal mucosa. *Aquacult. Nutri.* 6, 77–84. doi: 10.1046/j.1365-2095.2000.00129.x

Lara, C., Romina, M., Barbara, F., and Nicol,ò, M. (2017). Impact of Omega-3 fatty acids on the gut microbiota. *Int. J. Molecul. ences* 18:2645. doi: 10.3390/ijms18122645

Li, E., Xu, C., Wang, X., Wang, S., Zhao, Q., Zhang, M., et al. (2018a). Gut microbiota and its modulation for healthy farming of pacific white shrimp *Litopenaeus vannamei*. *Rev. Fish. ence Aquacult.* 26, 381–399. doi: 10.1080/23308249.2018.1440530

Li, S., Sang, C., Zhang, J., Li, Z., and Chen, N. (2018b). Molecular cloning, expression profiling of adipose triglyceride lipase (ATGL) and forkhead box

O1 (FoxO1), and effects of dietary carbohydrate level on their expression in hybrid grouper (*Epinephelus fuscoguttatus*♀× *E. lanceolatus*♂). *Aquaculture* 492, 103–112. doi: 10.1016/j.aquaculture.2018.03.062

Li, X., Piao, X., Kim, S., Liu, P., Wang, L., Shen, Y., et al. (2007). Effects of chito-oligosaccharide supplementation on performance, nutrient digestibility, and serum composition in broiler chickens. *Poult. Sci.* 86, 1107–1114. doi: 10.1093/ps/86.6.1107

Li, X., Zheng, S., Han, T., Song, F., and Wu, G. (2020). Effects of dietary protein intake on the oxidation of glutamate, glutamine, glucose and palmitate in tissues of largemouth bass (*Micropterus salmoides*). *Amino Acids* 52, 1491–1503. doi: 10.1007/s00726-020-02907-3

Liaqat, F., and Eltem, R. (2018). Chitooligosaccharides and their biological activities: a comprehensive review. *Carbohydr. Polym.* 184, 243–259. doi: 10.1016/j.carbpol.2017.12.067

Lin, S., Mao, S., Guan, Y., Luo, L., Luo, L., and Pan, Y. (2012). Effects of dietary chitosan oligosaccharides and Bacillus coagulans on the growth, innate immunity and resistance of koi (*Cyprinus carpio koi*). *Aquaculture* 342, 36–41. doi: 10.1016/j.aquaculture.2012.02.009

Little, T. J., Hultmark, D., and Read, A. F. (2005). Invertebrate immunity and the limits of mechanistic immunology. *Nat. Immunol.* 6, 651–654. doi: 10.1038/ni1219

Liu, B., Xu, L., Ge, X., Xie, J., Xu, P., Zhou, Q., et al. (2013). Effects of mannan oligosaccharide on the physiological responses, HSP70 gene expression and disease resistance of Allogynogenetic crucian carp (*Carassius auratus gibelio*) under Aeromonas hydrophila infection. *Fish Shellfish Immunol.* 34, 1395–1403. doi: 10.1016/j.fsi.2013.02.028

Liu, J. X., Xu, Q. H., Li, S., Yu, X. D., Liu, W. Y., Ouyang, G., et al. (2017). Transcriptional factors Eaf1/2 inhibit endoderm and mesoderm formation via suppressing TGF-β signaling. *Biochimica et Biophysica Acta (BBA)— Gene Regul. Mechan.*1860, 1103–1116. doi: 10.1016/j.bbagrm.2017.09.001

Liu, P., Piao, X. S., Kim, S. W., Wang, L., Shen, Y. B., Lee, H. S., et al. (2008). Effects of chito-oligosaccharide supplementation on the growth performance, nutrient digestibility, intestinal morphology, and fecal shedding of Escherichia coli and Lactobacillus in weaning pigs. *J. Anim. ence* 86, 2609–2618. doi: 10.2527/jas.2007-0668

Luo, L., Cai, X., He, C., Xue, M., Wu, X., and Cao, H. (2009). Immune response, stress resistance and bacterial challenge in juvenile rainbow trouts Oncorhynchus mykiss fed diets containing chitosan-oligosaccharides. *Curr. Zool.* 55, 416–422. doi: 10.1093/czoolo/55.6.416

Mahious, A. S., Gatesoupe, F. J., Hervi, M., Metailler, R., and Ollevier, F. (2006). Effect of dietary inulin and oligosaccharides as prebiotics for weaning turbot, Psetta maxima (*Linnaeus, C.* 1758). *Aquacult. Int.* 14, 219–229. doi: 10.1007/s10499-005-9003-4

Maqsood, S., Singh, P., Samoon, M. H., and Balange, A. K. (2010). Effect of dietary chitosan on non-specific immune response and growth of *Cyprinus carpio* challenged with *Aeromonas hydrophila*. *J. Card. Fail.* 2, 77–85. Available online at: https://www.researchgate.net/publication/286150928

Mei, G. Y., Carey, C. M., Tosh, S., and Kostrzynska, M. (2011). Utilization of different types of dietary fibres by potential probiotics. *Can. J. Microbiol.* 57:857. doi: 10.1139/w11-077

Mussatto, S. I., and Mancilha, I. M. (2007). Non-digestible oligosaccharides: a review. *Carbohydr. Polym.* 68, 587–597. doi: 10.1016/j.carbpol.2006.12.011

Naveed, M., Phil, L., Sohail, M., Hasnat, M., Baig, M. M. F. A., Ihsan, A. U., et al. (2019). Chitosan oligosaccharide (COS): an overview. *Int. J. Biol. Macromol.* 129, 827–843. doi: 10.1016/j.ijbiomac.2019.01.192

Ofek, I., Mirelman, D., and Sharon, N. (1977). Adherence of Escherichia coli to human mucosal cells mediated by mannose receptors. *Nature* 265, 623–625. doi: 10.1038/265623a0

Palframan, R. J., Gibson, G. R., and Rastall, R. A. (2002). Effect of pH and dose on the growth of gut bacteria on prebiotic carbohydrates in vitro. *Anaerobe* 8, 287–292. doi: 10.1006/anae.2002.0434

Pérez-Jiménez, A., Bañuelos-Vargas, I., López, L. M., and Peres, H. (2014). Effect of fishmeal replacement by soy protein concentrate with taurine supplementation on hepatic intermediary metabolism and antioxidant status of totoaba juveniles (*Totoaba macdonaldi*). *Comp. Biochem. Physiol. Part B Biochem. Molecul. Biol.* 170, 18–25. doi: 10.1016/j.cbpb.2014.01.003

Qingsen, S., Xindi, S., Chao, C., Jiejie, H., and Guoyun, L. (2016). Dietary fucoidan modulates the gut microbiota in mice by increasing the abundance of Lactobacillus and Ruminococcaceae. *Food Funct.* 7, 3224–3232. doi: 10.1039/C6FO00309E

Rajendran, S., Okolie, C. L., Udenigwe, C. C., and Mason, B. (2017). Structural features underlying prebiotic activity of conventional and potential prebiotic oligosaccharides in food and health. *J. Food Biochem.* 41:e12389. doi: 10.1111/jfbc.12389

Refstie, S., Landsverk, T., Bakke-Mckellep, A. M., Ring, E., Sundby, A., Shearer, K. D., et al. (2006). Digestive capacity, intestinal morphology, and microflora of 1-year and 2-year old Atlantic cod (*Gadus morhua*) fed standard or bioprocessed soybean meal. *Aquaculture* 261, 269–284. doi: 10.1016/j.aquaculture.2006.07.011

Ricardo, S., Alvaro, B., and José E. (2014). Growth and intestinal morphology of juvenile pacu *Piaractus mesopotamicus* (*Holmberg* 1887) fed dietary prebiotics (mannanoligosaccharides—MOS). *Anais da Academia Brasileira de Ciências* 86, 1517–1524. doi: 10.1590/0001-3765201420130088

Ring, E., Olsen, R. E., Gifstad, T., Dalmo, R. A., and Bakke, A. M. (2010). Prebiotics in aquaculture: a review. *Aquacult. Nutrit.* 16, 117–136. doi: 10.1111/j.1365-2095.2009.00731.x

Sahoo, B. R., Dikhit, M. R., Bhoi, G. K., Maharana, J., Lenka, S. K., Dubey, P. K., et al. (2015). Understanding the distinguishable structural and functional features in zebrafish TLR3 and TLR22, and their binding modes with fish dsRNA viruses: an exploratory structural model analysis. *Amino Acids* 47, 381–400. doi: 10.1007/s00726-014-1872-2

Salze, G., Mclean, E., Schwarz, M. H., and Craig, S. R. (2008). Dietary mannan oligosaccharide enhances salinity tolerance and gut development of larval cobia. *Aquaculture* 274, 148–152. doi: 10.1016/j.aquaculture.2007.11.008

Sang, H. M., Fotedar, R., and Filer, K. (2015). Effects of dietary mannan oligosaccharide on the survival, growth, immunity and digestive enzyme activity of freshwater crayfish, *Cherax destructor Clark* (1936). *Aquacult. Nutrit.* 17, e629–e635. doi: 10.1111/j.1365-2095.2010.00812.x

Saurabh, S., and Sahoo, P. K. (2010). Lysozyme: an important defence molecule of fish innate immune system. *Aquac. Res.* 39, 223–239. doi: 10.1111/j.1365-2109.2007.01883.x

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/AEM.01541-09

Shen, J., Liu, H., Tan, B., Dong, X., Yang, Q., Chi, S., et al. (2020). Effects of replacement of fishmeal with cottonseed protein concentrate on the growth, intestinal microflora, haematological and antioxidant indices of juvenile golden pompano (*Trachinotusovatus*). *Aquacult. Nutrit.* 26, 1119–1130. doi: 10.1111/anu.13069

Si, B., Wang, Y., Bao, N., Chen, F., Wang, L., Sun, M., et al. (2017). Effects of coated chitosan oligosaccharides on growth performance and immune response of sea cucumber (*Stichopus japonicus*). *J. Guangdong Ocean Univ.* 37, 28–34. doi: 10.3969/j.issn.1673-9159.2017.01.005

Skiba-Cassy, S., Geurden, I., Panserat, S., and Seiliez, I. (2016). Dietary methionine imbalance alters the transcriptional regulation of genes involved in glucose, lipid and amino acid metabolism in the liver of rainbow trout (*Oncorhynchusmykiss*). *Aquaculture* 454, 56–65. doi: 10.1016/j.aquaculture.2015.12.015

Song, S. K., Beck, B. R., Kim, D., Park, J., Kim, J., Kim, H. D., et al. (2014). Prebiotics as immunostimulants in aquaculture: a review. *Fish Shellfish Immunol.* 40, 40–48. doi: 10.1016/j.fsi.2014.06.016

Sun, L. W., Wen, H., Jiang, M., Fan, W. U., Liu, W., Tian, J., et al. (2011). Effect of dietary chitosan on growth performance, non-specific immunity and hematological parameters of juvenile GIFT Tilapia, *Oreochromisniloticus*. *J. Guangdong Ocean Univ.* 31, 43–49. Available online at: http://en.cnki.com.cn/Article_en/CJFDTOTAL-SHDX201103009.htm

Swiatkiewicz, S., Swiatkiewicz, M., Arczewska-Wlosek, A., and Jozefiak, D. (2015). Chitosan and its oligosaccharide derivatives (chito-oligosaccharides) as feed supplements in poultry and swine nutrition. *J. Anim. Physiol. Anim. Nutr.* 99, 1–12. doi: 10.1111/jpn.12222

Talpur, A. D., Munir, M. B., Mary, A., and Hashim, R. (2014). Dietary probiotics and prebiotics improved food acceptability, growth performance, haematology

and immunological parameters and disease resistance against Aeromonas hydrophila in snakehead (*Channastriata*) fingerlings. *Aquaculture* 426, 14–20. doi: 10.1016/j.aquaculture.2014.01.013

Tao, F., Du, Y., Li, J., Wei, Y., and Yao, P. (2007). Antioxidant activity of half N-acetylated water-soluble chitosan in vitro. *Euro. Food Res. Technol.* 225, 133–138. doi: 10.1007/s00217-006-0391-0

Tarnecki, A. M., Burgos, F. A., Ray, C. L., and Arias, C. R. (2017). Fish intestinal microbiome: diversity and symbiosis unravelled by metagenomics. *J. Appl. Microbiol.* 123, 2–17. doi: 10.1111/jam.13415

Torrecillas, S., Rivero-Ramírez, F., Izquierdo, M., Caballero, M., Makol, A., Suarez-Bregua, P., et al. (2018). Feeding European sea bass (*Dicentrarchuslabrax*) juveniles with a functional synbiotic additive (mannan oligosaccharides and Pediococcus acidilactici): an effective tool to reduce low fishmeal and fish oil gut health effects? *Fish Shellfish Immunol.* 81, 10–20. doi: 10.1016/j.fsi.2018.07.007

Vieira, T. F., Corrêa, R., Peralta, R. A., Peralta-Muniz-Moreira, R. F., Adelar, B., and Peralta, R. M. (2020). An overview of structural aspects and health beneficial effects of antioxidant oligosaccharides. *Curr. Pharm. Des.* 26, 1759–1777. doi: 10.2174/1381612824666180517120642

Waite, D. W., Inka, V., Christian, R., Parks, D. H., Ying, Z., Ken, T., et al. (2017). Comparative genomic analysis of the class epsilonproteobacteria and proposed reclassification to epsilonbacteraeota (phyl. *nov.). Front. Microbiol.* 8:19. doi: 10.3389/fmicb.2017.00682

Wan, J., Yang, K., Xu, Q., Chen, D., Yu, B., Luo, Y., et al. (2016). Dietary chitosan oligosaccharide supplementation improves foetal survival and reproductive performance in multiparous sows. *RSC Adv.* 6, 70715–70722. doi: 10.1039/C6RA13294D

Wan, M., Yin, P., Fang, W., Xie, S., 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 (*Litopenaeusvannamei*). *Aquacult. Nutri.* 24, 1845–1854. doi: 10.1111/anu.12823

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

Wanga, K. Z., Jianga, W. D., Wua, P., Liua, Y., and Jianga, J. (2018). Gossypol reduced the intestinal amino acid absorption capacity of young grass carp (*Ctenopharyngodonidella*). *Aquaculture* 492, 46–58. doi: 10.1016/j.aquaculture.2018.03.061

Wei, G., Cai, S., Wu, Y., Ma, S., and Huang, Y. (2020). Immune effect of Vibrio harveyi formalin-killed cells vaccine combined with chitosan oligosaccharide and astragalus polysaccharides in♀ *Epinephelusfuscoguttatus*×♂ *Epinepheluslanceolatus*. *Fish Shellfish Immunol.* 98, 186–192. doi: 10.1016/j.fsi.2020.01.015

Wei, H., Yang, M., Zhao, T., Wang, X., and Zhou, H. (2013). Functional expression and characterization of grass carp IL-10: An essential mediator of TGF-β1 immune regulation in peripheral blood lymphocytes. *Mol. Immunol.* 53, 313–320. doi: 10.1016/j.molimm.2012.08.021

Wink, D. A., Darbyshire, J. F., Nims, R. W., Saavedra, J. E., and Ford, P. C. (1993). Reactions of the bioregulatory agent nitric oxide in oxygenated aqueous media: Determination of the kinetics for oxidation and nitrosation by intermediates generated in the nitric oxide/oxygen reaction. *Chem. Res. Toxicol.* 6, 23–27. doi: 10.1021/tx00031a003

Wu, C., Lu, B., Wang, Y., Jin, C., and Ye, J. (2020). Effects of dietary vitamin D3 on growth performance, antioxidant capacities and innate immune responses in juvenile black carp *Mylopharyngodonpiceus*. *Fish Physiol. Biochem.* 46, 2243–2256. doi: 10.1007/s10695-020-00876-8

Wu, Y. V., Tudor, K. W., Brown, P. B., and Rosati, R. R. (1999). Substitution of Plant Proteins or Meat and Bone Meal for Fish Meal in Diets of Nile Tilapia. *North Am. J. Aquaculture* 61, 58-63. doi: 10.1577/1548-8454(1999)061<;0058:SOPPOM>2.0.CO;2

Wu,. M., S. B., Singh, J., Wang, C., and Chung, C., (2011). Antidiabetic and antisteatotic effects of the selective fatty acid synthase (FAS) inhibitor platensimycin in mouse models of diabetes. *Proc. Nat. Acad. Sci.* 108, 5378–5383. doi: 10.1073/pnas.1002588108

Xiaoyi, S., Hua, Y., Nose, K., Satoko, H., and Emir, Q. (2008). Decline in intestinal mucosal IL-10 expression and decreased intestinal barrier function in a mouse model of total parenteral nutrition. *Am. J. Physiol. Gastrointest. Liver Physiol.* 57, 139–147. doi: 10.1152/ajpgi.00386.2007

Xie, W., Xu, P., and Liu, Q. (2001). Antioxidant activity of water-soluble chitosan derivatives. *Bioorgan. Med. Chem. Lett.* 11, 1699–1701. doi: 10.1016/S0960-894X(01)00285-2

Yamamoto, T., Matsunari, H., Sugita, T., Furuita, H., Masumoto, T., Iwashita, Y., et al. (2012). Optimization of the supplemental essential amino acids to a fish meal-free diet based on fermented soybean meal for rainbow trout *Oncorhynchusmykiss*. *Fish. Sci.* 78, 359–366. doi: 10.1007/s12562-011-0456-2

Yan, L., and Xiao, Q. Z. (2006). Dietary glutamine supplementation improves structure and function of intestine of juvenile Jian carp (*Cyprinuscarpiovar. Jian)*—sciencedirect. *Aquaculture* 256, 389–394. doi: 10.1016/j.aquaculture.2006.02.011

Ye, G., Dong, X., Yang, Q., Chi, S., Liu, H., Zhang, H., et al. (2020). Low-gossypol cottonseed protein concentrate used as a replacement of fish meal for juvenile hybrid grouper (*Epinephelusfuscoguttatus*♀× *Epinepheluslanceolatus*♂): Effects on growth performance, immune responses and intestinal microbiota. *Aquaculture* 524:735309. doi: 10.1016/j.aquaculture.2020.735309

Yin, B., Liu, H., Tan, B., Dong, X., Chi, S., Yang, Q., et al. (2018). Cottonseed protein concentrate (CPC) suppresses immune function in different intestinal segments of hybrid grouper♀ *Epinephelusfuscoguttatus*×♂ *Epinepheluslanceolatu* via TLR-2/MyD88 signaling pathways. *Fish Shellfish Immunol.* 81, 318–328. doi: 10.1016/j.fsi.2018.07.038

Yin, B., Liu, H., Tan, B., Dong, X., Chi, S., Yang, Q., et al. (2020). Preliminary study of mechanisms of intestinal inflammation induced by plant proteins in juvenile hybrid groupers (♀ *Epinephelus fuscoguttatus* ×♂ *E. lanceolatu). Fish and Shellfish Immunol.* 106, 341–356. doi: 10.1016/j.fsi.2020.07.026

Yu, Z., He, X., Fu, D., and Zhang, Y. (2011). Two superoxide dismutase (SOD) with different subcellular localizations involved in innate immunity in *Crassostreahongkongensis*. *Fish Shellfish Immunol.* 31, 533–539. doi: 10.1016/j.fsi.2011.06.022

Zeng, Q., Tian, X., and Wang, L. (2017). Genetic adaptation of microbial populations present in high-intensity catfish production systems with therapeutic oxytetracycline treatment. *Sci. Rep.* 7, 1–13. doi: 10.1038/s41598-017-17640-3

Zhang, B. (2019). Dietary chitosan oligosaccharides modulate the growth, intestine digestive enzymes, body composition and nonspecific immunity of loach *Paramisgurnusdabryanus*. *Fish Shellfish Immunol.* 88, 359–363. doi: 10.1016/j.fsi.2019.03.006

Zhang, R. B., Cao, J. M., Huang, Y. H., Wang, G. X., Chen, X. Y., Yan, J., et al. (2012). Effect of dietary xylooligosaccharides on intestinal histology and microflora of tilapia(*Oreochromisniloticus*×*O.aureus*) and resistance against *Aeromonashydrophila*. *J. Shanghai Ocean Univ.* 21, 233–240. doi: 10.1007/s11783-011-0280-z

Zhou, S., Song, D., Zhou, X., Mao, X., Zhou, X., Wang, S., et al. (2019). Characterization of Bacillus subtilis from gastrointestinal tract of hybrid Hulong grouper (*Epinephelusfuscoguttatus*× *E.lanceolatus*) and its effects as probiotic additives. *Fish Shellfish Immunol.* 84, 1115–1124. doi: 10.1016/j.fsi.2018.10.058

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: 21 September 2021
doi: 10.3389/fmars.2021.740660

Effects of Low- and High-Level Gossypol and Sodium Butyrate Supplementation Under High-Level Gossypol Condition on Growth Performance and Intestinal Health of Hybrid Grouper (*Epinephelus fuscoguttatus*♀× *Epinephelus lanceolatus*♂)

OPEN ACCESS

Edited by:
Xiang-Jun Leng,
Shanghai Ocean University, China

Reviewed by:
Min Jin,
Ningbo University, China
Oksana B. Stoliar,
Ternopil Volodymyr Hnatyuk National Pedagogical University, Ukraine

***Correspondence:**
Hongyu Liu
liuhyu@gdou.edu.cn
Beiping Tan
bptan@126.com

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

Received: 13 July 2021
Accepted: 24 August 2021
Published: 21 September 2021

Citation:
Yin B, Liu H, Tan B, Dong X, Chi S, Yang Q and Zhang S (2021) Effects of Low- and High-Level Gossypol and Sodium Butyrate Supplementation Under High-Level Gossypol Condition on Growth Performance and Intestinal Health of Hybrid Grouper (Epinephelus fuscoguttatus♀× Epinephelus lanceolatus♂). Front. Mar. Sci. 8:740660. doi: 10.3389/fmars.2021.740660

Bin Yin[1,2,3], *Hongyu Liu*[1,2,3]*, *Beiping Tan*[1,2,3]*, *Xiaohui Dong*[1,2,3], *Shuyan Chi*[1,2,3], *Qihui Yang*[1,2,3] and *Shuang Zhang*[1,2,3]

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

The supplementation of gossypol in excess is noted to cause detrimental effects such as the reduction of antioxidant enzymes and disruption of lipid metabolism in animals. Studies regarding the effects of different levels of gossypol are very rare; thus, this study was conducted to evaluate the effects of low and high dietary levels of gossypol and of supplementation with 0.13 % sodium butyrate (NaB) under high gossypol conditions on the growth performance and intestinal health of hybrid grouper (*Epinephelus fuscoguttatus*♀×*Epinephelus lanceolatus*♂). Four treatments were used: Feed containing 40% fish meal was used as the control group [fishmeal (FM)], the FM diet plus 0.03% gossypol acetic acid (abbreviated as gossypol) as the low-level gossypol group (gL), FM + 0.15% gossypol was used as the high-level gossypol group (gH), and FM+0.15 % gossypol with 0.13 % NaB as the repair group (gHNaB). All diets were isonitrogenous and isolipidic. The results showed that the gL treatment significantly increased specific growth rate (SGR) and feed utilization; upregulated mRNA levels of distal intestinal transforming growth factor-β1 (*tgfβ1*), *jam*, *occludin*, *claudin3*, and *zo1*; and downregulated mRNA levels of *il8*, *ifnγ,* and *akt*. The gH treatment significantly reduced SGR and feed utilization; increased distal intestinal total nitric oxide synthase (NOS) activity and nitric oxide (NO) content; upregulated mRNA levels of distal intestinal *tnfα*, *il1β*, *il6*, *ifnγ*, *caspase2*, *caspase9*, and *akt*; and downregulated mRNA levels of *tgfβ1*, *jam*, and *zo1*. NaB supplementation significantly increased distal intestinal total NOS activity and NO content; downregulated distal intestinal *tnfα*, *il1β*, *ifnγ*, *pi3k p85,* and *akt* mRNA levels; and increased distal intestinal *tgfβ1*,

jam, *occludin*, and *zo1* mRNA levels. Above all, low- and high-level gossypol exhibited positive and negative effects on growth performance, distal intestinal anti-inflammatory capacity, and tight junctions, respectively, in hybrid groupers. NaB supplementation improved distal intestinal anti-inflammatory capacity and tight junctions in hybrid groupers to a certain extent.

Keywords: hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂), gossypol, sodium butyrate, intestinal inflammation, tight junction

INTRODUCTION

Hybrid grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatus*) is a coastal warm-water, broad-salt fish that is widely cultured in southern China and has high economic value. In 2017, the Ministry of Agriculture included groupers in the National Technical System of the Marine Fish Industry. It has been reported that the feed protein content requirement of hybrid grouper can reach 50% (Rahimnejad et al., 2015; Jiang et al., 2016), and the content of fishmeal (FM) in feed is also up to 50% or more. The global shortage of FM resources has seriously limited the development of the grouper industry. Therefore, finding suitable protein sources to replace FM has become a long-term research focus in aquatic animal nutrition.

Cottonseed meal is a by-product obtained from cottonseed after oil extraction and is used as a protein source for aquatic animal feed because it contains up to 40–45 % crude protein (Kumar et al., 2021). However, intake of high levels of cottonseed meal can cause negative effects, such as reduced growth performance, organ damage, and reduced reproductive capacity in fish (Mbahinzireki et al., 2015). These negative effects may be related to the presence of antinutritional factors, including gossypol. Gossypol is a polyphenolic compound isolated from the pigment gland of cottonseed, a natural yellow pigment, and is the main antinutritional factor present in cottonseed meal (Krogdahl et al., 2010; Bian et al., 2017). The active hydroxyl group of free gossypol combines with lysine, and this complex reduces the availability of lysine in the cotton meal. Gossypol can also form complexes with protein, affecting animal growth, while the active aldehyde group can be combined with iron ions, interfering with the synthesis of hemoglobin. This gossypol/iron interaction reduces the number of red blood cells in the animal body, thereby causing iron deficiency anemia in animals (Cao et al., 2018).

In laying hens, diets containing high levels of gossypol led to the disruption of lipid metabolism and reduced antioxidant function (Hou, 2014). In the rumen, the 0.1 mg/g addition of gossypol significantly increased the number of total bacteria abundance and fiber-degrading bacteria and improved the degradation rate of dry matter (Hou, 2012). These results, across multiple livestock species, indicate that the effects of gossypol vary depending on the concentration and species. Similarly, diets high in cottonseed have negative effects on fish (Deng et al., 2014): This is mainly due to the presence of gossypol, which has phenolic and carbonyl groups that can react with acids and amines, thus causing anorexia, diarrhea, and eventually death (Rinchard et al., 2003). Studies have shown that high-level gossypol inhibits the growth performance of rainbow trout and causes damage to the liver and spleen (Herman, 1970). The addition of 900 mg/kg of gossypol to the feed significantly reduced the weight gain (WG) and feed utilization of channel catfish (*Ictalurus puctatus*) (Yildirim et al., 2003), while the growth performance of gibel carp (*Carassius auratus gibelio*) was significantly reduced when dietary gossypol went above 300 mg/kg, with gossypol residue in the tissues increased (Jiang, 2011); at levels above 900 mg/kg, the liver function of the gibel carp was also damaged. When cottonseed protein concentrate (CPC) was used to replace FM, the growth of hybrid grouper (Yin et al., 2018) and golden pompano (*Trachinotus ovatus*) (Shen et al., 2020) was significantly increased at low-level substitution. We suspect that this facilitation effect is related to the low-level gossypol contained in the CPC. However, the mechanism of this low-level gossypol or CPC addition has rarely been investigated.

Currently, the solution to reduced fish growth rates caused by high levels of dietary plant protein is usually to supplement the feed with additives. Sodium butyrate (NaB) has been widely reported as an alternative to antibiotics in promoting growth, nutrient absorption, and immunity in aquatic animals. NaB can significantly improve the growth performance of grass carp (*Ctenopharyngodon idella*) (Liu et al., 2017) and tilapia (*Oreochromis niloticus*) (Ahmed and Sadek, 2015), maintain the normal morphology of intestinal epithelial cells (Claus et al., 2007), and improve the antioxidant capacity of gilthead seabream (*Sparus aurata*) (Robles et al., 2013). However, it has not been reported whether the addition of NaB after high-level gossypol can have a positive effect on hybrid grouper.

The health of fish intestines is closely related to growth. Antimicrobial peptides, lysozyme, and immunoglobulins in the fish intestine form an immune barrier. In addition, tightly connected molecules in the intestine are important for maintaining normal intestinal structure and nutrient absorption. Therefore, a systematic and in-depth study of gossypol is necessary for the intestinal health of aquatic animals and the sustainable development of aquaculture.

In a previous study in our laboratory, we found that CPC as a replacement for FM had a nonlinear, parabolic effect on specific growth rate (SGR) in hybrid grouper (Yin et al., 2018), which we speculate is closely related to the residual gossypol in CPC. Currently, there is a gap in research on gossypol in hybrid grouper. Therefore, in the present study, we investigated the effects of gossypol on the growth performance, serum immunity, and intestinal health of hybrid grouper, as well as the restorative

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

Ingredients/%	Diets			
	FM	**gL**	**gH**	**gHNaB**
Red fishmeal	40.00	40.00	40.00	40.00
Casein	11.54	11.54	11.54	11.54
Gelatin	2.89	2.89	1.71	1.71
Wheat flour	20.00	20.00	20.00	20.00
Fish oil	4.72	4.72	4.72	4.72
Soy lecithin	2.00	2.00	2.00	2.00
Calcium monophosphate	1.00	1.00	1.00	1.00
[a]Vitamin premix	0.20	0.20	0.20	0.20
[b]Mineral premix	0.50	0.50	0.50	0.50
Antioxidants	0.05	0.05	0.05	0.05
Choline chloride	0.50	0.50	0.50	0.50
Gossypol	0	0.03	0.15	0.15
Vitamin C	0.05	0.05	0.05	0.05
Cellulose microcrystalline	16.55	16.52	16.40	15.07
Microencapsulated sodium butyrate (NaB)	0	0	0	0.13
Proximate composition (% air dry matter)				
[c]Crude protein	47.56	47.25	47.36	48.09
[c]Crude lipid	10.55	9.97	10.44	10.53
[c]Moisture	9.08	9.21	9.00	9.17

[a]*Vitamin premix (g/kg mixture): vitamin B1, 17.00 g; vitamin B2, 16.67 g; vitamin B6, 33.33 g; vitamin B12, 0.07 g; vitamin K, 3.33 g; vitamin E, 66.00 g; retinyl acetate, 6.67 g; VD, 33.33 g, nicotinic acid, 67.33 g; D-calcium pantothenate, 40.67 g; biotin, 16.67; folic acid, 4.17 g; inositol, 102.04 g; cellulose, 592.72 g. All ingredients were diluted with corn starch to 1.00 kg.*

[b]*Mineral premix (g/kg mixture): $CaCO_3$, 350.00 g; $NaH_2PO_4{\cdot}H_2O$, 200.00 g; KH_2PO_4, 200.00 g; NaCl, 12.00 g; $MgSO_4{\cdot}7H_2O$, 10.00 g; $FeSO_4{\cdot}7H_2O$, 2.00 g; $MnSO_4{\cdot}7H_2O$, 2.00 g; $AlCl_3{\cdot}6H_2O$, 1.00 g; $CuCl_2{\cdot}2H_2O$, 1.00 g; KF, 1.00 g; $NaMoO_4{\cdot}2H_2O$, 0.50 g; $NaSeO_3$, 0.40 g; $CoCl_2{\cdot}6H_2O$, 0.10 g; KI, 0.10g; zeolite powder, 219.90 g. All ingredients were diluted with corn starch to 1.00 kg (obtained from Zhanjiang Yuehua Feed Co. Ltd., Zhanjiang, China).*

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

effect of NaB by supplementing low- and high-level gossypol, and supplementing the high-level gossypol with appropriate levels of NaB to provide a theoretical basis and reference for the efficient use of cottonseed meal in aquafeeds.

MATERIALS AND METHODS

Experimental Diets

The composition of the basal diet is presented in **Table 1**. Gossypol-acetic acid (1 mg = 0.8962 mg gossypol, abbreviated as gossypol) used in this study was obtained from Ci Yuan Biotechnology Co., Ltd. Shannxi (purity = 98.02 %) (Wanga et al., 2018). The group with 0.00% gossypol and 0.00% NaB was used as the control group (FM). The control group was supplemented with 0.03% gossypol as the low-level gossypol group (gL); 0.15 % gossypol as the high-level gossypol group (gH); and 0.15 % gossypol and 0.13 % NaB as the repair group (gHNaB). Four groups of isonitrogenous (48 %) and isolipidic (10 %) experimental diets were prepared. Red FM, casein, and gelatin were used as the main protein sources; fish oil and soy lecithin were used as the main lipid sources, and wheat flour was used as the carbohydrate source. After passing all the solid raw materials through a 0.25-mm sieve, all the raw materials were weighed according to the percentage in **Table 1** and mixed, and 30% of the mixture weight of water was added and mixed again. The final mixture was made into strips (2.0 mm diameter), naturally air-dried for 48 h, and then placed in a refrigerator at −20°C.

Feeding Trial

Hybrid grouper with an initial weight of 10.70 ± 0.09 g were purchased from a grouper hatchery in Zhanjiang, China. They were domesticated for 10 days in an outdoor concrete pond at the biological research base of Donghai Island, Guangdong Ocean University, China, using commercial feed to adapt to the base environment. A total of 480 hybrid grouper in healthy body condition and uniform size were randomly selected and divided into four treatment groups, each including four replicates with 30 fish per replicate. The culture experiments were conducted in 12 0.3-m^3 fiberglass tanks for 8 weeks. Apparent satiation feeding was performed daily at 7:30 and 16:30 and adjusted according to feeding conditions. Water temperature was 30.00±1.59°C, salinity was maintained at 35.48±1.24 g/kg, dissolved oxygen was kept above 7 mg/L, pH was maintained at 7.8–8.1, and ammonia nitrogen was kept below 0.03 mg/L during the breeding period.

Sample Collection

At the end of the feeding experiment, all fish were starved for 24 h. First, the fish in each tank were counted and weighed, and the data obtained were used to calculate the WG, SGR, and survival (SR) and combined with the weight of feed

consumed to calculate the feed coefficient ratio (FCR) (Yin et al., 2021a). For the intestine, part of the intestine near the cloacal pore, accounting for one-third of the total intestine of two randomly selected fish from each replicate, was stored in 4% formalin solution and used as paraffin-embedded TUNEL-stained sections; the distal intestine from four randomly selected fish from each replicate was stored at −80°C and used for damage indicator determination, and the distal intestine from two randomly selected fish from each replicate was stored at −80°C and used as PCR samples.

Distal Intestinal TUNEL Staining Observation

The slices were placed in xylene I for 10 min; xylene II for 10 min; xylene II for 10 min; anhydrous ethanol I for 5 min; anhydrous ethanol II for 5 min; anhydrous ethanol III for 5 min; and distilled water wash. An immunohistochemistry pen was used to draw circles around the tissues, and proteinase K working solution (stock solution: PBS = 1:9) was added dropwise inside the circles and incubated for 22 min at 37°C. The samples were washed three times for 5 min each with PBS (pH 7.4). Next, 0.1 % Triton (stock solution: PBS = 1:1,000) was added to the circles, incubated for 20 min at room temperature, and again washed three times with PBS for 5 min each time. After shaking the sections dry, equilibration buffer was added dropwise to the circles and incubated for 10 min at room temperature. The mixture [V(TDT enzyme): V(Dutp): V(equilibration buffer) = 1:5:50] was added to the circles and incubated for 2 h at 37°C. The incubation was kept moist during the incubation. After washing three times with PBS for 5 min each time, DAPI staining solution was added to the circles and incubated for 10 min at room temperature away from light. The sections were washed again using PBS three times for 5 mins each, shaken dry, and sealed with anti-fade mounting medium. Finally, images were observed and acquired under an ortho-fluorescence microscope (Nikon Eclipse C1) with a camera (Nikon Eclipse Ci-L), and a 20× object lens (CFI, Plan Fluor, N.A. 0.17, W. D. 2.1 mm).

Distal Intestinal Damage Indicators Determination

Nitric oxide (NO) (#A012-0-2) and total nitric oxide synthase (NOS) (#A014-2) were determined according to the instructions of the kits from Nanjing Jiancheng Institute of Biological Engineering. For NO, a standard curve was constructed using sodium nitrite. Equal volumes of double-distilled water, sodium nitrite standard solution, and intestinal supernatant samples were added to the blank, standard, and assay wells, respectively. The color developer agent was added to all wells and left for 15 min, and the OD was measured at 550 nm. For total NOS, equal volumes of double-distilled water and samples were added to the blank and assay wells, respectively. Then, the substrate buffer, accelerator, and color agent were added sequentially to all wells. A water bath at 37°C for 15 min was performed, followed by the addition of the wash solution and termination solution. After zeroing with double-distilled water, the OD value of

TABLE 2 | Primers used in this experiment for quantitative RT-PCR.

Gene name	Sequence (5′-3′)
Jam	F: CACGACAACGATGGCTCACCTC
	R: GCATTTCTGAAGGCGGCAATCTTG
Occluding	F: CTGTCACTGTCTATAAGCTACGCTC
	R: TCTTAACACTTTGCACATGAAGTGGA
claudin3	F: AAGCAAGGTCAACATGGCGGA
	R: GCGCTGCATGTGAAGTGTGATAG
claudin12	F: AGGGATCGCTGTGGCAACG
	R: CAGCCCGTCATACACGCTG
claudin15	F: ACTTCAGGACCAGGTCAAAGTTAGG
	R: CGATCCAGATTCAGCCAGAGCT
zo1	F: TGGAGCTGCGCTTACCTCAC
	R: GGTCAATGAGCACAGACACACAGT
Tnfα	F: AACTGTGTGTCCCCACTGCC
	R: CCACAGATGGCCCAGGTCAT
il1β	F: AAGGTGGACGCCAACAGACA
	R: GTTCACTGCAGGCTCAGGGA
tgfβ1	F: CTTCTCCTCCTCCTCGCTGC
	R: GATGTTGCTGAGGGCTTCGC
hepcidin	F: TGTCAATGACCCACTGAGCCTCG
	R: TCCACTGCAAACTGCTGGGC
il6	*F: CAATCCCAGCACCTTCCAC*
	R: CCTGACAGCCAGACTTCCTCT
il8	F: TGTGGCACTCCTGGTTCTCC
	R: GGGTTCACCTCCACCTGTCC
Ifnγ	F: CGATTCGGTCATCAAGAGCAT
	R: CTCCGTCACGACCGACACCA
caspase2	F: TCGGACATGATCTGTGGCTTTGC
	R: GGAGACGCAGTGTGGTGTTGAG
caspase3	F: TGGATCAACGTCTGTTTCCTTGTACTG
	R: GTTCATTGCCTTTCCCCGTGTTTTC
caspase6	F: AGAATCACTGAAGCCGCAGAAGC
	R: ATCATCATTGCCGTGACTCAGGAAG
caspase7	F: CATAACGGACACATACGGACACTCTG
	R: CTTCTTCTTCTTCCTCTGGCGTAAGC
caspase8	F: TCCTCCTCCCTTCTTGATCCAACTG
	R: AGCCTTCGCATCCTCCTGAGTC
caspase9	F: TTTGGGTGTAAGTGTCATCTGGGAAAG
	R: ACGCTATGCTGATTGTCTGCTCTTG
pi3k p85	F: GCCGAGGAGGAAGAGGATGTAGAC
	R: GAGGAGATGGTGGAGAAGGTGGAG
3pdk1	F: GGCAGCCATTACTGGAGCTTCTC
	R: TGCGAGCAGGAACAGATGACAAC
Akt	F: GGCAGGATGTGGTACAGAAGAAGC
	R: TGTCTGGAGGAGTGAGTGTGATGG
foxo4	F: GGGAGATACAAGGGAAGCAGTAACAC
	R: GCGGTGGTCAGCTTGATGTCTC
Fasl	F: CCTTCATCAAGAGCTGGCTGACTATC
	ACCTTCTCCTCCTTGTCTGACTCAC
β-actin	F: TACGAGCTGCCTGACGGACA
	R: GGCTGTGATCTCCTTCTGC

TABLE 3 | Growth parameters and feed utilization of juvenile hybrid grouper fed the experimental diets for 8 weeks.

	FM	gL	gH	gHNaB
IBW (g)	10.70 ± 0.08	10.66 ± 0.09	10.68 ± 0.10	10.77 ± 0.08
FBW (kg)	2.12 ± 0.07^{a}	2.39 ± 0.06^{a}	1.61 ± 0.09^{b}	1.53 ± 0.082^{b}
WG (%)	576.97 ± 13.32^{b}	649.40 ± 17.33^{c}	452.84 ± 20.38^{a}	416.97 ± 12.97^{a}
SGR (%/day)	2.73 ± 0.03^{b}	2.88 ± 0.03^{c}	2.44 ± 0.05^{a}	2.35 ± 0.04^{a}
SR (%)	95.83 ± 1.60	97.50 ± 0.83	89.17 ± 1.59	90.84 ± 5.84
FCR	0.97 ± 0.03^{b}	0.76 ± 0.03^{a}	1.63 ± 0.10^{c}	1.55 ± 0.02^{c}

Value show means ± SE (n = 4); Significance was evaluated by one-way ANOVA followed by Tukey's multiple range tests. FM, control diet; gL, containing 0.03% gossypol diet, gH, containing 0.15% gossypol diet, gHNaB, containing 0.15% gossypol and 0.13% NaB diet. IBW, initial body weight; FBW, final body weight; WG, weight gain; SGR, specific growth rate; SR, survival; FCR, feed coefficient ratio. ^{a,b,c} Mean values among all treatments with different letters were significantly different when the interaction was significant ($P < 0.05$).

each sample was determined using a UV-Vis spectrophotometer (UV-2450, Shimadzu, Japan) at 530 nm using a 1-cm optical diameter cuvette.

Real-Time Quantitative PCR Analysis

After the extraction of total RNA from the distal intestine using the traditional Trizol (TRI reagent solution, Invitrogen, Carlsbad, CA, United States), the quality and quantity of the total RNA were evaluated by 1% agarose gel electrophoresis with an electrophoresis instrument and A260: 280 values with a NanoDrop 2000 spectrophotometer (Thermo Fisher, United States), respectively. Total RNA was reverse-transcribed to cDNA using PrimeScript^{TM} RT-PCR Kit (Takara, Kusatsu, Japan) according to the manufacturer's instructions. Real-time quantitative PCR was performed using SYBR GreenPro Taq HS qPCR Kit II (Accurate Biology, China) on an Applied Biosystems 7500 Real-Time PCR System. The full-length transcriptome sequence of the distal intestine of hybrid grouper (Zhang et al., 2021) was the basis for designing the primers (**Table 2**) used in this experiment (accession nos.: PRJNA664623 and PRJNA664416). β-Actin was used as a housekeeping gene, and all the CT values were analyzed using the $2^{-\Delta\Delta CT}$ method according to Livak and Schmittgen (2001).

Statistical Analysis

To statistically analyze the data from this experiment, SPSS Statistics (v.22, SPSS Inc., Chicago, IL, United States) was used. After all the data were examined by joint hypotheses test to ensure that equal deviation standards were obtained, they were subjected to one-way ANOVA followed by Tukey's multiple range tests to determine significant differences among treatment groups using SPSS v. 22 (IBM, United States) at a significance level of $P < 0.05$. The results are presented as mean ± standard error (SEM).

RESULTS

Growth Performance

The growth performance and feed utilization of the hybrid grouper are listed in **Table 3**. Compared with FM, WG and SGR were significantly higher in the gL and significantly lower in the gH treatments. NaB supplementation did not significantly increase WG and SGR, and WG and SGR in gHNaB were not significantly different from those in gH. The FCR was significantly decreased in the gL and significantly increased in the gH compared with the FM, and no significant difference was found between gH and gHNaB. For SR, there was no significant difference in SR among the four groups.

Distal Intestinal Damage Indicator

To investigate the effects of different levels of gossypol and supplementation of NaB under high-level gossypol conditions on the distal intestinal damage conditions, the NO level and total NOS activity were determined (**Figure 1**). Compared with FM, the addition of low-level gossypol had no significant effect on NO content or total NOS activity. The addition of high-level gossypol significantly increased NO content and total NOS activity. Compared with gH, the NO content and total NOS activity in gHNaB were significantly lower.

Distal Intestinal Immune- and Tight Junction-Associated Gene Expression

To investigate the effects of different levels of gossypol and supplementation of NaB under high-level gossypol condition on the distal intestinal inflammation and tight junction, the mRNA levels of *tnfα*, *il1β*, *tgfβ1*, *hepcidin*, *il6*, *il8*, *ifnγ*, *jam*, *occludin*, *claudin3*, *claudin12*, *claudin15*, and *zo1* in the distal intestine of hybrid grouper were determined (**Figure 2**). Compared with the FM, the addition of low-level gossypol significantly downregulated the mRNA levels of *il8* and *ifnγ* and significantly upregulated the mRNA levels of *tgfβ1*, *jam*, *occludin*, *claudin3*, and *zo1*. The addition of high-level gossypol significantly upregulated the mRNA levels of *tnfα*, *il1β*, *il6*, *il8*, and *ifnγ* and significantly downregulated the mRNA levels of *tgfβ1*, *jam*, and *zo1*. Compared with gH, the mRNA levels of *tnfα*, *il1β*, *il8*, and *ifnγ* were significantly downregulated, and *jam*, *occludin*, and *zo1* were significantly upregulated in gHNaB. There was no significant difference in the mRNA levels of *claudin12* or *claudin15* among the four groups.

Distal Intestinal TUNEL Staining and Apoptosis-Associated Gene Expression

To investigate the effects of different levels of gossypol and supplementation of NaB under high-level gossypol condition on distal intestinal apoptosis, the TUNEL staining (**Figure 3A**) and mRNA levels of *caspase2*, *caspase3*, *caspase6*, *caspase7*,

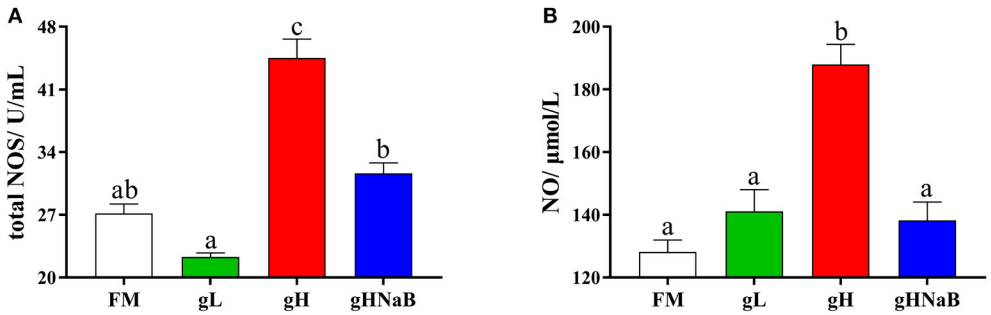


FIGURE 1 | Effects of different treatments on the NO **(A)** content and total NOS **(B)** activity in the distal intestine of hybrid grouper. Values were means of eight replicates ($n = 8$), and were represented as mean ± standard error. [a,b,c] Mean values with unlike letters were significantly different ($P < 0.05$).

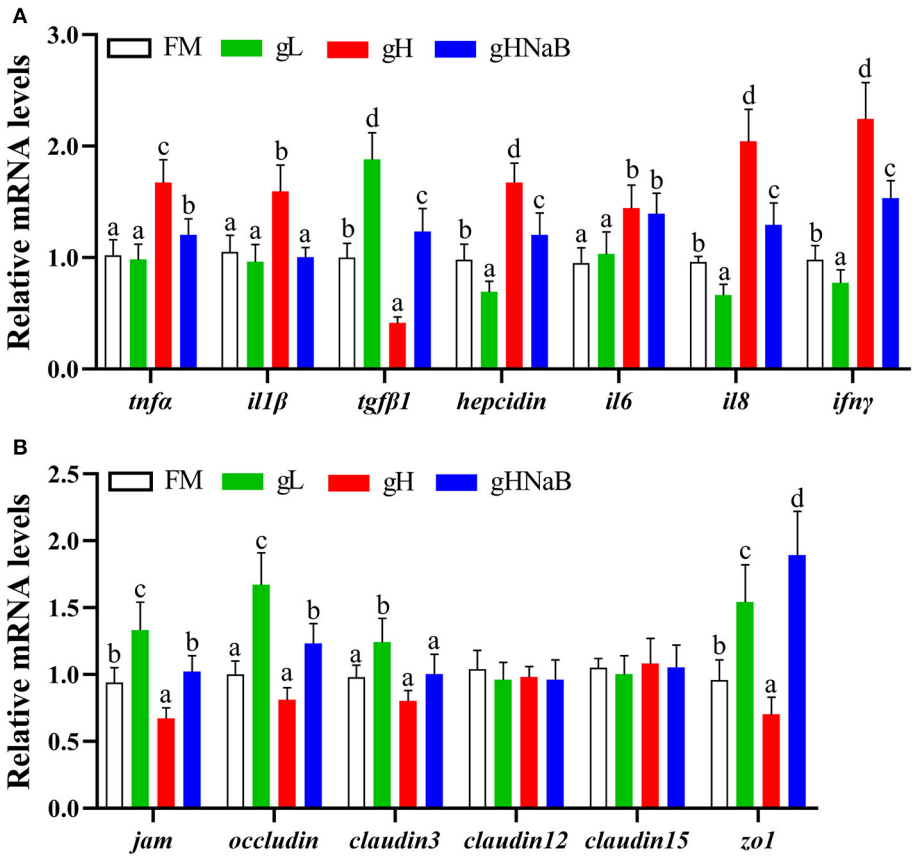


FIGURE 2 | Effects of different treatments on the mRNA levels of immune-related genes **(A)**, including *tnfα*, *il1β*, *tgfβ1*, *hepcidin*, *il6*, *il8* and *ifnγ*, and tight junction-related genes **(B)**, including *jam*, *occludin*, *claudin3*, *claudin12*, *claudin15* and *zo1* in the distal intestine of hybrid grouper. Values were means of eight replicates ($n = 8$), and were represented as mean ± standard error. [a,b,c,d] Mean values with unlike letters were significantly different ($P < 0.05$).

caspase8, *caspase9*, *pi3k p85*, *3pdk1*, *akt*, *foxo4,* and *fasl* in the distal intestine of hybrid grouper were determined (**Figure 3B**). TUNEL staining showed no apoptotic cells in any of the four groups. Compared with FM, the addition of low-level gossypol significantly downregulated the mRNA levels of *akt*. The addition of high-level gossypol significantly upregulated the mRNA levels of *caspase2*, *caspase3*, *caspase9*, *pi3k p85*, and *akt* and significantly downregulated the mRNA levels of *foxo4*. Compared with gH, NaB supplementation significantly downregulated the mRNA levels of *pi3k p85*, *3pdk1*, and *akt*. There were no significant differences among the four groups in the mRNA levels of *caspase6*, *caspase7*, *caspase8*, and *fasl*.

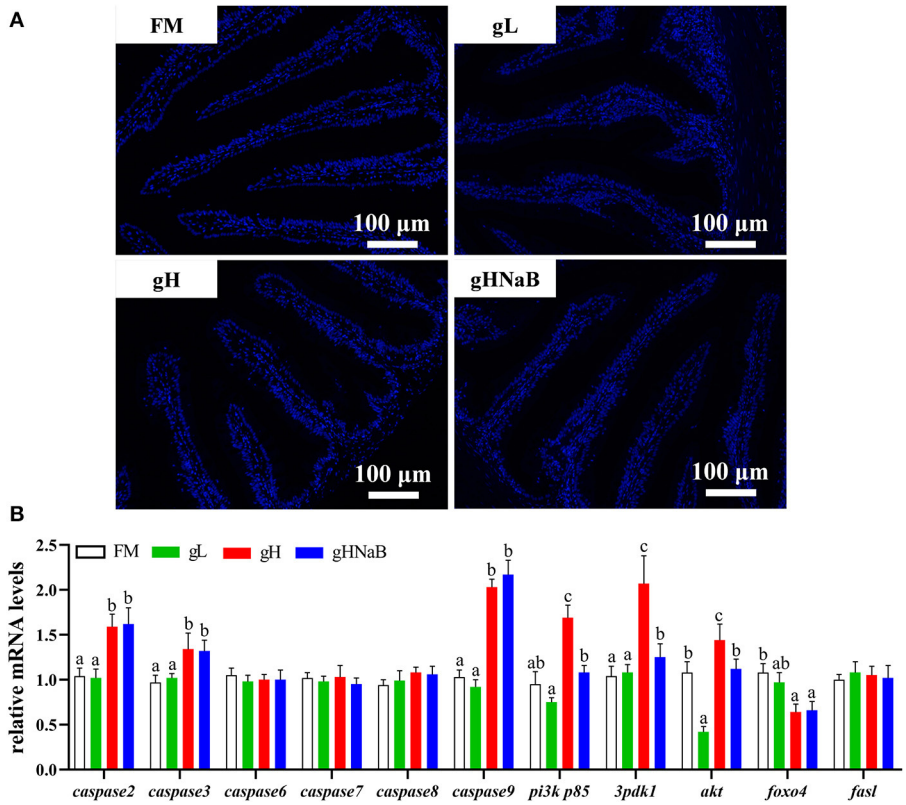


FIGURE 3 | Effects of different treatments on the TUNEL staining **(A)** and mRNA levels of apoptosis-related genes **(B)**, including *caspase2*, *caspase3*, *caspase6*, *caspase7*, *caspase8*, *caspase9*, *pi3k p85*, *3pdk1*, *akt*, *foxo4,* and *fasl* in the distal intestine of hybrid grouper. Values were means of eight replicates ($n = 8$) and were represented as Mean ± standard error. ^{a,b,c} Mean values with unlike letters were significantly different ($P < 0.05$).

DISCUSSION

Cottonseed meal is a by-product of cottonseed oil extraction and has a high crude protein content and a rich variety of amino acids, which can effectively alleviate the shortage of protein feed resources in China (Mbahinzireki et al., 2015). However, cottonseed meal contains gossypol, which is toxic to aquatic animals at high levels, which greatly limits the utilization of cottonseed meal in aquatic feed. Therefore, in the present study, the effects of gossypol on hybrid grouper and the protective effect of NaB were investigated by exploring low- and high-level gossypol while supplementing NaB in the background of high-level gossypol.

The present study indicated that the addition of low-level gossypol to the feed significantly increased WG and SGR of hybrid grouper, similar to the experimental results for Nile tilapia (*Oreochromis niloticus*) (Lim et al., 2003). However, when high levels of gossypol were added, hybrid grouper showed the exact opposite growth performance to Nile tilapia. The reason for this difference may be related to fish species, as Nile tilapia may have a higher tolerance to gossypol than hybrid grouper. Different levels of tolerance to gossypol have been reported between fish species: The addition of gossypol at or above 1,175 mg/kg to the feed significantly reduced the SGR of grass carp (Wanga et al., 2018), while a study on channel catfish found that the addition of 1,500 mg/kg gossypol significantly reduced WG and feed utilization, similar to the results of the present experiment. Interestingly, the addition of gossypol had no significant effect on SR in either grass carp, channel catfish, or hybrid grouper, suggesting that gossypol might not directly affect fish mortality. To alleviate the negative effects on the growth of hybrid grouper caused by high-level gossypol, we added an appropriate dose of NaB under conditions of high-level gossypol addition. Unfortunately, NaB supplementation could not alleviate the negative effect of gossypol (**Table 3**).

The fish intestine is the main site where fish store food, digest, and absorb nutrients (Gu et al., 2013; Sun et al., 2021). Oxidative damage can destroy intestinal structural integrity (Wang et al., 2016). Maintaining normal antioxidant capacity is essential for maintaining normal intestinal cell function (Chen et al., 2018). Multiple organs in animals can be profoundly affected by the feed. To further verify whether this effect exists in the intestine, we determined the NO and total NOS in the distal intestine. NOS is a key enzyme in the process of NO synthesis, which is abundantly expressed in various inflammatory diseases, and it can promote inflammation and act as an inflammatory mediator (Al-Harbi et al., 2019). The results indicated that high-level gossypol may modulate the inflammatory process through total

NOS. NaB supplementation reduced total NOS to the control level, which could be a side effect of the repairing effect of NaB on intestinal inflammation in hybrid grouper, which may be related to total NOS.

Nitric oxide reacts with superoxide anions to generate peroxynitrite anions, which are strong oxidants that cause damage to intestinal epithelial cells. We found that high-level gossypol significantly increased the distal intestinal NO content, suggesting that high-level gossypol induced intestinal damage and disrupted distal intestinal barrier function. NaB supplementation may alleviate this injury by reducing NO levels, which may be related to the fact that NaB can reduce NO production by inhibiting the NF-κB pathway (Liu et al., 2012). Since there are few studies on NOS and NO in aquatic animals, the modulation of NOS and NO in the distal intestine of hybrid grouper by gossypol and NaB still needs further investigation.

Fish intestinal health is closely related to intestinal structural integrity and immune barrier function, and harmful or toxic substances in feed would usually disrupt the intestinal structural integrity and the immune barrier. Fish intestinal inflammatory cytokines are closely related to intestinal health, and inflammatory cytokines play different roles in intestinal inflammation (Tian et al., 2017). Inflammatory factors are usually classified into two types: proinflammatory factors, such as *tnfα*, *il1β*, *il8*, and *ifnγ* (Li et al., 2018b; Carriero et al., 2020), whose upregulation further exacerbates inflammation, and anti-inflammatory factors, such as *tgfβ1* and *hepcidin* (Li et al., 2017; Yan et al., 2019), whose upregulation enhances the intestinal anti-inflammatory capacity. In addition, *hepcidin* and *il6*, which often show high expression in intestinal inflammation, have different roles from proinflammatory factors in the progression of intestinal inflammation, and their upregulation is usually associated with a protective effect on the intestinal response to inflammation (Kuhn et al., 2018). Cotton seed meal can cause intestinal inflammation by upregulating the transcription of pro-inflammatory factors and downregulating anti-inflammatory factors (Liu et al., 2020). CPC substitution for FM upregulated intestinal *tnfα* and *il1β* expression in hybrid grouper (Yin et al., 2018) and downregulated TGF-β3 and IL-10 transcription in silver sillago (*Sillago sihama* Forsskál, 1775) (Liu et al., 2020). Studies in grass carp showed that excess gossypol upregulated the mRNA expression of *tnfα*, *ifnγ2*, *il1β*, and *il6* in the intestines of grass carp (Wang et al., 2019). Few studies have examined the effects of low levels of gossypol on intestinal immunity in aquatic animals, and we speculate that the promotion of growth of hybrid grouper by low-level gossypol may be closely related to the upregulation of the anti-inflammatory factor *tgfβ1* and the downregulation of the expression of the inflammatory factors *il8* and *ifnγ*. Supplementation with high-level gossypol significantly reduced the immunity of the distal intestine of hybrid groupers and induced the development of intestinal inflammation; similar results were also found in grass carp (Wang et al., 2019). After further supplementation with NaB, we found that proinflammatory factors showed an overall downward trend, such as *tnfα*, *il1β*, *il8*, and *ifnγ*, while *tgfβ1* was upregulated, suggesting that the supplementation of NaB in the feed could improve the distal intestinal anti-inflammatory capacity of hybrid grouper. Supplementation of 0.2 % NaB in the diet of turbot (*Scophthalmus maximus* L.) (Liu et al., 2019) can alleviate intestinal inflammation by decreasing the expression of *tnfα*, in line with the results of this experiment. However, after NaB supplementation, hybrid grouper still needed to express high-level *il6*, indicating that a certain degree of inflammation might still exist in the intestine, which also indicates that the repair of the intestine by NaB was incomplete.

In fish, intestinal health depends in part on intestinal barrier function, which is closely related to the structural integrity and tight junctions of the intestine (Al-Sadi and Ma, 2007; Al-Sadi et al., 2008; Yin et al., 2021b). Nutrient absorption interacts closely with the intestine to maintain the normal structure and nutrient absorption function of the intestine. Tight junction proteins, such as *jam*, claudins, *occludin*, and zonula occludens 1 (*zo1*), are the major membrane proteins of tight junctions that control the decellularization gap between epithelial cells, thus preventing the decellularization of intestinal bacteria and other antigens from spreading between epithelial cells (Zhao et al., 2014). *Jam* is a single transmembrane protein between epithelial cells and a protein that appears early in the formation of cell junctions and recruits other tight junctions (Hamazaki et al., 2002). The main function of *occludin* is to regulate tight junctions (Mclaughlin et al., 2004). *claudin3* is involved in the formation of tight junction barriers and reduced expression of *claudin3* results in reduced cell barrier function (Blasig et al., 2011). In this experiment, low-level gossypol promoted growth by upregulating the expression of *jam*, *occludin*, *claudin3,* and *zo1* and improving the tight junctions of the distal intestine and the efficiency of nutrient absorption. In contrast, the expressions of *jam*, *occludin*, *claudin3,* and *zo1* indicated that high-level gossypol disrupted the distal intestinal tight junctions of hybrid grouper. The expression of *zo1*, *occludin*, and *claudin3*c in the proximal, middle, and distal intestines of mid-growth grass carp was negatively affected by gossypol when the level of gossypol in the feed reached 243.94 mg/kg or higher (Wang, 2019), which was consistent with the results of the present study. The expression of *jam*, *occludin*, and *zo1* was upregulated after supplementation with NaB, while the expression of *claudin3* was not significantly changed, indicating that NaB was incomplete for the repair of tight junctions in the distal intestine of hybrid grouper, but still had a certain enhancement effect on tight junctions. Combined with the growth performance, we speculate that this incomplete repair effect may not be reflected in the growth performance, and therefore, the growth performance of the NaB group was not significantly improved. Gossypol was found to be the main cause of apoptosis induction in organ cells.

Gossypol can exacerbate apoptosis in grass carp intestinal epithelial cells by inducing DNA fragmentation (Wang, 2019) and induces apoptosis by interacting with mitochondrial caspases (Oliver et al., 2005). However, there are exceptions; as observed by TUNEL staining, the addition of 680 mg/kg gossypol to the feed did not cause apoptosis in carp hepatocytes (Zhang, 2019). There are no reports to date on the effect of gossypol on

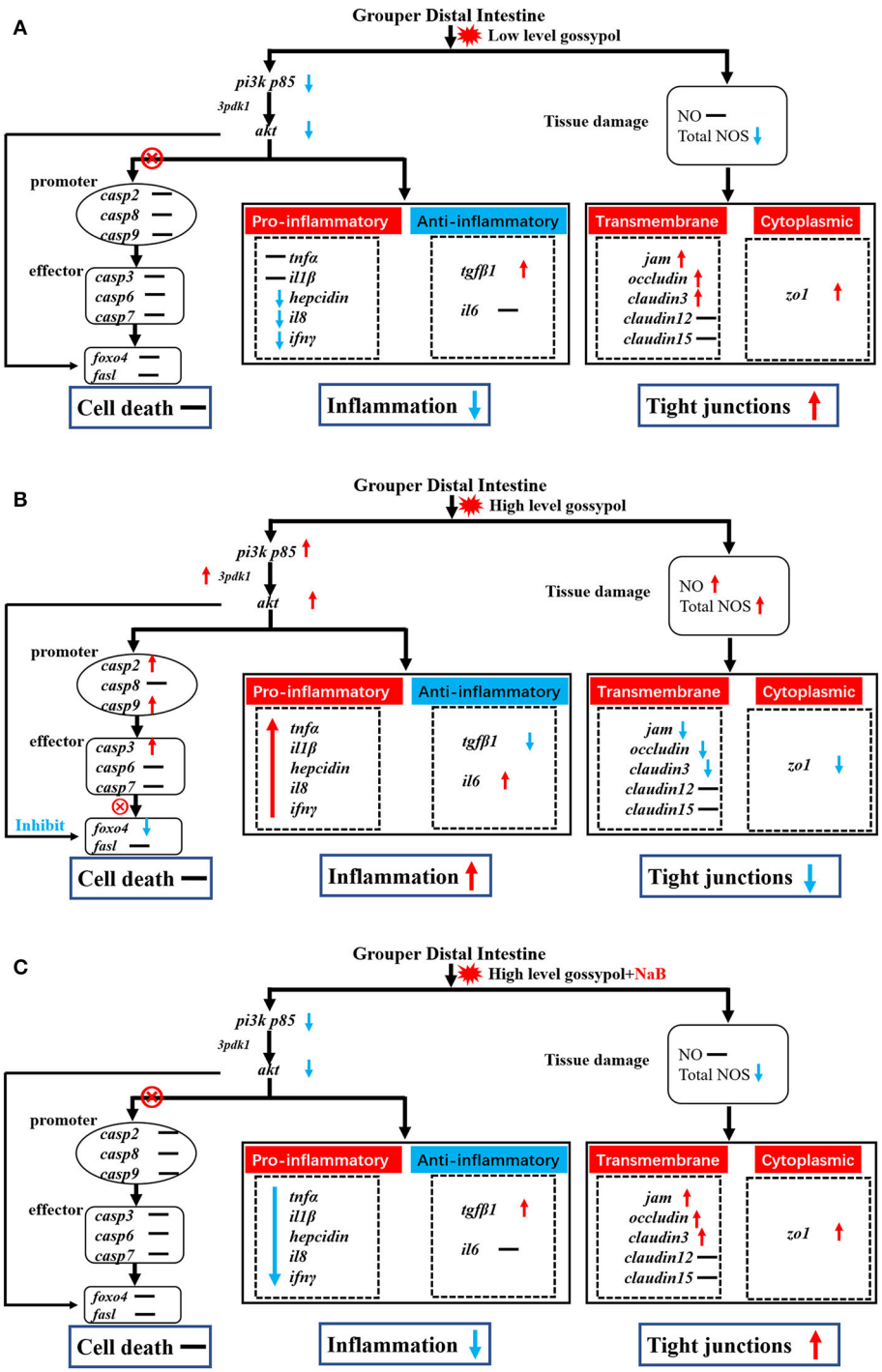


FIGURE 4 | Potential signal transduction in the distal intestine of hybrid grouper. Casp: caspase. → , through; ⊗ → , not through; red arrow, upregulation; blue arrow, downregulation. **(A)** group gL; **(B)** group gH; **(C)** group gHNaB.

apoptosis in the intestinal tract of hybrid groupers. Therefore, we further verified the genes related to apoptosis in the distal intestine of hybrid groupers at the transcriptional level and observed apoptosis by TUNEL staining. The expression of genes associated with apoptosis revealed that low-level gossypol did not significantly affect apoptosis-related genes (**Figure 4A**). Apoptosis is regulated by multiple factors. The promoters of *caspase2*, *caspase8*, and *caspase9*, when tightly bound to

pro-apoptotic signals, activate the downstream effectors *caspase3*, *caspase6,* and *caspase7* to perform apoptotic functions (Degterev and Yuan, 2008; Fuchs and Steller, 2011; Kaufmann et al., 2012). *pi3k*/*akt* can regulate apoptosis not only directly through the promoters, but also by direct interaction with *foxo4* and *fasl*, which promote or inhibit the onset of apoptosis (Kim et al., 2012; Li et al., 2018a; Zhang et al., 2020). There are several ways to regulate apoptosis, and inhibition of the *pi3k*/*akt* pathway can promote apoptosis to some extent (Zheng et al., 2012). Combined with the results of this experiment, apoptotic signals were transduced normally from *pi3k*/*akt* to promoters and effectors at high-level gossypol, yet high expression of *pi3k*/*akt* inhibited the expression of *foxo4*, blocking the transduction of apoptotic signals and inhibiting apoptosis (**Figure 4B**). Activated *pi3k*/*akt* could inhibit apoptosis mediated by FOXO transcription factors, and similarly, blocking *pi3k*/*akt* signaling can also activate FOXO-mediated apoptosis (Qi et al., 2020). This may suggest that this process may also be present in the intestine of hybrid grouper, but due to the inhibition of apoptosis by high expression of *pi3k*/*akt* via *foxo4*. Therefore, we speculate that no significant apoptosis occurred in the intestine of the hybrid grouper. When we supplemented with NaB, the expression of *pi3k p85* and *akt* was downregulated, but the expression of promoter *caspase2*, *caspase8,* and *caspase9*, and effector *caspase3*, *caspase6,* and *caspase7* were not significantly changed (**Figure 4C**). This incomplete transduction process may imply that the regulation of apoptosis by *pi3k*/*akt* through the promoter is blocked, and the exact reason needs to be further explored.

To visually verify the results regarding apoptosis at the transcriptional level, we further observed distal intestinal TUNEL staining of the hybrid grouper. From the TUNEL staining, it is interesting to note that we did not observe any apoptosis in any of the groups, suggesting that low- and high-level gossypol is not the cause of apoptosis in the intestine of the hybrid grouper. Few studies have reported the effects of gossypol on apoptosis in intestinal cells. However, studies in the macrophage cell line RAW264.7 cells found that gossypol induced the onset of apoptosis through a caspase-dependent mitochondrial signaling pathway (Deng et al., 2013). This difference in results may be due to species differences or may be caused by the different response mechanisms of different cells to gossypol.

CONCLUSION

In this study, appropriate low-level gossypol improved growth performance, distal intestinal anti-inflammatory capacity, and tight junctions, while high-level gossypol decreased the above indicators. NaB supplementation under exposure to high-level gossypol partially improved intestinal anti-inflammatory capacity and tight junctions; however, the positive effects of NaB could not be reflected in growth performance in hybrid grouper.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Review Board of Guangdong Ocean Unviersity.

AUTHOR CONTRIBUTIONS

HL and BT designed the study. BY conducted the study and analyzed the data. XD participated in the interpretation of the results. BY wrote the manuscript. QY, SC, and SZ purchased the reagent supplies. HL revised the manuscript. All authors have actively contributed to the study, read and approved the final manuscript.

FUNDING

This work was supported by the National Key R&D Program of China (2019YFD0900200), the National Natural Science Foundation of China (No. 31772864), and Natural Science Foundation of Guangdong Province (2018A030313154 and 2020A1515011129).

ACKNOWLEDGMENTS

We would like to thank the Shanghai Menon Animal Nutrition Technology Co., Ltd. (Shanghai, China) for kindly providing 30% microencapsulated NaB in this experiment.

REFERENCES

Ahmed, H. A., and Sadek, K. (2015). Impact of dietary supplementation of sodium butyrate and/or protexin on the growth performance, some blood parameters, and immune response of *Oreochromis niloticus*. *Int. J. Agric. Innov. Res.* 3, 985–991. doi: 10.1016/0003-9969(77)90069-3

Al-Harbi, N. O., Imam, F., Al-Harbi, M. M., Al-Shabanah, O. A., Alotaibi, M. R., As Sobeai, H. M., et al. (2019). Rutin inhibits carfilzomib-induced oxidative stress and inflammation via the NOS-mediated NF-κB signaling pathway. *Inflammopharmacology* 27, 817–827. doi: 10.1007/s10787-018-0550-5

Al-Sadi, R., Ye, D. M., Dokladny, K., and Ma, T. Y. (2008). Mechanism of IL-1beta-induced increase in intestinal epithelial tight junction permeability. *J. Immunol.* 180, 5653–5661. doi: 10.4049/jimmunol.180.8.5653

Al-Sadi, R. M., and Ma, T. Y. (2007). IL-1 causes an increase in intestinal epithelial tight junction permeability. *J. Immunol.* 178, 4641–4649. doi: 10.4049/jimmunol.178.7.4641

Bian, F. Y., Jiang, H. W., Man, M. S., Mai, K. S., Zhou, H. H., Xu, W., et al. (2017). Dietary gossypol suppressed postprandial TOR signaling and elevated ER stress pathways in turbot (*Scophthalmus maximus* L.). *Am. J. Physiol. Endocrinol. Metab.* 312, E37–E47. doi: 10.1152/ajpendo.00285.2016

Blasig, I. E., Bellmann, C., Cording, J., Vecchio, G. D., Zwanziger, D., Huber, O., et al. (2011). Occludin protein family: oxidative stress and reducing conditions. *Antioxid. Redox Signaling* 15, 1195–1219. doi: 10.1089/ars.2010.3542

Cao, S. Y., Wang, G. M., Ge, F., Li, X. H., Zhu, Q. Q., Ge, R. S., et al. (2018). Gossypol inhibits 5α-reductase 1 and 3α-hydroxysteroid dehydrogenase: its

possible use for the treatment of prostate cancer. *Fitoterapia* 133, 102–108. doi: 10.1016/j.fitote.2018.12.024

Carriero, M. M., Henrique-Silva, F., Meira, C. M., Gato, I. M. Q., Caetano, A. R., Lobo, F. P., et al. (2020). Molecular characterization and gene expression analysis of the pro-inflammatory cytokines IL-1β and IL-8 in the South American fish *Piaractus mesopotamicus* challenged with *Aeromonas dhakensis*. *Genet. Mol. Biol.* 43:e20200006. doi: 10.1590/1678-4685-gmb-2020-0006

Chen, J. L., Yu, B., Chen, D. W., Huang, Z. Q., Mao, X. B., Ping, Z., et al. (2018). Chlorogenic acid improves intestinal barrier functions by suppressing mucosa inflammation and improving antioxidant capacity in weaned pigs. *J. Nutr. Biochem.* 59, 84–92. doi: 10.1016/j.jnutbio.2018.06.005

Claus, R., Günthner, D., and Letzgub, H. (2007). Effects of feeding fat-coated butyrate on mucosal morphology and function in the small intestine of the pig. *J. Anim. Physiol. Anim. Nutr.* 91, 312–318. doi: 10.1111/j.1439-0396.2006.00655.x

Degterev, A., and Yuan, J. Y. (2008). Expansion and evolution of cell death programmes. *Nat. Rev. Mol. Cell Biol.* 9, 378–390. doi: 10.1038/nrm2393

Deng, J. M., Zhang, X., Long, X. W., Tao, L. L., Wang, Z., Niu, G. Y., et al. (2014). Effects of dietary cholesterol supplementation on growth and cholesterol metabolism of rainbow trout (*Oncorhynchus mykiss*) fed diets with cottonseed meal or rapeseed meal. *Fish Physiol. Biochem.* 40, 1827–1838. doi: 10.1007/s10695-014-9971-2

Deng, S. J., Yan, H., Yi, J. N., Lu, Y., Wei, Q., Guo, C. Z., et al. (2013). Gossypol acetic acid induces apoptosis in RAW264.7 cells via a caspase-dependent mitochondrial signaling pathway. *J. Vet. Sci.* 14, 281–289. doi: 10.4142/jvs.2013.14.3.281

Fuchs, Y., and Steller, H. (2011). Programmed cell death in animal development and disease. *Cell* 147, 742–758. doi: 10.1016/j.cell.2011.10.033

Gu, J. N., Krogdahl, Å., Sissener, N. H., Kortner, T. M., Gelencser, E., Hemre, G.-I., et al. (2013). Effects of oral Bt-maize (MON810) exposure on growth and health parameters in normal and sensitised Atlantic salmon, *Salmo salar* L. *Br. J. Nutr.* 109, 1408–1423. doi: 10.1017/S000711451200325X

Hamazaki, Y., Itoh, M., Sasaki, H., Furuse, M., and Tsukita, S. (2002). Multi-PDZ domain protein 1 (MUPP1) is concentrated at tight junctions through its possible interaction with claudin-1 and junctional adhesion molecule. *J. Biol. Chem.* 277, 455–461. doi: 10.1074/jbc.M109005200

Herman, R. L. (1970). Effects of gossypol on rainbow trout *Salmo guirdneri* Richardson. *J. Fish Biol.* 2, 293–303. doi: 10.1111/j.1095-8649.1970.tb03288.x

Hou, C. Y. (2012). *The Effect of Gossypol on Rumen Microbial Population and Fiber Degradability*. Hohhot: Inner Mongolia Agricultural University.

Hou, N. N. (2014). *Effects of Gossypol on Hepatic Lipid Metabolism and Antioxidant Function of Laying Hens*. Tai'an: Shandong Agriculture University.

Jiang, C. Q. (2011). *Study on rational utilizing rapeseed meal and cottonseed meal in Carassius auratus gibelio*. Shanghai Ocean University.

Jiang, S., Wu, X., Luo, Y., Wu, M., Lu, S., Jin, Z., et al. (2016). Optimal dietary protein level and protein to energy ratio for hybrid grouper (*Epinephelus fuscoguttatus* ♀× *Epinephelus lanceolatus* ♂) juveniles. *Aquaculture* 465, 28–36. doi: 10.1016/j.aquaculture.2016.08.030

Kaufmann, T., Strasser, A., and Post, P. J. (2012). Fas death receptor signalling: roles of Bid and XIAP. *Cell Death Differ.* 19, 42–50. doi: 10.1038/cdd.2011.121

Kim, J. H., Choi, J. S., and Lee, B. H. (2012). PI3K/Akt and MAPK pathways evoke activation of FoxO transcription factor to undergo neuronal apoptosis in brain of the silkworm *Bombyx mori* (Lepidoptera: Bombycidae). *Cell Mol. Biol.* 58, OL1780–1785. doi: 10.1170/209

Krogdahl, Å., Penn, M., Thorsen, J., Refstie, S., and Bakke, A. M. (2010). Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids. *Aquacult. Res.* 41, 333–344. doi: 10.1111/j.1365-2109.2009.02426.x

Kuhn, K. A., Schulz, H. M., Regner, E. H., Severs, E. L., Hendrickson, J. D., Mehta, G., et al. (2018). Bacteroidales recruit IL-6-producing intraepithelial lymphocytes in the colon to promote barrier integrity. *Mucosal Immunol.* 11, 357–368. doi: 10.1038/mi.2017.55

Kumar, M., Tomar, M., Punia, S., Grasso, S., and Amarowicz, R. (2021). Cottonseed: a sustainable contributor to global protein requirements. *Trends Food Sci. Technol.* 111, 100–113. doi: 10.1016/j.tifs.2021.02.058

Li, S., Yuan, H., Liu, Y. Y., Zhao, Y. J., He, X. X., Zhang, L. L., et al. (2018a). Ipatasertib, a novel Akt inhibitor, induces transcription factor FoxO3a and NF-κB directly regulates PUMA-dependent apoptosis. *Cell Death Dis.* 9:911. doi: 10.1038/s41419-018-0943-9

Li, S. A., Jiang, W. D., Feng, L., Liu, Y., Wu, P., Jiang, J., et al. (2018b). Dietary myo-inositol deficiency decreased intestinal immune function related to NF-kappa B and TOR signaling in the intestine of young grass carp (*Ctenopharyngodon idella*). *Fish Shellfish Immunol.* 76, 333–346. doi: 10.1016/j.fsi.2018.03.017

Li, Y. X., Hu, H. B., Liu, J. T., Yang, P., Zhang, Y. J., Ai, Q. H., et al. (2017). Dietary soya allergen β-conglycinin induces intestinal inflammatory reactions, serum-specific antibody response and growth reduction in a carnivorous fish species, turbot *Scophthalmus maximus* L. *Aquacult. Res.* 48, 4022–4037. doi: 10.1111/are.13224

Lim, C., Aksoy, M., and Klesius, P. (2003). Levels of dietary gossypol affect growth and bacterial resistance of Nile tilapia. *Global Aquacult.* 6, 42–43.

Liu, H., Dong, X. H., Tan, B. P., Du, T., Zhang, S., Yang, Y. Z., et al. (2020). Effects of fish meal replacement by low-gossypol cottonseed meal on growth performance, digestive enzyme activity, intestine histology and inflammatory gene expression of silver sillago (*Sillago sihama* Forsskál) (1775). *Aquacult. Nutr.* 26, 1724–1735. doi: 10.1111/anu.13123

Liu, M. M., Guo, W., Wu, F., Qu, Q. C., Tan, Q. S., and Gong, W. B. (2017). Dietary supplementation of sodium butyrate may benefit growth performance and intestinal function in juvenile grass carp (*Ctenopharyngodon idellus*). *Aquac. Res.* 48, 4102–4111. doi: 10.1111/are.13230

Liu, T., Li, J., Liu, Y., Xiao, N., Suo, H., Xie, K., et al. (2012). Short-chain fatty acids suppress lipopolysaccharide-induced production of nitric oxide and proinflammatory cytokines through inhibition of NF-κB pathway in RAW264.7 cells. *Inflammation* 35, 1676–1684. doi: 10.1007/s10753-012-9484-z

Liu, Y., Chen, Z. C., Dai, J. H., Yang, P., Xu, W. Q., Ai, Q. H., et al. (2019). Sodium butyrate supplementation in high-soybean meal diets for turbot (*Scophthalmus maximus* L.): Effects on inflammatory status, mucosal barriers and microbiota in the intestine. *Fish Shellfish Immunol.* 88, 65–75. doi: 10.1016/j.fsi.2019.02.064

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

Mbahinzireki, G. B., Dabrowski, K., Lee, K. J., El-Saidy, D., and R., W.E. (2015). Growth, feed utilization and body composition of tilapia (*Oreochromis* sp.) fed with cottonseed meal-based diets in a recirculating system. *Aquacult. Nutr.* 7, 189–200. doi: 10.1046/j.1365-2095.2001.00172.x

Mclaughlin, J., Padfield, P. J., Burt, J. P. H., and O'neill, C. A. (2004). Ochratoxin A increases permeability through tight junctions by removal of specific claudin isoforms. *Am. J. Physiol. Cell Physiol.* 287, 1412–1417. doi: 10.1152/ajpcell.00007.2004

Oliver, C. L., Miranda, M. B., Shangary, S., Land, S., Wang, S. M., and Johnson, D. E. (2005). (-)-Gossypol acts directly on the mitochondria to overcome Bcl-2- and Bcl-X(L)-mediated apoptosis resistance. *Mol. Cancer Ther.* 4, 23–31. doi: 10.1186/1476-4598-4-23

Qi, M., Gao, S. Q., Liu, J. Q., Han, Y. L., and Zheng, L. R. (2020). Inhibitory effects of Akt on neuronal FoxO4 activation after subarachnoid hemorrhage *in vivo* and *in vitro*. *bioRxiv*. doi: 10.1101/2020.01.20.912436

Rahimnejad, S., Bang, I.-C., Park, J.-Y., Sade, A., Choi, J., and Lee, S.-M. (2015). Effects of dietary protein and lipid levels on growth performance, feed utilization and body composition of juvenile hybrid grouper, *Epinephelus fuscoguttatus* × *E. lanceolatus*. *Aquaculture* 446, 283–289. doi: 10.1016/j.aquaculture.2015.05.019

Rinchard, J., Lee, K., Dabrowski, K., Ciereszko, A., Blom, J. H., and Ottobre, J. S. (2003). Influence of gossypol from dietary cottonseed meal on haematology, reproductive steroids and tissue gossypol enantiomer concentrations in male rainbow trout (*Oncorhynchus mykiss*). *Aquacult. Nutr.* 9, 275–282. doi: 10.1046/j.1365-2095.2003.00253.x

Robles, R., Lozano, A., B., Sevilla, A., Márquez, L., Nuez-Ortín, W., et al. (2013). Effect of partially protected butyrate used as feed additive on growth and intestinal metabolism in sea bream (*Sparus aurata*). *Fish Physiol. Biochem.* 39, 1567–1580. doi: 10.1007/s10695-013-9809-3

Shen, J. F., Liu, H. Y., Tan, B. P., Dong, X. H., Yang, Q. H., Chi, S. Y., et al. (2020). Effects of replacement of fishmeal with cottonseed protein concentrate on the growth, intestinal microflora, haematological and antioxidant indices of juvenile golden pompano (*Trachinotus ovatus*). *Aquacult. Nutr.* 26, 1119–1130. doi: 10.1111/anu.13069

Sun, C. Y., Liu, Y., Feng, L., Jiang, W. D., Wu, P., Jiang, J., et al. (2021). Xylooligosaccharide supplementation improved growth performance and prevented intestinal apoptosis in grass carp. *Aquaculture* 535:736360. doi: 10.1016/j.aquaculture.2021.736360

Tian, L., Zhou, X. Q., Jiang, W. D., Liu, Y., Wu, P., Jiang, J., et al. (2017). Sodium butyrate improved intestinal immune function associated with NF-kappa B and p38MAPK signalling pathways in young grass carp (*Ctenopharyngodon idella*). *Fish Shellfish Immunol.* 66, 548–563. doi: 10.1016/j.fsi.2017.05.049

Wang, B., Feng, L., Chen, G. F., Jiang, W. D., Liu, Y., Kuang, S. Y., et al. (2016). Jian carp (*Cyprinus carpio* var. Jian) intestinal immune responses, antioxidant status and tight junction protein mRNA expression are modulated via Nrf2 and PKC in response to dietary arginine deficiency. *Fish Shellfish Immunol.* 51, 116–124. doi: 10.1016/j.fsi.2015.10.032

Wang, K. Z. (2019). *Effects of Gossypol on the Intestinal Structure and Immune Barrier and the Related Mechanisms in Grass Carp (Ctenopharyngodon idella)*. Ya'an: Sichuan Agriculture University.

Wang, K. Z., Feng, L., Jiang, W. D., Wu, P., Liu, Y., Jiang, J., et al. (2019). Dietary gossypol reduced intestinal immunity and aggravated inflammation in on-growing grass carp (*Ctenopharyngodon idella*). *Fish Shellfish Immunol.* 86, 814–831. doi: 10.1016/j.fsi.2018.12.014

Wanga, K. Z., Jianga, W. D., Pei, W., Yang, L., and Jiang, J. (2018). Gossypol reduced the intestinal amino acid absorption capacity of young grass carp (*Ctenopharyngodon idella*). *Aquaculture* 492, 46–58. doi: 10.1016/j.aquaculture.2018.03.061

Yan, L. C., Feng, L., Jiang, W. D., Wu, P., Y., L., et al. (2019). Dietary taurine supplementation to a plant protein source-based diet improved the growth and intestinal immune function of young grass carp (*Ctenopharyngodon idella*). *Aquacult. Nutr.* 25, 873–896. doi: 10.1111/anu.12907

Yildirim, M., Lim, C., Wan, P. J., and Klesius, P. H. (2003). Growth performance and immune response of channel catfish (*Ictalurus puctatus*) fed diets containing graded levels of gossypol–acetic acid. *Aquaculture* 219, 751–768. doi: 10.1016/S0044-8486(03)00062-0

Yin, B., Liu, H. Y., Tan, B. P., Dong, X. H., Chi, S. Y., Yang, Q. H., et al. (2018). Cottonseed protein concentrate (CPC)suppresses immune function in different intestinal segments of hybrid grouper ♀*Epinephelus fuscoguttatus*×♂*Epinephelus. lanceolatu* via TLR-2/MyD88 signaling pathways. *Fish Shellfish Immunl.* 81, 318–328. doi: 10.1016/j.fsi.2018.07.038

Yin, B., Liu, H. Y., Tan, B. P., Dong, X. H., Chi, S. Y., Yang, Q. H., et al. (2021a). MHC II-PI3K/Akt/mTOR signaling pathway regulates intestinal immune response induced by soy glycinin in hybrid grouper: protective effects of sodium butyrate. *Front. Immunol.* 11:615980. doi: 10.3389/fimmu.2020.615980

Yin, Z. Y., Liu, Q. D., Liu, Y. T., Gao, S. N., L., H. Y., et al. (2021b). Early life intervention using probiotic clostridium butyricum improves intestinal development, immune response, and gut microbiota in large yellow croaker (*Larimichthys crocea*) larvae. *Front. Immunol.* 12:640767. doi: 10.3389/fimmu.2021.640767

Zhang, J. J., Wang, J. Q., Xu, X. Y., Yang, J. Y., Wang, Z., Jiang, S., et al. (2020). Red ginseng protects against cisplatin-induced intestinal toxicity by inhibiting apoptosis and autophagy via the PI3K/AKT and MAPK signaling pathways. *Food Function* 11, 1–34. doi: 10.1039/D0FO00469C

Zhang, M. D. (2019). *The Toxicity and Mechanism of Hepatotoxicity Induced by Gossypol Acetate in Cyprinus carpio*. Henan: Henan Normal University.

Zhang, W., Tan, B. P., Deng, J. M., Dong, X. H., Yang, Q. H., Chi, S. Y., et al. (2021). The single-molecule long-read sequencing of intestine after soy meal-induced enteritis in juvenile pearl gentian grouper, *Epinephelus fuscoguttatus*♀ × *Epinephelus lanceolatus*♂. *Front. Mar. Sci.* 8:688601. doi: 10.3389/fmars.2021.688601

Zhao, J., Feng, L., Liu, Y., Jiang, W. D., Wu, P., Jiang, J., et al. (2014). Effect of dietary isoleucine on the immunity, antioxidant status, tight junctions and microflora in the intestine of juvenile Jian carp (*Cyprinus carpio* var. Jian). *Fish Shellfish Immunol.* 41, 663–673. doi: 10.1016/j.fsi.2014.10.002

Zheng, J., Hu, J. D., Chen, Y. Y., Chen, B. Y., and Liu, T. B. (2012). Baicalin induces apoptosis in leukemia HL-60/ADR cells via possible down-regulation of the PI3K/Akt signaling pathway. *Asian Pac. J. Cancer Prev.* 13, 1119–1124. doi: 10.7314/APJCP.2012.13.4.1119

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: 03 November 2021
doi: 10.3389/fmars.2021.764042

Effects of a Single Cell Protein (*Methylococcus capsulatus*, Bath) in Pacific White Shrimp (*Penaeus vannamei*) Diet on Growth Performance, Survival Rate and Resistance to *Vibrio parahaemolyticus*, the Causative Agent of Acute Hepatopancreatic Necrosis Disease

Orapint Jintasataporn[1], Srinoy Chumkam[2], Supawit Triwutanon[3], Allan LeBlanc[4] and Jarin Sawanboonchun[4]*

[1] Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand, [2] Faculty of Agricultural Technology, Valaya Alongkorn Rajabhat University Under the Royal Patronage, Pathum Thani, Thailand, [3] Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen, Nakhon Pathom, Thailand, [4] Calysta, Inc., San Mateo, CA, United States

OPEN ACCESS

Edited by:
Xiang-Jun Leng, Shanghai Ocean University, China

Reviewed by:
Chunxiao Zhang, Jimei University, China
Li Li, Ocean University of China, China

***Correspondence:**
Orapint Jintasataporn
ffisora@ku.ac.th

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

Received: *24 August 2021*
Accepted: *14 October 2021*
Published: *03 November 2021*

Citation:
Jintasataporn O, Chumkam S, Triwutanon S, LeBlanc A and Sawanboonchun J (2021) Effects of a Single Cell Protein (Methylococcus capsulatus, Bath) in Pacific White Shrimp (Penaeus vannamei) Diet on Growth Performance, Survival Rate and Resistance to Vibrio parahaemolyticus, the Causative Agent of Acute Hepatopancreatic Necrosis Disease. Front. Mar. Sci. 8:764042. doi: 10.3389/fmars.2021.764042

The efficacy of a single cell protein (SCP) methanotroph (*Methylococcus capsulatus*, Bath) bacteria meal (FeedKind®, Calysta, Menlo Park, CA, United States), in Pacific white shrimp (*Penaeus vannamei*) diets was studied to determine growth performance, survival rate and disease resistance against *Vibrio parahaemolyticus* causing Acute Hepatopancreatic Necrosis Disease (AHPND). The growth trial was assigned in a completely randomized design (CRD) with four treatments and 5 replicates of each, T1: a fishmeal-based control containing 15% fish meal and 3 diets with graded levels of methanotroph bacteria meal, namely T2: 5% methanotroph bacteria meal, T3: 10% methanotroph bacteria meal, and T4: 15% methanotroph bacteria meal. Shrimp were fed *ad libitum* for 6 weeks on trial diets to assess growth. Subsequent to the growth trial, three replicates of the same groups were exposed to *V. parahaemolyticus* by a single bath challenge and held for a further 15 days on the same diets as the growth study to assess survival and resistance. No significant differences ($p > 0.05$) in survival or in growth performance, including final weight, weight gain, specific growth rate, feed consumption or feed conversion ratio of white shrimp fed feeds containing methanotroph bacteria meal or control diets for 6 weeks. Immune markers such as hemocyte counts, phenoloxidase, superoxide dismutase and lysozyme activity were similar across all groups after the 6-week feeding trial. In a *V. parahaemolyticus* challenge, methanotroph bacteria meal in the diet significantly promoted the survival rate, and the reduction of *Vibrio* sp. in the hepatopancreas of white shrimp. Hemocyte count and phenoloxidase activity showed no significant differences ($p > 0.05$) between

diet treatment groups, but hemolymph protein was significantly higher ($p < 0.05$) in shrimp fed diets containing 15% methanotroph bacteria meal after challenge. The *Vibrio* colony counts from hepatopancreas in the treatment groups were all significantly lower than the control ($p < 0.05$). The findings show that methanotroph bacteria meal can entirely replace fishmeal in white shrimp diets and the 15% inclusion of methanotroph bacteria meal in shrimp diet shows no adverse effects on growth performance, feed utilization and survival rate. In addition, shrimp fed methanotroph bacteria meal diets exhibited improved survival rates to an AHPND challenge.

Keywords: single cell protein (SCP), *Penaeus vannamei*, *Vibrio parahaemolyticus* (AHPND), FeedKind, functional feeds

INTRODUCTION

The increase in aquaculture in recent years has led to a concomitant increase in demand for fishmeal with approximately 75% of the global production of fishmeal in 2018 utilized in aquaculture (FAO, 2018). Fishmeal is used in aquaculture feeds because it has all the nutritional requirements aquatic animals need (Swick et al., 1995; Samocha et al., 2004; Tacon et al., 2009). However, it can be an expensive ingredient because of high demand and inelastic supply (Samocha et al., 2004) and thus one option for feed mills is to substitute fishmeal with alternative proteins, but these frequently do not match the nutritional requirements of shrimp and can have variable or unbalanced nutritional profiles (Malcorps et al., 2019).

Animal sources of protein such as meat and bone meal, and poultry by-product meal, are used to replace fishmeal; but growth may be lower because of low lipid quality and differing nutritional profiles (Tan et al., 2005; Gamboa-Delgado et al., 2014). Swine meat meal has been used to replace 35% of the protein contribution of fishmeal but, at higher levels, caused nutritional imbalances in *Penaeus vannamei* (Hernández et al., 2008). Alternative proteins such as yeast, fungi, microalgae, and bacteria can serve as single cell protein sources for animals and several studies have investigated the utility of these proteins in a variety of animal feeds (Qui and Davis, 2017a,b; Qui et al., 2017; Linder, 2019; Glencross et al., 2020; Jones et al., 2020); their use in aquaculture species have been recently reviewed by Glencross et al. (2020).

Methanotrophs are gram-negative bacteria that use methane as their sole source of carbon and energy and have been identified for their potential use as a protein source in feeds for a variety aquaculture species (Berge et al., 2005; Øverland et al., 2006; Aas et al., 2007; Øverland et al., 2011; Biswas et al., 2020; Chen et al., 2021). Whilst studies have investigated the use of bacterial derived single cell protein feed additives from *Corynebacterium ammoniagenes* (Hamidoghli et al., 2019) and purple non-sulfur bacteria *Rhodobacter sphaeroides* and *Afifella marina* (Chumpol et al., 2018) in shrimp diets, to date only one published study has evaluated the use of *Methylococcus capsulatus* as an alternative protein source for shrimp feed formulations with up to 45% of the fish meal replaced with FeedKind® followed by exposure to *Vibrio parahaemolyticus* by injection (Chen et al., 2021). The current study builds on the results obtained by Chen et al. (2021) by replacing up to 100% of the fish meal in the diet with *M. capsulatus*-derived protein and by exposing shrimp to the AHPND bacterium through a more natural, bath route.

FeedKind® (Calysta, Menlo Park, CA, United States) is a bacterial biomass composed primarily of *M. capsulatus* produced *via* a continuous aerobic fermentation. Other bacterial strains present in the culture population that support the growth of *M. capsulatus* include *Ralstonia* sp. (DB3), *Aneurinibacillus* sp. (DB4), and *Brevibacillus agri* (DB5). The harvested biomass is centrifuged, heat inactivated, and spray dried. The nutrient content and amino acid profile of methanotroph bacteria meal, FeedKind®, is comparable to traditional proteins used in animal feeds such as fishmeal (Biswas et al., 2020) and has been shown to be a viable protein source for popular species of aquaculture fish including rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*) and yellowtail (*Seriola quinqueradiata*) (Berge et al., 2005; Øverland et al., 2006; Biswas et al., 2020). An additional benefit of producing methanotroph bacteria meal, is that it utilizes less than 0.01% of the land and around 10% of blue water compared with that used to produce soy protein, enhancing its sustainability credentials. Recently, Chen et al. (2021) reported that replacement of fishmeal with methanotroph bacteria meal did not negatively impact the growth or feed conversation rates of shrimp. Chen et al. (2021) also found that at higher dietary levels of methanotroph bacteria meal, shrimp increased oxidation levels in the hepatopancreas and increased the height of the mucosal folds in the gut, improved gut microbiota, and increased resistance to a *V. parahaemolyticus* challenge *via* intraperitoneal injection.

Several problematic bacterial diseases of shrimp have been reported, some of which have become globally widespread *via* the rapid expansion of shrimp farming (Thitamadee et al., 2016). Whilst *Vibrio* species are common in the normal microbiota of shrimp ponds, many are pathogenic to shrimp (Yang et al., 2014; Anandaraja et al., 2017), including *V. parahaemolyticus*, *Vibrio harveyi*, *Vibrio owensii*, *Vibrio campbellii*, and *Vibrio alginolyticus* (Soto-Rodriguez et al., 2012, 2015; Kondo et al., 2015; Liu et al., 2015; Dong et al., 2017; Wu et al., 2019).

Acute Hepatopancreatic Necrosis Disease (AHPND), also known as Early Mortality Syndrome (EMS), is a major concern for shrimp production caused by the bacterium *V. parahaemolyticus* with outbreaks occurring routinely in Southeast Asia shrimp farms in which mortality rates exceed 70%

and global annual losses are estimated at more than US$1 billion (Zorriehzahra and Banaederakhshan, 2015). *V. parahaemolyticus* is transmitted orally and infects the digestive organs and hepatopancreas of shrimp (Lightner et al., 2012). China detected the first outbreak of EMS in 2009 with the disease subsequently identified in Vietnam and Malaysia in 2010 and 2011, respectively (Tran et al., 2013). Following the first outbreak recorded in Thailand in 2011, the country has continued to be impacted by outbreaks since (Chucherd, 2013; Putth and Polchana, 2016). In the past antibiotics were commonly used to treat EMS and other *Vibrio* diseases, but these have been widely prohibited (Liu et al., 2017). To enhance shrimp health, many farmers "top-dress" feeds at the farm with additives to improve health outcomes, but this practice is of limited efficacy and is problematic (FAO, 2019). On the other hand, methanotroph bacteria meal is blended in the feed during manufacture at the feed mill, providing a potentially more efficient method for delivering an immune stimulant.

This study was designed to determine the effects of methanotroph bacteria meal (FeedKind®) on growth performance, survival rate and resistance to *V. parahaemolyticus* challenge of Pacific white shrimp, *P. vannamei*. As an inactivated gram-negative bacterial biomass, it was theorized that FeedKind® may impart an immune response in shrimp.

MATERIALS AND METHODS

All research was conducted at the Nutrition and Aquafeed Laboratory, Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand.

Experimental Diets

Shrimp feed ingredients were ground to 150–250 μm, mixed, and then water was added at 25% before passing through a Hobart mincer to form feed pellets. Pellets were then placed in a hot air oven at 60°C for 8–10 h to dry. The proximate compositions of trial feed such as moisture, protein, lipid, fiber, ash, energy, calcium, and phosphorus were analyzed as described by Association of Official Analytical Chemists (AOAC) (2000). All diets were sieved to suitable particle sizes of 1.5–2 mm for use in the trial. Samples of every batch of feed were kept at room temperature to be used later for verifications if needed. The formulations for each treatment diet are shown in **Table 1**. Soybean and poultry meal levels were adjusted accordingly to ensure crude protein levels were equivalent across all treatments.

Experimental Design

Growth Trials

Shrimp were obtained from a private farm in the Samutsongkarm province, Thailand, acclimated under trial conditions for 10 days, and fed three times daily with a commercial feed containing 35% CP followed by a further 7–8 days feeding with the trial diet prior to the start of the study. Five hundred individual shrimp were randomly allocated in batches of 25 to one of twenty 500 L tanks containing 300 L of brackish water (15–20 PSU). Twenty percent of the water in the static tanks was replaced every 2 days to maintain water quality in the range pH 7–8, DO > 5.0 mg/L, temperature 27–31°C, salinity 15–20 PSU, alkalinity >100 mg/L, and ammonia <1.0 mg/L. Shrimp were fed to satiation three times daily; 2 h after feeding, any uneaten feed was siphoned out of the tank, collected using a plankton net, dried at 65°C for 24 h before being weighed to calculate feed intake. Shrimp were fed with experimental diet as described above containing either 15% fishmeal (T1: Control) or methanotroph bacteria meal (FeedKind®) to replace fishmeal at 33% (T2: 5% FeedKind®), 66% (T3: 10% FeedKind®), or 100% (T4: 15% FeedKind®) with five replicates for each treatment. The diets were blinded to the technicians and randomly assigned to individual tanks. The amounts fed to each treatment tank were recorded. Shrimp were fed test diets for 6 weeks. At the end of the 6-week trial, material was collected for histological analysis, for immune measurements and for Vibrio spp. counts as described below.

Vibrio parahaemolyticus Challenge

After 6 weeks of the experimental feeding trial, thirty shrimp from each treatment in the growth trial were moved to 100L challenge test aquariums to form three replicates, each containing 10 shrimp for each diet to give a total of 120 shrimp used in the challenge trial. The *V. parahaemolyticus* (AHPND) virulent strain from Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen, Nakhon Pathom, Thailand was cultured on tryptic soy agar (Difco) supplemented with 1.5% NaCl (w/v) for 24 h at 35°C. After 24 h of growth, bacterial colonies were transferred to tryptic soy broth (Difco) supplemented with 1.5% NaCl and incubated for 24 h at 35°C. Next, the bacterial culture was centrifuged at 1,000 rpm for 15 min at room temperature. The supernatant was removed, and the bacterial pellet was resuspended in saline solution and then added to the tanks on Day 0 to reach a concentration of 5.8×10^4 CFU/ml in the water column of each challenge test aquarium. The concentration used was based on prior results obtained in our laboratory for this strain. A negative control (unchallenged) group was included in the study. Challenged shrimp were immersed in the *Vibrio* solution and continued to be fed test diets for 15 days before termination when haemolymph was taken for immune measurements and hepatopancreas taken for *V. parahaemolyticus* assessment as described below.

Data Collection

Growth Measurements

Measurements of performance parameters during the experimental period were recorded. To determine growth performance factors, the live weight of shrimp was taken to determine average shrimp weight at day 0 and at days 14, 28, and 42, to determine overall weight gain in the growth trials. Specific growth rate (SGR) for each replicate was determined using the formula:

$$\frac{(Ln\ W6wk) - (Ln\ W0wk)}{days\ of\ treatment\ period} \times 100$$

TABLE 1 | Compositions of treatment diets expressed as percentages (where CP = crude protein).

Ingredients	T1: Control	T2: FeedKind 5%	T3: FeedKind 10%	T4: FeedKind 15%
Fishmeal, SE Asia, 64% CP	15.0	10.0	5.0	0.0
Methanotroph bacteria meal (FeedKind), 71% CP	0.0	5.0	10.0	15.0
Krill meal, 54% CP	2.0	2.0	2.0	2.0
Squid liver meal, 43% CP	5.0	5.0	5.0	5.0
Poultry meal, 63% CP	5.0	5.0	5.0	4.4
Soybean meal United States, 48% CP	28.2	28.2	29.0	29.3
Soybean meal fermented, 53% CP	6.0	6.0	5.0	4.7
Wheat gluten meal, 78% CP	1.0	0.4	0.0	0.0
Wheat flour	26.3	27.0	27.7	28.5
Fish oil	1.2	1.2	1.2	1.3
Soybean oil	1.0	1.0	1.0	1.0
Soy lecithin	1.7	1.5	1.4	1.2
Vitamin premix[1]	2.0	2.0	2.0	2.0
DL-Methionine	0.2	0.2	0.2	0.2
Mineral premix[2]	2.5	2.5	2.5	2.5
Mono calcium phosphate, MCP	0.5	0.5	0.5	0.5
Tuna Hydrolysate	2.0	2.0	2.0	2.0
Polymethylcarbamide	0.5	0.5	0.5	0.5
Total (%)	100	100	100	100
Proximate composition by AOAC (2000)				
Moisture (%)	3.77	3.77	3.36	3.60
Ash (%)	9.60	8.94	8.78	8.32
Crude protein (%)	38.35	38.64	38.70	38.77
Lipid (%)	7.60	7.31	6.97	6.63
Calcium (%)	1.66	1.48	1.28	1.10
Phosphorus (%)	1.12	1.08	1.06	0.98
Fiber (%)	5.54	5.73	6.20	6.38
Energy (MJ/Kg)	19.08	19.49	19.06	19.47

[1] *Containing 3,500,000 IU Vitamin A, 1,500,000 IU Vitamin D3, 75 g Vitamin E, 15 g Vitamin K3, 12.5 g Vitamin B1, 10 g Vitamin B2, 12.5 g Vitamin B6, 0.01 g Vitamin B12, 50 g Niacin, 40 g Pantothenic acid, 0.5 g Biotin, 5 g Folic acid, and 100 g Vitamin C per kg.*
[2] *Contains 12.5 g copper, 15 g iron, 15 g manganese, 0.5 g iodine, 0.1 g cobalt, 50 g zinc, 0.175 g selenium per kg.*

where W6wk is the average weight of the shrimp at week 6 and W0wk is the initial average weight of shrimp in each group. Feed conversion ratio (FCR) was determined by dividing total feed consumption by total weight gain for each treatment. Survival rate for each treatment group was also calculated.

Immune Parameters

The hemolymph of fifty shrimp per experimental treatment (ten shrimp per replicate) randomly selected at the end of the growth trial and all shrimp per treatment at the end of the *V. parahaemolyticus* trial were collected using 10 percent (w/v) sodium citrate as an anticoagulant. The hemolymph were taken from the pericardial cavity using a 1-mL syringe, pooled, and stored at −20 until analysis. Measurements including total hemocyte count, hemolymph protein level, and phenol oxidase activity were determined according to the method of Encarnacion et al. (2012), lysozyme activity by turbidity method as described by Shugar (1952), superoxide dismutase enzyme activity and total glutathione using Sigma-Aldrich Assay Kits (19160-1KT-F and CS0260-1KT, respectively).

Histology

Histological examinations of hepatopancreas health were conducted using five shrimp from each tank (replicate) in the growth trial. Shrimp were randomly collected and immersed in ice water for stunning before removal of small portions of hepatopancreas tissue, injected with Davidson's fixative and then transferred to 70% ethanol before being processed to wax blocks, then sectioned using a microtome, with sections being stained with hematoxylin and eosin (H&E) according to Bell and Lightner (1988). The number of B-cells, R-cells, and other cell types were counted from up to 10 tubules per slide.

Bacterial Methods

Vibrio bacteria counts from hepatopancreas of shrimp after 42 days of the growth trial and 15 days after *V. parahaemolyticus* challenge were conducted. Two shrimp from each replicate of each treatment were randomly sampled and then surface body sterilized with 70% ethanol, scarified to collect the hepatopancreas which was then homogenized in 0.85 saline water. The solution was spread on the TCBS Agar (Difco) with 1.5% NaCl (w/v) for 24 h at 35°C. After 24 h of growth, bacterial colonies were counted and recorded.

Statistical Analysis

This study was conducted in completely randomized design (CRD). All data were analyzed by one-way analysis of variance (ANOVA). If an ANOVA resulting from the analyses was significant then least square means were used to test pairwise differences among treatment. The Duncan's Multiple Range Test was used to determine the significant difference test. Statistical tests were considered significant at an alpha = 0.05 and the alphabetical notation was used to mark the differences at these significance levels. Residuals were checked for normality using quantile to quantile plots and Bartlett tests were used to assess the homogeneity of variances. The data in percentage of mortality among methanotroph bacterial meal replaced for fishmeal in the growth trial and challenge assays were transformed following the method of Sokal and Rohlf (1995) and then subjected to ANOVA. Differences were considered statistically significant if $p < 0.05$.

RESULTS

Growth Trial

Growth Parameters

The initial average weight of pacific white shrimp was 1.17 ± 0.09 g. The growth performance of white shrimp fed different levels of methanotroph bacteria meal for 6 weeks showed no significant differences ($p > 0.05$) in terms of final weight, weight gain, specific growth rate, feed consumption, and feed conversion ratio when compared to the control diet (see **Table 2**). Survival rate was not significantly different ($p > 0.05$) among treatments.

Growth Trial – Immune Parameters

The immune parameters of white shrimp fed different levels of methanotroph bacteria meal or control diet are presented in **Table 3**. No significant differences ($p > 0.05$) were noted in hemocyte counts, hemolymph protein, phenoloxidase activity, lysozyme activity, superoxide dismutase activity, and amount of total glutathione between treatments. However, *Vibrio* sp. counts in the hemolymph and in the hepatopancreas following plate cultures were significantly reduced in shrimp fed methanotroph bacteria meal compared with control diet prior to challenge with *V. parahaemolyticus* (**Table 3**).

Growth Trial - Histology

Histological sections of hepatopancreases of shrimp fed diets containing different concentrations of methanotroph bacteria meal, showed that blister-like cells (B-cell) percentage was highest in shrimp fed diet T2 (5% FeedKind) but lowest in those fed the control diet. Resorptive-cells (R-cell) percentages were highest in shrimp fed the control diet and in shrimp fed diet T4 (15% FeedKind) (**Table 4**).

B-cell size was largest in the control group compared to other groups. Degeneration and change of lumen structure (note the star-like shaped seen is normal) were found in groups fed diets T2 and T3 in which 33 and 66%, respectively, of the fishmeal was replaced with methanotroph bacteria meal. Representative histological images are shown in **Figure 1**.

Vibrio parahaemolyticus Challenge Trial

Vibrio parahaemolyticus Challenge – Survival

The survival rate of shrimp immersed in *V. parahaemolyticus* EMS 5.8×10^4 CFU/ml for 15 days after being fed feeds containing different levels of single cell protein for 6 weeks are presented in **Table 5**. Mortalities were noted in the control diet group at day 9 post-infection. The results showed that on day 9 to day 15 after immersion, shrimp fed methanotroph bacteria meal at 10 and 15% in diets T3 and T4 had a significantly higher survival rate ($p < 0.05$) than diets T2 and T1 control, respectively. No mortalities were noted in shrimp fed diets containing 15% methanotroph bacteria meal.

Vibrio parahaemolyticus Challenge – Immune Parameters

The measured immune parameters of white shrimp collected after the 15-day challenge with *V. parahaemolyticus* are presented in **Table 6**. Hemocyte count and phenoloxidase activity showed no significant differences ($p > 0.05$) between diet treatment groups, but hemolymph protein was significantly higher ($p < 0.05$) in shrimp fed diet T4 containing 15% methanotroph bacteria meal. The *Vibrio* colony counts from hepatopancreas in the treatment groups were all significantly lower than the control ($p < 0.05$), and the colony counts from the group fed diet T4 were further reduced and significantly different from diet T2.

TABLE 2 | Growth performance of white shrimp (n = 25 for each replicate and each time period) fed different level of methanotroph bacteria meal for 6 weeks.

	Periods	T1: Control	T2: FeedKind 5%	T3: FeedKind 10%	T4: FeedKind 15%	*P*-Value
Production (g/tank)	0 weeks	29.82 ± 1.81	29.02 ± 1.81	29.32 ± 2.60	28.34 ± 2.82	0.783
	6 weeks	106.31 ± 8.43	110.35 ± 4.79	111.80 ± 9.0	115.30 ± 7.84	0.351
Average weight (g/ind.)	0 week	1.19 ± 0.07	1.16 ± 0.07	1.17 ± 0.10	1.13 ± 0.11	0.783
	6 weeks	4.98 ± 0.35	4.93 ± 0.23	4.99 ± 0.07	5.15 ± 0.25	0.54
Weight gain (g/ind.)	6 weeks	3.78 ± 0.37	3.77 ± 0.28	3.81 ± 0.10	4.01 ± 0.29	0.498
Specific growth rate (%BW/d)	6 weeks	3.40 ± 0.25	3.44 ± 0.24	3.45 ± 0.20	3.61 ± 0.28	0.562
Feed consumed (g/ind.)	6 weeks	4.77 ± 0.36	4.54 ± 0.27	4.63 ± 0.10	4.56 ± 0.30	0.565
Feed conversion ratio	6 weeks	1.26 ± 0.09	1.21 ± 0.13	1.21 ± 0.05	1.15 ± 0.15	0.438
Survival Rate (%)	6 weeks	85.60 ± 6.07	89.60 ± 2.19	89.60 ± 6.07	89.60 ± 3.58	0.474

Mean values in row show no significant differences (p > 0.05) between treatments.

TABLE 3 | Immune parameters (n = 10 per replicate) and *Vibrio* counts (n = 2 per replicate) of white shrimp fed different level of methanotroph bacteria meal for 6 weeks under pre-challenge conditions.

	T1: Control	T2: FeedKind 5%	T3: FeedKind 10%	T4: FeedKind 15%	*P*-Value
Hemocyte count ($\times 10^5$ cell/ml)	25.40 ± 2.97	24.80 ± 2.77	26.40 ± 3.05	25.60 ± 3.36	0.87
Hemolymph protein (mg/dl)	4.64 ± 0.95	5.32 ± 0.59	5.36 ± 0.82	5.72 ± 1.39	0.395
Phenoloxidase activity (unit/min/mg Protein)	56.24 ± 14.68	40.41 ± 7.93	50.89 ± 20.91	51.73 ± 14.62	0.435
Lysozyme activity (unit/ml)	403.33 ± 25.17	370.00 ± 72.11	370.00 ± 10.00	383.33 ± 40.41	0.761
Superoxide dismutase activity (unit/ml)	26.13 ± 3.13	25.95 ± 2.38	26.37 ± 3.78	23.92 ± 3.00	0.753
Glutathione (nM/ml)	37.83 ± 4.20	35.03 ± 1.80	36.90 ± 1.40	41.57 ± 6.07	0.284
Vibrio bacterial counts from hepatopancreas (Log CFU/ml)	2.61 ± 0.31[a]	2.39 ± 0.32[a]	1.16 ± 0.28[b]	1.38 ± 0.66[b]	0.001

Values with different superscript letters in row indicate significant differences ($p < 0.05$) between treatments.

TABLE 4 | Number and percentage of B-cell and R-cell from hepatopancreas of shrimp fed different levels of methanotroph bacteria meal for 6 weeks under pre-challenge conditions.

Group	Number of cells		Percentage	
	B-cell	R-cell	B-cell	R-cell
T1: Control diet	6.17	3.83	56.13	33.79
T2: FeedKind 5%	5.75	1.50	69.94	17.81
T3: FeedKind 10%	6.40	2.40	63.72	24.20
T4: FeedKind 15%	6.17	3.17	60.61	30.88

DISCUSSION

This study has successfully shown that Pacific white shrimp fed diets where fishmeal is replaced with the single cell protein methanotroph bacteria meal, FeedKind, for 6 weeks shows comparable levels of growth and survival to shrimp fed standard feeds containing 15% fishmeal (T1). Specifically, feed conversion ratios, specific growth rates and overall increases in weight are greater in shrimp fed FeedKind compared with those fed standard diets. Importantly, methanotroph bacteria meal reduces *Vibrio* spp. loads in the hepatopancreas and improves the survival rate when shrimp are exposed to a *V. parahaemolyticus* challenge. In agreement with Chen et al. (2021), this study has shown the substitution of fishmeal with methanotroph bacteria meal did not affect uptake of feed nor have any other detrimental effects such as antinutritional properties or impacts on a range of immune measures.

These results are in broad agreement with studies showing dietary supplementation of fishmeal with single cell protein shows no significant differences in growth and feed efficiency in fish species such as Atlantic salmon (Berge et al., 2005), Atlantic halibut (Aas et al., 2007), and Japanese yellowtail (Biswas et al., 2020). Other research on single cell protein as replacement of fishmeal in shrimp diets has shown they can be used to replace fishmeal, partially or fully, e.g., purple non-sulfur bacteria (Chumpol et al., 2018), *C. ammoniagenes* (Hamidoghli et al., 2019), and KnipBio Meal (Tlusty et al., 2017). Previous studies have found that disease resistance parameters can be promoted in shrimp by using nutrition supplements such as biofloc feed (Ekasari et al., 2014) and organic acids and essential oils (He et al., 2017). The positive effect on the immune capacity, disease resistance and gut microbiota of *P. vannamei* have also been shown to be improved by using single cell protein (Chumpol et al., 2018; Chen et al., 2021). Furthermore, shrimp fed methanotroph bacteria meal have been shown to have increased oxidation levels in the hepatopancreas, increased mucosal fold height in the gut, improved gut microbiota, and an overall improvement in general disease resistance factors (Chen et al., 2021).

The digestive gland or hepatopancreas of crustaceans is used for monitoring cultured shrimp health and serves as a sensitive indicator for metabolism, ecdysis phase, nutritional status, and disease state in various shrimp species. The hepatopancreas is the site of digestion, nutrient absorption, reserve nutrient storage, and synthesis and secretion of digestive enzymes and is composed of numerous blinded tubules, with each tubule consisting of different epithelial cell types, i.e., E-cell (embryonic), R-cell (resorptive), F-cell (fibrillary), and B-cell (blister like). Histological analysis of the hepatopancreas has been used as a practical means for assessing the nutritional condition in the shrimp culture (Díaz et al., 2010; Vogt, 2020). In the current study, B-cell types are more numerous and smaller in shrimp fed FeedKind compared with those fed control diets suggesting B-cell types may be produced in greater numbers in shrimp fed FeedKind® diets. The increased number of these secretory cells that function as primary producers of digestive enzymes and antioxidants, suggests that FeedKind®-fed shrimp are better able to accumulate nutrients from the diet and to transport digested material compared with those fed control diets. Conversely, R-cells which function as the main site for lipid and glycogen storage, do not show a clear pattern of size distribution between treatments suggesting that diet has little impact on these cells.

In the current study, improved survival rates of shrimp fed diets with total or partial replacement of fishmeal with methanotroph bacteria meal for 6 weeks followed by a *V. parahaemolyticus* bath challenge was demonstrated. Specifically, no mortality was observed in the diet with 15% FeedKind (replacement of 100% of fishmeal) while only 76% of shrimp challenged with *V. parahaemolyticus* and fed the control fishmeal-based diet survived. In comparison, only 50% of Pacific shrimp fed a diet containing up to 10.5% FeedKind® and 13.75% fishmeal survived a challenge with *V. parahaemolyticus* by injection compared to 25% survival when fed control diets without bacterial meal (Chen et al., 2021). The differences observed in shrimp survival between the current study and that

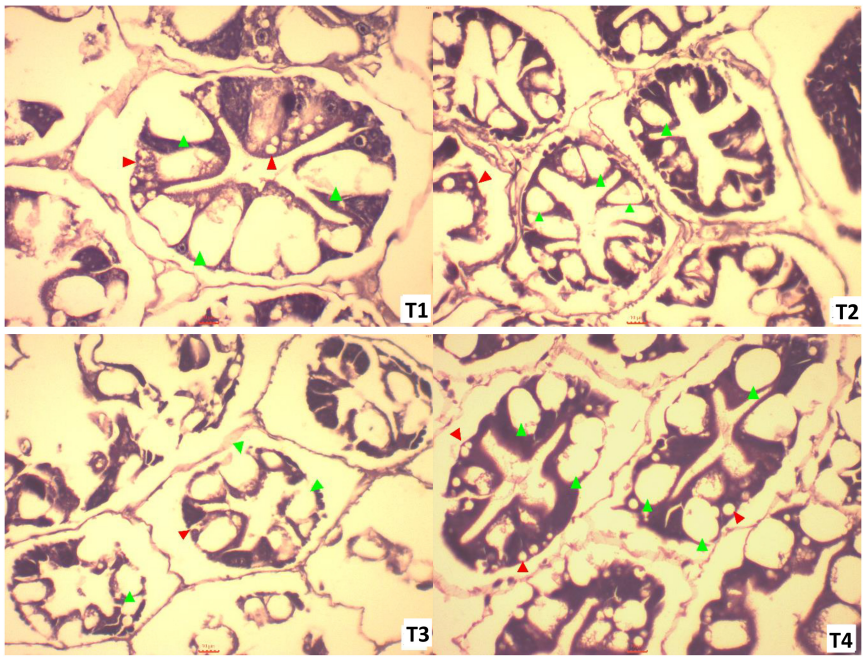


FIGURE 1 | Light micrograph of hepatopancreas of shrimp fed different concentration of methanotroph bacteria meal for 6 weeks under pre-challenge conditions (T1: Control diet, T2: FeedKind 5%, T3: FeedKind 10%, T4: FeedKind 15%; green arrow = B-cell, red arrow = R-cell; scale bar = 10 μm, ×400).

TABLE 5 | Survival rate of white shrimp following immersion challenge by *V. parahaemolyticus* (AHPND) at 5.8 × 10^4 CFU/ml for 15 days after being fed different levels of methanotroph bacteria meal diets for 6 weeks.

Days after challenge	T1: Control	T2: FeedKind 5%	T3: FeedKind 10%	T4: FeedKind 15%	*P*-Value
Day 0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	.....
Day 3	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	.....
Day 6	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	.....
Day 9	86.7 ± 5.77^{b}	93.3 ± 5.77^{ab}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	0.011
Day 12	83.3 ± 5.77^{b}	90.0 ± 10.00^{ab}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	0.019
Day 15	76.7 ± 5.77^{c}	86.7 ± 5.77^{b}	96.7 ± 5.77^{a}	100.0 ± 0.0^{a}	0.002

Values with different superscript letters in row indicate significant differences ($p < 0.05$) between treatments.

TABLE 6 | Immune parameters in white shrimp fed different levels of methanotroph bacteria meal after 15 days of immersion by *Vibrio parahaemolyticus*.

	T1: Control	T2: FeedKind 5%	T3: FeedKind 10%	T4: FeedKind 15%	*P*-Value
Hemocyte count (×10^5 cell/ml)	12.67 ± 1.53^{a}	11.67 ± 2.08^{a}	13.00 ± 3.00^{a}	16.33 ± 3.79^{a}	0.256
Hemolymph protein (mg/dl)	4.00 ± 1.21^{b}	4.13 ± 0.55^{b}	4.15 ± 0.44^{b}	6.76 ± 0.37^{a}	0.004
Phenoloxidase activity (unit/min/mg Protein)	23.14 ± 10.10^{a}	24.42 ± 3.14^{a}	24.72 ± 6.04^{a}	20.84 ± 0.83^{a}	0.859
Vibrio bacterial counts from hepatopancreas (Log CFU/ml)	3.70 ± 0.02^{a}	2.65 ± 0.17^{b}	2.13 ± 0.18^{bc}	1.47 ± 0.82^{c}	0.001

Values with different superscript letters in a row indicate significant differences ($p < 0.05$) between treatments.

of Chen et al. (2021) can be partly explained by the challenge method (IP vs. bath) and by the challenge dose. Using the bath challenge method in the current study is a more natural route of exposure and is more comparable to infection processes on the farm. Comparison between doses used in different studies and by different exposure routes is rarely considered. However, Joshi et al. (2014) exposed *P. vannamei* to 10^8 CFU/ml *V. parahaemolyticus* by the bath route or to 10^3 CFU per shrimp by injection with mortality outcomes being similar, although characteristic disease was not induced in shrimp exposed *via* the IP route. Unlike Chen et al. (2021), typical clinical signs of AHPND were noted in shrimp exposed to *V. parahaemolyticus* in the current study, supporting the view that a bath exposure route is preferable to demonstrate the impact of AHPND on the host. In addition, the control diets used by Chen et al. (2021) differed significantly from those in the present study. Whereas the prior work included peanut meal as a source of protein, this ingredient is not commonly used in shrimp feeds outside

of China, and therefore was not included in the current study. Similarly, the prior study included brewer's yeast, which, as a single cell product containing beta glucans and nucleic acids, may have also influenced the shrimp immune response alongside the methanotroph bacteria meal (Chen et al., 2021).

It is recognized that feeding shrimp with diets containing different levels of methanotroph bacteria meal for 6 weeks prior to a *V. parahaemolyticus* challenge led to a substantial reduction in *Vibrio* spp. numbers in the hepatopancreas as well as lower numbers of *V. parahaemolyticus* post-challenge leading to improved survival. Reduced *Vibrio* spp. levels and increased expression of anti-lipopolysaccharide factor (ALF) have been identified in the intestine of shrimp fed diets containing bacterial protein meal (Chen et al., 2021). The authors speculate that bacterial protein meal may stimulate toll-like receptors (TLRs) present in the shrimp digestive tract, which then activate an innate immune response and up-regulate the production of antimicrobial peptides or other enzymes increasing the ability of white shrimp to resist a bacterial pathogen. ALFs are known to bind to gram-negative bacterial cell walls and disrupt cellular function causing cell death and lysis (Zhan et al., 2015) and thus may play an important role in reducing the impact of *V. parahaemolyticus* in shrimp fed diets containing FeedKind. Further study is necessary to validate this hypothesis and possibly to identify the specific pathway and antimicrobial function stimulated by methanotroph bacteria meal protein. In the current study, although *Vibrio* sp. counts in the hemolymph and in the hepatopancreas were significantly reduced in shrimp fed methanotroph bacteria meal compared with control diet, no significant differences were noted in hemocyte counts, hemolymph protein, phenoloxidase activity, lysozyme activity, superoxide dismutase activity or amount of total glutathione between treatments suggesting that these measures are not sufficiently sensitive in detecting differences in immune responses prior to a disease challenge. However, in shrimp challenged with *V. parahaemolyticus* and fed a diet where 100% of the fish meal was replaced with FeedKind, haemolymph protein was significantly higher compared with other groups. Although the current study did not attempt determine which haemolymph proteins were amplified, it is known that a wide range of these antimicrobial peptides, which perform a number of functions on host defense, have been reported in crustaceans (Fredrick and Ravichandran, 2012). It is likely that the decrease in *V. parahaemolyticus* and improved survival rates in animals fed FeedKind® is a direct result of the noted increase in haemolymph proteins.

The current study has shown that fish meal can be entirely replaced in shrimp diets with limited impact on growth and survival of shrimp; additional studies should validate the results of the current study under field conditions to confirm that complete replacement of fishmeal with methanotroph bacteria meal protein, followed by a natural *V. parahaemolyticus* challenge leads to improved survival.

CONCLUSION

The efficacy of single cell protein, methanotroph bacteria meal protein in Pacific white shrimp diets was studied to determine growth performance, survival rate and disease resistance against *V. parahaemolyticus*. Trials of this novel protein source for replacing fishmeal in shrimp diets have shown that the protein does not affect the growth feed efficiency, or survival of shrimp reared under experimental conditions. Additionally, shrimp in this study demonstrated an increased tolerance to disease when challenged with *V. parahaemolyticus*, the causative agent of Early Mortality Syndrome (EMS), indicating methanotroph bacteria meal protein, FeedKind® protein, may help promote a robust immune response.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

OJ: study design, conception, data analysis, and writing manuscript. SC and ST: data collection, data analysis, and writing manuscript. AL and JS: study design, conception, and writing manuscript.

FUNDING

Calysta, Inc. funded this research and contributed to the drafting of this manuscript.

REFERENCES

Aas, T. S., Hatlen, B., Grisdale-Helland, B., Terjesen, B. F., Penn, M., Bakke-McKellep, A. M., et al. (2007). Feed intake, growth and nutrient utilization in Atlantic halibut (*Hippoglossus hippoglossus*) fed diets containing a bacterial protein meal. *Aquac. Res.* 38, 351–360. doi: 10.1111/j.1365-2109.2007.01672.x

Anandaraja, R., Sridhar, R., Balachandran, C., Palanisammi, A., Ramesh, S., and Nagarajan, K. (2017). Pathogenicity profile of *Vibrio parahaemolyticus* in farmed pacific white shrimp, *Penaeus vannamei*. *Fish Shellfish Immunol.* 67, 368–381. doi: 10.1016/j.fsi.2017.06.020

Association of Official Analytical Chemists (AOAC) (2000). *Official Methods of Analysis of AOAC International*, 17th Edn. Gaithersburg, MD: AOAC International.

Bell, T. A., and Lightner, D. V. (1988). *A Handbook of Normal Penaeid Shrimp Histology: World Aquaculture Society*. Louisiana: World Aquaculture Society.

Berge, G. M., Baeverfjord, G., Skrede, A., and Storebakken, T. (2005). Bacterial protein grown on natural gas as protein source in diets for Atlantic salmon, *Salmo salar*, in saltwater. *Aquaculture* 244, 233–240. doi: 10.1016/j.aquaculture.2004.11.017

Biswas, A., Takakuwa, F., Yamada, S., Matsuda, A., Saville, R. M., LeBlanc, A., et al. (2020). Methanotroph (*Methylococcus capsulatus*, Bath) bacteria meal as

an alternative protein source for Japanese yellowtail, *Seriola quinqueradiata*. *Aquaculture* 529:735700. doi: 10.1016/j.aquaculture.2020.735700

Chen, M., Chen, X.-Q., Tian, L.-X., Liu, Y.-J., and Niu, J. (2020). Enhanced intestinal health, immune responses and ammonia resistance in Pacific white shrimp (*Litopenaeus vannamei*) fed dietary hydrolyzed yeast (*Rhodotorula mucilaginosa*) and Bacillus licheniformis. *Aquac. Rep*. 17:100385. doi: 10.1016/j.aqrep.2020.100385

Chen, Y., Chi, S., Shuang, Z., Dong, X., Yang, Q., Liu, H., et al. (2021). Replacement of fishmeal with methane-utilizing bacteria products in the diets of Pacific white shrimp (*Litopenaeus vannamei*). *Aquaculture* 541:736801. doi: 10.1016/j.aquaculture.2021.736801

Chucherd, N. (2013). The case study of EMS in Thailand. *Asian Aquac. Network Pract. Maga.* 4, 14–19.

Chumpol, S., Kantachote, D., Nitoda, T., and Kanzaki, H. (2018). Administration of purple non-sulfur bacteria as single cell protein by mixing with shrimp feed to enhance growth, immune response and survival in white shrimp (*Litopenaeus vannamei*) cultivation. *Aquaculture* 489, 85–95. doi: 10.1016/j.aquaculture.2018.02.009

Díaz, A. C., Sousa, L. G., and Petriella, A. M. (2010). Functional cytology of the hepatopancreas of *Palaemonetes argentines* (Crustacea, Decapoda, Caridea) under osmotic stress. *Brazilian Arch. Biol. Technol.* 53, 599–608. doi: 10.1590/S1516-89132010000300013

Dong, X., Wang, H., Xie, G., Zou, P., Guo, C., Liang, Y., et al. (2017). An isolate of *Vibrio campbellii* carrying the pirVP gene causes acute hepatopancreatic necrosis disease. *Emerg. Microbes Infect.* 6, 1–3. doi: 10.1038/emi.2016.131

Ekasari, J., Azhar, M. H., Surawidjaja, E. H., Nuryati, S., De Schryver, P., and Bossier, P. (2014). Immune response and disease resistance of shrimp fed biofloc grown on different carbon sources. *Fish Shellfish Immunol.* 41, 332–339. doi: 10.1016/j.fsi.2014.09.004

Encarnacion, A. B., Fagutao, F., Jintasataporn, O., Worawattanamateekul, W., Hirono, I., and Ohshima, T. (2012). Application of ergothioneine-rich extract from an edible mushroom *Flammulina velutipes* for melanosis prevention in shrimp, *Penaeus monodon* and *Litopenaeus vannamei*. *Food Res. Internatl.* 45, 232–237. doi: 10.1016/j.foodres.2011.10.030

FAO (2018). *The State of World Fisheries and Aquaculture 2018: Meeting the Sustainable Development Goals*. Italy: Food and Agriculture Organization of the United Nations. 210.

FAO (2019). *Report of the FAO Workshop on the on-Farm Feeding and Feed Management in Aquaculture*. Rome: Food and Agriculture Organization of the United Nations., 8.

Fredrick, W. S., and Ravichandran, S. (2012). Hemolymph proteins in marine crustaceans. *Asian Pac. J. Trop. Biomed.* 2, 496–502. doi: 10.1016/S2221-1691(12)60084-7

Gamboa-Delgado, J., Castañeda-Solís, J. D., Nieto-López, M. G., Villarreal-Cavazos, D., and Cruz-Suárez, L. E. (2014). Isotopic evaluation of the nutritional contribution of poultry by-product meal and fishmeal to the growth of pacific white shrimp, *Litopenaeus vannamei*. *J. World Aquac. Soc.* 45, 430–438. doi: 10.1111/jwas.12134

Glencross, B. D., Huyben, D., and Schrama, J. W. (2020). The application of single-cell ingredients in aquaculture feeds—a review. *Fishes* 5:22. doi: 10.3390/fishes5030022

Hamidoghli, A., Yun, H., Won, S., Kim, S., Farris, N. W., and Bai, S. C. (2019). Evaluation of a single-cell protein as a dietary fishmeal substitute for whiteleg shrimp *Litopenaeus vannamei*. *Fish. Sci.* 85, 147–155. doi: 10.1007/s12562-018-1275-5

He, W., Rahimnejad, S., Wang, L., Song, K., Lu, K., and Zhang, C. (2017). Effects of organic acids and essential oils blend on growth, gut microbiota, immune response and disease resistance of Pacific white shrimp (*Litopenaeus vannamei*) against *Vibrio parahaemolyticus*. *Fish Shellfish Immunol.* 70, 164–173. doi: 10.1016/j.fsi.2017.09.007

Hernández, C., Olvera-Novoa, M. A., Aguilar-Vejar, K., González-Rodríguez, B., and de la Parra, I. A. (2008). Partial replacement of fishmeal by porcine meat meal in practical diets for Pacific white shrimp (*Litopenaeus vannamei*). *Aquaculture* 277, 244–250. doi: 10.1016/j.aquaculture.2008.02.016

Jones, S. W., Karpol, A., Friedman, S., Maru, B. T., and Tracy, B. P. (2020). Recent advances in single cell protein use as a feed ingredient in aquaculture. *Curr. Opin. Biotechnol.* 61, 189–197. doi: 10.1016/j.copbio.2019.12.026

Joshi, J., Srisala, J., Troung, V. H., Chen, I., Nuangsaeng, B., Suthienkul, O., et al. (2014). Variation in *Vibrio parahaemolyticus* isolates from a single Thai shrimp farm experiencing an outbreak of acute hepatopancreatic necrosis disease (AHPND). *Aquaculture* 42, 297–302. doi: 10.1016/j.aquaculture.2014.03.030

Kondo, H., Van, P. T., Dang, L. T., and Hirono, I. (2015). Draft genome sequence of non-*Vibrio parahaemolyticus* acute hepatopancreatic necrosis disease strain KC13.17.5, isolated from diseased shrimp in Vietnam. *Genome Announcements* 3:e978. doi: 10.1128/genomeA.00978-15

Lightner, D. V., Redman, R. M., Pantoja, C. R., Noble, B. L., and Loc, T. (2012). Early mortality syndrome affects shrimp in Asia. *Global Aquac. Adv.* 2012:40.

Linder, T. (2019). Making the case for edible microorganisms as an integral part of a more sustainable and resilient food production system. *Food Security* 11, 265–278. doi: 10.1007/s12571-019-00912-3

Liu, L., Xiao, J., Xia, X., Pan, Y., Yan, S., and Wang, Y. (2015). Draft genome sequence of vibrio owensii strain SH-14, which causes shrimp acute hepatopancreatic necrosis disease. *Genome Announcements* 3:e1395. doi: 10.1128/genomeA.01395-15

Liu, X., Steele, J. C., and Meng, X. Z. (2017). Usage, residue, and human health risk of antibiotics in Chinese aquaculture: a review. *Environ. Poll.* 223, 161–169. doi: 10.1016/j.envpol.2017.01.003

Malcorps, W., Kok, B., Land, M. V., Fritz, M., van Doren, D., Servin, K., et al. (2019). The sustainability conundrum of fishmeal substitution by plant ingredients in shrimp feeds. *Sustainability* 11:1212. doi: 10.3390/su11041212

Øverland, M., Borge, G. I., Vogt, G., Schøyen, H. F., and Skrede, A. (2011). Oxidative stability and sensory quality of meat from broiler chickens fed a bacterial meal produced on natural gas. *Poultry Sci.* 90, 201–210. doi: 10.3382/ps.2010-00784

Øverland, M., Romarheim, O. H., Hovin, M., Storebakken, T., and Skrede, A. (2006). Apparent total tract digestibility of unprocessed and extruded diets containing basic and autolyzed bacterial protein meal grown on natural gas in mink and rainbow trout. *Animal Feed Sci. Technol.* 129, 237–251. doi: 10.1016/j.anifeedsci.2005.12.017

Putth, S., and Polchana, J. (2016). "Current status and impact of early mortality syndrome (EMS)/acute hepatopancreatic necrosis disease (AHPND) and hepatopancreatic microsporidiosis (HPM) outbreaks on Thailand s shrimp farming," in *Addressing Acute Hepatopancreatic Necrosis Disease (AHPND) and Other Transboundary Diseases for Improved Aquatic Animal Health in Southeast Asia: Proceedings of the ASEAN Regional Technical Consultation on EMS/AHPND and Other Transboundary Diseases for Improved Aquatic Animal Health in Southeast Asia*, eds R. V. Pakingking Jr., E. G. T. de Jesus-Ayson and B. O. Acosta (Iloilo: Aquaculture Department, Southeast Asian Fisheries Development Center), 79–87.

Qui, X., and Davis, D. A. (2017a). Effects of dietary phytase supplementation on growth performance and apparent digestibility coefficients of Pacific white shrimp *Litopenaeus vannamei*. *Aquacu. Nutr.* 23, 942–951.

Qui, X., and Davis, D. A. (2017b). Evaluation of flash dried yeast as a nutritional supplement in plant-based practical diets for Pacific white shrimp *Litopenaeus vannamei*. *Aquac. Nutr.* 23, 1244–1253. doi: 10.1111/anu.12499

Qui, X., Tian, H., and Davis, D. A. (2017). Evaluation of a high protein distiller's dried grains product as a protein source in practical diets for Pacific white shrimp *Litopenaeus vannamei*. *Aquaculture* 480, 1–10. doi: 10.1016/j.aquaculture.2017.07.038

Samocha, T., Davis, D. A., Saoud, I. P., and DeBault, K. (2004). Substitution of fishmeal by co-extruded soybean poultry by-product meal in practical diets for the Pacific white shrimp, *Litopenaeus vannamei*. *Aquaculture* 231, 197–203. doi: 10.1016/j.aquaculture.2003.08.023

Shugar, D. (1952). The measurement of lysozyme activity and the ultra-violet inactivation of lysozyme. *Biochim. Biophys. Acta.* 8, 302–309. doi: 10.1016/0006-3002(52)90045-0

Sokal, R. R., and Rohlf, F. J. (1995). *Biometry: The Principles and Practice of Statistics in Biological Research*, 3rd Edn. New York: W.H. Freeman and Co.

Soto-Rodriguez, S. A., Gomez-Gil, B., Lozano, R., del Rio-Rodríguez, R., Diéguez, A. L., and Romalde, J. L. (2012). Virulence of vibrio harveyi responsible for the "bright-red" syndrome in the pacific white shrimp *Litopenaeus vannamei*. *J. Invertebr. Pathol.* 109, 307–317. doi: 10.1016/j.jip.2012.01.006

Soto-Rodriguez, S. A., Gomez-Gil, B., Lozano-Olvera, R., Betancourt-Lozano, M., and Morales-Covarrubias, M. S. (2015). Field and experimental evidence of *Vibrio parahaemolyticus* as the causative agent of acute hepatopancreatic

necrosis disease of cultured shrimp (*Litopenaeus vannamei*) in Northwestern Mexico. *Appl. Environ. Microbiol.* 81, 1689–1699. doi: 10.1128/AEM.03610-14

Swick, R. A., Akiyama, D. M., Boonyaratpalin, M., and Creswell, D. C. (1995). *Use of Soybean Meal and Synthetic Methionine in Shrimp Feed*. St. Louis, MO: American Soybean Association, Technical Bulletin, AQ43–AQ1995.

Tacon, A. G. J., Metian, M., and Hasan, M. R. (2009). *Feed Ingredients and Fertilizers for Farmed Aquatic Animals. Sources and Composition. FAO Fisheries and Aquaculture technical paper, 540*. Italy: FAO.

Tan, B., Mai, K., Zheng, S., Zhou, Q., Liu, L., and Yu, Y. (2005). Replacement of fishmeal by meat and bone meal in practical diets for the white shrimp *Litopenaeus vannamei* (Boone). *Aquac. Res.* 36, 439–444.

Thitamadee, S., Prachumwat, A., Srisala, J., Jaroenlak, P., Salachan, P. V., Sritunyalucksana, K., et al. (2016). Review of current disease threats for cultivated penaeid shrimp in Asia. *Aquaculture* 452, 69–87. doi: 10.1016/j.aquaculture.2015.10.028

Tlusty, M., Rhyne, A., Szczebak, J. T., Bourque, B., Bowen, J. L., Burr, G., et al. (2017). A transdisciplinary approach to the initial validation of a single cell protein as an alternative protein source for use in aquafeeds. *PeerJ* 5:e3170. doi: 10.7717/peerj.3170

Tran, L., Nunan, L., Redman, R. M., Mohney, L. L., Pantoja, C. R., Fitzsimmons, K., et al. (2013). Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. *Dis. Aquat. Orgs.* 105, 45–55. doi: 10.3354/dao02621

Vogt, G. (2020). Cytopathology and immune response in the hepatopancreas of decapod crustaceans. *Dis. Aquat. Orgs.* 138, 41–88. doi: 10.3354/dao03443

Wu, C. C., Lin, C. L., Huang, C. Y., Hsieh, S., Liu, C. H., and Hsieh, S. L. (2019). α-Phellandrene enhances the immune response and resistance against *Vibrio alginolyticus* in white shrimp (*Litopenaeus vannamei*). *Fish Shellfish Immunol.* 84, 1108–1114. doi: 10.1016/j.fsi.2018.11.013

Yang, Y. T., Chen, I. T., Lee, C. T., Chen, C. Y., Lin, S. S., Hor, L. I., et al. (2014). Draft genome sequences of four strains of *Vibrio parahaemolyticus*, three of which cause early mortality syndrome/acute hepatopancreatic necrosis disease in shrimp in China and Thailand. *Genome Announcements* 2:e816. doi: 10.1128/genomeA.00816-14

Zhan, W., He, L., Wei, X., Wang, X., and Tang, X. (2015) An anti-lipopolysaccharide factor in *Litopenaeus vannamei* participates in the immune defense against WSSV and *Vibrio anguillarum*. *J. Crust. Biol.* 35, 670–675. doi: 10.1163/1937240x-00002364

Zorriehzahra, M. J., 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: AL and JS are employed by and own company stock in Calysta, Inc., the supplier of the bacterial protein meal FeedKind used in these studies.

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: 12 November 2021
doi: 10.3389/fmars.2021.764964

Effects of Dietary Inclusion of *Clostridium autoethanogenum* Protein on the Growth Performance and Liver Health of Largemouth Bass (*Micropterus salmoides*)

Qisheng Lu[1,2†], *Longwei Xi*[1,2†], *Yulong Liu*[1,2], *Yulong Gong*[1,2], *Jingzhi Su*[1,2], *Dong Han*[1,2,3*], *Yunxia Yang*[1], *Junyan Jin*[1], *Haokun Liu*[1], *Xiaoming Zhu*[1] and *Shouqi Xie*[1,2,4]

[1] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences (CAS), Wuhan, China, [2] College of Advanced Agricultural Sciences, University of Chinese Academy of Sciences, Beijing, China, [3] Hubei Engineering Research Center for Aquatic Animal Nutrition and Feed, Wuhan, China, [4] The Innovative Academy of Seed Design, Chinese Academy of Sciences (CAS), Wuhan, China

OPEN ACCESS

Edited by:
Jin Niu,
Sun Yat-sen University, China

Reviewed by:
Guangzhen Jiang,
Nanjing Agricultural University, China
Min Jin,
Ningbo University, China

***Correspondence:**
Dong Han
hand21cn@ihb.ac.cn

[†]These authors have contributed equally to this work and share first authorship

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

Received: *26 August 2021*
Accepted: *08 October 2021*
Published: *12 November 2021*

Citation:
Lu Q, Xi L, Liu Y, Gong Y, Su J, Han D, Yang Y, Jin J, Liu H, Zhu X and Xie S (2021) Effects of Dietary Inclusion of Clostridium autoethanogenum Protein on the Growth Performance and Liver Health of Largemouth Bass (Micropterus salmoides). Front. Mar. Sci. 8:764964. doi: 10.3389/fmars.2021.764964

Clostridium autoethanogenum protein (CAP) is a novel protein source for aqua-feeds. The present study aimed to investigate the effects of dietary CAP on growth performance, immunity, and liver health status of juvenile largemouth bass (*Micropterus salmoides*). Four isonitrogenous and isolipid experimental diets were formulated to replace 0% (D1, control), 25% (D2), 50% (D3), and 75% (D4) of fish meal by CAP. Fish (15.05 ± 0.08 g) were randomly fed one of four experimental diets for 8 weeks. The results showed that weight gain (WG), specific growth rate (SGR), feeding rate (FR), viscerosomatic index (VSI), and hepatosomatic index (HSI) of the D4 group were significantly lower than D1, D2, and D3 groups ($P < 0.05$). With the increase of substitution level, the total antioxidant capacity (T-AOC) of liver tissue was significantly decreased, while the plasma alkaline phosphatase (AKP) activity was significantly increased ($P < 0.05$). Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were significantly higher in D3 and D4 groups than in D1 and D2 groups ($P < 0.05$). Replacing 50 or 75% fish meal by CAP significantly induced the transcription level of apoptosis-promoting genes (*bcl-2*-associated death protein [*bad*] and *bcl-2*-assoxicated × protein; *bag* [*bax*]), anti-apoptosis-related genes (tumor protein 53 *[p53*] and b-cell lymphoma-2 [*bcl-2*]), and the apoptotic *Caenorhabditis elegans* (*C. elegans*) death gene-3 like caspases (cysteine-aspartic proteases-3 [*caspase-3*], cysteine-aspartic proteases-8 [*caspase-8*], cysteine-aspartic proteases-9 [*caspase-9*], and cysteine-aspartic proteases-10 [*caspase-10*]) in liver, while suppressed the gene expression of the inflammatory factors [interleukin-1β (*il-1β*), interleukin-8 (*il-8*), and tumor necrosis factor, *tnf*] in head kidney. At the same time, dietary inclusion of CAP elevated the protein expression of bcl-2, autophagy microtubule-associated protein light chain 3A/B (LC3A/B-I), and LC3A/B-II by inhibiting the phosphorylation of the mammalian target of rapamycin (mTOR; $P < 0.05$). Moreover, the apoptosis rate of the D3 and D4 groups was significantly increased ($P < 0.05$). Taken

together, these results indicated that the optimal level of CAP-replacing fish meal should be <50% that has no negative effect on the growth performance and liver health of juvenile largemouth bass. In addition, excessive CAP inclusion may damage liver health by activating autophagy and apoptosis signaling pathways.

Keywords: growth performance, liver health, apoptosis, largemouth bass, *Clostridium autoethanogenum* protein

INTRODUCTION

Fish meal is the preferred protein for aqua-feed because of its balanced nutrition and good palatability (Henry et al., 2015). With the rapid development of the aquaculture industry and the unsustainability of fish resources, an alternative protein source for fish meal is increasingly important (Hardy et al., 2018). Many research efforts have aimed to replace fish meal with soybean meal or other plant proteins (e.g., Kaushik et al., 1995; Sitjà-Bobadilla et al., 2005; Sahlmann et al., 2019), but disadvantages, such as imbalance of amino acid profile (Mambrini et al., 1999), anti-nutritional factors (Francis et al., 2001), and unpalatability (Luo et al., 2010), have limited the further application of plant proteins. In recent years, some new alternative proteins, such as single-cell protein (SCP), have attracted much attention because of the high efficiency and stability of the production and less anti-nutritional factors (Davies and Wareham, 1988).

Clostridium autoethanogenum protein (CAP) is a new type of bacterial protein produced by using *Clostridium autoethanogenum* (CA), which has no toxic genes (Utturkar et al., 2015). Meanwhile, the crude protein content of CAP is more than 72%, which has a similar essential amino acid profile to fish meal, with considerable amounts of carbohydrate, lipid, and vitamin (Simpson et al., 2016). As a pretty novel SCP, current preliminary studies only evaluate the effects of the supplementation with CAP on the growth, digestion, and antioxidant capacity of grass carp (*Ctenopharyngodon idella*), (Wei et al., 2018), Jian carp (*Cyprinus carpio var. Jian*), (Li M. Y. et al., 2021), tilapia (GIFT: *Oreochromis niloticus*), (Maulu et al., 2020, 2021), and black sea bream (*Acanthopagrus schlegelii*), (Chen Y. et al., 2020), but few studies to date have focused on the liver health of fish and the regulation mechanism of dietary inclusion of CAP, especially in high inclusion levels.

Largemouth bass (*Micropterus salmoides*), a typical carnivorous fish, has become an important freshwater fish species in China, with a total cultured production of 477, 808 tons in 2019 (Fishery Bureau, 2020). In the past few years, researchers have devoted their energy to the effect of SCP on the growth of largemouth bass, such as yeast hydrolysate (Zhou et al., 2018; Gong et al., 2019) and symbiotic (Yang et al., 2020). CAP includes renewable energy, environmentally friendly ingredients, and has a high crude protein content. Therefore, the present study investigated four levels of CAP to replace fish meal to study their effects on growth performance and the way to affect liver health of largemouth bass, to provide a reference for the application of CAP in aquatic feed.

MATERIALS AND METHODS

Diet Preparation

The formulation of the experimental diets was formulated in this study (**Table 1**). CAP was purchased from Hebei Shoulang New Energy Technology Co., Ltd., (Tangshan, Hebei, China). Four experimental diets were formulated to include 0, 8.1, 16.2, and 24.3% of CAP replacing 0% (D1), 25% (D2), 50% (D3), and 75% (D4) of dietary fish meal, respectively. D1, where the fish meal content was 40% and was used as the control diet. All the diets were made in puffing feed, and the extruded diets were processed through an extruder (TSE65S; Modern Yanggong Machinery S&T Development Co., Ltd. Beijing, China). All the wet diets were oven-dried at 60°C and stored at −20°C until used.

Fish and Trial Management

Largemouth bass were purchased from a fish farm (Ezhou, Hubei, China). All fish were cultured in an experimental system with a D1 diet for 2 weeks to adapt to the rearing conditions. Before the trial, all fish have fasted 24 h for gastric emptying. Then, 24 similar-size largemouth bass (initial weight: 15.05 ± 0.08 g) were randomly selected, weighed, and put into each tank (volume: 200 L). Three replicates were randomly distributed for one experimental diet. During the experiment, fish were fed to apparent satiation twice a day (08:30 and 16:30) and daily feed intake was recorded. Uneaten feed was taken out and recorded. The feeding trial lasted for 8 weeks. During the whole trial, the water temperature was kept at 26.0 ± 1.0°C. Water-dissolved oxygen content was maintained at 6.4 ± 0.5 mg/L, and ammonia-N was lower than 0.5 mg/L. The photoperiod was 12-h light/12-h dark, with the light period from 08:00 to 20:00.

Sample Collection

Twenty-four hours after the last feeding, the fish in each experimental group were counted and batched weighed. Six fish per tank were randomly selected and anesthetized (MS-222; Sigma, St Louis, MO, USA at 10 mg L^{-1}) for sampling. Three fish in each tank were randomly used to measure the physical index. Three fish per tank were selected to collect blood samples from a caudal vein with heparinized syringes. Plasma was centrifuged (3,000 × g for 10 min) at 4°C and then the supernatant was collected, immediately frozen in liquid nitrogen, and stored at −80°C for further analysis. Three fish per tank were chosen randomly, and liver and head kidney tissues were rapidly sampled, placed in liquid nitrogen, and stored at −80°C for subsequent analysis. Two fish per tank were sampled for the analysis of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL).

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

Ingredient (g/kg dry matter)	D1	D2	D3	D4
Fishmeal[a]	400.0	300.0	200.0	100.0
Clostridium autoethanogenum protein[b]	0.0	81.0	162.0	243.0
Blood meal[c]	40.0	40.0	40.0	40.0
Gluten[d]	50.0	50.0	50.0	50.0
Soybean meal[e]	100.0	100.0	100.0	100.0
Soybean protein concentrate[f]	130.0	130.0	130.0	130.0
Cassava starch[g]	110.0	110.0	110.0	110.0
Fish oil	35.0	38.8	42.5	46.2
Soybean oil	35.0	38.8	42.5	46.2
Vitamin and mineral additives[h]	10.0	10.0	10.0	10.0
Monocalcium phosphate	15.0	15.0	15.0	15.0
Choline chloride	1.0	1.0	1.0	1.0
Microcrystalline cellulose	74.0	85.4	97.0	108.6
Proximate composition				
Crude protein	499.3	495.5	500.6	505.0
Lipid	90.9	82.6	88.2	86.2

[a]*Fishmeal: from Superprime, TASA Fish Product Co. Ltd, Peru.* [b]*Clostridium autoethanogenum protein: from Hebei Shoulang New Energy Technology Co., Ltd.* [c]*Blood meal: from Beijing Yangyuan Veterinary Medicine Technology Co., Ltd, Beijing, China.* [d]*Gluten: from Henan Midaner Trading Co., Ltd, Xinzheng, Henan, China.* [e]*Soybean meal: from Qingdao Bohai Agricultural Development Co., Ltd, Qingdao, China.* [f]*Soybean protein concentrate: from Yihai grain and oil industry Co., Ltd, Taizhou, Jiangsu, China.* [g]*Cassava starch: from Wuhan Yiteng Starch Co., Ltd, Wuhan, China.* [h]*Vitamin and mineral additives: from Guangdong Nutriera Group, Guangzhou, China.*

Biochemical Analysis

The activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), reduced glutathione (GSH), the content of malondialdehyde (MDA), and total antioxidant capacity (T-AOC) in the liver tissue were determined according to the instruction of the commercial kits. In addition, the plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (AKP) were tested using commercial kits according to the instructions of the manufacturer. All the commercial kits were from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNAs of liver and head kidney tissues were extracted according to the instruction of TRIzol Reagent (Ambion, Life Technologies, Carlsbad, CA, USA). M-MLV First-Strand Synthesis Kit (Invitrogen, Shanghai, China) was used to obtain cDNA by reversing transcription of total RNA. Real-time PCR was carried out with LightCycle® 480 II system using LightCycle 480 SYBR Green I Master Mix (Roche, Switzerland). The reactions of real-time PCR were based on that described by Su et al. (2017). In the present study, 18s and beta-actin (β-actin) were chosen as internal references for normalization. The primers used for qRT-PCR in largemouth bass were showed in **Table 2**. Each sample was run in duplicate, and the results were calculated with the means.

TABLE 2 | Real-time PCR primers sequences.

Gene	Primers	Primer sequence	Sources
β-*actin*	F (forward primer) R (reverse primer)	AAAGGGAAATCGTGCGTGAC AAGGAAGGCTGGAAGAGGG	Sun et al., 2020
18s	F R	CGGCTACCACATCCAAGGAA CCTGTATTGTTATTTTTCGTC ACTACCT	Sun et al., 2020
il-1β	F R	CGTGACTGACAGCAAAAAGA GG GATGCCCAGAGCCACAGTTC	Yu et al., 2019
il-8	F R	CGTTGAACAGACTGGGAGAGATG AGTGGGATGGCTTCATTATCT TGT	Yu et al., 2019
tnf	F R	CTTCGTCTACAGCCAGGCAT CG TTTGGCACACCGACCTCACC	Yu et al., 2019
bad	F R	CACATTTCGGATGCCACTAT TTCTGCTCTTCTGCGATTGA	Yin et al., 2020
bax	F R	TCTTCACTCAGTCCCACAAA ATACCCTCCCAGCCACC	Yin et al., 2020
bag	F R	ATGACCCGAGACACGACAC CATAACCTGGGCGAAGAAT	Yin et al., 2020
p53	F R	AGATTGAATGGTGGTGGG GTTCTGGCGGACTGGA	XM_03873 0857.1
bcl-2	F R	CCATCCACGACGAACCTG GGCGTATCGCTGCTCAAACT	Yin et al., 2020
caspase-3	F R	GCTTCATTCGTCTGTGTTC CGAAAAAGTGATGTGAGGTA	Yu et al., 2019
caspase-8	F R	GAGACAGACAGCAGACAACCA TTCCATTTCAGCAAACACATC	Yin et al., 2020
caspase-9	F R	CTGGAATGCCTTCAGGAGACG GG GGGAGGGGCAAGACAACA GGGTG	Yin et al., 2020
caspase-10	F R	CAAACCACTCACAGCGTCTAC AT TGGTTGGTTGAGGACAGAGAG GG	Yin et al., 2020

Western Blot

Liver tissues were cell lysed by RIPA lysis buffer (Beyotime Biotechnology, China) containing protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche, Basel, Switzerland). Proteins (40 mg) were separated on sodium dodecyl sulfate–polyacrylamide (SDS-PAGE) gels and then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked for 1 h by using 5% skimmed milk in TBST buffer (20 mM TrisHCl, 150 mM sodium chloride, 0.1% Tween 20, pH 7.5) and then were incubated overnight at 4°C by using the following specific primary antibodies: mammalian target of rapamycin phosphorylation (P-mTOR) Antibody (1:1,000, #2,971; Cell signaling, Danvers, MA, USA), mammalian target of rapamycin (mTOR) Antibody (1:1,000, #2,972; Cell signaling, Danvers, MA, USA), BCL-2 Antibody (1:1,000, ab32,124; Abcam), or autophagy microtubule-associated protein light chain 3A/B (LC3A/B) Antibody (1:1,000, #12,741; Cell

signaling, Danvers, MA, USA). After washing, membranes were incubated with a secondary antibody: glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1,000, ab8,245; Abcam). Wherein, GAPDH was used as an internal reference protein. The bands were acquired by ImageQuant LAS 4000mini (GE Healthcare Life Sciences) and quantified using Image J software (National Institutes of Health).

Histological Analysis in the Liver Tissue

To observe the pathological changes of the liver tissue, the tissues were fixed into 4% paraformaldehyde and were embedded in paraffin and cut into 4 μm sections. The method of TUNEL technique was adopted (Lu et al., 2019). The relative area (%) of the apoptotic nucleus was calculated by Image-Pro Plus 6.0.

Statistical Analysis

All data are presented as mean ± SE ($n \geq 3$), and one-way ANOVA was used to detect the significance of the differences between groups in SPSS 20 (SPSS Inc., Chicago, IL, USA) for windows. The significant difference level was considered $P < 0.05$.

TABLE 3 | The effects of dietary CAP inclusion on growth performance, feed utilization, and biometric parameters of juvenile largemouth bass.

	D1	D2	D3	D4
IBW (g)	15.03 ± 0.07	15.09 ± 0.10	14.97 ± 0.08	15.12 ± 0.07
FBW (g)	59.41 ± 2.59^{b}	65.56 ± 2.11^{b}	61.56 ± 0.51^{b}	49.30 ± 2.40^{a}
WG (%)	297.54 ± 23.61^{b}	330.14 ± 13.12^{b}	297.52 ± 2.18^{b}	219.57 ± 23.52^{a}
SGR (%/d)	2.45 ± 0.08^{b}	2.62 ± 0.05^{b}	2.52 ± 0.01^{b}	2.11 ± 0.10^{a}
FCR	0.76 ± 0.03^{b}	0.73 ± 0.03^{b}	0.89 ± 0.02^{c}	0.58 ± 0.06^{a}
FR (% BW/d)	1.57 ± 0.08^{b}	1.57 ± 0.09^{b}	1.84 ± 0.04^{b}	1.07 ± 0.15^{a}
HSI (%)	2.60 ± 0.20^{b}	2.46 ± 0.14^{b}	2.49 ± 0.19^{b}	1.72 ± 0.21^{a}

CAP, Clostridium autoethanogenum protein; initial body weight (IBW, g) = initial body weight/initial number of fish; final body weight (FBW, g) = final body weight/final number of fish; weight gain (WG, %) = 100 × (final body weight − initial body weight)/initial body weight; specific growth rate (SGR, %/day) = 100 × (Ln final individual weight − Ln initial individual weight)/number of feeding days; Feed conversion ratio (FCR) = feed consumed/weight gain; feeding rate (FR, % BW/d) = dry weight of feed × 100/[(initial body weight + final body weight)/2]/days; hepatosomatic index (HSI, %) = (liver weight/body weight) × 100. Values were presented as means ± SEM (n = 3), and bars with different letters indicated significantly differences (P < 0.05).

RESULTS

Growth Performance and Morphometric Parameters

The results of growth, feed utilization, and morphological indices of largemouth bass fed experimental diets were shown in **Table 3**. After 8-week feeding, there was no significant difference between group D2 and the control group D1 from the perspective of various growth indicators. However, weight gain (WG), specific growth rate (SGR), feeding rate (FR), and hepatosomatic index (HSI) of the D4 group were significantly lower than in D1, D2, and D3 groups ($P < 0.05$), while there were no significant differences of growth performance between the three groups ($P > 0.05$). Besides, the feed conversion ratio (FCR) of the D1 and D2 groups also was significantly higher than D3 and D4 groups ($P < 0.05$).

Biochemical Parameters

The non-specific immunity-related parameters of plasma in largemouth bass were presented in **Figure 1**. The content of AKP in plasma increased significantly with dietary CAP concentrations ($P < 0.05$). Plasma ALT and AST levels were significantly lower in the D1 and D2 groups than in D3 and D4 groups ($P < 0.05$), and there were no significant differences between D1 and D2 groups ($P > 0.05$).

Anti-Oxidant-Related Parameters in the Liver Tissue

Figure 2 showed the activities of anti-oxidant enzymes. At the end of the 8-week feeding trial, the effects of dietary CAP inclusion on the anti-oxidation were compared among D1, D2, D3, and D4 groups. When the replacement level of fishmeal was exceeded 25%, the activities of T-AOC and SOD in the liver tissues were reduced significantly ($P < 0.05$). The activity of GSH-Px in liver tissues of group D2 was highest among all groups ($P < 0.05$), but there were no significant differences among the other three groups ($P > 0.05$). The MDA content of groups D3 and D4 was significantly higher than group D1 and D2 ($P < 0.05$), while there were no significant differences between D1 and D2 groups. Meanwhile, no significant difference between CAT and GSH was found among all groups ($P > 0.05$).

Gene Expression Related to Immunity in the Head Kidney Tissue

To investigate the immune status of CAP inclusion in the body, the mRNA expression of related genes was performed and shown in **Figure 3**. Compared with the control group D1, the transcriptional expressions of major inflammatory factors of interleukin-1β (il-1β), interleukin-8 (*il-8*), and tumor necrosis factor (*tnf*) were significantly downregulated in head kidney tissues of D3 and D4 groups ($P < 0.05$), while there was no difference in the expression of *il-1*β and *tnf* between D1 and D2 groups ($P > 0.05$).

Transcriptional and Protein Expression of Apoptosis and Autophagy in the Liver Tissue

Dietary inclusion of CAP had a significant effect on mRNA expression of apoptosis in the liver tissue of largemouth bass (**Figures 4A–I**). Results showed that the mRNA expressions of apoptosis-promoting genes (*bcl-2*-associated death protein [*bad*] and *bcl-2*-assoxicated *x* protein; *bag* [*bax*]) and anti-apoptosis-related genes (tumor protein 53 [*p53*] and b-cell lymphoma-2 [*bcl-2*]) in D3 and D4 groups were significantly higher than D1 and D2 groups, while there is no significant difference between D1 and D2 groups ($P < 0.05$). Moreover, the apoptotic *C. elegans* death gene-3 like caspases (cysteine-aspartic proteases-3 [*caspase-3*], cysteine-aspartic proteases-8 [*caspase-8*], cysteine-aspartic proteases-9 [*caspase-9*], and cysteine-aspartic

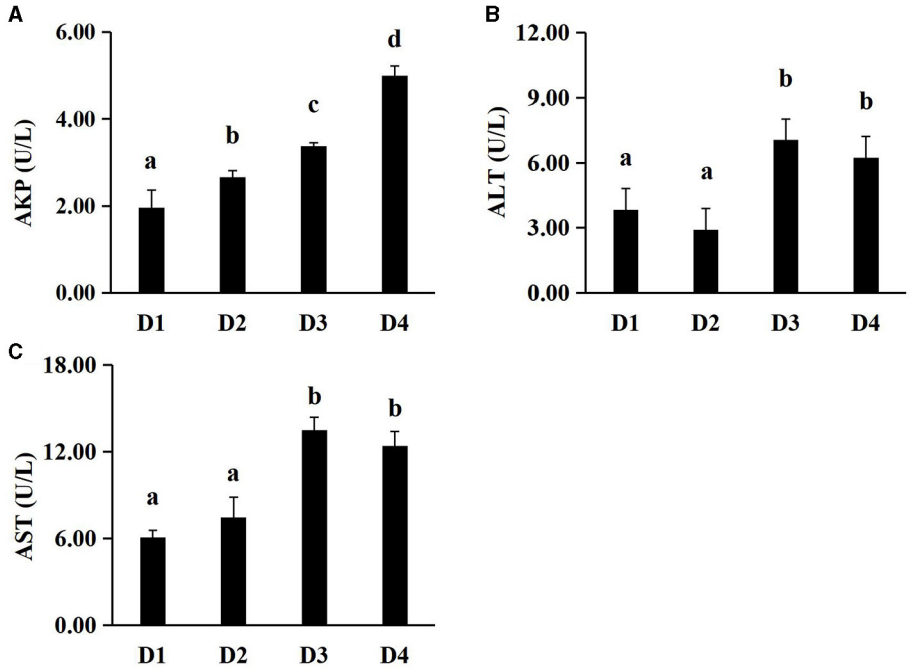


FIGURE 1 | Effects of dietary CAP inclusion on plasma AKP, ALT and AST in largemouth bass after an 8-week feeding trial. **(A)** AKP, alkaline phosphatase; **(B)** ALT, alanine aminotransferase; **(C)** AST, aspartate aminotransferase. Values were presented as means ± SEM ($n = 3$ at least), and bars with different letters indicated significant differences ($P < 0.05$). CAP, *Clostridium autoethanogenum* protein.

proteases-10 [*caspase-10*]) had the same phenomenon. To further understand the effects of dietary CAP on apoptosis and autophagy of juvenile largemouth bass, the expression of related proteins was detected and shown in **Figures 4J–N**. Dietary CAP inclusion significantly inhibited TOR phosphorylation (P-mTOR/mTOR) in largemouth bass ($P < 0.05$). Compared with the control group D1, the expressions of the apoptosis-related protein (bcl-2) and autophagy-related protein (LC3A/B-I *and* LC3A/B-II) were significantly induced in D3 and D4 groups ($P < 0.05$).

Histological Analysis

The histologic results of liver tissues are shown in **Figure 5A**. Apoptosis was stimulated by the grade levels of CAP inclusion, as the apoptotic rate (**Figure 5B**) of D3 and D4 groups was significantly higher than other groups ($P < 0.05$), while there was no significant difference between D1 and D2 groups ($P > 0.05$).

DISCUSSION

Clostridium autoethanogenum protein, a kind of SCP, is a by-product of ethanol production. In the production process, clean energy sources, such as ethanol and bacterial protein, are finally obtained through five processes of gas pretreatment, fermentation, distillation dehydration, bacterial separation, spray drying, and sewage treatment, and about 1,500 t of bacterial protein can be obtained for each 10,000 t of ethanol production, thereby having great development potential. The utilization of CAP in aquatic feeds greatly promotes the conversion and reuse of industrial wastes and reduces the emission of harmful gases. However, as CAP belongs to microbial fermentation products, and CA has not yet entered the feed additive catalog of China, further research is needed to comprehensively evaluate the biological safety of CAP.

In the present study, compared with the control group, the CAP replacement fish meal level of 25 and 50% groups had no significant effect on the growth performance (WG, SGR, FR, and HSI) of largemouth bass. This is similar to the study in grass carp that dietary CAP lower than 50 g/kg has no negative effect on the growth performance of the fish (Wei et al., 2018). Similarly, In the pearl gentian grouper (♀*epinephelus fuscoguttatus* × ♂*epinephelus lanceolatu*) and largemouth bass (*Micropterus salmoides*), cottonseed protein concentrate (CPC) could replace 24% fish meal, and a composite mixture of shrimp hydrolysate and plant proteins could take the place of 30% fish meal without affecting growth performance (Chen G. et al., 2020; Sla et al., 2020). This may be related to the fact that bacterial proteins are highly digestible and easy to use. However, when the level of CAP replacement fish meal exceeds 50%, it will have a negative impact on the growth performance of largemouth bass. On the one hand, the content of lysine in CAP is low, and lysine has a great influence on the growth center (Chen Y. et al., 2020; Wang et al., 2020). Therefore, the relative lack of lysine content in feeds with high CAP replacement levels may cause the growth performance

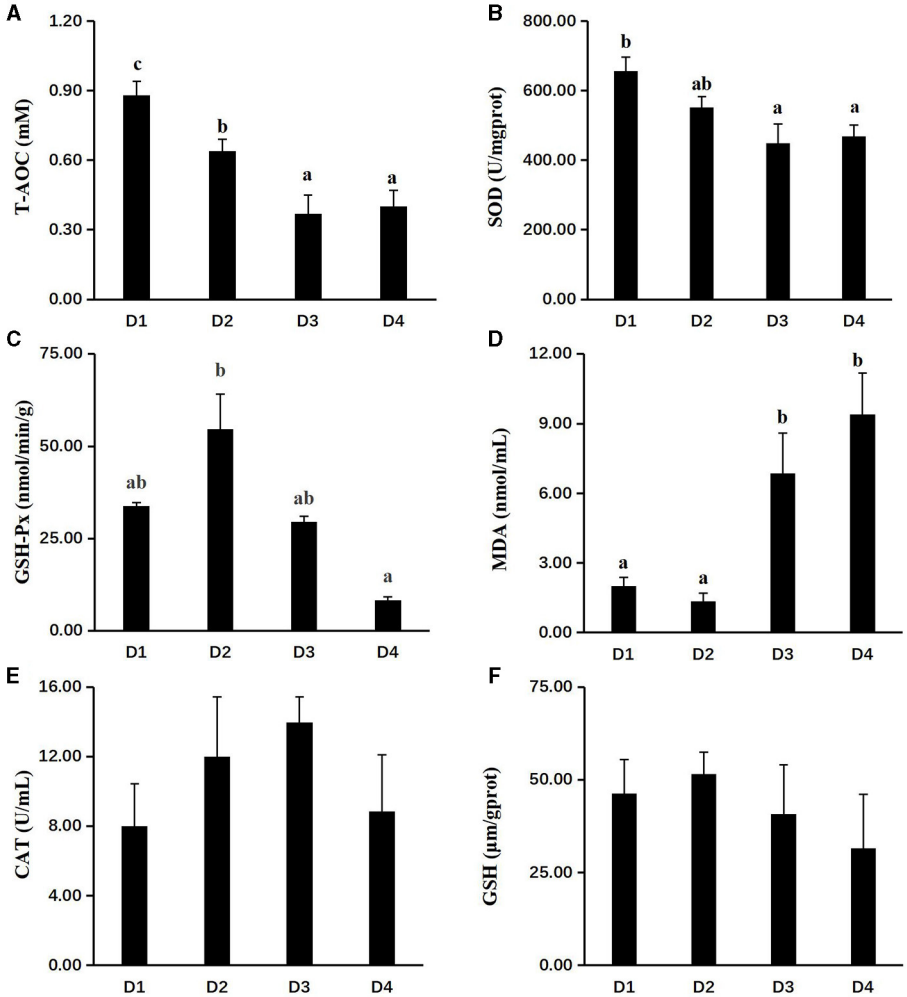


FIGURE 2 | Effects of dietary CAP inclusion on anti-oxidative capacity in liver. **(A)** T-AOC, total antioxidant capacity; **(B)** SOD, superoxide dismutase; **(C)** GSH-Px, glutathione peroxidase; **(D)** MAD, malondialdehyde; **(E)** CAT, catalase; and **(F)** GSH, reduced glutathione. Values were presented as means ± SEM ($n = 3$ at least), and bars with different letters indicated significant differences ($P < 0.05$). CAP, *Clostridium autoethanogenum* protein.

of the D4 group to decline. On the other hand, palatability may be an important reason why CAP cannot completely replace fish meal. Similar results were reported in a study on rainbow trout (*Oncorhynchus mykiss Walbaum*), which is replacing fish meal with *Methylobacterium extorquens* SCP (Hardy et al., 2018). Therefore, according to the results of this experiment, the level of dietary CAP replacement fish meal should be <50% and will not negatively affect the growth performance of largemouth bass. Importantly, the health status of fish may be a better explanation for the difference in growth performance.

Alanine aminotransferase, AST, and AKP are important indicators to measure liver health. Normal blood has relatively low contents of ALT, AST, and AKP. However, when the corresponding cells are damaged, the permeability of the cell membrane will be increased, and ALT, AST, and AKP in the cytoplasm will be released into the blood, resulting in an increase in blood contents, and suggesting extensive damage to the liver parenchyma. In the present study, ALT and AST in plasma were not significantly variational when the replacement of fishmeal was 25%, compared with the control group D1, but their contents in plasma increased significantly after the fishmeal was replaced by more than 25%. Similar results also appeared in other SCP substitution experiments (e.g., Abdel-Tawwab et al., 2010; Omar et al., 2012; Zhang et al., 2018). With the increase of substitution level, the plasma AKP content increased significantly. Anders and Stefan also found that in the experiment of feeding rainbow trout with bacterial protein instead of fish meal, excessive replacement may impair liver health (Anders and Stefan, 1993). These results implied that when the replacement amount of CAP exceeds 50%, it may affect the liver health of largemouth bass.

Apoptosis, a marker of DNA fragmentation (Rhee et al., 2013), is a process in which cells end their lives voluntarily,

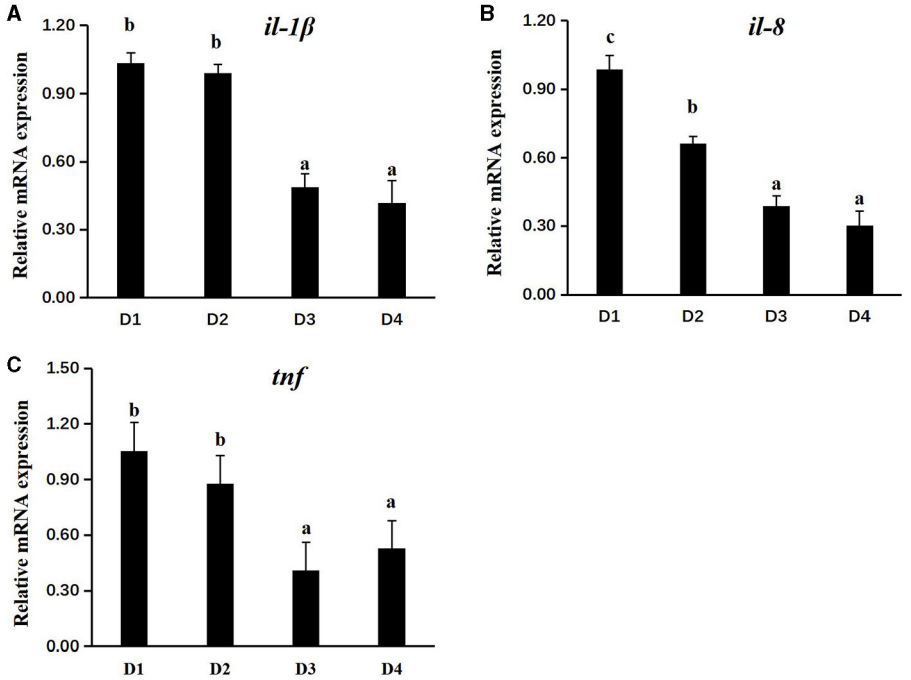


FIGURE 3 | Effects of dietary CAP on the transcriptional level of immune-related genes in the head kidney (*il-1*β, *il-8*, and *tnf*) of juvenile largemouth bass. **(A)** *il-1*β, interleukin-1β; **(B)** *il-8,* interleukin-8; and **(C)** *tnf*, tumor necrosis factor. Values were presented as means ± SEM ($n = 3$ at least), and bars with different letters indicated significantly differences ($P < 0.05$). CAP, *Clostridium autoethanogenum* protein.

and it is the basic to maintain cell homeostasis. Apoptosis can be activated by a variety of cellular signals, such as increased intracellular Ca^{2+} concentration, reactive oxygen species (ROS), such as hydroxyl radicals caused by oxidative damage (ROS), growth factors, and hormone stimulation caused by oxidative damage (Gump and Thorburn, 2011). As this study showed, juvenile largemouth bass fed with the D2 group did not have a significant effect on the level of apoptosis compared with the control group, while D3 and D4 groups had higher DNA fragmentation, supporting that high-level CAP could increase the level of apoptosis in largemouth bass. The induction effect of CAP on apoptosis may be correlated with the caspase-dependent pathway. The caspase-dependent pathway is involved in the modulation of apoptosis (Ji et al., 2011). Among the caspases, *caspase-3* and *caspase-9* have been identified as a key executor of cell apoptosis (Li et al., 1997; Deng et al., 2009). In the cell apoptosis pathway which is independent of the death receptor, activated *caspase-9* initiated a protease cascade leading to the rapid activation of *caspase-3* (Li et al., 1997). Moreover, the activation of the intrinsic apoptotic pathway leads to changes in gene expression, such as anti-apoptotic (*bcl-2*, *bcl-xl*, and *bag*) and pro-apoptotic factors (*bax* and bad), which respond to death signals (Breckenridge and Xue, 2004). Compared with D1 and D2 groups, significant upregulation in *caspase-3* and *caspase-9* mRNA expression was observed in D3 and D4 groups, respectively, which noted that excessive CAP replacement level could induce the upregulation of *caspase-3* and *caspase-9* mRNA expressions. On the one hand, a significant positive correlation was observed between *caspase-9* mRNA level and the mRNA levels of *caspase-3*, *caspase-8*, and *caspase-10*, indicating that excessive CAP upregulated *caspase-9* mRNA expression partly through increasing *caspase-3*, *caspase-8*, and *caspase-10* mRNA expressions. On the other, excessive protein replacement level of CAP can lead to increased mRNA expressions of pro-apoptotic (*bax* and *bad*) and anti-apoptotic (*bcl-2*, *p53*, and *bag*)-related factors. These results are consistent with research reported in Atlantic salmon (*Salmo salar*) that soybean meal activated *caspase-3* and induced apoptosis (Bakke-McKellep et al., 2007). Autophagy, in a conservative way, at a basic level is necessary to maintain cell self-explanation and participate in the pathophysiological processes of the immune system and removal of micro-organisms, tumors, and other diseases (Jing and Lim, 2012; Ryter et al., 2014). At present, studies have found that mTOR inhibits autophagy and apoptosis (Li L. et al., 2021). Besides, many classical apoptotic signaling pathways or proteins have been found to have complex interactions with the regulation of autophagy (Su et al., 2013). Both LC3A/B-I and LC3A/B-II were considered molecular markers of autophagy in cells, and the content of LC3-II was directly proportional to the degree of autophagy. It is worth pondering that CAP inhibits the mTOR signaling pathway and thus activates the autophagy and apoptosis pathway. It is possible that a high level of CAP substitutes for fish meal and lacks some essential amino acids, resulting in energy deficiency and activation of autophagy and apoptosis pathway (Liu et al., 2013).

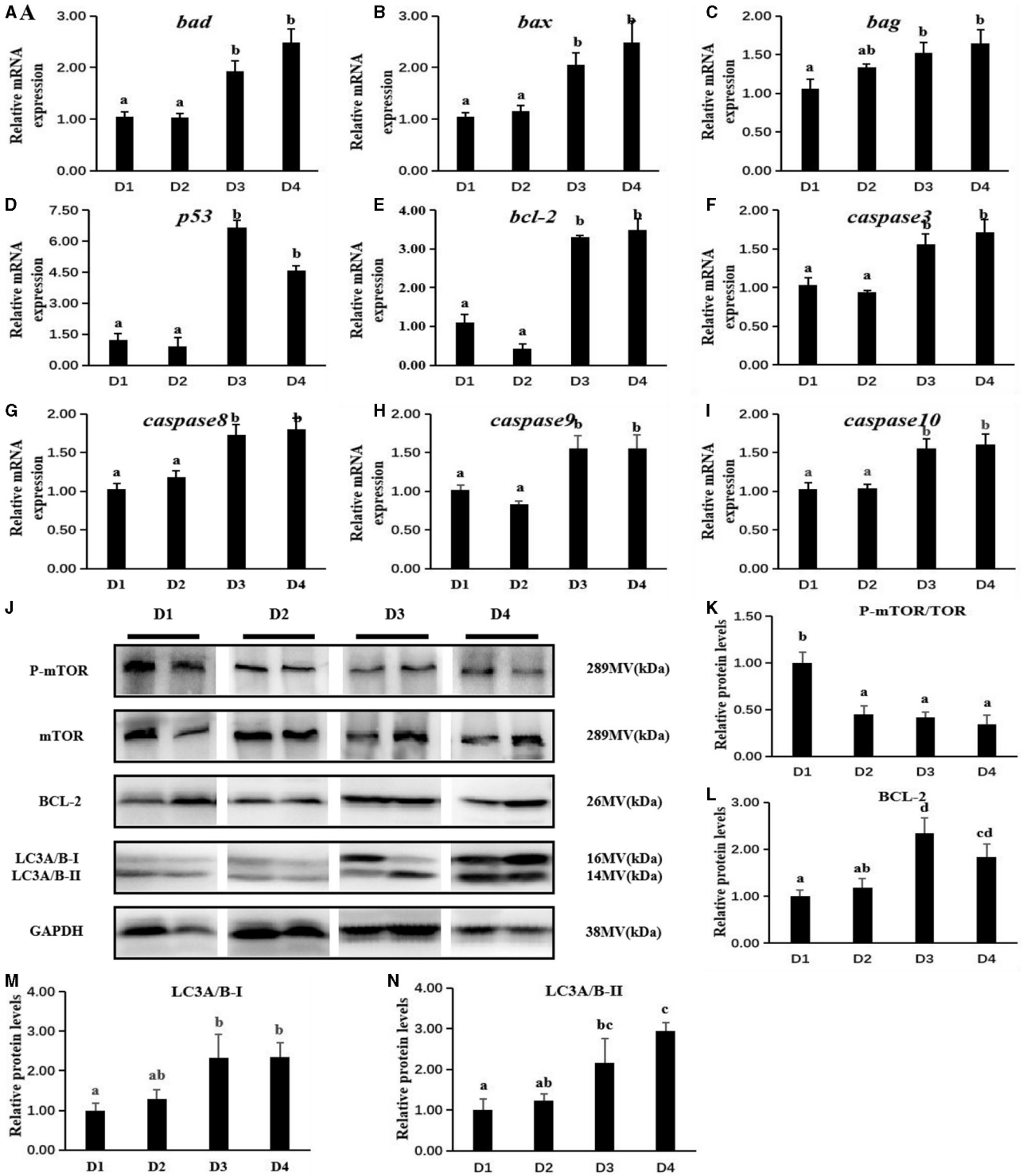


FIGURE 4 | Effects of dietary CAP inclusion on the expression of apoptosis-related genes **(A–I)** and autophagy-related proteins **(J–N)** of largemouth bass in liver tissue after an 8-week feeding trial. *bad*, *bcl-2*-associated death protein; *bax, bcl-2*-associcated *x* protein; *bag*, bcl-2-associated athanogene; *p53*, tumor protein 53; *bcl-2*, b-cell lymphoma-2; *caspase3*, cysteine-aspartic proteases-3; *caspase8*, cysteine-aspartic proteases-8; *caspase9*, cysteine-aspartic proteases-9; *caspase10*, cysteine-aspartic proteases-10; P-mTOR, mammalian target of rapamycin phosphorylation; mTOR, mammalian target of rapamycin; LC3A/B, autophagy microtubule-associated protein light chain 3A/B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Values were presented as means ± SEM ($n = 3$ at least), and bars with different letters indicated significant differences ($P < 0.05$). CAP, *Clostridium autoethanogenum* protein.

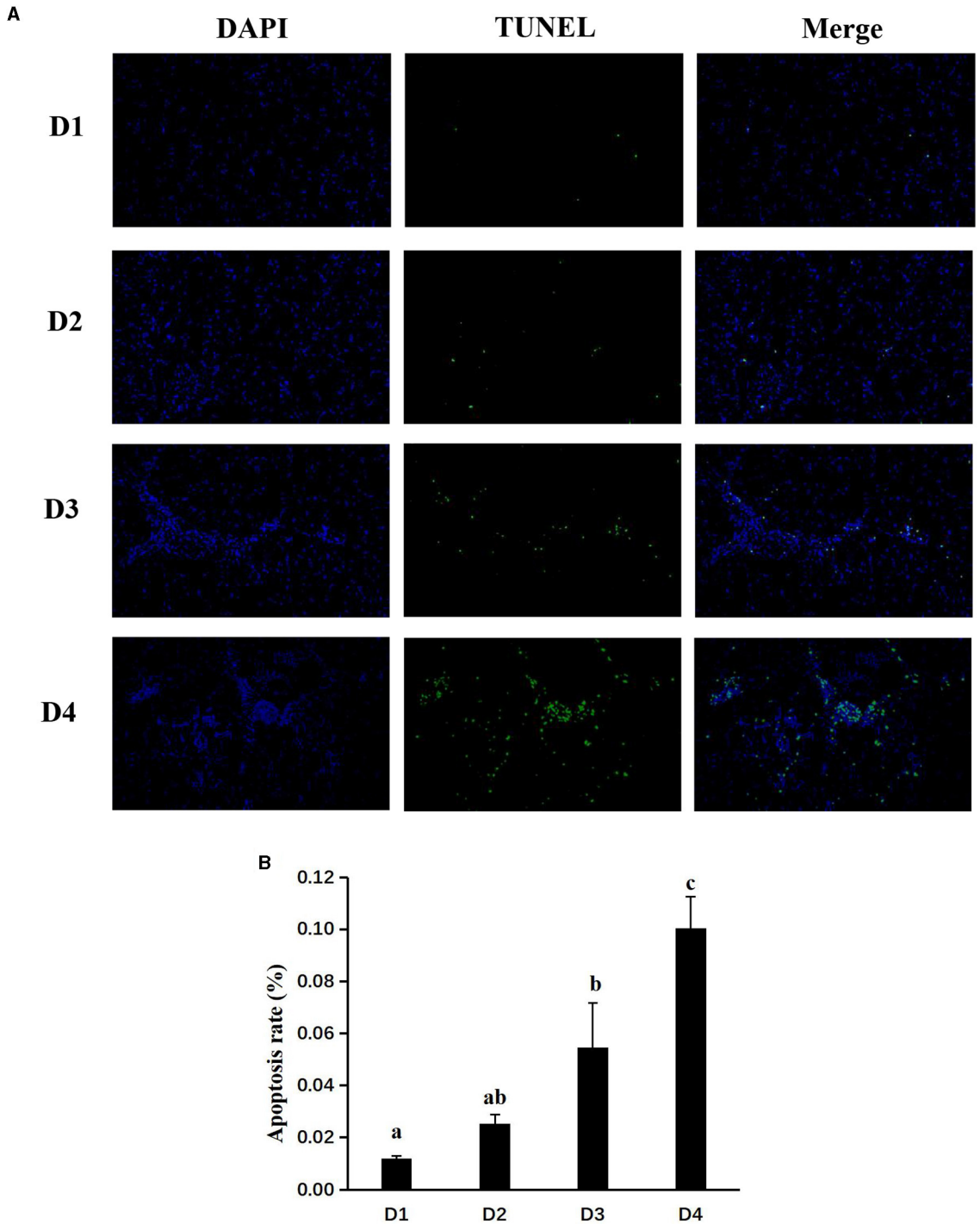


FIGURE 5 | Effects of dietary CAP inclusion on TUNEL **(A)** and apoptosis rate **(B)** in liver tissues of largemouth bass Values were presented as means ± SEM ($n = 3$ at least), and bars with different letters indicated significant differences ($P < 0.05$). CAP, *Clostridium autoethanogenum* protein; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Most importantly, apoptosis and autophagy are consequences of exceeding ROS (Jin et al., 2011). Studies showed that elevated ROS levels caused oxidative damage in various organs, such as the liver tissue of fish (Jiang et al., 2011). The antioxidant enzyme system, consisting of SOD, GSH-Px, CAT, GSH, and T-AOC, is an antioxidant defense mechanism of the body

that is closely related to health and can prevent the damage caused by reactive oxygen species and maintain the balance between the generation and removal of free radicals. When the oxidation-antioxidant balance tilts toward the oxidation direction, oxidative stress will occur and cause dysfunction and oxidative damage (Zeng et al., 2017). In the present experiment, it was found that the increased substitution level of CAP did not cause changes in the liver tissue of CAT and GSH activity. This is similar to the previous study that yeast culture has no effect on the antioxidant capacity of gibel carp (*Carassius auratus gibelio CAS III*), (Zhang et al., 2018). However, it caused a significant decrease in T-AOC, a comprehensive indicator of antioxidant activity. MDA, the final product of lipid peroxidation, is usually used to evaluate the health of the body (Fujioka and Shibamoto, 2005) and measure endogenous oxidative damage (Ding et al., 2015). When CAP substitutes for fishmeal at 25%, there was no difference between the MDA content in the liver compared with the control group D1, but when it exceeded 25%, the MDA content in the liver increased significantly. It showed that high levels of CAP substitution affected the antioxidant capacity of juvenile largemouth bass, leading to the accumulation of free radicals, which damaged liver health. Inversely, replacing fish meal with CAP in feed for juvenile black sea bream did not affect the antioxidant capacity of the liver tissue (Chen Y. et al., 2020). This conflicting result may be due to different fish and protein-processing methods used, which may have an impact on the availability and digestibility of nutrients.

As we all know, autophagy and apoptosis also belong to the category of immune homeostasis. Apoptosis is a programmed cell death that occurs cell-autonomously. The most important feature of immune autophagy is the capture and degradation of intracellular microorganisms by autophagy (Faure and Lafont, 2013). Autophagy occurs inside of cells, mainly to clean up the internal environment of cells. When the body is damaged by microbial adaptation mechanisms, autophagy can easily capture the pathogenic microorganisms invading cells through the autophagic aptamers known as sequestosome 1/p62 sample receptors (*slr*), (Deretic, 2012). *Slr* is involved in the release of pro-inflammatory factor signals through the *tnf* receptor-associated factor or by activating *caspase-8* to promote cell death. Common pro-inflammatory factors are *il-1*β, *il-8*, and *tnf*, which are also markers of inflammation and participate in host defense against microbial pathogens (Secombes et al., 2001; Corripio-Miyar et al., 2007). *Il-1*β signal initiates the production and secretion of pro-inflammatory mediators, such as *il-8*, which can respond to inflammatory stimuli through several cell types. An exception is *il-8* expression, which has been reported to affect the *tnf* expression (Giraudo et al., 1998). Notably, studies have shown that autophagy is related to the activation of inflammatory factors (Shi et al., 2012). In the present study, the head kidney *il-1*β, *il-8*, and *tnf* expressions were downregulated in the CAP substitution groups D3 and D4. Similarly, dietary supplementation of 25% yeast had significantly downregulated the expression of *il-1*β and *il-8* in the Atlantic salmon (*Salmo salar*), (Sahlmann et al., 2019). *Il-1*β promotes rapid response to infection by inducing a cascade of reactions leading to inflammation. These results indicated that more than 50% replacement of fish meal by CAP in the diet might result in decreased immunity of largemouth bass, which was consistent with the above results in the present study.

CONCLUSIONS

In conclusion, the present results indicated that replacing <50% of dietary fish meal by CAP had no negative effects on growth performance, anti-oxidation capacity, immune response, autophagy, and apoptosis system of largemouth bass. However, excessive substitution by CAP may damage liver health by inhibiting mTOR phosphorylation to activate the autophagy and apoptosis pathways. Together, the utilization of CAP in aqua-feeds will promote the conversion and reuse of industrial waste, reduce the emission of harmful gases, and has a huge potentiality.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Institute of Hydrobiology, Chinese Academy of Science.

AUTHOR CONTRIBUTIONS

DH designed the experiment and revised the manuscript. QL and LX performed the experiment and drafted the manuscript. YL, JS, and HL performed the statistical analysis. YY contributed to the sample analysis. JJ, HL, XZ, and SX provided suggestions on the experimental design and contributed to the manuscript modification. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by the National Key R&D Program of China (2018YFD0900400 and 2019YFD0900200), China Agriculture Research System (CARS-46), the National Natural Science Foundation of China (31672670, 31972771, and 31972805), the Southern Marine Science and Engineering Guangdong Laboratory (Zhanjiang), (ZJW-2019-06), Fund Project in State Key Laboratory of Freshwater Ecology and Biotechnology (2019FBZ02 and 2019FBZ05), Hubei High-tech Innovation and Business Incubation Center (2019-02-055), and Science and Technology Project of Wuhan (2019020701011459).

ACKNOWLEDGMENTS

The authors thank Guanghan Nie for technical support with the research system.

REFERENCES

Abdel-Tawwab, M., Mousa, A. A., and Mohammed, M. A. (2010). Use of live baker's yeast, *Saccharomyces cerevisiae*, in practical diet to enhance the growth performance of galilee tilapia, *Sarotherodon galilaeus* (L.), and its resistance to environmental copper toxicity. *J. World Aquac. Soc.* 41, 214–223. doi: 10.1111/j.1749-7345.2010.00361.x

Anders, K., and Stefan, A. (1993). Nutritive value of two bacterial strains of single-cell protein for rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 109, 119–130. doi: 10.1016/0044-8486(93)90209-H

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

Breckenridge, D. G., and Xue, D. (2004). Regulation of mitochondrial membrane permeabilization by BCL-2 family proteins and caspases. *Curr. Opin. Cell Biol.* 16, 647–652. doi: 10.1016/j.ceb.2004.09.009

Chen, G., Yin, B., Liu, H., Tan, B., Dong, X., Yang, Q., et al. (2020). Effects of fishmeal replacement with cottonseed protein concentrate on growth, digestive proteinase, intestinal morphology and microflora in pearl gentian grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatu*). *Aquac. Res.* 51, 2870–2884. doi: 10.1111/are.14626

Chen, Y., Sagada, G., Xu, B., Chao, W., Zou, F., Ng, W., et al. (2020). Partial replacement of fishmeal with *clostridium autoethanogenum* single-cell protein in the diet for juvenile black sea bream (*acanthopagrus schlegelii*). *Aquac. Res.* 51, 1000–1011. doi: 10.1111/are.14446

Corripio-Miyar, Y., Bird, S., Tsamopoulos, K., and Secombes, C. J. (2007). Cloning and expression analysis of two pro-inflammatory cytokines, IL-1 beta and IL-8, in haddock (*Melanogrammus aeglefinus*). *Mol. Immunol.* 44, 1361-1373. doi: 10.1016/j.molimm.2006.05.010

Davies, S. J., and Wareham, H. (1988). A preliminary evaluation of an industrial single cell protein in practical diets for tilapia (*Oreochromis mossambicus peters*). *Aquaculture* 73, 189–199. doi: 10.1016/0044-8486(88)90053-1

Deng, J., Yu, L., Liu, C., Yu, K., and Zhou, B. (2009). Hexbromocyclododecane-induced developmental toxicity and apoptosis in zebra fish embryos. *Aquat. Toxicol.* 93, 29–36. doi: 10.1016/j.aquatox.2009.03.001

Deretic, V. (2012). Autophagy as an innate immunity paradigm: expanding the scope and repertoire of pattern recognition receptors. *Curr. Opin. Immunol.* 24, 21–31. doi: 10.1016/j.coi.2011.10.006

Ding, Z., Zhang, Y., Ye, J., Du, Z., and Kong, Y. (2015). An evaluation of replacing fish meal with fermented soybean meal in the diet of *Macrobrachium nipponense*: growth, non-specific immunity, and resistance to *Aeromonas hydrophila*. *Fish Shellfish Immunol.* 44, 295–301. doi: 10.1016/j.fsi.2015.02.024

Faure, M., and Lafont, F. (2013). Pathogen-induced autophagy signaling in innate immunity. *J. Innate Immun.* 5, 456–470. doi: 10.1159/000350918

Fishery Bureau (2020). *China Fishery Statistical Yearbook 2020*. Beijing: China Agriculture Press.

Francis, G., Makkar, H., and Becker, K. (2001). Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture* 199, 197–227. doi: 10.1016/S0044-8486(01)00526-9

Fujioka, K., and Shibamoto, T. (2005). Improved malonaldehyde assay using headspace solid-phase microextraction and its application to the measurement of the antioxidant activity of phytochemicals. *J. Agric. Food Chem.* 53, 4708–4713. doi: 10.1021/jf050297q

Giraudo, E., Primo, L., Audero, E., Gerber, H. P., Koolwijk, P., Soker, S., et al. (1998). Tumor necrosis factor-alpha regulates expression of vascular endothelial growth factor receptor-2 and of its co-receptor neuropilin-1 in human vascular endothelial cells. *J. Biol. Chem.* 273, 22128–22135. doi: 10.1074/jbc.273.34.22128

Gong, Y. L., Yang, F., Hu, J. P., Liu, C., Liu, H. K., Han, D., et al. (2019). Effects of dietary yeast hydrolysate on the growth, antioxidant response, immune response and disease resistance of largemouth bass (*Micropterus salmoides*). *Fish Shellfish Immunol.* 94, 548–557. doi: 10.1016/j.fsi.2019.09.044

Gump, J. M., and Thorburn, A. (2011). Autophagy and apoptosis: what is the connection? *Trends Cell Biol.* 21, 387–392. doi: 10.1016/j.tcb.2011.03.007

Hardy, R. W., Patro, B., Pujol-Baxley, C., Marx, C. J., and Feinberg, L. (2018). Partial replacement of soybean meal with *Methylobacterium extorquens* single-cell protein in feeds for rainbow trout (*Oncorhynchus mykiss walbaum*). *Aquac. Res.* 49, 2218–2224. doi: 10.1111/are.13678

Henry, M., Gasco, L., Piccolo, G., and Fountoulaki, E. (2015). Review on the use of insects in the diet of farmed fish: past and future. *Anim. Feed Sci. Technol.* 203, 1–22. doi: 10.1016/j.anifeedsci.2015.03.001

Ji, H., Li, J., and Liu, P. (2011). Regulation of growth performance and lipid metabolism by dietary *n*-3 highly unsaturated fatty acids in juvenile grass carp, *Ctenopharyngodon idellus*. *Comp. Biochem. Physiol. B,. Biochem. Mol. Biol.* 159, 49–56. doi: 10.1016/j.cbpb.2011.01.009

Jiang, W. D., Wu, P., Kuang, S. Y., Liu, Y., Jiang, J., Hu, K., et al. (2011). Myo-inositol prevents copper-induced oxidative damage and changes in antioxidant capacity in various organs and the enterocytes of juvenile Jian carp (*cyprinus carpio var. jian). Aquat. Toxicol.* 105, 543–551. doi: 10.1016/j.aquatox.2011.08.012

Jin, Y. X., Zheng, S. S., Pu, Y., Shu, L. J., Sun, L. W., Liu, W. P., et al. (2011). Cypermethrin has the potential to induce hepatic oxidative stress, DNA damage and apoptosis in adult zebrafish (*Danio rerio*). *Chemosphere* 82, 398–404. doi: 10.1016/j.chemosphere.2010.09.072

Jing, K., and Lim, K. (2012). Why is autophagy important in human diseases? *Exp. Mol. Med.* 44, 69–72. doi: 10.3858/emm.2012.44.2.028

Kaushik, S. J., Cravedi, J. P., Lalles, J. P., Sumpter, J., Fauconneau, B., and Laroche, M. (1995). Partial or total replacement of fish meal by soybean protein on growth, protein utilization, potential estrogenic or antigenic effects, cholesterolemia and flesh quality in rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* 133, 257–274. doi: 10.1016/0044-8486(94)00403-B

Li, L., Wang, J., Feng, L., Fan, J. T., Wang, J., Tan, N. H., et al. (2021). *Rubioncolin C*, a natural naphthohydroquinone dimer isolated from Rubia yunnanensis, inhibits the proliferation and metastasis by inducing ROS-mediated apoptotic and autophagic cell death in triple-negative breast cancer cells. *J. Ethnopharmacol.* 277:114184. doi: 10.1016/j.jep.2021.114184

Li, M. Y., Liang, H. L., Xie, J., Chao, W., Zhou, F. Q., Ge, X. P., et al. (2021). Diet supplemented with a novel *Clostridium autoethanogenum* protein have a positive effect on the growth performance, antioxidant status and immunity in juvenile Jian carp (*Cyprinus carpio var. Jian). Aquac. Rep.* 19:100572. doi: 10.1016/j.aqrep.2020.100572

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., et al. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91, 479–489. doi: 10.1016/S0092-8674(00)80434-1

Liu, G., Bi, Y., Wang, R., and Wang, X. (2013). Self-eating and self-defense: autophagy controls innate immunity and adaptive immunity. *J. Leukoc. Biol.* 93, 511–519. doi: 10.1189/jlb.0812389

Lu, D. L., Ma, Q., Wang, J., Li, L. Y., Han, S. L., Limbu, S. M., et al. (2019). Fasting enhances cold resistance in fish through stimulating lipid catabolism and autophagy. *J. Physiol.* 597, 1585-1603. 13/JP277091 doi: 10.1113/JP277091

Luo, L., Xue, M., Wu, X. F., Cai, X. F., and Liang, Y. M. (2010). Partial or total replacement of fishmeal by solvent-extracted cottonseed meal in diets for juvenile rainbow trout (*Oncorhynchus mykiss*). *Aquac. Nutr.* 12, 418–424. doi: 10.1111/j.1365-2095.2006.00443.x

Mambrini, M., Roem, A. J., Carvèdi, J. P., Lallès, J. P., and Kaushik, S. J. (1999). Effects of replacing fish meal with soy protein concentrate and of DL-methionine supplementation in high-energy, extruded diets on the growth and nutrient utilization of rainbow trout, *Oncorhynchus mykiss*. *J. Anim. Sci.* 77, 2990–2999. doi: 10.2527/1999.77112990x

Maulu, S., Liang, H. L., Ge, X. P., Yu, H., Huang, D. Y., and Ke, J. (2021). Effect of dietary *Clostridium autoethanogenum* protein on growth, body composition, plasma parameters and hepatic genes expression related to growth and AMPK/TOR/PI3K signaling pathway of the genetically improved farmed tilapia (GIFT: *Oreochromis niloticus*) juveniles. *Anim. Feed Sci. Technol.* 276:114914. doi: 10.1016/j.anifeedsci.2021.114914

Maulu, S., Liang, H. L., Ke, J., Ren, M. C., Ge, X. P., Huang, D. Y., et al. (2020). Dietary *Clostridium autoethanogenum* protein modulates intestinal absorption, antioxidant status, and immune response in GIFT (*Oreochromis niloticus*) juveniles. *Aquac. Res.* 52, 5787-5799. doi: 10.1111/are.15454

Omar, S. S., Merrifield, D. L., Kühlwein, H., Williams, P., and Davies, S. J. (2012). Biofuel derived yeast protein concentrate (YPC) as a novel feed ingredient in carp diets. *Aquaculture* 330–333, 54–62. doi: 10.1016/j.aquaculture.2011.12.004

Rhee, J. S., Yu, I. T., Kim, B. M., Jeong, C. B., Lee, K. W., Kim, M. J., et al. (2013). Copper induces apoptotic cell death through reactive oxygen species-triggered oxidative stress in the intertidal copepod *Tigriopus japonicus*. *Aquat. Toxicol.* 132–133, 182–189. doi: 10.1016/j.aquatox.2013.02.013

Ryter, S. W., Mizumura, K., and Choi, A. M. (2014). The impact of autophagy on cell death modalities. *Int. J. Cell Biol.* 2014:502676. doi: 10.1155/2014/502676

Sahlmann, C., Djordjevic, B., Lagos, L., and Mydland, L. T., Øverland, M. (2019). Yeast as a protein source during smoltification of Atlantic salmon (*Salmo salar L.*), enhances performance and modulates health. *Aquaculture* 513:734396. doi: 10.1016/j.aquaculture.2019.734396

Secombes, C. J., Wang, T., Hong, S., Peddie, S., Crampe, M., Laing, K. J., et al. (2001). Cytokines and innate immunity of fish. *Dev. Comp. Immunol.* 25, 713–723. doi: 10.1016/S0145-305X(01)00032-5

Shi, C. S., Shenderov, K., Huang, N. N., Kabat, J., Abu-Asab, M., Fitzgerald, K. A., et al. (2012). Activation of autophagy by inflammatory signals limits IL-1β production by targeting ubiquitinated inflammasomes for destruction. *Nat. Immunol.* 13, 255–263 doi: 10.1038/ni.2215

Simpson, S., Allen, W. E., Conrado, R. J., and Molloy, S. (2016). *Gas Fermentation for the Production of Protein or Feed*. U.S. Patent No 0338380A1. Skokie, IL: U.S. Patent and LanzaTech, Inc.

Sitjà-Bobadilla, A., Pe?a-Llopis, S., Gómez-Requeni, P., Médale, F., Kaushik, S., and Pérez-Sánchez, J. (2005). Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*). *Aquaculture* 249, 387–400. doi: 10.1016/j.aquaculture.2005.03.031

Sla, B., Min, D. A., Hq, A., and Nca, C. (2020). 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

Su, J. Z., Gong, Y. L., Cao, S. P., Lu, F., Han, D., Liu, H. K., et al. (2017). Effects of dietary Tenebrio molitor meal on the growth performance, immune response and disease resistance of yellow catfish (*Pelteobagrus fulvidraco*). *Fish Shellfish Immunol.* 69, 59–66. doi: 10.1016/j.fsi.2017.08.008

Su, M., Mei, Y., and Sinha, S. (2013). Role of the crosstalk between autophagy and apoptosis in cancer. *J. Oncol.* 2013:102735. doi: 10.1155/2013/102735

Sun, J. L., Zhao, L. L., Liao, L., Tang, X. H., Cui, C., Liu, Q., et al. (2020). Interactive effect of thermal and hypoxia on largemouth bass (*Micropterus salmoides*) gill and liver: aggravation of oxidative stress, inhibition of immunity and promotion of cell apoptosis. *Fish Shellfish Immunol.* 98, 923–936. doi: 10.1016/j.fsi.2019.11.056

Utturkar, S. M., Klingeman, D. M., Bruno-Barcena, J. M., Chinn, M. S., Grunden, A. M., Kopke, M., et al. (2015). Sequence data for *Clostridium autoethanogenum* using three generations of sequencing technologies. *Sci. Data.* 2:150014. doi: 10.1038/sdata.2015.14

Wang, W., Xu, Y., Chi, S., Yang, P., and Song, F. (2020). Dietary lysine regulates body growth performance via the nutrient-sensing signaling pathways in largemouth bass (*Micropterus salmoides*). *Front. Mar. Sci.* 7:595682. doi: 10.3389/fmars.2020.595682

Wei, H., Huan, Y. U., Chen, X., Chao, W., Zou, F., Chen, P., et al. (2018). Effects of soybean meal replaced by *Clostridium autoethanogenum* protein on growth performance, plasma biochemical indexes and hepatopancreas and intestinal histopathology of grass carp (*Ctenopharyngodon idllus*). *Chinese. J. Anim. Nutr.* 30, 4190–4201.

Yang, P., Yang, W., He, M., Li, X., and Leng, X. (2020). Dietary synbiotics improved the growth, feed utilization and intestinal structure of largemouth bass (*Micropterus salmoides*) juvenile. *Aquac. Nutr.* 26, 590–600. doi: 10.1111/anu.13020

Yin, P., Xie, S. W., Zhuang, Z. X., He, X. S., Tang, X. P., Tian, L. X., et al. (2020). Dietary supplementation of bile acid attenuate adverse effects of high-fat diet on growth performance, antioxidant ability, lipid accumulation and intestinal health in juvenile largemouth bass (*Micropterus salmoides*). *Aquaculture* 531:735864. doi: 10.1016/j.aquaculture.2020.735864

Yu, H. H., Zhang, L. L., Chen, P., Liang, X. F., Cao, A. Z., Han, J., et al. (2019). Dietary bile acids enhance growth, and alleviate hepatic fibrosis induced by a high starch diet via AKT/FOXO1 and cAMP/AMPK/SREBP1 pathway in *Micropterus salmoides*. *Front. Physiol.* 10:1430. doi: 10.3389/fphys.2019.01430

Zeng, J., Chen, Y., Ding, R., Feng, L., Fu, Z. H., Yang, S., et al. (2017). Isoliquiritigenin alleviates early brain injury after experimental intracerebral hemorrhage via suppressing ROS-and/or NF-κB-mediated NLRP3 inflammasome activation by promoting Nrf2 antioxidant pathway. *J. Neuroinflammation* 14:119. doi: 10.1186/s12974-017-0895-5

Zhang, P. Y., Cao, S. P., Zou, T., Han, D., Liu, H. K., Jin, J. Y., et al. (2018). Effects of dietary yeast culture on growth performance, immune response and disease resistance of gibel carp (*Carassius auratus gibelio CAS III*). *Fish Shellfish Immunol.* 82, 400–407. doi: 10.1016/j.fsi.2018.08.044

Zhou, M., Liang, R., Mo, J., Yang, S., Gu, N., Wu, Z., et al. (2018). Effects of brewer's yeast hydrolysate on the growth performance and the intestinal bacterial diversity of largemouth bass (*Micropterus salmoides*). *Aquaculture* 484, 139–144. doi: 10.1016/j.aquaculture.2017.11.006

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: 29 November 2021
doi: 10.3389/fmars.2021.778301

Evaluation of Methanotroph (*Methylococcus capsulatus*, Bath) Bacteria Meal (FeedKind®) as an Alternative Protein Source for Juvenile Black Sea Bream, *Acanthopagrus schlegelii*

*Bingying Xu[1], Yuechong Liu[1], Kai Chen[1], Lei Wang[1], Gladstone Sagada[1], Arnaud Fabrice Tegomo[2,3], Yifei Yang[4], Yuxiao Sun[1], Lu Zheng[1], Sami Ullah[1] and Qingjun Shao[1,2]**

[1] College of Animal Sciences, Zhejiang University, Hangzhou, China, [2] Ocean Academy, Zhejiang University, Zhoushan, China, [3] Ocean College, Zhejiang University, Zhoushan, China, [4] School of Veterinary Science, The University of Queensland, Gatton, QLD, Australia

OPEN ACCESS

Edited by:
Min Gu,
Shandong University, Weihai, China

Reviewed by:
Hualiang Liang,
Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences, China
Khor Waiho,
University of Malaysia Terengganu, Malaysia

***Correspondence:**
Qingjun Shao
qjshao@zju.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: 16 September 2021
Accepted: 26 October 2021
Published: 29 November 2021

Citation:
Xu B, Liu Y, Chen K, Wang L, Sagada G, Tegomo AF, Yang Y, Sun Y, Zheng L, Ullah S and Shao Q (2021) Evaluation of Methanotroph (Methylococcus capsulatus, Bath) Bacteria Meal (FeedKind®) as an Alternative Protein Source for Juvenile Black Sea Bream, Acanthopagrus schlegelii. Front. Mar. Sci. 8:778301. doi: 10.3389/fmars.2021.778301

Single-cell proteins are attracting growing attention as viable alternatives for fishmeal (FM) in aquatic feed. Methanotroph (*Methylococcus capsulatus*, Bath) bacteria meal FeedKind® (FK) is a type of single cell protein with high protein content (75.14%) and desirable amino acids profile, produced by *Methylococcus capsulatus* (Bath) living on methane consumption. The present study evaluated the potential of replacing FM with FK in the diet of black sea bream (*Acanthopagrus schlegelii*). Five iso-energetic and iso-nitrogenous diets were designed with FK replacing 0, 4.13, 8.27, 16.53, and 24.80% FM protein in the basal diet (40% FM content), respectively. All the diets were fed to three replicates of fish (initial weight 6.56 ± 0.02 g) for 70 days. After the feeding trial, replacing dietary 8.27% FM protein with FK significantly improved the weight gain and specific growth rate of fish ($P < 0.05$), while other groups showed no significant difference in the growth performance ($P > 0.05$). The fish fed diets with 8.27 and 16.53% replacement levels exhibited significantly increased feeding rates. The 8.27% FK diet significantly increased the whole-body and muscle crude protein contents, apparent digestibility of crude lipid, foregut, and midgut amylase activities. The microvillus density in the midgut of fish fed the 24.80% FK diet significantly increased. The diet with 8.27% FK increased the serum triglyceride content of the fish, while the 24.80% FK diet reduced the serum triglyceride, total cholesterol, and low-density lipoprotein cholesterol contents of the fish. In conclusion, the results indicated that replacing dietary FM protein with up to 24.80% FK had no adverse effects on the growth of black sea bream, whilst replacing 8.27% FM protein with FK enhanced its growth performance and feed utilization.

Keywords: black sea bream, FeedKind®, single-cell protein, growth, feed utilization, histology

INTRODUCTION

Meals obtained from animal products such as fish, cattle, and poultry are readily available and provide a variety of nutritional profiles for farmed aquatic animals. Marine proteins, such as those derived from fish, shrimp, and squid, have superior nutritional values, but their production has raised ecological and economic concerns, especially fish meal (FM) (Olsen and Hasan, 2012; Gamboa-Delgado and Márquez-Reyes, 2018; Kim et al., 2019). Plant meals are the most commonly used protein sources to replace animal proteins in feed (Kaushik et al., 2004; De Francesco et al., 2007; Cruz-Suárez et al., 2009). However, inherent characteristics of plant meals, such as the presence of anti-nutritional factors and lack of some essential amino acids (e.g., methionine, lysine), have either limited their use or required extra processing and costs (Francis et al., 2001; Gatlin et al., 2007; Miao et al., 2018). Among the unconventional sources of nutrients that have been intensely studied, single-cell protein (SCP), a bulk of dried cells that can also be termed as bioprotein, microbial protein or biomass, including microalgae, yeast, fungal, and bacterial proteins (BP), are attracting growing attention as sustainable protein sources for substituting animal- and plant-derived ingredients in aquafeeds (Anupama and Ravindra, 2000; Sánchez-Muros et al., 2014; Henry et al., 2015; Adeoye et al., 2021; Alloul et al., 2021; Jannathulla et al., 2021; Maulu et al., 2021).

In addition to high protein content (60–82%, dry matter), SCPs contain amino acid profiles similar to FM and provide fatty acids, nucleic acids, vitamins, and minerals that can support the growth and normal physiological functions of aquatic animals (Matassa et al., 2016; Gamboa-Delgado and Márquez-Reyes, 2018; Wang et al., 2020a). BPs generally contains higher methionine content (up to 3%) than algal or fungal SCPs (Erdman et al., 1977; Anupama and Ravindra, 2000). Many studies with BPs have been conducted on white leg shrimp (*Penaeus vannamei*) (Tlusty et al., 2017; Hamidoghli et al., 2019; Alloul et al., 2021), Florida pompano (*Trachionotus carolinus*) (Rhodes et al., 2015), tilapia (*Oreochromis niloticus*) (Maulu et al., 2021), Atlantic salmon (*Salmo salar*) (Storebakken et al., 2004; Berge et al., 2005; Aas et al., 2006a; Romarheim et al., 2011), rainbow trout (*Oncorhynchus mykiss*) (Perera et al., 1995b; Aas et al., 2006b; Øverland et al., 2006; Hardy et al., 2018), Atlantic halibut (*Hippoglossus hippoglossus*) (Aas et al., 2007), Japanese yellowtail (*Seriola quinqueradiata*) (Biswas et al., 2020), and African catfish (*Clarias gariepinus*) (Adeoye et al., 2021). Although based on different feed formulas, they have demonstrated that various BPs could partially or even wholly replace FM or soybean meal (SBM) in the diet without adverse effects on the growth performance or health status of various aquatic species.

FeedKind® (FK) (Calysta, Inc., Menlo Park, CA, United States) is a BP product derived from Bath. It is produced by continuous aerobic fermentation of the bacteria with methane as the sole carbon and energy source in a proprietary fermenter. The harvested biomass is subsequently centrifuged, heat inactivated, and spray dried (Biswas et al., 2020). FeedKind® has high contents of crude protein and lipid, with a well-balanced amino acids profile comparable to the FM (**Table 1**). Biswas et al. (2020) found that FK could replace 30% of the dietary FM protein without impacting the growth performance or feed efficiency of Japanese yellowtail.

TABLE 1 | The nutritional composition of FeedKind® and fishmeal (%, dry matter).

Nutritional components	Fishmeal	FeedKind®
Crude protein	71.76	75.14
Crude lipid	8.03	8.31
Ash	17.53	7.20
Phosphorus	2.17	1.57
Essential amino acids		
Arginine	4.27	4.75
Histidine	0.69	1.58
Iso-leucine	3.00	3.27
Leucine	5.13	5.80
Lysine	4.21	4.32
Methionine	2.27	1.90
Phenylalanine	3.11	3.27
Threonine	2.92	3.38
Valine	3.79	4.11
Non-essential amino acids		
Alanine	4.12	5.17
Aspartic acid	5.56	6.96
Cystine	1.55	0.42
Glutamic acid	8.87	8.23
Glycine	4.55	3.59
Proline	4.50	2.43
Serine	4.25	2.64
Tyrosine	1.99	2.00
Total amino acids	64.78	63.82

As a popular aquaculture species in Southeast Asia, black sea bream (*Acanthopagrus schlegelii*) is adaptive to the intensive aquaculture on account of its characteristics of fast growth rate, high disease resistance, and tolerance to a wide range of environment (Hong and Zhang, 2003; Wang et al., 2020b). Previous study on the diet of black sea bream showed that *Clostridium autoethanogenum* protein (CAP) could replace FM up to 58.20% without adverse effects on the growth performance, antioxidative status, and digestive enzymes activities (Chen et al., 2020). It showed a higher possible replacement level than that in largemouth bass (*Micropterus salmoides*) (150 g/kg) (Yang et al., 2021). Meanwhile, there is still limited knowledge on how dietary BPs may affect black sea bream. The purpose of this study was to evaluate the potential of using FK as an alternative for FM in the diet of black sea bream based on the growth performance, feed utilization, digestive enzymes activities, intestinal and hepatic histology, and serum biochemical and antioxidative/oxidative parameters.

MATERIALS AND METHODS

Experimental Diets

The methanotroph bacteria meal (FeedKind®, FK) was provided by Calysta, Inc., Menlo Park, CA, United States.

Five isonitrogenous (44.8% crude protein) and isoenergetic (21 kJ/g gross energy) diets were formulated with FM protein substituted by graded levels of FK protein at 0 (FK0), 4.13 (FK4.13), 8.27 (FK8.27), 16.53 (FK16.53), and 24.80% (FK24.80) (**Table 2**). Crystalline DL-methionine, L-lysine, and L-arginine were added to maintain sufficient and balanced levels of these essential amino acids, according to the recommended levels from previous studies on black sea bream (Zhou et al., 2010a,b, 2011a,b). Taurine was added according to the recommended level by Tong et al. (2020). Non-essential amino acids were added to balance the protein level. $Ca(H_2PO_4)_2$ was added to meet the available phosphorus contents required in black sea bream (Shao et al., 2008). The yttrium oxide (Y_2O_3) was supplemented at 0.1% for determining apparent digestibility.

After pulverizing and sifting through a 178-μm sieve, all the solid ingredients were weighed before mixing thoroughly with the lipid ingredients. The mixture was pelletized into 2.5-mm-diameter pellets using a pelletizer (Modle HKJ-218; Huarui, Wuxi, China). The pellets were then steamed for 10 min, dried for 72 h at 24°C before being stored at –20°C for subsequent feeding.

TABLE 2 | Feed formula and proximate composition of the experimental diets.

Ingredients (%)	FK0	FK4.13	FK8.27	FK16.53	FK24.80
Fishmeal[1]	40.00	38.21	36.42	32.84	29.26
FeedKind[2]	0.00	1.50	3.00	6.00	9.00
Soy protein concentrate	5.00	5.00	5.00	5.00	5.00
Fermented soybean meal	5.00	5.00	5.00	5.00	5.00
Squid liver meal	3.00	3.00	3.00	3.00	3.00
Chicken meat meal	4.00	4.00	4.00	4.00	4.00
Fish oil	3.00	3.13	3.26	3.52	3.78
Corn oil	5.00	4.99	4.98	4.96	4.94
Soy lecithin	2.00	2.00	2.00	2.00	2.00
Wheat flour	21.00	21.00	21.00	21.00	21.00
α-Starch	2.54	2.41	2.29	2.03	1.78
50% L-carnitine	0.30	0.30	0.30	0.30	0.30
Vitamin premix[3]	0.30	0.30	0.30	0.30	0.30
Mineral premix[4]	0.50	0.50	0.50	0.50	0.50
Sodium carboxymethyl cellulose	0.50	0.50	0.50	0.50	0.50
Carrageenan	0.20	0.20	0.20	0.20	0.20
DL-Methionine	0.41	0.42	0.43	0.45	0.46
L-Lysine	0.64	0.65	0.65	0.67	0.68
L-Arginine	0.30	0.30	0.31	0.31	0.31
Taurine	0.25	0.26	0.27	0.29	0.32
Non-essential amino acids[5]	0.57	0.54	0.51	0.45	0.40
$Ca(H_2PO_4)_2$	2.32	2.32	2.32	2.32	2.32
α-Cellulose	0.47	0.77	1.06	1.66	2.25
Zeolite powder	2.50	2.50	2.50	2.50	2.50
Y_2O_3	0.10	0.10	0.10	0.10	0.10
Antiseptic	0.10	0.10	0.10	0.10	0.10
Total	100.00	100.00	100.00	100.00	100.00
Proximate composition (%, dry matter)					
Crude protein	45.13	45.03	45.18	44.62	43.98
Crude lipid	14.99	15.16	15.28	15.76	15.52
Ash	13.65	13.36	13.15	12.62	12.13
Gross energy (kJ/g)	20.81	20.89	20.96	21.12	21.12

[1]Provided by Zhejiang Jin Jia Feed Co., Ltd, Hangzhou, China.
[2]Provided by Calysta, Inc., California, United States.
[3]Vitamin premix (mg/kg): α-tocopherol, 80; retinyl acetate, 40; cholecalciferol, 0.1; menadione, 15; niacin, 165; riboflavin, 22; pyridoxine HC1, 40; thiamin mononitrate, 45; D-Ca pantothenate, 102, folic acid, 10; vitamin B12, 0.9; inositol, 450; ascorbic acid, 150; Na menadione bisulfate, 15; thiamin, 5; choline chloride, 320 and p-aminobenzoic acid, 50.
[4]Mineral premix (mg/kg): Na_2SiO_3, 0.4; $CaCO_3$, 544.9; $NaH_2PO_4{\cdot}H_2O$, 200; KH_2PO_4, 200; $MgSO_4{\cdot}7H_2O$, 10; $MnSO_4{\cdot}H_2O$, 4; $CuCl_2{\cdot}2H_2O$, 2; $ZnSO_4{\cdot}7H_2O$, 12; $FeSO_4{\cdot}7H_2O$, 12; NaCl, 12; KI, .1; $CoCl_2{\cdot}6H_2O$, .1; $Na_2MoO_4{\cdot}2H_2O$, .5; $AlCl_3{\cdot}6H_2O$, 1; and KF, 1.
[5]Non-essential amino acids: Aspartic acid:Glycine = 1:1, for balancing the protein levels among all the groups.

Experimental Fish and Feeding Trial

Black sea bream was provided by a fish farm (Zhoushan, China). The feeding trial was conducted in the Xixuan Fishery Science and Technology Island (Zhoushan, China). Fish were acclimated to the experimental conditions for 2 weeks in a plastic pond (5 m × 3 m × 1 m) before the feeding trial. After acclimation, 450 fish (6.56 ± 0.02 g) were randomly selected and divided into 15 fiberglass tanks. Each treatment included three replicates with 30 fish in each tank (filled with 420 L of water). Sand filtrated seawater was supplied to all the tanks at 2 L/min. The tanks were kept under natural photoperiod and continuously aerated with air stones. Water temperature was maintained at 26 ± 2°C; salinity, 27 ± 1 g/L; pH, 7.7 ± 0.1; and dissolved oxygen ≥ 5 mg/L. The fish were fed to apparent satiation two times daily at 08:00 and 16:00 for 70 days. Feces were removed 2 h after each feeding session.

Sampling

Before the feeding experiment, 30 fish were randomly collected and stored at −20°C for determining the initial whole-body crude protein content. From the 8th week, the fish feces were collected before 07:00 every day following the method of Wang et al. (2020c). Briefly, after siphoning, the feces were precipitated, filtrated, and finally collected in sealed bags and stored at −20°C for determining apparent digestibility coefficient. After the last feeding, all the fish were fasted for 24 h, anaesthetized with MS-222 (60 mg/L), and individually measured for final body weight and length. Five fish from each tank were randomly selected and preserved at −20°C for whole-body proximate composition analysis. Pooled blood was drawn from the caudal vein of the rest of the fish with 1 ml syringes. The blood samples were settled at 4°C for 2 h before being centrifuged at 10,000 *g* for 15 min to get the serum for biochemical analyses. Subsequently, ten fishes were dissected on ice to orderly separate the viscera, liver, and intraperitoneal fat, and then, weighed for calculating the somatic indexes. The dorsal muscle was removed for proximate composition analysis. The gastrointestinal tract was divided into stomach, foregut, midgut, and hindgut for determining the respective digestive enzyme activities. All the samples were stored at −20°C until analyses. Histological samples from the liver and midgut were collected from three fishes per tank, separated into two small parts, then respectively, fixed in 10% formalin and 2.5% glutaraldehyde solution (4°C).

Proximate Compositions

The proximate compositions of experimental feed, fish whole-body, muscle, and feces were determined following the standard protocols of the Association of Official Analytical Chemists (AOAC, 1995). Moisture was determined by drying the sample to constant weight at 105°C in an oven. Crude protein content was determined by Kjeldahl method (N × 6.25), and crude lipid content was determined by Soxhlet extraction method with diethyl ether. Ash content was determined by combusting the sample at 550°C for 8 h in a muffle furnace. The amino acid compositions of FM, FK, and fish feces were assayed by an automatic amino acid analyzer (Hitachi L-8900, Tokyo, Japan) after acid hydrolysis. The feed and fecal samples were dried, ground, and digested with acid. After filtration and dilution, the yttrium (Y) content was determined by an inductively coupled plasma mass spectrometer (PerkinElmer ELAN DRC-e, Waltham, MA, United States).

Biochemical Assays

The supernatants of the stomach and intestine samples were obtained following the procedure of Zhou et al. (2020). Briefly, the tissues were homogenized in 9 vol (v/w) of 0.86% physiological saline. The homogenate was then centrifuged at 2,500 *g* for 10 min at 4°C before collecting the supernatants, which were used to determine the digestive enzymes activities. The serum was used to assay the contents of glucose (GLU), triglyceride (TG), total cholesterol (T-CHO), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), and malondialdehyde (MDA), as well as the activities of glutamic pyruvic transaminase (GPT), glutamic oxalacetic transaminase (GOT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px). The biochemical assays of serum and tissues were determined using assay kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the instructions of the manufacturer.

Histological Analysis of Midgut and Liver

After serial dehydration in graded alcohol, the midgut and liver tissues fixed in 10% formalin were embedded in paraffin and sectioned to 5-μm thickness. The slices were then subjected to hematoxylin and eosin (H&E) staining, and observed under a light microscope (Olympus BX61, Tokyo, Japan). After fixing with 2.5% glutaraldehyde for more than 4 h, the midgut samples were postfixed with 1% OsO_4 for 1.5 h. After double fixation, the samples were first dehydrated by graded ethanol, and then, dried in Hitachi Model HCP-2 critical point dryer (Tokyo, Japan). The dehydrated samples were coated with gold-palladium (Hitachi Model E-1010 ion sputter, Tokyo, Japan), and observed in scanning electron microscope (Hitachi Model SU-8010 SEM, Tokyo, Japan). The villus height and microvillus density were measured using Image-Pro Plus 6.0 software. The number of microvilli per unit area was counted in five randomly selected non-overlapping fields of view (Wang Y. et al., 2019).

Statistical Analysis

All the data were processed by IBM SPSS Statistics 24.0 and presented as mean ± standard error (SEM). Levene's test was used to determine the normality and homogeneity of variances. Independent-sample Kruskal-Wallis test followed by Bonferroni

TABLE 3 | Growth performance and feed utilization of black sea bream fed with different diets[1].

Index	FK0	FK4.13	FK8.27	FK16.53	FK24.80
SR[2] (%)	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
WG[3] (%)	483.28 ± 5.47^{b}	505.07 ± 2.06^{ab}	570.16 ± 12.94^{a}	536.13 ± 5.84^{ab}	501.47 ± 4.53^{ab}
SGR[4] (%/day)	2.52 ± 0.01^{b}	2.57 ± 0.01^{ab}	2.72 ± 0.03^{a}	2.64 ± 0.01^{ab}	2.56 ± 0.01^{ab}
CF[5] (g/cm^3)	2.84 ± 0.04	2.92 ± 0.04	2.92 ± 0.04	2.93 ± 0.02	2.86 ± 0.04
VSI[6] (%)	9.35 ± 0.17	9.32 ± 0.16	9.17 ± 0.32	9.11 ± 0.18	8.76 ± 0.26
HSI[7] (%)	2.55 ± 0.12	2.47 ± 0.12	2.56 ± 0.06	2.59 ± 0.04	2.46 ± 0.05
IFR[8] (%)	3.38 ± 0.18	3.08 ± 0.15	2.84 ± 0.21	2.86 ± 0.25	2.76 ± 0.24
FR[9] (%/day)	2.45 ± 0.08^{b}	2.54 ± 0.06^{ab}	2.65 ± 0.04^{a}	2.62 ± 0.04^{a}	2.59 ± 0.02^{ab}
FCR[10]	1.27 ± 0.03	1.23 ± 0.02	1.22 ± 0.01	1.23 ± 0.02	1.26 ± 0.01
PER[11]	1.74 ± 0.04	1.81 ± 0.02	1.82 ± 0.01	1.77 ± 0.05	1.76 ± 0.02
PPV[12] (%)	37.22 ± 0.67	37.50 ± 0.85	38.04 ± 0.25	37.59 ± 0.83	37.48 ± 0.21

[1] *Values are presented as mean ± SEM (n = 3).*
[2] *SR (survival rate, %) = (final fish number/initial fish number) × 100.*
[3] *WG (weight gain, %) = (final weight – initial weight)/initial weight × 100.*
[4] *SGR (specific growth rate, %/day) = (ln final weight – ln initial weight) × 100/days.*
[5] *CF (condition factor, g/cm^3) = body weight/body length^3 × 100.*
[6] *VSI (viscerosomatic index, %) = viscera weight/body weight × 100.*
[7] *HSI (hepatosomatic index, %) = liver weight/body weight × 100.*
[8] *IFR (intraperitoneal fat ratio, %) = (intraperitoneal fat weight/body weight) × 100.*
[9] *FR (feeding rate, %/day) = dry feed intake/[(final weight + initial weight)/2]/days × 100.*
[10] *FCR (feed conversion rate) = dry feed intake/weight gain.*
[11] *PER (protein efficacy ratio) = weight gain/total protein intake.*
[12] *PPV (protein productive value, %) = protein gain/total protein intake × 100.*
[a,b] *Means in the same column with different superscripts are significantly different (P < 0.05).*

adjust was performed when data were not homogeneous. A one-way analysis of variance (ANOVA) followed by Duncan multiple-range test was performed to determine the statistically significant differences among different groups. The significance level was set as $P < 0.05$.

RESULTS

Growth Performance and Feed Utilization

As shown in **Table 3**, no significant differences were found in survival rate (SR), condition factor (CF) (ANOVA, $F_{4,10} = 1.561$, $P = 0.258$), viscerosomatic index (VSI) (ANOVA, $F_{4,10} = 0.650$, $P = 0.640$), hepatosomatic index (HSI) (ANOVA, $F_{4,10} = 0.456$, $P = 0.766$), and intraperitoneal fat ratio (IFR) (ANOVA, $F_{4,10} = 1.072$, $P = 0.420$) of black sea bream among all the treatments. With the replacement level of FM by dietary FK increasing, the weight gain (WG) and specific growth rate (SGR) of fish initially increased, and then decreased after 8.27% replacement level. The WG (ANOVA, $F_{4,10} = 103.298$, $P = 0.000$) and SGR (ANOVA, $F_{4,10} = 101.470$, $P = 0.000$) of fish in the FK8.27 group were significantly higher than the FK0 group, but no significant differences were obtained among the other FK inclusion groups and the FK0 group. Compared with the FK0 group, the fish in the FK8.27 and FK16.53 groups showed a significantly increased feeding rate (FR) (ANOVA, $F_{4,10} = 2.481$, $P = 0.088$). The feed conversion rate (FCR) (Kruskal-Wallis, $F_{4,10} = 5.284$, $P = 0.259$), protein efficacy ratio (PER) (ANOVA, $F_{4,10} = 1.141$, $P = 0.384$), and protein productive value (PPV) (ANOVA, $F_{4,10} = 0.185$, $P = 0.942$) among all the groups showed no significant differences.

Whole-Body and Muscle Proximate Composition

As shown in **Table 4**, the whole-body crude protein content of fish in the FK8.27 group was significantly higher than other groups (ANOVA, $F_{4,10} = 6.764$, $P = 0.005$). Compared with the FK0 group, the whole-body ash content was significantly lower in the FK inclusion groups (ANOVA, $F_{4,10} = 7.650$, $P = 0.004$). Moisture (ANOVA, $F_{4,10} = 1.615$, $P = 0.234$) and crude lipid contents (Kruskal-Wallis, $F_{4,10} = 5.681$, $P = 0.224$) in the whole body of the fish were not significantly affected by dietary FK inclusion. Moisture in the muscle of the fish in the FK24.80 group was significantly higher than other groups (ANOVA, $F_{4,10} = 7.262$, $P = 0.005$). A significant difference was observed in the muscle crude protein content of fish between the FK8.27 and FK0 groups (Kruskal-Wallis, $F_{4,10} = 11.732$, $P = 0.019$). Replacing 24.80% FM protein with FK in the diet significantly decreased muscle crude lipid content than in other treatments (ANOVA, $F_{4,10} = 11.267$, $P = 0.000$).

Apparent Digestibility and Gastrointestinal Digestive Enzyme Activities

As listed in **Table 5**, the ADC of dry matter and crude lipid increased with increasing dietary FK protein replacement level to 8.27% then, decreased. The ADC of dry matter (ANOVA, $F_{4,10} = 1.805$, $P = 0.198$), and crude lipid (ANOVA, $F_{4,10} = 2.955$, $P = 0.075$) in the FK8.27 group were significantly higher than in the FK24.80 and FK0 groups, respectively. There was no significant difference in the ADC of crude protein among all the groups (Kruskal-Wallis, $F_{4,10} = 3.523$, $P = 0.474$).

The digestive enzyme activities in the gastrointestinal tract of all the treatments are presented in **Table 6**. Compared with the FK24.80 group, the FK8.27, and FK16.53 groups exhibited enhanced trypsin (ANOVA, $F_{4,10} = 3.292$, $P = 0.030$) and lipase (ANOVA, $F_{4,10} = 2.970$, $P = 0.042$) activities in the foregut. The foregut amylase activity in the FK8.27 and FK16.53 groups was also significantly higher than the other groups (ANOVA, $F_{4,10} = 6.995$, $P = 0.001$). The midgut amylase activity in the FK8.27 group was significantly higher than in the FK0 group (ANOVA, $F_{4,10} = 1.557$, $P = 0.217$), but no significant differences were found in the midgut trypsin (ANOVA, $F_{4,10} = 0.359$, $P = 0.835$) and lipase activities (ANOVA, $F_{4,10} = 0.458$, $P = 0.765$) among the treatments. The digestive enzyme activities in the stomach and hindgut were not significantly affected by dietary FK inclusion.

Serum Biochemical and Antioxidative/Oxidative Parameters

The effects of dietary FK on serum biochemical and antioxidative/oxidative parameters of black sea bream are listed in **Table 7**. The serum TG concentration increased with increasing dietary FK protein replacement level to 8.27%, then decreased (ANOVA, $F_{4,10} = 10.405$, $P = 0.000$). The serum T-CHO content of fish in the FK24.80 group was lower than in the FK0, FK4.13, and FK8.27 groups (ANOVA, $F_{4,10} = 2.819$, $P = 0.048$). Replacing 24.80% FM protein with FK in the diet significantly decreased the serum LDL-C level (Kruskal-Wallis, $F_{4,10} = 12.323$, $P = 0.015$). The serum GPT activity of fish in the FK8.27 group was significantly lower than in the FK24.80 group (ANOVA, $F_{4,10} = 2.752$, $P = 0.055$). No significant differences in glucose (ANOVA, $F_{4,10} = 0.927$, $P = 0.472$), HDL-C (Kruskal-Wallis, $F_{4,10} = 3.504$, $P = 0.477$), and MDA contents (ANOVA, $F_{4,10} = 0.280$, $P = 0.888$), as well as GOT (ANOVA, $F_{4,10} = 0.435$, $P = 0.781$), SOD (ANOVA, $F_{4,10} = 0.275$, $P = 0.891$), and GSH-Px (ANOVA, $F_{4,10} = 1.384$, $P = 0.272$) activities were found in the fish serum among all the groups.

Intestinal and Hepatic Histological Observation

The midgut histological structures of fish in the FK0, FK8.27, and FK24.80 groups are presented in **Figure 1**. The experimental diets did not affect the integrity of midgut intestinal mucosa morphology, with no visible damage. Each mucosal fold was composed of a simple lamina propria with abundant goblet cells and intraepithelial leucocytes (IELs). Different from the other two groups, the FK24.80 group showed expansion of central lacteal in the lamina propria of some villus. The villus height of fish fed with the FK8.27 diet was significantly higher than the FK24.80 diet, whereas both groups showed similar villus height to the control group (ANOVA, $F_{4,10} = 4.047$, $P = 0.039$). The intestinal microvilli

TABLE 4 | Whole-body and muscle proximate composition of black sea bream fed with different diets[1].

Index (%)	FK0	FK4.13	FK8.27	FK16.53	FK24.80
Whole body					
Moisture	65.76 ± 0.06	66.13 ± 0.67	64.91 ± 0.24	65.10 ± 0.40	65.19 ± 0.11
Crude protein	17.56 ± 0.02^{b}	17.60 ± 0.05^{b}	17.86 ± 0.03^{a}	17.71 ± 0.08^{b}	17.65 ± 0.04^{b}
Crude lipid	11.91 ± 0.13	12.56 ± 0.76	13.53 ± 0.21	13.38 ± 0.10	12.90 ± 0.04
Ash	4.73 ± 0.01^{a}	4.57 ± 0.05^{b}	4.54 ± 0.05^{b}	4.54 ± 0.03^{b}	4.50 ± 0.01^{b}
Muscle					
Moisture	73.45 ± 0.17^{b}	73.40 ± 0.16^{b}	73.43 ± 0.12^{b}	73.37 ± 0.06^{b}	74.13 ± 0.03^{a}
Crude protein	20.13 ± 0.01^{b}	20.56 ± 0.04^{ab}	21.02 ± 0.07^{a}	20.77 ± 0.23^{ab}	20.32 ± 0.07^{ab}
Crude lipid	4.43 ± 0.11^{ab}	4.29 ± 0.18^{b}	4.73 ± 0.06^{a}	4.41 ± 0.10^{ab}	3.63 ± 0.08^{c}
Ash	1.61 ± 0.01	1.61 ± 0.01	1.61 ± 0.02	1.60 ± 0.03	1.64 ± 0.03

[1]*Values are presented as mean ± SEM (n = 3).*
[a,b,c]*Means in the same column with different superscripts are significantly different (P < 0.05).*

TABLE 5 | Apparent digestibility coefficients of dry matter, protein, and lipid of black sea bream fed with different diets[1].

Index (%)	FK0	FK4.13	FK8.27	FK16.53	FK24.80
Apparent digestibility coefficient					
Dry matter[2]	80.58 ± 0.94^{ab}	81.07 ± 0.81^{ab}	81.75 ± 0.80^{a}	79.68 ± 0.39^{ab}	78.15 ± 1.04^{b}
Crude protein[3]	85.80 ± 1.13	86.35 ± 0.63	88.41 ± 0.98	86.63 ± 0.50	85.57 ± 0.15
Crude lipid[3]	90.96 ± 1.26^{b}	94.58 ± 0.72^{ab}	94.97 ± 1.03^{a}	92.21 ± 0.99^{ab}	91.61 ± 0.44^{ab}

[1]*Values are presented as mean ± SEM (n = 3).*
[2]*Apparent digestibility coefficients of dry matter (ADC, %) = (1 − dietary Y_2O_3/fecal Y_2O_3) × 100.*
[3]*Apparent digestibility of nutrient in feed (%) = [1 − (dietary Y_2O_3/fecal Y_2O_3) × (nutrient content in feces/nutrient content in feed)] × 100.*
[a,b]*Means in the same column with different superscripts are significantly different (P < 0.05).*

TABLE 6 | Digestive enzyme activities in gastrointestinal tract of black sea bream fed with different diets[1].

Parameters	FK0	FK4.13	FK8.27	FK16.53	FK24.80
Stomach					
Pepsin (U/mgprot)	8.19 ± 0.51	9.06 ± 0.24	9.68 ± 0.48	8.91 ± 0.61	8.83 ± 0.15
Lipase (U/gprot)	0.62 ± 0.07	0.62 ± 0.09	0.62 ± 0.08	0.62 ± 0.08	0.69 ± 0.06
Amylase (U/mgprot)	1.43 ± 0.15	1.53 ± 0.21	1.59 ± 0.22	1.39 ± 0.16	1.38 ± 0.21
Foregut					
Trypsin (U/mgprot)	3538.03 ± 327.09^{ab}	3411.90 ± 258.56^{ab}	4117.65 ± 437.49^{a}	3797.30 ± 407.68^{a}	2454.55 ± 272.38^{b}
Lipase (U/gprot)	0.85 ± 0.05^{ab}	0.92 ± 0.11^{ab}	1.09 ± 0.08^{a}	1.08 ± 0.06^{a}	0.81 ± 0.07^{b}
Amylase (U/mgprot)	4.30 ± 0.27^{b}	4.42 ± 0.21^{b}	6.48 ± 0.70^{a}	5.95 ± 0.21^{a}	4.15 ± 0.49^{b}
Midgut					
Trypsin (U/mgprot)	5,580.22 ± 409.61	5,898.89 ± 378.71	6,115.81 ± 424.16	5,643.15 ± 269.79	5,607.06 ± 486.78
Lipase (U/gprot)	2.02 ± 0.22	2.15 ± 0.14	2.35 ± 0.19	2.12 ± 0.12	2.10 ± 0.19
Amylase (U/mgprot)	5.41 ± 0.55^{b}	6.49 ± 0.43^{ab}	7.22 ± 0.63^{a}	6.79 ± 0.55^{ab}	6.34 ± 0.51^{ab}
Hindgut					
Trypsin (U/mgprot)	4,009.93 ± 144.65	4,181.68 ± 210.97	4,637.95 ± 410.95	4,102.46 ± 273.67	4,230.18 ± 413.16
Lipase (U/gprot)	2.02 ± 0.09	2.02 ± 0.14	2.06 ± 0.15	2.08 ± 0.19	2.08 ± 0.15
Amylase (U/mgprot)	5.21 ± 0.19	5.35 ± 0.33	5.15 ± 0.33	5.56 ± 0.44	4.89 ± 0.42

[1]*Values are presented as mean ± SEM (n = 3).*
[a,b]*Means in the same column with different superscripts are significantly different (P < 0.05).*

in all the groups were arranged neatly and tightly. When the FK protein replacement level increased to 24.8%, the midgut microvillus presented a higher density (ANOVA, $F_{4,10} = 7.258$, $P = 0.009$). For all the fish sampled, the nuclei of hepatocytes had normal and spherical shapes, with clear hepatocyte boundaries (**Figure 2**).

DISCUSSION

The present study assessed the viability of using methanotroph bacteria meal as an FM alternative in the formulated diet of black sea bream. Among the experimental diets, the levels of taurine and essential amino acids were balanced and sufficient

TABLE 7 | Serum biochemical and antioxidative/oxidative parameters of black sea bream fed with different diets[1].

Parameters[2]	FK0	FK4.13	FK8.27	FK16.53	FK24.80
GLU (mmol/L)	10.82 ± 0.88	10.57 ± 1.01	9.71 ± 0.64	9.66 ± 1.49	8.57 ± 0.73
TG (mmol/L)	2.84 ± 0.15^{b}	3.04 ± 0.20^{b}	3.66 ± 0.21^{a}	3.26 ± 0.11^{ab}	2.17 ± 0.18^{c}
T-CHO (mmol/L)	9.63 ± 0.44^{a}	9.76 ± 0.22^{a}	9.52 ± 0.43^{a}	9.24 ± 0.38^{ab}	8.13 ± 0.47^{b}
HDL-C (mmol/L)	3.02 ± 0.22	2.89 ± 0.06	3.35 ± 0.22	3.22 ± 0.20	2.90 ± 0.15
LDL-C (mmol/L)	4.58 ± 0.24^{a}	4.33 ± 0.17^{ab}	4.14 ± 0.13^{ab}	3.95 ± 0.22^{ab}	3.33 ± 0.06^{b}
GPT (U/L)	2.15 ± 0.23^{ab}	2.06 ± 0.27^{ab}	1.67 ± 0.29^{b}	2.08 ± 0.27^{ab}	2.84 ± 0.24^{a}
GOT (U/L)	7.88 ± 0.87	6.56 ± 1.28	8.13 ± 0.28	7.10 ± 0.90	7.32 ± 1.12
SOD (U/ml)	142.18 ± 7.03	142.04 ± 5.52	134.54 ± 4.65	138.56 ± 8.39	135.55 ± 5.27
GSH-Px (U/ml)	165.63 ± 24.37	200.97 ± 19.85	225.86 ± 22.95	214.68 ± 11.53	210.72 ± 13.42
MDA (nmol/ml)	11.07 ± 1.48	10.15 ± 0.81	10.20 ± 1.23	11.63 ± 0.98	10.85 ± 1.11

[1]Values are presented as mean ± SEM (n = 3).
[2]GLU, glucose; TG, triglyceride; T-CHO, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; GPT, glutamic pyruvic transaminase; GOT, glutamic oxalacetic transaminase; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde.
[a,b,c]Means in the same column with different superscripts are significantly different (P < 0.05).

for the growth of black sea bream. The improved WG and SGR of fish in the FK8.27 group may be ascribed to the synergistic effects of combining appropriate levels of two ingredients, i.e., FM and FK, with high nutritional values and complementary amino acids in the diet (Adeoye et al., 2021). Similar results were also obtained in African catfish fed diet with 30% FM protein replaced by a BP product (Yenprotide manufactured by Yenher Agro-Products Sdn. Bhd., Malaysia) (Adeoye et al., 2021), Atlantic salmon fed diets with 18 and 36% bacterial protein meal containing a mix of Bath, *Alcaligenes acidovorans*, *Bacillus brevis*, and *Bacillus firmus* (BPM) (Aas et al., 2006a), as well as Nile tilapia (*Oreochromis nilotica*) and Malaysian Mahseer (*Tor tambroides*) fed with diets incorporating 1:2 (w/w) of phototrophic purple bacteria (*Rhodovulum sulfidophilum* and *Marichromatium* sp., respectively) (Banerjee et al., 2000; Chowdhury et al., 2016). Chowdhury et al. (2016) reported that the extracellular enzymes of phototrophic purple bacteria could benefit the early digestion and metabolism of fish. These enzymes may be responsible for the improved fish growth. Further studies on the functions of extracellular enzymes in FK are needed to verify this speculation. Furthermore, the improved growth of fish fed the FK8.27 diet in this study could be explained by the higher FR with unchanged levels of feed utilization (FCR, PER, and PPV) of the fish. Good attractability and palatability of diet can promote the onset and continuation of feed ingestion in most aquatic animals, and thus increase feed intake (Grasso and Basil, 2002; Rønnestad et al., 2013). Low molecular weight (<1,000 Da) substances such as amino acids and nucleotides can stimulate the olfactory and gustatory sensory cells of fish (Gamboa-Delgado and Márquez-Reyes, 2018). Previous studies reported that dietary supplementation of nucleotides improved the feed intake, growth, immune response, and stress tolerance in fish (Rumsey et al., 1992; Li and Gatlin, 2006) and shrimp (Li et al., 2007; Biswas et al., 2012). Microorganisms have high nucleotide content, making them efficient palatability agents (Gamboa-Delgado and Márquez-Reyes, 2018). The increased FR of black sea bream fed the FK diet can be attributed to improved palatability caused by a high nucleotide level.

In the present study, replacing FM protein up to 24.80% with FK protein in the diet did not significantly affect the growth performance and feed utilization of black sea bream, which may be because the substitution level of FK was not too high. In our previous study of black sea bream, no significant effects were found on the growth performance of fish fed diets with up to 58.20% FM replaced by CAP (Chen et al., 2020). Similar results were also obtained in Pacific white shrimp (*Litopenaeus vannamei*, 50% FM replacement) and smallmouth grunt (*Haemulon chrysargyreum*, 30% FM replacement) fed with *Methylobacterium extorquens* BP diets (Tlusty et al., 2017), African catfish fed with 60 or 100% BP diets (Adeoye et al., 2021), and rainbow trout fed with 27% BPM diet (Aas et al., 2006b). Nevertheless, in the study of Storebakken et al. (2004), dietary 50% FM amino acids replaced by BPM exhibited negative effects on the growth of Atlantic salmon. Aas et al. (2007) found that Atlantic halibut fed 9% BPM diet performed similarly as the control group, whereas the growth performance and feed utilization were reduced in the fish fed 18% BPM diet. Reduced growth performance was also found in Japanese yellowtail when more than 50% dietary FM was replaced by FK (Biswas et al., 2020). Apart from the differences of fish species and FM content in the basal diets of different experiments, compared to the present study, the adverse results may be because high levels of dietary BPs could reduce the digestibility and absorption of nitrogen, total amino acids, as well as several essential and non-essential amino acids (Perera et al., 1995a,b; Storebakken et al., 2004; Øverland et al., 2006). Hence, overuse of BPs in diets may limit the amino acids supply for fish growth. In addition, Sharif et al. (2021) proposed that the adverse effects on the fish growth may be due to the high concentration of nucleic acids in the BPs. Although dietary nucleic acids may improve the growth and immunity of fish, a high dietary level (10%) can affect the palatability of diets, increase the uric acid level in the serum and adversely affect feed intake, growth and feed utilization of fish (Tacon and Cooke, 1980; Li and Gatlin, 2006). FK contains 9% nucleic acids (Calysta data), and thus FK24.80 contributed less than 1% nucleic acids to the feed. Based on the unchanged FR, there was no apparent palatability-mediated aversion for the FK24.80 diet in the present study. Therefore, it can be inferred that the nucleic acids in the experimental diets had no negative effects on the feed intake and growth of black sea bream. The

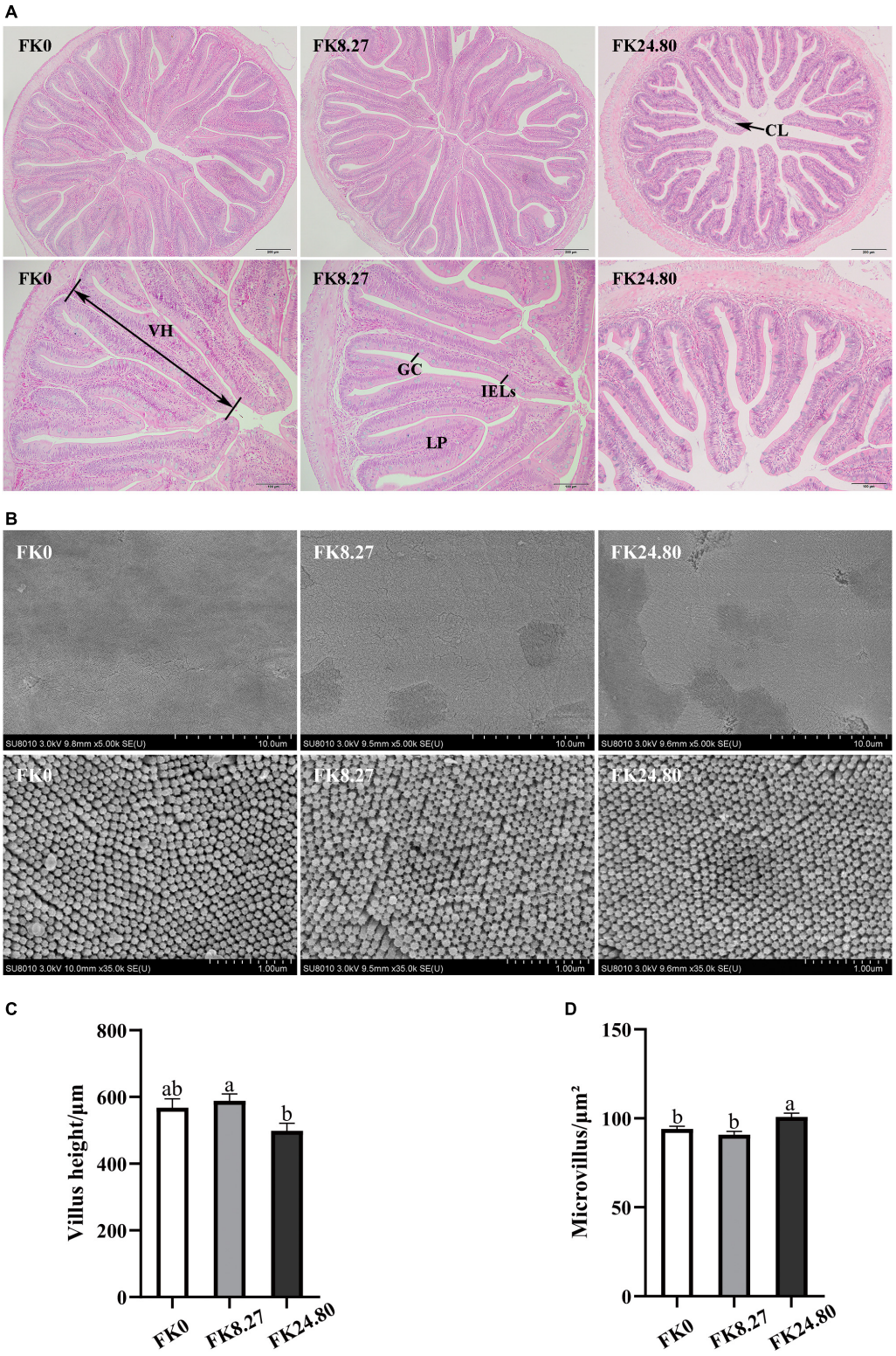


FIGURE 1 | Intestinal histomorphological observation and statistics of black sea bream fed FK0, FK8.27, and FK24.80 diets for 10 weeks. **(A)** Representative histomorphological images from hematoxylin and eosin-stained midgut transverse section (100×/200×), single arrows indicate vacuoles in the lamina propria; **(B)** representative scanning electron microscope images of ultrastructure of the midgut epithelium surface (5.00 k×/35.0 k×); **(C)** mean (±SEM) height of villus in midgut ($n = 3$); **(D)** mean (±SEM) number of microvillus per μm^2 in midgut ($n = 3$). ^{a,b}Means with different superscripts are significantly different ($P < 0.05$). VH, villus height; LP, lamina propria; GC, goblet cell; IELs, intraepithelial leucocytes; CL, central lacteal.

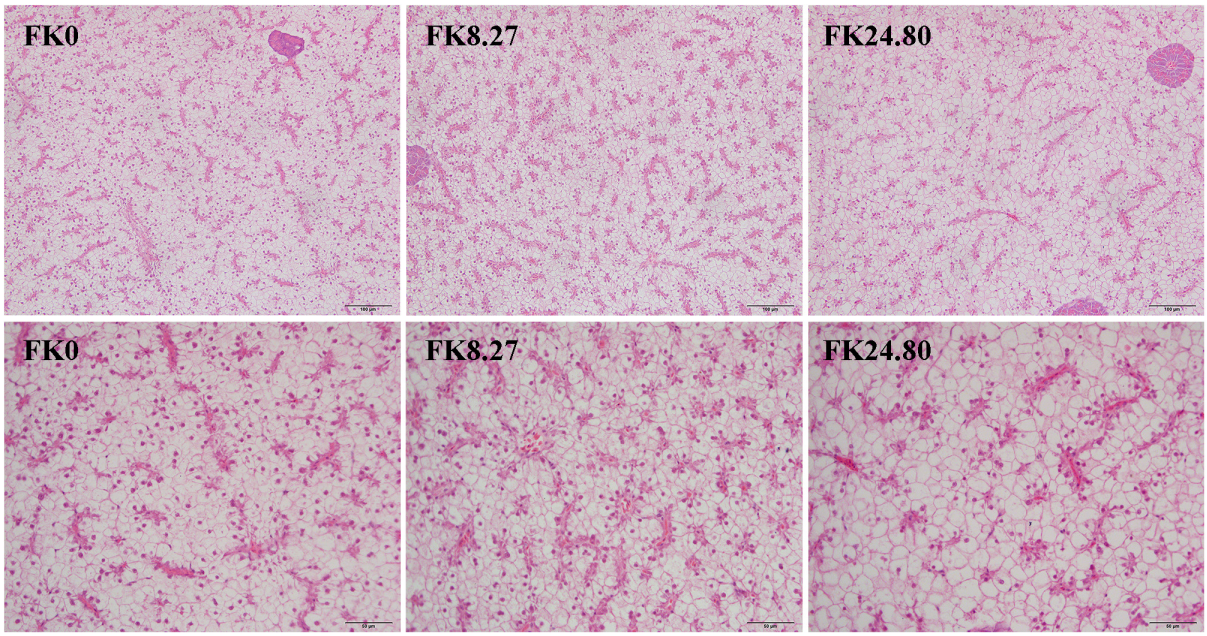


FIGURE 2 | Representative histomorphological images from hematoxylin and eosin-stained liver of black sea bream fed FK0, FK8.27, and FK24.80 diets for 10 weeks (200×/400×).

effects of FK replacement levels higher than 24.80% on black sea bream could be further studied to determine the maximum dietary FK concentration that can maintain the normal growth of fish. On the other hand, low digestibility ascribed to microbial cell walls and membranes impeding enzymatic digestion could also account for the poor performance at relatively high BP inclusion levels (Kiessling and Askbrandt, 1993). Previous studies found that the ADCs of dry matter and energy significantly decreased as the dietary SCP level increased in sea bass (*Dicentrarchus labrax*) (Oliva-Teles and Gonçalves, 2001), and Atlantic salmon (Berge et al., 2005). Rumsey et al. (1991) demonstrated that the energy and nitrogen digestibility of rainbow trout increased after removing all the yeast cell wall components and separating nitrogen into amino acids and nucleic acids fractions. In this study, the FK24.80 group also presented the lowest ADC of dry matter, though no significant differences in the ADCs of dry matter and crude protein were found between FK inclusion groups and the control. Further study on the FK and its autolysate as dietary protein sources is needed to explain the factors affecting its digestion, especially at a high inclusion level.

In the present study, dietary 8.27% FM protein replaced by FK significantly increased the crude protein content in the whole-body and muscle of black sea bream. This suggests that the combination of FM and appropriate level of FK in the diet could contribute to superior growth performance and nutrient absorption. Adeoye et al. (2021) also found that catfish fed with 30% BP included diet had higher body protein content than the control. In addition, the foregut and midgut amylase activities of fish in the FK8.27 group were higher than those in the control group, indicating that the capacity of the fish to digest carbohydrates was enhanced and more protein was saved for growth. With the increase of FK replacement level, the whole-body and muscle crude lipid contents of fish first increased and then decreased, with the fish in the FK8.27 group showing the highest crude lipid content. This result could be related to the higher lipase activity in the foregut and the ADC of crude lipid of fish fed the FK8.27 diet, which suggests enhanced lipid digestion of fish. Many studies have also demonstrated the protein-sparing effect of lipid (Yigit et al., 2002; Ai et al., 2004; Aliyu-Paiko et al., 2010; Li et al., 2017; Wang L. et al., 2019). Furthermore, the lipid composition of FK is different from that of the FM, being rich in phospholipids, consisting mainly of 16:0 and 16:1 fatty acids (Calysta data). Tocher et al. (2008) proposed that dietary phospholipids could improve the digestion and absorption of lipids and other nutrients, and facilitate the transport efficiency of fatty acids and lipids from the intestine to the rest of the body by promoting lipoprotein synthesis. The higher serum TG content in the FK8.27 group also indicates a more active lipid metabolism of the fish. It could be speculated that the combination of FM and suitable level of FK in the diet could benefit the digestion and retention of lipids in the body of the fish. Similar results were found in rainbow trout (Hauptman et al., 2014), red drum (*Sciaenops ocellatus*) (Rosales et al., 2017), and sunshine bass (female white bass *Morone chrysops* × male striped bass *M. saxatilis*) (Gause and Trushenski, 2011). In addition, Adeoye et al. (2021) and Hamidoghli et al. (2019) hypothesized thathigher lipid contents could be ascribed to an attempt to compensate for the imbalance of BP amino acids by promoting protein deamination, resulting in the non-nitrogenous or carbonaceous components of the diet being deposited as lipids. However, the muscle crude lipid content of fish in the FK24.80 group was instead lower than the other groups in this study. This

may be due to BPs non-starch polysaccharides (NSP) content, which is assumed to interfere with nutrients digestion and absorption by increasing digesta viscosity (Storebakken et al., 1998; Leenhouwers et al., 2006; Duan et al., 2017). The specific mechanisms need further exploration.

In the present study, black sea bream fed with representative levels of dietary FK were taken for histological observation, including FK0, FK8.27, and FK24.80. The midgut was chosen because of its highest digestive enzyme activities among the three parts of the intestine. The results showed that dietary FK did not negatively affect the integrity of fish midgut intestinal mucosa and hepatic morphology. No significant differences in midgut villus height were observed between the FK inclusion groups and the control group, while the microvillus in the FK24.80 group presented a higher density than the FK0 and FK8.27 groups. Similarly, previous studies reported that dietary BPs did not damage the intestinal morphology of Atlantic salmon (Storebakken et al., 2004; Berge et al., 2005) and African catfish (Adeoye et al., 2021). The increased microvillus density provided a larger nutrient absorptive surface in the fish fed the FK24.80 diet, which may compensate for the relatively lower villus height. Dietary nucleotides, which increased with the increasing FK levels, were demonstrated to influence gut motility, thus increasing the transit time of digesta and may stimulate the increase of microvilli density (Kim et al., 1968). Furthermore, dietary nucleotides and phospholipids could improve intestinal health and ameliorate intestinal injury (Sturm and Dignass, 2002; Li and Gatlin, 2006), which may benefit the growth of microvilli in the FK24.80 group.

Serum biochemical parameters are widely used as indicators of the general nutritional condition and physiological status of fish (Congleton and Wagner, 2006). In this study, the measured parameters, such as GLU, HDL-C, and MDA contents, GPT, GOT, SOD, and GSH-Px activities, did not show any obvious changes in fish fed the FK included diets. Similar results were found in African catfish fed dietary BP obtained from fermentation of agricultural wastes (Adeoye et al., 2021), Japanese yellowtail fed dietary FK (Biswas et al., 2020), and black sea bream fed dietary CAP (Chen et al., 2020). Serum TG, T-CHO, and LDL-C are indicators relating to lipid metabolism of fish. In the present study, the serum TG concentration increased with increasing dietary FK protein replacement level to 8.27% then decreased. Replacing 24.80% FM protein with FK in the diet significantly decreased the serum TG, T-CHO, and LDL-C levels of fish. This result was consistent with the gut lipase activity, suggesting that the lipid hydrolysis level increased first and then decreased with the increasing dietary FK levels. It may also be related to the dietary lipid composition, as FK had a different fatty acid profile from FM. Studies found that dietary phospholipids could lower plasma lipoprotein levels, and bioactive components in bacterial meal lipids could lower blood cholesterol (Øverland et al., 2010). Nevertheless, referring to other studies on black sea bream, the values of these parameters were all within the normal ranges (Jin et al., 2017, 2019; Wang et al., 2020b). These results manifested that feeding FK to black sea bream did not elicit apparent adverse effects on the fish physiological health and antioxidative/oxidative status.

CONCLUSION

In conclusion, this study found that dietary FM protein can be partly (24.80%) replaced with FK without adverse impacts on growth performance, feed utilization, intestinal and hepatic histology, serum biochemical and antioxidative/oxidative parameters in black sea bream. The combination of dietary FM and appropriate FK level (e.g., FK8.27) can contribute to superior growth performance and nutrient absorption of black sea bream. Further research on the higher dietary replacement level in various aquatic species and the bioactive components (e.g., nucleic acids, phospholipids, NSPs) is essential for evaluating the nutritional value and exploring the functional mechanism of SCP products.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at Zhejiang University.

AUTHOR CONTRIBUTIONS

QS designed the study and provided the fund support. BX conducted the experiment, analyzed the data, and wrote the manuscript. YL and KC participated in the feeding trial and subsequent analysis. LW, GS, AT, and YS helped to take samples and revised the manuscript. YY, LZ, and SU helped to analyze the samples and results. All the authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by China National Ministry of Science and Technology (Funding No. 2020YFD0900801).

ACKNOWLEDGMENTS

We are grateful to the Key Lab of Mari-culture and Breeding of Zhejiang Province and China-Norwegian Joint Laboratory of Nutrition and Feed for Marine Aquaculture for providing the rearing system and logistical support during the feeding trial. Thanks for the technical support for light microscopy by the Experimental Teaching Center of Zhejiang University College of Animal Sciences and the Core Facility Platform of Zhejiang University School of Medicine. Thanks for the technical support for electron microscopy by Bio-Ultrastructure Analysis Lab of Analysis Center of Agrobiology and Environmental Sciences, Zhejiang University.

REFERENCES

Aas, T. S., Grisdale-Helland, B., Terjesen, B. F., and Helland, S. J. (2006a). Improved growth and nutrient utilisation in Atlantic salmon (Salmo salar) fed diets containing a bacterial protein meal. *Aquaculture* 259, 365–376. doi: 10.1016/j.aquaculture.2006.05.032

Aas, T. S., Hatlen, B., Grisdale-Helland, B., Terjesen, B. F., Bakke-McKellep, A. M., and Helland, S. J. (2006b). Effects of diets containing a bacterial protein meal on growth and feed utilisation in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 261, 357–368. doi: 10.1016/j.aquaculture.2006.07.033

Aas, T. S., Hatlen, B., Grisdale-Helland, B., Terjesen, B. F., Penn, M., Bakke-McKellep, A. M., et al. (2007). Feed intake, growth and nutrient utilization in Atlantic halibut (*Hippoglossus hippoglossus*) fed diets containing a bacterial protein meal. *Aquac. Res.* 38, 351–360. doi: 10.1111/j.1365-2109.2007.01672.x

Adeoye, A. A., Akegbejo-samsons, Y., Fawole, F. J., Olatunji, P. O., Muller, N., Wan, A. H. L., et al. (2021). From waste to feed: dietary utilisation of bacterial protein from fermentation of agricultural wastes in African catfish (*Clarias gariepinus*) production and health. *Aquaculture* 531:735850. doi: 10.1016/j.aquaculture.2020.735850

Ai, Q., Mai, K., Li, H., Zhang, C., Zhang, L., Duan, Q., et al. (2004). Effects of dietary protein to energy ratios on growth and body composition of juvenile Japanese seabass, *Lateolabrax japonicus*. *Aquaculture* 230, 507–516. doi: 10.1016/j.aquaculture.2003.09.040

Aliyu-Paiko, M., Hashim, R., and Shu-Chien, A. C. (2010). Influence of dietary lipid/protein ratio on survival, growth, body indices and digestive lipase activity in Snakehead (*Channa striatus*, Bloch 1793) fry reared in re-circulating water system. *Aquac. Nutr.* 16, 466–474. doi: 10.1111/j.1365-2095.2009.00683.x

Alloul, A., Wille, M., Lucenti, P., Bossier, P., Van Stappen, G., Vlaeminck, S. E. et al. (2021). Purple bacteria as added-value protein ingredient in shrimp feed: *Penaeus vannamei* growth performance, and tolerance against Vibrio and ammonia stress. *Aquaculture* 530:735788. doi: 10.1016/j.aquaculture.2020.735788

Anupama, and Ravindra, P. (2000). Value-added food: single cell protein. *Biotechnol. Adv.* 18, 459–479. doi: 10.1016/S0734-9750(00)00045-48

AOAC (1995). *Official Methods of Analysis of the Association of Official Analytical Chemists*. Washington, DC: Association of Official Analytical Chemists.

Banerjee, S., Azad, S. A., Vikineswary, S., Selvaraj, O. S., and Mukherjee, T. K. (2000). Phototrophic bacteria as fish feed supplement. *Asian-Australasian J. Anim. Sci.* 13, 991–994. doi: 10.5713/ajas.2000.991

Berge, G. M., Baeverfjord, G., Skrede, A., and Storebakken, T. (2005). Bacterial protein grown on natural gas as protein source in diets for Atlantic salmon. Salmo salar, saltwater. *Aquaculture* 244, 233–240. doi: 10.1016/j.aquaculture.2004.11.017

Biswas, A., Takakuwa, F., Yamada, S., Matsuda, A., Saville, R. M., LeBlanc, A., et al. (2020). Methanotroph (*Methylococcus capsulatus*, Bath) bacteria meal as an alternative protein source for Japanese yellowtail, Seriola quinqueradiata. *Aquaculture* 529:735700. doi: 10.1016/j.aquaculture.2020.735700

Biswas, G., Korenaga, H., Nagamine, R., Kono, T., Shimokawa, H., Itami, T., et al. (2012). Immune stimulant effects of a nucleotide-rich baker's yeast extract in the kuruma shrimp, *Marsupenaeus japonicus*. *Aquaculture* 366-367, 40–45. doi: 10.1016/j.aquaculture.2012.09.001

Chen, Y., Sagada, G., Xu, B., Chao, W., Zou, F., Ng, W. K., et al. (2020). Partial replacement of fishmeal with *Clostridium autoethanogenum* single-cell protein in the diet for juvenile black sea bream (*Acanthopagrus schlegelii*). *Aquac. Res.* 51, 1000–1011. doi: 10.1111/are.14446

Chowdhury, A. J. K., Zakaria, N. H., Abidin, Z. A. Z., and Rahman, M. M. (2016). Phototrophic purple bacteria as feed supplement on the growth, feed utilization and body compositions of Malaysian Mahseer, Tor tambroides juveniles. *Sains Malaysiana* 45, 135–140.

Congleton, J. L., and Wagner, T. (2006). Blood-chemistry indicators of nutritional status in juvenile salmonids. *J. Fish Biol.* 69, 473–490. doi: 10.1111/j.1095-8649.2006.01114.x

Cruz-Suárez, L. E., Tapia-Salazar, M., Villarreal-Cavazos, D., Beltran-Rocha, J., Nieto-López, M. G., Lemme, A., et al. (2009). Apparent dry matter, energy, protein and amino acid digestibility of four soybean ingredients in white shrimp *Litopenaeus vannamei* juveniles. *Aquaculture* 292, 87–94. doi: 10.1016/j.aquaculture.2009.03.026

De Francesco, M., Parisi, G., Pérez-Sánchez, J., Gómez-Réqueni, P., Médale, F., Kaushik, S. J., et al. (2007). Effect of high-level fish meal replacement by plant proteins in gilthead sea bream (*Sparus aurata*) on growth and body/fillet quality traits. *Aquac. Nutr.* 13, 361–372. doi: 10.1111/j.1365-2095.2007.00485.x

Duan, Y., Zhang, Y., Dong, H., Wang, Y., Zheng, X., and Zhang, J. (2017). Effect of dietary *Clostridium butyricum* on growth, intestine health status and resistance to ammonia stress in Pacific white shrimp *Litopenaeus vannamei*. *Fish Shellfish Immunol.* 65, 25–33. doi: 10.1016/j.fsi.2017.03.048

Erdman, M. D., Bergen, W. G., and Adinarayana Reddy, C. (1977). Amino acid profiles and presumptive nutritional assessment of single cell protein from certain lactobacilli. *Appl. Environ. Microbiol.* 33, 901–905. doi: 10.1128/aem.33.4.901-905.1977

Francis, G., Makkar, H. P. S., and Becker, K. (2001). Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture* 199, 197–227.

Gamboa-Delgado, J., and Márquez-Reyes, J. M. (2018). Potential of microbial-derived nutrients for aquaculture development. *Rev. Aquac.* 10, 224–246. doi: 10.1111/raq.12157

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

Gause, B., and Trushenski, J. (2011). Replacement of fish meal with ethanol yeast in the diets of sunshine bass. *N. Am. J. Aquac.* 73, 97–103. doi: 10.1080/15222055.2011.544939

Grasso, F. W., and Basil, J. A. (2002). How lobsters, crayfishes, and crabs locate sources of odor: current perspectives and future directions. *Curr. Opin. Neurobiol.* 12, 721–727. doi: 10.1016/S0959-4388(02)00388-384

Hamidoghli, A., Yun, H., Won, S., Kim, S. K., Farris, N. W., and Bai, S. C. (2019). Evaluation of a single-cell protein as a dietary fish meal substitute for whiteleg shrimp *Litopenaeus vannamei*. *Fish. Sci.* 85, 147–155. doi: 10.1007/s12562-018-1275-1275

Hardy, R. W., Patro, B., Pujol-Baxley, C., Marx, C. J., and Feinberg, L. (2018). Partial replacement of soybean meal with Methylobacterium extorquens single-cell protein in feeds for rainbow trout (*Oncorhynchus mykiss* Walbaum). *Aquac. Res.* 49, 2218–2224. doi: 10.1111/are.13678

Hauptman, B. S., Barrows, F. T., Block, S. S., Gibson Gaylord, T., Paterson, J. A., Rawles, S. D., et al. (2014). Evaluation of grain distillers dried yeast as a fish meal substitute in practical-type diets of juvenile rainbow trout. *Oncorhynchus mykiss*. *Aquaculture* 432, 7–14. doi: 10.1016/j.aquaculture.2014.03.026

Henry, M., Gasco, L., Piccolo, G., and Fountoulaki, E. (2015). Review on the use of insects in the diet of farmed fish: past and future. *Anim. Feed Sci. Technol.* 203, 1–22. doi: 10.1016/j.anifeedsci.2015.03.001

Hong, W., and Zhang, Q. (2003). Review of captive bred species and fry production of marine fish in China. *Aquaculture* 227, 305–318. doi: 10.1016/S0044-8486(03)00511-518

Jannathulla, R., Sravanthi, O., Moomeen, S., Gopikrishna, G., and Dayal, J. S. (2021). Microbial products in terms of isolates, whole-cell biomass, and live organisms as aquafeed ingredients: production, nutritional values, and market potential—a review. *Aquac. Int.* 29, 623–650. doi: 10.1007/s10499-021-00644-642

Jin, M., Pan, T., Cheng, X., Zhu, T. T., Sun, P., Zhou, F., et al. (2019). Effects of supplemental dietary L-carnitine and bile acids on growth performance, antioxidant and immune ability, histopathological changes and inflammatory response in juvenile black seabream (*Acanthopagrus schlegelii*) fed high-fat diet. *Aquaculture* 504, 199–209. doi: 10.1016/j.aquaculture.2019.01.063

Jin, M., Yuan, Y., Lu, Y., Ma, H., Sun, P., Li, Y., et al. (2017). Regulation of growth, tissue fatty acid composition, biochemical parameters and lipid related genes expression by different dietary lipid sources in juvenile black seabream, *Acanthopagrus schlegelii*. *Aquaculture* 479, 25–37. doi: 10.1016/j.aquaculture.2017.05.017

Kaushik, S. J., Covès, D., Dutto, G., and Blanc, D. (2004). Almost total replacement of fish meal by plant protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchus labrax*. *Aquaculture* 230, 391–404. doi: 10.1016/S0044-8486(03)00422-428

Kiessling, A., and Askbrandt, S. (1993). Nutritive value of two bacterial strains of single-cell protein for rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 109, 119–130. doi: 10.1016/0044-8486(93)90209-H

Kim, S. W., Less, J. F., Wang, L., Yan, T., Kiron, V., Kaushik, S. J., et al. (2019). Meeting global feed protein demand: challenge, opportunity, and strategy. *Annu. Rev. Anim. Biosci.* 7, 221–243. doi: 10.1146/annurev-animal-030117-014838

Kim, T. S., Shulman, J., and Levine, R. A. (1968). Relaxant effect of cyclic adenosine 3′, 5′-monophosphate on the isolated rabbit ileum. *J. Pharmacol. Exp. Ther.* 163, 36–42.

Leenhouwers, J. I., Adjei-Boateng, D., Verreth, J. A. J., and Schrama, J. W. (2006). Digesta viscosity, nutrient digestibility and organ weights in African catfish (*Clarias gariepinus*) fed diets supplemented with different levels of a soluble non-starch polysaccharide. *Aquac. Nutr.* 12, 111–116. doi: 10.1111/j.1365-2095.2006.00389.x

Li, P., and Gatlin, D. M. (2006). Nucleotide nutrition in fish: current knowledge and future applications. *Aquaculture* 251, 141–152. doi: 10.1016/j.aquaculture.2005.01.009

Li, P., Lawrence, A. L., Castille, F. L., and Gatlin, D. M. (2007). Preliminary evaluation of a purified nucleotide mixture as a dietary supplement for Pacific white shrimp *Litopenaeus vannamei* (Boone). *Aquac. Res.* 38, 887–890. doi: 10.1111/j.1365-2109.2007.01761.x

Li, W., Wen, X., Huang, Y., Zhao, J., Li, S., and Zhu, D. (2017). Effects of varying protein and lipid levels and protein-to-energy ratios on growth, feed utilization and body composition in juvenile *Nibea diacanthus*. *Aquac. Nutr.* 23, 1035–1047. doi: 10.1111/anu.12471

Matassa, S., Boon, N., Pikaar, I., and Verstraete, W. (2016). Microbial protein: future sustainable food supply route with low environmental footprint. *Microb. Biotechnol.* 9, 568–575. doi: 10.1111/1751-7915.12369

Maulu, S., Liang, H., Ge, X., Yu, H., Huang, D., and Ke, J. (2021). Effect of dietary *Clostridium autoethanogenum* protein on growth, body composition, plasma parameters and hepatic genes expression related to growth and AMPK / TOR / PI3K signaling pathway of the genetically improved farmed tilapia (GIFT: *Oreochromis niloticus*) juveniles. *Animal Feed Sci. Technol.* 276:114914. doi: 10.1016/j.anifeedsci.2021.114914

Miao, S., Zhao, C., Zhu, J., Hu, J., Dong, X., and Sun, L. (2018). Dietary soybean meal affects intestinal homoeostasis by altering the microbiota, morphology and inflammatory cytokine gene expression in northern snakehead. *Sci. Rep.* 8:113. doi: 10.1038/s41598-017-18430-18437

Oliva-Teles, A., and Gonçalves, P. (2001). Partial replacement of fishmeal by brewers yeast (*Saccaromyces cerevisae*) in diets for sea bass (*Dicentrarchus labrax*) juveniles. *Aquaculture* 202, 269–278. doi: 10.1016/S0044-8486(01)00777-773

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

Øverland, M., Romarheim, O. H., Hovin, M., Storebakken, T., and Skrede, A. (2006). Apparent total tract digestibility of unprocessed and extruded diets containing basic and autolyzed bacterial protein meal grown on natural gas in mink and rainbow trout. *Anim. Feed Sci. Technol.* 129, 237–251. doi: 10.1016/j.anifeedsci.2005.12.017

Øverland, M., Tauson, A. H., Shearer, K., and Skrede, A. (2010). Evaluation of methane-utilising bacteria products as feed ingredients for monogastric animals. *Arch. Anim. Nutr.* 64, 171–189. doi: 10.1080/17450391003691534

Perera, W. M. K., Carter, C. G., and Houlihan, D. F. (1995a). Apparent absorption efficiencies of amino acids in rainbow trout, *Oncorhynchus mykiss* (Walbaum), fed diets containing bacterial single-cell protein. *Aquac. Nutr.* 1, 95–103. doi: 10.1111/j.1365-2095.1995.tb00024.x

Perera, W. M. K., Carter, C. G., and Houlihan, D. F. (1995b). Feed consumption, growth and growth efficiency of rainbow trout (*Oncorhynchus mykiss* (Walbaum) fed on diets containing a bacterial single-cell protein. *Br. J. Nutr.* 73, 591–603. doi: 10.1079/bjn19950061

Rhodes, M. A., Zhou, Y., and Davis, D. A. (2015). Use of dried fermented biomass as a fish meal replacement in practical diets of Florida pompano, *Trachinotus carolinus*. *J. Appl. Aquac.* 27, 29–39. doi: 10.1080/10454438.2014.959834

Romarheim, O. H., Øverland, M., Mydland, L. T., Skrede, A., and Landsverk, T. (2011). Bacteria grown on natural gas prevent soybean meal-induced enteritis in atlantic salmon. *J. Nutr.* 141, 124–130. doi: 10.3945/jn.110.128900

Rønnestad, I., Yúfera, M., Ueberschär, B., Ribeiro, L., Sæle, Ø, and Boglione, C. (2013). Feeding behaviour and digestive physiology in larval fish: current knowledge, and gaps and bottlenecks in research. *Rev. Aquac.* 5, S59–S98. doi: 10.1111/raq.12010

Rosales, M., Castillo, S., Pohlenz, C., and Gatlin, D. M. (2017). Evaluation of dried yeast and threonine fermentation biomass as partial fish meal replacements in the diet of red drum *Sciaenops ocellatus*. *Anim. Feed Sci. Technol.* 232, 190–197. doi: 10.1016/j.anifeedsci.2017.08.014

Rumsey, G. L., Hughes, S. G., Smith, R. R., Kinsella, J. E., and Shetty, K. J. (1991). Digestibility and energy values of intact, disrupted and extracts from brewer's dried yeast fed to rainbow trout (*Oncorhynchus mykiss*). *Anim. Feed Sci. Technol.* 33, 185–193. doi: 10.1016/0377-8401(91)90059-90052

Rumsey, G. L., Winfree, R. A., and Hughes, S. G. (1992). Nutritional value of dietary nucleic acids and purine bases to rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 108, 97–110. doi: 10.1016/0044-8486(92)90321-B

Sánchez-Muros, M. J., Barroso, F. G., and Manzano-Agugliaro, F. (2014). Insect meal as renewable source of food for animal feeding: a review. *J. Clean. Prod.* 65, 16–27. doi: 10.1016/j.jclepro.2013.11.068

Shao, Q., Ma, J., Xu, Z., Hu, W., Xu, J., and Xie, S. (2008). Dietary phosphorus requirement of juvenile black seabream, *Sparus macrocephalus*. *Aquaculture* 277, 92–100. doi: 10.1016/j.aquaculture.2008.01.029

Sharif, M., Zafar, M. H., Aqib, A. I., Saeed, M., Farag, M. R., and Alagawany, M. (2021). Single cell protein: sources, mechanism of production, nutritional value and its uses in aquaculture nutrition. *Aquaculture* 531:735885. doi: 10.1016/j.aquaculture.2020.735885

Storebakken, T., Baeverfjord, G., Skrede, A., Olli, J. J., and Berge, G. M. (2004). Bacterial protein grown on natural gas in diets for Atlantic salmon, Salmo salar, in freshwater. *Aquaculture* 241, 413–425. doi: 10.1016/j.aquaculture.2004.07.024

Storebakken, T., Kvien, I. S., Shearer, K. D., Grisdale-Helland, B., Helland, S. J., and Berge, G. M. (1998). The apparent digestibility of diets containing fish meal, soybean meal or bacterial meal fed to Atlantic salmon (Salmo salar): evaluation of different faecal collection methods. *Aquaculture* 169, 195–210. doi: 10.1016/S0044-8486(98)00379-372

Sturm, A., and Dignass, A. U. (2002). Modulation of gastrointestinal wound repair and inflammation by phospholipids. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 1582, 282–288. doi: 10.1016/S1388-1981(02)00182-188

Tacon, A. G. J., and Cooke, D. J. (1980). Nutritional value of dietary nucleic acids to trout. *Nutr. Rep. Int.* 22, 631–640.

Tlusty, M., Rhyne, A., Szczebak, J. T., Bourque, B., Bowen, J. L., Burr, G., et al. (2017). A transdisciplinary approach to the initial validation of a single cell protein as an alternative protein source for use in aquafeeds. *PeerJ* 2017:e3170. doi: 10.7717/peerj.3170

Tocher, D. R., Bendiksen, E. Å, Campbell, P. J., and Bell, J. G. (2008). The role of phospholipids in nutrition and metabolism of teleost fish. *Aquaculture* 280, 21–34. doi: 10.1016/j.aquaculture.2008.04.034

Tong, S., Wang, L., Kalhoro, H., Volatiana, J. A., and Shao, Q. (2020). Effects of supplementing taurine in all-plant protein diets on growth performance, serum parameters, and cholesterol 7α-hydroxylase gene expression in black sea bream, *Acanthopagrus schlegelii*. *J. World Aquac. Soc.* 51, 990–1001. doi: 10.1111/jwas.12611

Wang, C., Chuprom, J., Wang, Y., and Fu, L. (2020a). Beneficial bacteria for aquaculture: nutrition, bacteriostasis and immunoregulation. *J. Appl. Microbiol.* 128, 28–40. doi: 10.1111/jam.14383

Wang, L., Xu, B., Sagada, G., Ng, W. K., Chen, K., Zhang, J., et al. (2020b). Dietary berberine regulate lipid metabolism in muscle and liver of black sea bream (*Acanthopagrus schlegelii*) fed normal or high-lipid diets. *Br. J. Nutr.* 125, 481–493. doi: 10.1017/S0007114520003025

Wang, L., Yin, N., Sagada, G., Hua, Y., Li, H., Zhang, J., et al. (2020c). Partial replacement of fishmeal with corn gluten meal, pea protein isolate and their mixture in diet of black sea bream (*Acanthopagrus schlegelii*) juveniles: effects on growth performance, feed utilization and haematological parameters. *Aquac. Res.* 51, 2071–2083. doi: 10.1111/are.14558

Wang, L., Zhang, W., Gladstone, S., Ng, W. K., Zhang, J., and Shao, Q. (2019). Effects of isoenergetic diets with varying protein and lipid levels on the growth, feed utilization, metabolic enzymes activities, antioxidative status and serum biochemical parameters of black sea bream (*Acanthopagrus schlegelii*). *Aquaculture* 513:734397. doi: 10.1016/j.aquaculture.2019.734397

Wang, Y., Li, C., Pan, C., Liu, E., Zhao, X., and Ling, Q. (2019). Alterations to transcriptomic profile, histopathology, and oxidative stress in liver of pikeperch

(*Sander lucioperca*) under heat stress. *Fish Shellfish Immunol.* 95, 659–669. doi: 10.1016/j.fsi.2019.11.014

Yang, P., Li, X., Song, B., He, M., Wu, C., and Leng, X. (2021). The potential of Clostridium autoethanogenum, a new single cell protein, in substituting fish meal in the diet of largemouth bass (*Micropterus salmoides*): growth, feed utilization and intestinal histology. *Aquac. Fish.* (in press). doi: 10.1016/j.aaf.2021.03.003

Yigit, M., Yardim, Ö, and Koshio, S. (2002). The protein sparing effects of high lipid levels in diets for rainbow trout (*Oncorhynchus mykiss*, W. 1792) with special reference to reduction of total nitrogen excretion. *Isr. J. Aquac. - Bamidgeh* 54, 79–88. doi: 10.46989/001c.20314

Zhou, F., Shao, J., Xu, R., Ma, J., and Xu, Z. (2010a). Quantitative L-lysine requirement of juvenile black sea bream (*Sparus macrocephalus*). *Aquac. Nutr.* 16, 194–204. doi: 10.1111/j.1365-2095.2009.00651.x

Zhou, F., Xiong, W., Xiao, J. X., Shao, Q. J., Bergo, O. N., Hua, Y., et al. (2010b). Optimum arginine requirement of juvenile black sea bream, Sparus macrocephalus. *Aquac. Res.* 41, 418–430. doi: 10.1111/j.1365-2109.2009.02474.x

Zhou, F., Shao, Q. J., Xiao, J. X., Peng, X., Ngandzali, B. O., Sun, Z., et al. (2011a). Effects of dietary arginine and lysine levels on growth performance, nutrient utilization and tissue biochemical profile of black sea bream, *Acanthopagrus schlegelii*, fingerlings. *Aquaculture* 319, 72–80. doi: 10.1016/j.aquaculture.2011.06.001

Zhou, F., Xiao, J. X., Hua, Y., Ngandzali, B. O., and Shao, Q. J. (2011b). Dietary L-methionine requirement of juvenile black sea bream (*Sparus macrocephalus*) at a constant dietary cystine level. *Aquac. Nutr.* 17, 469–481. doi: 10.1111/j.1365-2095.2010.00823.x

Zhou, L., Li, H., Qin, J. G., Wang, X., Chen, L., Xu, C., et al. (2020). Dietary prebiotic inulin benefits on growth performance, antioxidant capacity, immune response and intestinal microbiota in Pacific white shrimp (*Litopenaeus vannamei*) at low salinity. *Aquaculture* 518:734847. doi: 10.1016/j.aquaculture.2019.734847

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 Physiology

ORIGINAL RESEARCH
published: 21 December 2021
doi: 10.3389/fphys.2021.764987

Effects of Replacement of Dietary Fishmeal by Cottonseed Protein Concentrate on Growth Performance, Liver Health, and Intestinal Histology of Largemouth Bass (*Micropterus salmoides*)

Yulong Liu[1,2], Qisheng Lu[1,2], Longwei Xi[1,2], Yulong Gong[1,2], Jingzhi Su[1,2], Dong Han[1,2,3], Zhimin Zhang[1], Haokun Liu[1], Junyan Jin[1], Yunxia Yang[1], Xiaoming Zhu[1] and Shouqi Xie[1,2,4]*

[1] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China, [2] College of Advanced Agricultural Sciences, University of Chinese Academy of Sciences, Beijing, China, [3] Hubei Engineering Research Center for Aquatic Animal Nutrition and Feed, Wuhan, China, [4] The Innovative Academy of Seed Design, Chinese Academy of Sciences, Wuhan, China

OPEN ACCESS

Edited by:
Shiwei Xie,
Guangdong Ocean University, China

Reviewed by:
Xiaofang Liang,
Chinese Academy of Agricultural Sciences (CAAS), China
Mansour Torfi Mozanzadeh,
South Iran Aquaculture Research Center, Iran
Nan Bai,
Shandong University, China

***Correspondence:**
Dong Han
hand21cn@ihb.ac.cn

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

Received: 26 August 2021
Accepted: 25 November 2021
Published: 21 December 2021

Citation:
Liu Y, Lu Q, Xi L, Gong Y, Su J, Han D, Zhang Z, Liu H, Jin J, Yang Y, Zhu X and Xie S (2021) Effects of Replacement of Dietary Fishmeal by Cottonseed Protein Concentrate on Growth Performance, Liver Health, and Intestinal Histology of Largemouth Bass (Micropterus salmoides). Front. Physiol. 12:764987. doi: 10.3389/fphys.2021.764987

An 8-week feeding trial was conducted to explore the effects of replacement of dietary fishmeal by cottonseed protein concentrate (CPC) on growth performance, liver health, and intestine histology of largemouth bass. Four isoproteic and isolipidic diets were formulated to include 0, 111, 222, and 333 g/kg of CPC, corresponding to replace 0% (D1), 25% (D2), 50% (D3), and 75% (D4) of fishmeal. Two hundred and forty largemouth bass (15.11 ± 0.02 g) were randomly divided into four groups with three replicates per group. During the experiment, fish were fed to apparent satiation twice daily. Results indicated that CPC could replace up to 50% fishmeal in a diet for largemouth bass without significant adverse effects on growth performance. However, weight gain rate (WGR), specific growth rate (SGR), feed efficiency (FE), and condition factor (K) of the largemouth bass were significantly decreased when 75% of dietary fishmeal that was replaced by CPC. The whole body lipid content was increased with the increasing of dietary CPC levels. Oil red O staining results indicated that fish fed the D4 diet showed an aggravated fat deposition in the liver. Hepatocytes exhibited serious degeneration, volume shrinkage, and inflammatory cells infiltration in the D4 group. Intestinal villi appeared shorter and sparse with severe epithelial damage in the D4 group. The transcription levels of anti-inflammatory cytokines, such as transforming growth factor β (*tgf*-β), interleukin 10 (*il-10*), and interleukin 11 β (*il-11*β), were downregulated in the D4 group. The lipid metabolism-related genes carnitine palmitoyl transferase 1 (*cpt1*), peroxisome proliferator-activated receptor α (*ppar*α), and target of rapamycin (TOR) pathway were also significantly downregulated in the D4 group. It was concluded that

suitable replacement of fishmeal by less than 222 g CPC/kg diet had a positive effect on growth performance of fish, but an excessive substitution of 75% fishmeal by CPC would lead to the suppressed growth, liver inflammation, and intestinal damage of largemouth bass.

Keywords: cottonseed protein concentrate, growth, inflammation, intestinal histology, TOR pathway

INTRODUCTION

Fishmeal is known as the preferential protein ingredient in aquafeeds due to its high digestible protein content, balanced amino acid profile, and fewer anti-nutritional factors (NRC, 2011). However, the rapid development of aquaculture and aquafeeds increases the imbalance between supply and demand of fishmeal because of the unsustainable fisheries resources and the increasing price of fishmeal (Naylor et al., 2009; Turchini et al., 2018). Therefore, finding new alternative and efficient fishmeal substitutes is becoming more and more concerning (Hardy, 2010; FAO, 2018). So far, plant proteins have been widely reported as the fishmeal substitute in aquafeeds, such as soybean meal (Lim and Dominy, 1990), rapeseed meal (Cheng et al., 2010), cottonseed meal (Lim, 2010), and peanut meal (Liu et al., 2012). However, due to the existence of anti-nutritional factors, unbalanced amino acid profile, and low feed availability, plant proteins often cause many negative impacts on different fishes (Glencross et al., 2020).

As an important plant protein, cottonseed meal has a relatively balanced amino acid profile and is often widely used as a fishmeal substitute. However, the presence of gossypol, a main anti-nutritional factor in cottonseed meals, brought many adverse effects on the growth and health of fish and severely limited its utilization in aquafeeds (Anderson et al., 2016; Wan et al., 2018). Thus, removing anti-nutritional factors from cottonseed meals will make it better use in aquafeeds. With the recent development of cottonseed processing, the cottonseed protein concentrate (CPC) was obtained by low-temperature extraction with low levels of anti-nutritional factors, such as extremely low gossypol levels (Liu et al., 2020). Therefore, compared to the traditional cottonseed meal, CPC is an excellent plant protein to replace fishmeal in aquafeeds. As a new type of non-grain protein source, CPC was mainly evaluated in replacing dietary fishmeal for the marine fish species in a few studies. It was reported that replacing fishmeal with 60% CPC did not show adverse effects on growth performance and intestinal health of juvenile golden pompano (*Trachinotus ovatus*) (Shen et al., 2020). A study in pearl gentian grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatu* ♂) found that CPC replacing up to 24% of fishmeal exhibited no negative effects on the growth and intestinal morphology of the fish (Chen et al., 2020). It was also observed that dietary inclusion of CPC suppressed growth performance and immune function of hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatu* ♂) (Yin et al., 2018). When fed with a full plant protein diet with soybean protein concentrate and CPC, Japanese seabass (*Lateolabrax japonicas*) was induced abnormal metabolism in the liver and a fatty liver (Zhang et al., 2019). However, very little information on replacing fishmeal with CPC has been published in freshwater fish species, especially carnivorous fish that usually need a large content of dietary fishmeal.

Largemouth bass (*Micropterus salmoides*), a typical freshwater carnivorous fish, is one of the most important commercial cultivated fish in China with an annual production of more than 0.6 million tons in 2020 (He et al., 2020; China Fishery Statistical Yearbook, 2021). The dietary protein requirement for largemouth bass is about 48%, and it strongly depends on fishmeal, usually up to 40–50% (Huang et al., 2017). Therefore, alternative low-cost protein sources for largemouth bass are important for reducing feed prices and increasing the income of fishermen (Zhao et al., 2021). Till now, only one recent study evaluated the combination use of CPC and poultry by-product meal for replacing fishmeal in largemouth bass and mainly focused on the effects of the supplementation with CPC on growth performance and environmental impacts (Wang et al., 2021), but the investigation of replacing fishmeal with CPC on liver health and intestinal health of largemouth bass, especially at high replacing levels, has not been reported. In the present study, the effects of graded levels of dietary CPC on growth performance, liver health, and intestinal histology were conducted in largemouth bass to evaluate the feasibility for CPC replacing of fishmeal.

MATERIALS AND METHODS

Experimental Diets

All ingredients and proximate compositions of the experimental diets are shown in **Table 1**. Four isonitrogenous and isolipidic experimental diets were formulated to include 0, 111, 222, and 333 g/kg of CPC, corresponding to replace 0% (D1), 25% (D2), 50% (D3), and 75% (D4) of dietary fishmeal. The designed protein and lipid content of the experimental diets are based on Huang et al. (2017) and Li et al. (2020), which can satisfy the requirement of largemouth bass. D1 group was used as the control diet with 40% of fishmeal. All diets were processed through an extruder (TSE65S; Modern Yanggong Machinery S&T development CO., LTD., Beijing, China) and made into 2 mm diameter floating pellets. All diets were dried at 60°C in an oven and stored at 4°C. The composition of the CPC used in this experiment is shown in **Table 2**. **Table 3** shows the amino acids profile of CPC and the experimental diets.

Fish and Feeding Trial

Largemouth bass was purchased from a commercial fish farm (Ezhou, Hubei, China). All fish were fed the control D1 diet for 2 weeks to acclimate the experimental condition. The experiment

TABLE 1 | Formulation and composition of the experimental diets for largemouth bass.

Ingredients (g/kg dry matter)	Diets[1]			
	D1	D2	D3	D4
Fish meal[2]	400	300	200	100
Blood meal[3]	40	40	40	40
Gluten[4]	50	50	50	50
Soybean meal[5]	100	100	100	100
Soybean protein concentrate[6]	130	130	130	130
Cottonseed protein concetrated[7]	0	111	222	333
Cassava starch[8]	110	110	110	110
Fish oil[9]	32.6	35.8	39.0	42.2
Soybean oil	32.6	35.8	39.0	42.2
Vitamin and mineral additives[10]	10	10	10	10
Monocalcium phosphate	15	15	15	15
Choline chloride	1	1	1	1
Microcrystalline cellulose	78.8	61.4	44.0	26.6
Proximate chemical compositions (g/kg dry matter)				
Moisture	21.1	21.0	23.7	20.2
Crude protein	504.3	498.3	497.4	505.4
Crude lipid	83.2	89.7	83.3	85.5
Free gossypol (mg/kg)	–	80	151	284

[1]D1–D4: 0, 25, 50, and 75% of the fish meal was replaced by cottonseed protein concentrate.
[2]Fish meal: From Superprime, TASA Fish Product Co. Ltd, Peru.
[3]Blood meal: From Beijing Yangyuan Veterinary Medicine Technology Co., Ltd, Beijing, China.
[4]Gluten: From Henan Midaner Trading Co., Ltd, Xinzheng, Henan, China.
[5]Soybean meal: From Qingdao Bohai Agricultural Development Co., Ltd, Qingdao, China.
[6]Soybean protein concentrate: From Yihai grain and oil industry Co., Ltd, Taizhou, Jiangsu, China.
[7]Cottonseed protein concetrated: From Xinjiang Jinlan Plant Protein Co., Ltd, Xinjiang, China.
[8]Cassava starch: From Wuhan Yiteng Starch Co., Ltd, Wuhan, China.
[9]Fish oil: Peru anchovy oil, purchased from Coland Feed Co., Ltd., Wuhan, China.
[10]Vitamin and mineral additives: From Guangdong Nutriera Group, Guangzhou, China.

TABLE 2 | Proximate composition of cottonseed protein concentrate used in the experimental diets (g/kg dry matter).

Proximate composition	Cottonseed protein concentrate
Moisture	16.7
Crude protein	615.1
Crude lipid	23.6
Ash	81.2
Free gossypol (mg/kg)	709

was conducted in an indoor recirculating system with 12 circular plastic tanks (volume 140 L). At the beginning of the experiment, all fish were deprived of feed for 24 h. Two hundred and forty healthy fish with an initial body weight of 15.11 ± 0.02 g were randomly divided into 4 groups with 3 replicates in each group and 20 fish per replicate.

During the experiment, fish were fed to apparent satiation twice daily at 08:30 and 16:30 for 8 weeks. Daily feed intake was recorded, and uneaten feed was taken out and recorded. This experiment was conducted under 12 h light: 12 h dark, and aeration was supplied to each tank 24 h per day. The water temperature was maintained at 27.0°C ± 1.0°C, pH 7–8, dissolved oxygen > 5.0 mg/L, and ammonia-N < 0.5 mg/L.

TABLE 3 | Amino acid composition of the experimental diets (g/kg dry matter).

	D1	D2	D3	D4	CPC
Essential amino acids (EAAs)					
Met	5.80	6.46	5.12	2.71	2.96
Lys	29.63	32.35	27.21	25.26	24.68
Thr	16.88	17.92	15.96	15.85	18.08
Ile	18.33	19.25	16.94	16.74	18.46
His	14.21	14.52	14.41	15.07	17.81
Val	19.94	19.87	19.60	20.26	22.95
Leu	35.78	37.23	34.07	33.78	34.08
Arg	19.42	17.16	20.91	23.96	41.85
Phe	22.52	21.71	22.52	24.45	33.33
Non-essential amino acids (NEAAs)					
Asp	37.09	37.25	36.47	37.41	44.87
Ser	19.06	19.13	18.80	19.89	24.16
Glu	70.77	68.74	73.14	84.61	95.16
Gly	22.14	23.15	20.91	20.25	24.54
Ala	23.17	24.39	21.25	20.34	22.56
Cys	1.78	0.48	1.53	2.67	3.83
Pro	24.47	23.71	22.83	23.37	24.03
Tyr	12.07	12.62	11.78	12.13	14.33

D1–D4: 0, 25, 50, and 75% of the fish meal was replaced by cottonseed protein concentrate. CPC, cottonseed protein concentrate.

Sampling

At the end of the feeding trial, all fish were deprived of diets for 24 h. Then, all fish in each tank were bulk weighted to calculate mean final body weight (FBW), weight gain rate (WGR), specific growth rate (SGR), and feed efficiency (FE). Two fish from each tank were randomly selected and frozen at − 20°C for the analysis of body composition. Six fish from each tank were anesthetized with 60 mg/L MS-222, fish body weight and length, viscera, and liver weight of the three fish were recorded to calculate condition factor (K), hepatosomatic index (HSI), and viscerosomatic index (VSI). Three fish from each tank were selected, and liver samples near to the bile were collected, fast-frozen in liquid nitrogen, and stored at − 80°C for mRNA expression, enzyme analysis, and western blot analysis. Another three fish from each tank were sampled, and the liver tissues were collected and stored at − 20°C to determine the fat content of the liver. The liver and mid-intestine tissues from the three fish per tank were sampled, approximately 5 mm × 5 mm × 5 mm tissues were fixed in 4% paraformaldehyde (Boerfu Biotechnology Co., Ltd., Wuhan, China) and stored at 4°C no longer than 48 h for histological analysis.

Biochemical Assays

Analysis of the proximate composition of the experimental diets and fish samples was executed according to an AOAC protocol

(Association of Official Analytical Chemists [AOAC], 2003). Moisture content was determined by oven at 105°C until constant weight. Ash content was measured by incineration in a muffle furnace (Muffle furnace, Yingshan, Hubei, China) at 550°C for 12 h. Crude protein content was measured by the Kjeldahl method using a Kjeltec analyzer unit (Foss Tecator, Höganäs, Sweden), and crude lipid content was measured by the Soxtec system (Soxtec System HT6, Tecator, Höganäs, Sweden). The amino acid contents of all experimental diets were determined after acid hydrolysis in 6 N HCl for 24 h at 11°C according to the method of Tu et al. (2015) and then were analyzed using Hitachi L-8800 Amino Acid Analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan). Free gossypol levels of the experimental diets and CPC were determined according to the aniline method (Bian et al., 2016). Free gossypol was extracted in the presence of 3-amino-1-propanol with a mixture of 2-propanol and hexane. The extracted free gossypol was converted into gossypol-aniline with aniline. Finally, the absorbance of the compound was measured using a spectrophotometer at the wavelength of 440 nm. Liver samples were freeze-dried (Alpha 1-4 LD-plus, Christ, Osterode, Germany), and then the liver lipid content was determined by chloroform/methanol (V/V, 2:1) extraction method.

Activities of alkaline phosphatase (AKP), catalase (CAT), total superoxide dimustase (T-SOD), and malondialdehyde (MAD) content of liver tissues were measured according to the instructions of the commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China).

Histological Analysis

The liver and mid-intestine tissues fixed by 4% paraformaldehyde were dehydrated in a series of ethanol, embedded in paraffin, and cut into 5 μm sections. The sections were stained following the protocols of hematoxylin and eosin (H&E). Frozen liver sections were fixed in formalin and stained with oil red O. Then the samples were observed and photographed by using a microscope system (DM1000, Leica Microsystems, Germany). The image was analyzed by Image-Pro Plus 6.0.

Quantitative Real-Time PCR Analysis

Total RNA of the liver was extracted using TRIzol reagent and electrophoresed on an agarose gel to evaluate the integrity. The concentration of extracted RNA was spectrophotometrically quantified with Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, United States). In total, 1 μg of extracted total RNA was reverse transcribed to cDNA using an M-MLV First-Strand Synthesis Kit (Invitrogen, Shanghai, China), and the obtained cDNA was used for PCR. The quantitative real-time PCR was performed in LightCycle§ 480 II system in a 6 μl reaction volume containing LightCycle 480 SYBR Green I Master Mix (Roche, Switzerland). The final reaction volume of 6 μl includes 2 μl cDNA, 0.24 μl forward and reverse primer, 0.52 μl ddH_2O, and 3 μl LightCycle 480 SYBR Green I Master Mix. Negative controls were done in the same template which template was replaced with ddH_2O. The qPCR was conducted with the following condition: 95°C for 5 min followed by 40 cycles with 10 s at 95°C, 20 s at Tm, and 10 s at 75°C. After PCR final cycle, the melted curve was performed to confirm the amplification of a single product with 0.5°C increment from 65°C to 95°C. β-*actin* and *ef-1α*, expressed very stable in largemouth bass liver tissue, were chosen as the endogenous reference for normalization. The primers used for transcriptional expression were obtained from previous literatures (**Table 4**). The results were calculated according to the method of Su et al. (2020).

Western Blot Analysis

The western blot analysis was carried out according to the method described by Yang et al. (2018). Liver tissues were lysed by RIPA lysis buffer (Beyotime Biotechnology, China) with protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche, Basel, Switzerland). Equal amounts of protein were separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to poly(vinylidene fluoride) (PVDF) membranes. The PVDF membranes were blocked for 1 h with 5% milk in TBST buffer and then incubated with anti-phospho-m-target of rapamycin (TOR) (1:1000, #2971; CST, Danvers, MA, United States), anti-mTOR Antibody (1:1000, #2972; CST, Danvers, MA, United States), anti-phospho-ribosomal protein (S6)$^{Ser235/236}$ (1:1000, #4858; CST, Danvers, MA, United States), anti-S6 (1:1000, #2217; CST, Danvers, MA, United States), and anti-GAPDH (1:1000, ab8245; Abcam). Horseradish peroxidase-labeled secondary antibodies were used

TABLE 4 | Primers used in quantitative real-time PCR analysis.

Gene name	Primers	Sequence 5′-3′	Sources
β-*actin*	F	ATCGCCGCACTGGTTGTTGAC	Chen et al., 2012
	R	CCTGTTGGCTTTGGGGTTC	
ef-1α	F	TGCTGCTGGTGTTGGTGAGTT	Yu et al., 2019
	R	TTCTGGCTGTAAGGGGGCTC	
tgf-β	F	GCTCAAAGAGAGCGAGGATG	Yu et al., 2019
	R	TCCTCTACCATTCGCAATCC	
il-10	F	CGGCACAGAAATCCCAGAGC	Yu et al., 2019
	R	CAGCAGGCTCACAAAATAAACATCT	
il-11β	F	TTCCCAACAGACAGATGAAGAACTC	Yu et al., 2019
	R	TGCCTGTGTTCAGCCAGTCAA	
tnf-α	F	CTTCGTCTACAGCCAGGCATCG	Yu et al., 2019
	R	TTTGGCACACCGACCTCACC	
il-15	F	GTATGCTGCTTCTGTGCCTGG	Yu et al., 2019
	R	AGCGTCAGATTTCTCAATGGTGT	
cpt1	F	CATGGAAAGCCAGCCTTTAG	Yu et al., 2019
	R	GAGCACCAGACACGCTAACA	
pparα	F	CCACCGCAATGGTCGATATG	Yu et al., 2019
	R	TGCTGTTGATGGACTGGGAAA	
Fasn	F	TGTGGTGCTGAACTCTCTGG	Yu et al., 2019
	R	CATGCCTAGTGGGGAGTTGT	
Tor	F	TCAGGACCTCTTCTCATTGGC	Li et al., 2021
	R	CCTCTCCCACCATGTTTCTCT	
s6	F	GCCAATCTCAGCGTTCTCAAC	Li et al., 2021
	R	CTGCCTAACATCATCCTCCTT	

F, forward primer; R, reverse primer; ef-1α, elongation factor 1α; tgf-β, transforming growth factor β; il-10, interleukin 10; il-11β, interleukin 11 β; tnf-α, tumor necrosis factor α; il-15, interleukin; cpt1, carnitine palmitoyl transferase 1; pparα, peroxisome proliferator-activated receptor α; tor, target of rapamycin; s6, ribosomal protein.

to generate a chemiluminescent signal that was detected by ImageQuant LAS 4000mini (GE Healthcare Life Sciences). GAPDH was used as a control.

Calculation and Statistical Analysis

The WGR, SGR, FE, K, VSI, and HSI were calculated as follows:

$$\text{WGR}(\%) = 100 \times (\text{FBW} - \text{initial body weight})/\text{initial body weight}.$$

$$\text{SGR}(\%/\text{d}) = 100 \times [\text{LnFBW} - \text{Ln IBW}]/\text{days}.$$

$$\text{FE}(\%) = 100 \times \text{bodyweightgain}/\text{dry feed consumed}.$$

$$\text{K}(\text{g}/\text{cm}^3) = 100 \times \text{FBW}/(\text{bodylength})^3.$$

$$\text{VSI}(\%) = 100 \times \text{visceraweight}/\text{whole body weight}.$$

$$\text{HSI}(\%) = 100 \times \text{liverweight}/\text{whole body weight}.$$

All data were analyzed by SPSS 20 (SPSS Inc., Chicago, IL, United States) for windows. Before one-way ANOVA tested the differences among groups, all data were tested normal distribution and homogeneity of variances. After ANOVA identified the differences, Duncan's multiple range testing was used to identify the significant differences between group means. Effects with a probability of $P < 0.05$ were considered statistically significant. All data are presented as means ± SE.

RESULTS

Growth Performance

Effects of replacement of fishmeal with CPC on growth performance, feed utilization, and morphological indices of largemouth bass are shown in **Table 5**. The WGR in the D4 group was significantly lower ($P < 0.05$) than other groups, and there was no significant difference between D2 or D3 and D1 group ($P > 0.05$). And D4 group had the lowest SGR than other groups. Similar results were found in SGR and FE ($P < 0.05$). In addition, compared to the other groups, replacing fishmeal with CPC by 75% (D4 group) significantly decreased ($P < 0.05$) K and HSI of largemouth bass. But no significant differences were observed in VSI of largemouth bass fed different CPC diets (**Table 5**).

Body Composition

A significant increase ($P < 0.05$) of body lipid content was observed in the D4 group compared to the control D1 group, but no significant change ($P > 0.05$) of body lipid content was found between the D2 or D3 and D1 groups (**Table 6**). No changes of crude protein and moisture contents ($P > 0.05$) were found in largemouth bass fed different CPC diets. D4 group had a lower ash content ($P < 0.05$) than D1 and D2 groups (**Table 6**).

Similar to the results of body lipid content, the liver lipid content of largemouth bass increased with increasing dietary CPC levels, and the D4 group exhibited a significantly higher ($P < 0.05$) content of liver lipid than other groups (**Figure 1**).

TABLE 5 | The growth performance of largemouth bass fed different cottonseed protein concentrate diets for 8 weeks.

Items	Groups			
	D1	D2	D3	D4
IBW (g)	15.2 ± 0.09	15.1 ± 0.02	15.0 ± 0.02	15.2 ± 0.02
FBW (g)	63.1 ± 1.1^{b}	63.1 ± 1.3^{b}	63.5 ± 0.01^{b}	54.9 ± 2.0^{a}
WGR (%)	316.4 ± 7.6^{b}	317.4 ± 8.4^{b}	322.0 ± 0.6^{b}	261.8 ± 12.6^{a}
SGR (%/d)	2.7 ± 0.03^{b}	2.7 ± 0.04^{b}	2.70 ± 0.003^{b}	2.4 ± 0.07^{a}
FE (%)	119.7 ± 1.0^{b}	119.9 ± 0.4^{b}	118.8 ± 0.7^{b}	107.2 ± 2.1^{a}
K (g/cm^3)	2.2 ± 0.02^{b}	2.2 ± 0.01^{b}	2.2 ± 0.03^{b}	2.1 ± 0.05^{a}
VSI (%)	8.1 ± 0.2	8.4 ± 0.3	8.1 ± 0.2	8.5 ± 0.5
HSI (%)	2.9 ± 0.2^{b}	3.0 ± 0.2^{b}	2.7 ± 0.2^{ab}	2.3 ± 0.2^{a}

The results are presented as means ± SE values in the same column with different superscripts are significantly different (P < 0.05).

TABLE 6 | Effects of replacing fish meal with cottonseed protein concentrated on body composition of largemouth bass.

Items	Groups			
	D1	D2	D3	D4
Crude protein (%)	17.19 ± 0.12	17.29 ± 0.15	17.24 ± 0.10	16.98 ± 0.10
Crude lipid (%)	5.94 ± 0.29^{a}	6.34 ± 0.21^{ab}	6.86 ± 0.43^{ab}	7.16 ± 0.33^{b}
Ash (%)	3.71 ± 0.07^{b}	3.85 ± 0.24^{b}	3.68 ± 0.06^{ab}	3.39 ± 0.06^{a}
Moisture (%)	72.37 ± 0.42	71.80 ± 0.14	71.63 ± 0.35	71.95 ± 0.40

The results are presented as means ± SE values in the same column with different superscripts were significantly different (P < 0.05).

Histomorphology

Through the oil red O staining of the liver, it was found that fish in the D4 group showed an aggravated fat deposition (**Figure 2**). The hepatocytes in fish fed the control D1 diet showed clear boundary with vacuolated cytoplasm and no obvious vacuolar degeneration and inflammatory cells infiltration (**Figure 3**). However, with the increasing dietary CPC levels, largemouth bass a exhibited different degree of hepatocyte degeneration and inflammatory cells infiltration. Compared to the control D1 group, the hepatocytes of fish in the D4 group exhibited severe abnormalities with a disappeared boundary of hepatocytes, hepatocytes volume shrinkage, nucleus pyknosis, aggravated vacuolar degeneration, and inflammatory cells infiltration (**Figure 3**).

As shown in **Figure 4**, the structure of the mid-intestine in the D1, D2, or D3 group is normal. However, when 75% of dietary fishmeal was replaced by CPC, the intestinal villi of largemouth bass appeared shorter and sparse with severe epithelial damage in the D4 group. D4 group exhibited significantly lower ($P < 0.05$) villus height and width than the D1 group (**Table 7**).

Liver Antioxidant Enzymes

Fish in the D2 group had the highest activity of liver AKP, and the D4 group had the lowest activity of AKP among all groups (**Figure 5**). The activities of CAT and T-SOD in the D4 group were significantly higher ($P < 0.05$) than other groups. No

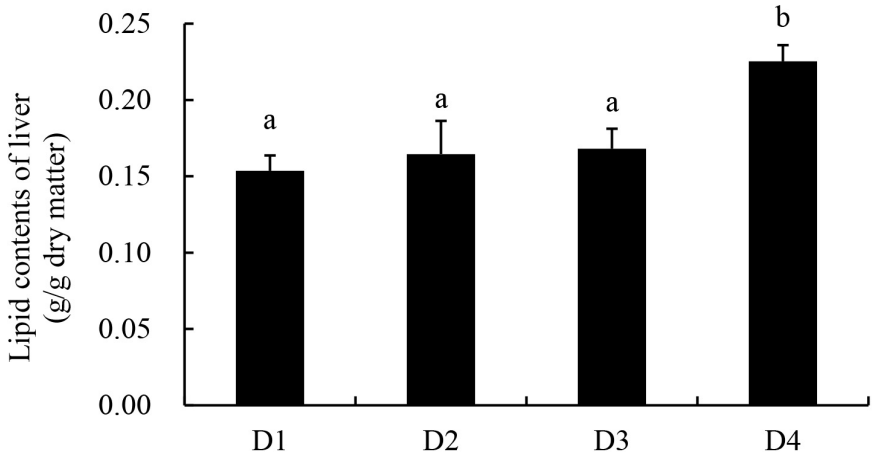


FIGURE 1 | Lipid content of liver tissues in largemouth bass fed different cottonseed protein concentrate diets for 8 weeks. Bars assigned with different letters were significantly different ($P < 0.05$).

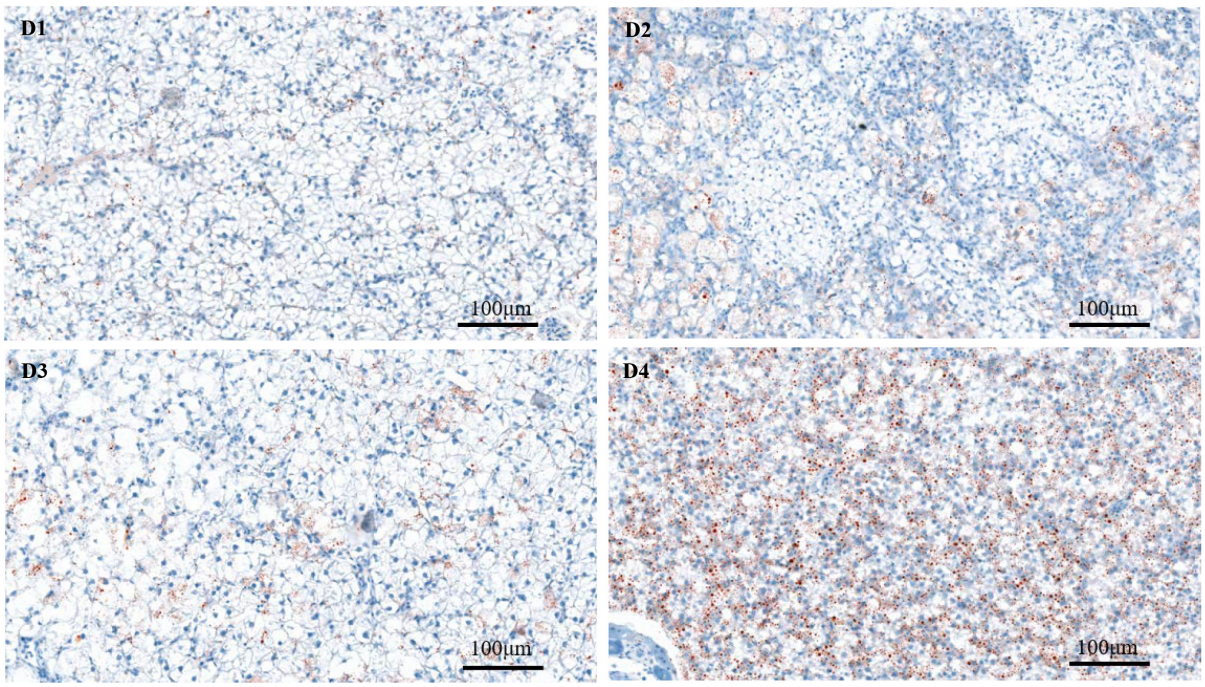


FIGURE 2 | Oil Red O staining (200×) of fat deposition of live tissues in largemouth bass fed different cottonseed protein concentrate diets for 8 weeks. Red points represent lipid droplets. Scale bar: 100 μm. D1–D4: 0, 25, 50, and 75% of the fish meal was replaced by cottonseed protein concentrate.

significant difference in the MDA content was found ($P > 0.05$) in largemouth bass fed different CPC diets (**Figure 5**).

Gene Expression and Protein Level in Liver Tissues

The transcriptional level of inflammation-related genes in liver tissues is shown in **Figure 6**. Anti-inflammatory-related cytokines transforming growth factor β (*tgf*-β), interleukin 10 (*il-10*), and interleukin 11 β (*il-11*β) were significantly induced in the D2 group, but were significantly downregulated in the D4 group ($P < 0.05$). However, the mRNA expression of pro-inflammatory cytokines *tnf*-α and *il-15* was not affected by the replacement of fishmeal by CPC ($P > 0.05$).

The relative expression of lipid metabolism-related genes in the liver is presented in **Figure 7**. The genes related to lipolysis peroxisome proliferator-activated receptor α (*ppar*α) and carnitine palmitoyl transferase 1 (*cpt1*) were downregulated with the increasing dietary CPC levels. The transcriptional level of *cpt1* was significantly higher ($P < 0.05$) in the D2 group

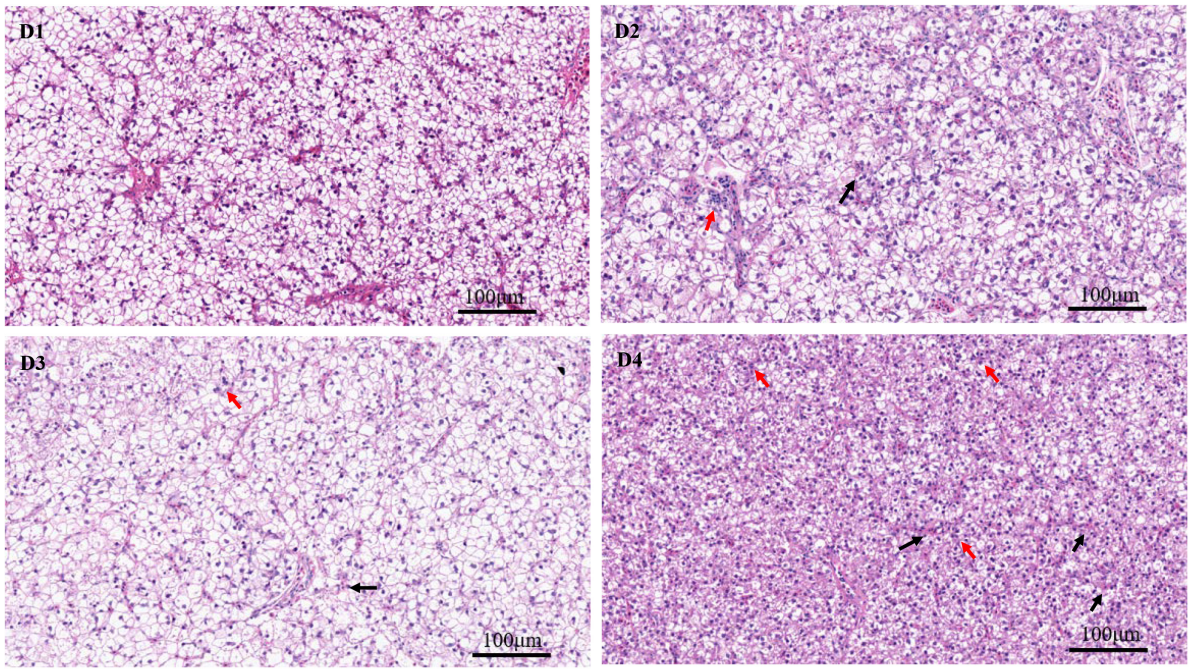


FIGURE 3 | H & E staining (200×) of liver histomorphology in largemouth bass fed different cottonseed protein concentrate diets for 8 weeks (black arrows show the inflammatory cells infiltration, and red arrows show the nucleus pyknosis). Scale bar: 100 μm. D1–D4: 0, 25, 50, and 75% of the fish meal was replaced by cottonseed protein concentrate.

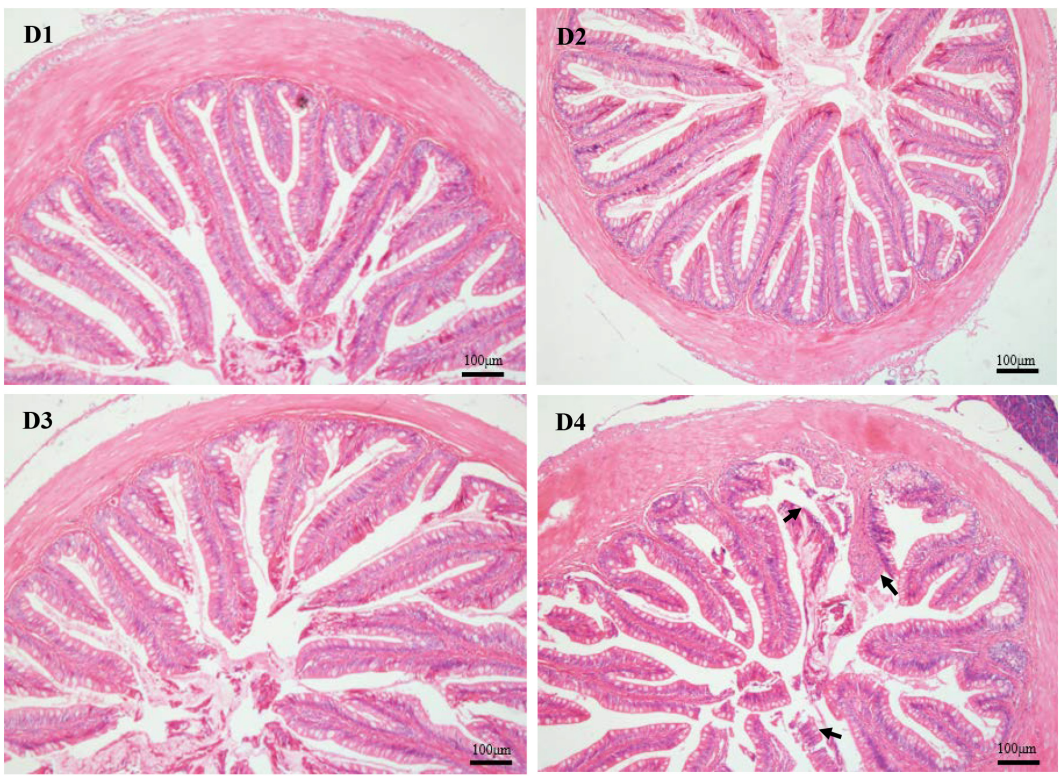


FIGURE 4 | H & E staining (100×) of mid-intestinal histology in largemouth bass fed different cottonseed protein concentrate diets for 8 weeks (black arrows show the epithelial damage). Scale bar: 100 μm. D1–D4: 0, 25, 50, and 75% of the fish meal was replaced by cottonseed protein concentrate.

TABLE 7 | Histological parameters of mid-intestinal in largemouth bass fed different cottonseed protein concentrate diets.

Groups	Villus height (μm)	Villus width (μm)
D1	564.43 ± 20.67^{b}	110.81 ± 3.89^{b}
D2	566.98 ± 15.60^{b}	99.93 ± 6.01^{ab}
D3	519.32 ± 13.41a^{b}	99.99 ± 2.45^{ab}
D4	476.63 ± 8.48^{a}	96.12 ± 3.52^{a}

The results are presented as means ± SE values in the same column with different superscripts were significantly different (P < 0.05).

than in the other groups. However, the relative expression of *fasn* was not significantly affected ($P > 0.05$) by the replacing of fishmeal with CPC.

The relative expression of *TOR* and *S6*was downregulated in fish with increasing dietary CPC levels (**Figures 8A,B**). Compared to the control D1 group, the D2 group did not exhibit a negative regulation ($P > 0.05$) of gene expression of *TOR* and *S6*. However, D4 group had the lowest level of relative expression of *tor* among all groups (**Figures 8A,B**). The corresponding protein levels of TOR and S6 were also detected. The phosphorylation activation of TOR and S6 was significantly downregulated ($P < 0.05$) in the D4 group (**Figures 8C–E**).

DISCUSSION

Many studies have been conducted to investigate the effects of replacing dietary fishmeal with plant protein on the growth performance of fish (Yin et al., 2018; Abbasi et al., 2020; Shen et al., 2020; Yaghoubi et al., 2020). In the present study, dietary replacement of 25% or 50% of dietary fishmeal by CPC had no negative effects on growth performance, but dietary substitution of 75% fishmeal by CPC significantly decreased the growth performance of the fish. The present results were supported by previous results that no adverse effects on growth performance were found in juvenile golden pompano when dietary fishmeal was replaced by CPC at a moderate level, but high substitution led to a significant negative effect on the growth performance of fish (Shen et al., 2020). It was also reported that in hybrid grouper, WGR and SGR were significantly declined when over 48% of dietary fishmeal was replaced by CPC (Yin et al., 2018). These findings indicated that a suitable replacement level of CPC in the diet showed no negative effects and even had positive effects on growth performance. It has been reported that a suitable CPC level in the diet may improve the amino acids profile in feed and make the proportion of amino acids more reasonable and suitable for aquatic animals (Elangovan and Shim, 2000; Yin et al., 2018). However, the high incorporation of CPC in diets impaired the growth performance of largemouth bass. Dietary CPC inclusion was often accompanied by poor palatability, imbalanced amino acid composition, and anti-nutritional factors (Chen et al., 2020; He et al., 2021). When replacing fishmeal with a high level, it would significantly affect the growth of fish (Yin et al., 2018; He et al., 2021). Our research results demonstrated that 111–222 g/kg CPC is the suitable replacement level for largemouth bass.

The liver is the most important organ of body metabolism (Tamura and Shimomura, 2005). In the present study, the lipid

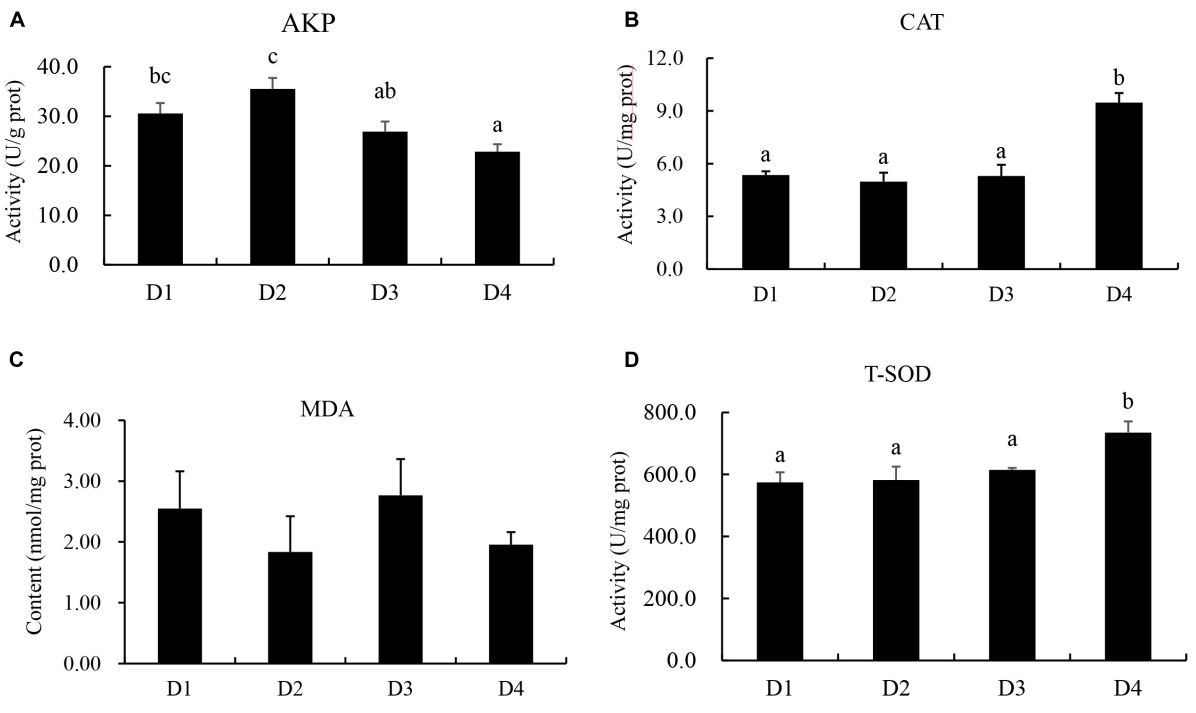


FIGURE 5 | Effects of replacing fish meal with cottonseed protein concentrate on liver antioxidant capacities of largemouth bass. **(A)** AKP, alkaline phosphatase. **(B)** CAT, catalase. **(C)** MDA, malondialdehyde. **(D)** T-SOD, total superoxide dimustase. Bars assigned with different letters were significantly different ($P < 0.05$).

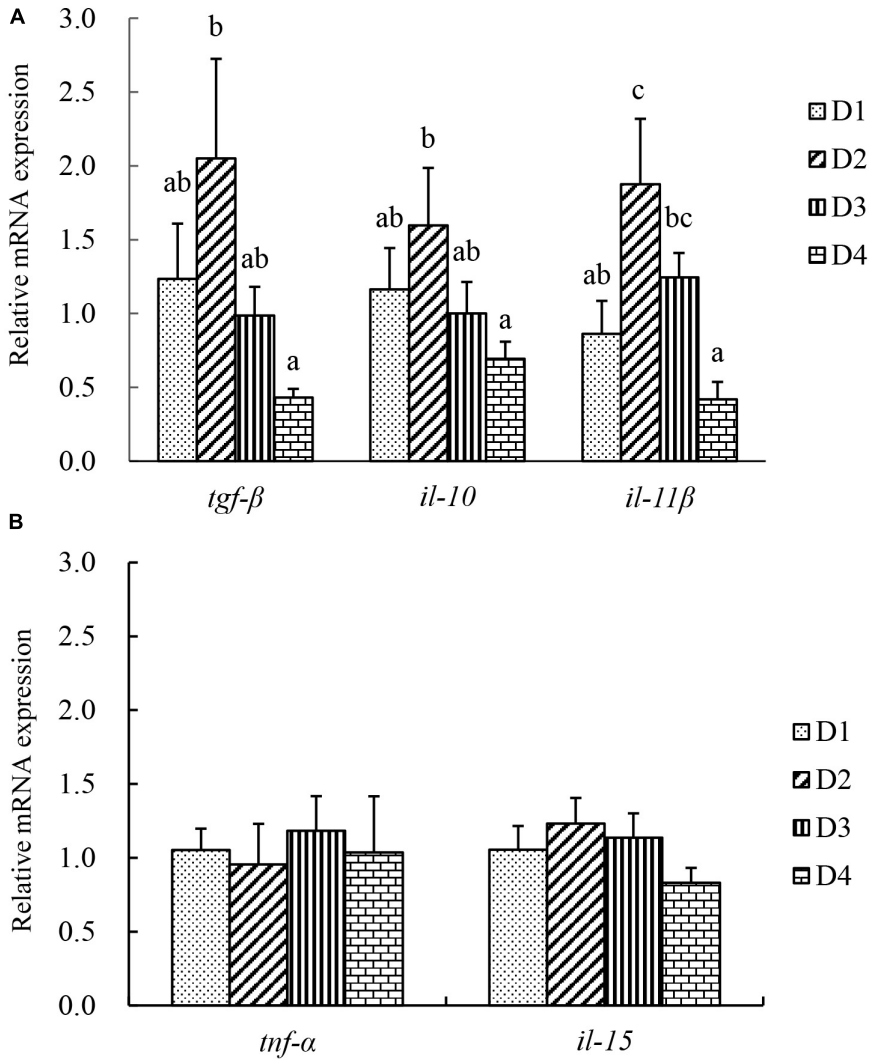


FIGURE 6 | The transcriptional level of inflammation-related genes in liver of largemouth bass fed different cottonseed protein concentrate diets for 8 weeks. **(A)** Anti-inflammatory related cytokines, transforming growth factor β (*tgf*-β), interleukin 10 (*il-10*), and interleukin 11β (*il-11*β). **(B)** Pro-inflammatory related cytokines, tumor necrosis factor α (*tnf*-α), and interleukin (*il-15*). Bars assigned with different letters were significantly different ($P < 0.05$).

deposition in liver tissues of largemouth bass was significantly increased in the D4 group. Similar results were reported that there was obvious fat deposition in liver tissues of hybrid grouper fed diet with the replacement of 36% fishmeal by CPC (Yin et al., 2018). Zhang et al. (2019) also reported that fatty liver was induced in Japanese seabass fed a full plant protein diet of soybean protein and CPC which was explained that a full plant protein diet caused nutrient and energy metabolic disorder and then induced fatty liver. In this study, 75% CPC replacement level might induce metabolic disorder in the liver. This was supported by the lipid metabolism-related gene expression (*cpt1*, *pparα*, and *fasn*) and histological results. In the present study, the D4 group with replacement of 75% fishmeal by CPC had significant downregulation of TOR signaling, lipolysis-related genes, and anti-inflammatory cytokine genes, which indicated that the metabolism of fish was significantly affected by the high substitution of CPC. In addition, from the histomorphology of the liver, we found that compared to the control D1 group, the hepatocytes of largemouth bass fed diet with the replacement of 75% fishmeal by CPC exhibited serious degeneration, volume shrinkage, and inflammatory cells infiltration. The results were well agreed with a previous study that juvenile turbot fed diet with 45% of fishmeal replaced by cottonseed meal led to smaller liver cell and liver damage (Bian et al., 2016). From the mid-intestinal histology, abnormal shorter and sparse with severe epithelial damage in fish was observed in the D4 group. Consistently, many researchers have reported that high plant proteins inclusion diet-induced intestinal structure damage in juvenile turbot (Bian et al., 2016), Japanese seabass (Wang et al., 2016), and largemouth bass (He et al., 2020). Therefore, all these indicated that 75% of dietary fishmeal replaced by CPC might induce abnormal liver fat deposition, liver, and intestinal

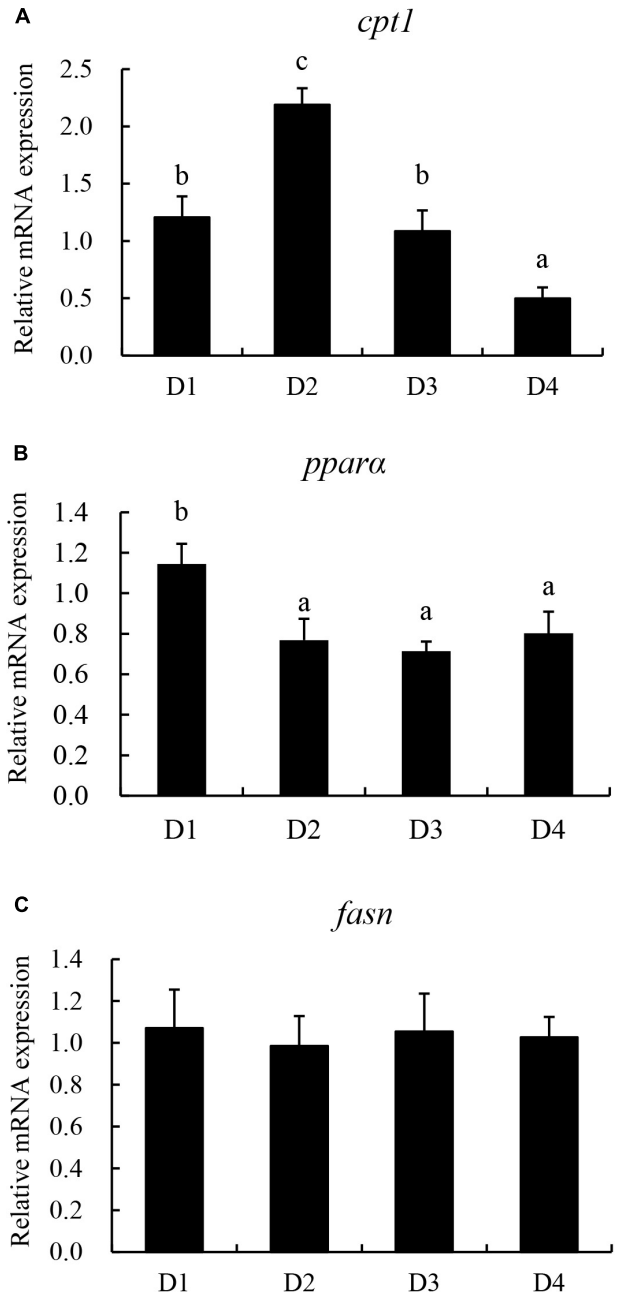


FIGURE 7 | Effects of replacing fishmeal with cottonseed protein concentrate on the transcriptional expression of lipid metabolism-related genes in liver of largemouth bass. **(A)** Carnitine palmitoyl transferase 1 (*cpt1*). **(B)** Peroxisome proliferator-activated receptor α (*pparα*). **(C)** Fatty acid synthase (*fasn*). Bars assigned with different letters were significantly different ($P < 0.05$).

damage of largemouth bass which would be closely related to the decreased growth of the fish.

Transforming growth factor β, il-10, and il-11β are the anti-inflammatory cytokines, and tnf-α and il-15 are two important pro-inflammatory cytokines, which play an important role in inflammatory processes and immune response to protect the liver from cell injury and initiate tissue regeneration (Yu et al., 2019; Zhang et al., 2019). In the present study, the gene expressions of *tgf*-β, *il-10*, and *il-11*β were significantly downregulated with the increasing dietary CPC levels, and these genes exhibited the highest transcriptional levels in the D2 group. Anti-inflammatory cytokines can prevent abnormal expression of immune response (Hoseinifar et al., 2018). The present results observed upregulation of anti-inflammatory cytokines in the D2 group (substitution of 25% fishmeal) and downregulation of anti-inflammatory cytokines in the D4 group (substitution of 75% fishmeal), together with the no changes of pro-inflammatory cytokines among all groups. All these indicated that low substitution of fishmeal by CPC caused an improved immune response of largemouth bass, but excessive substitution led to a significant decline in immune status which would also be closely related to the decreased growth and tissue damage of the fish.

In mammals and fish, the TOR signaling pathway, such as *tor*, *s6,* and so on, plays a key role in sensing nutrients and regulates organismal growth and homeostasis by coordinating the anabolism and catabolism (Lansard et al., 2010; Roux and Topisirovic, 2012; Bian et al., 2017). Furthermore, there is a positive correlation between the phosphorylation activation of the TOR pathway and growth in fish (Song et al., 2016; Bian et al., 2017). In the present study, the transcriptional and protein levels of *tor* and *s6* were significantly downregulated in fish of the D4 group (high substitution of 75% fishmeal). Similar results were reported by Zhou et al. (2018) that high dietary inclusion of plant protein decreased the relative expression of TOR pathway-related genes. Some studies have shown that gossypol and imbalanced amino acids can suppress TOR signaling (Tu et al., 2015; Bian et al., 2017). In the present study, high dietary inclusion of CPC contained high gossypol and imbalanced amino acids, which negatively regulated the TOR pathway. This indicated that the impaired TOR signaling pathway is one reason for the negative effect on growth performance in largemouth bass fed D4 diet.

In this study, the content of whole body protein and moisture were not affected by different dietary CPC inclusion. Similar results were reported in *Oncorhynchus mykiss* (Zhao et al., 2021) and *Litopenaeus vannamei* (Wan et al., 2018), which found that whole body protein and moisture contents were not significantly changed among CPC substitution groups. In the present study, whole body lipid content of largemouth bass was increased with the increasing dietary CPC levels. The result was consistent with previous results that the replacement of fishmeal by low-gossypol cottonseed meal increased whole body lipid content of juvenile southern flounder (*Paralichthys lethostigma*) and high dietary inclusion of low-gossypol cottonseed meal impaired the liver function of the fish (Alam et al., 2018). The present results of the increase of lipid content in whole body and liver tissues of fish with the increasing dietary CPC levels could be due to the significant downregulation of lipolysis genes and no change of lipogenesis in fish fed diets with high substitutions by CPC.

Compared to fishmeal, plant proteins are characterized as a high carbohydrate source (Zhang et al., 2019). High dietary carbohydrates often caused lipid metabolism disorder and caused lipid deposition in the liver (Zhang et al., 2020). In the current study, high dietary CPC along with high carbohydrates maybe

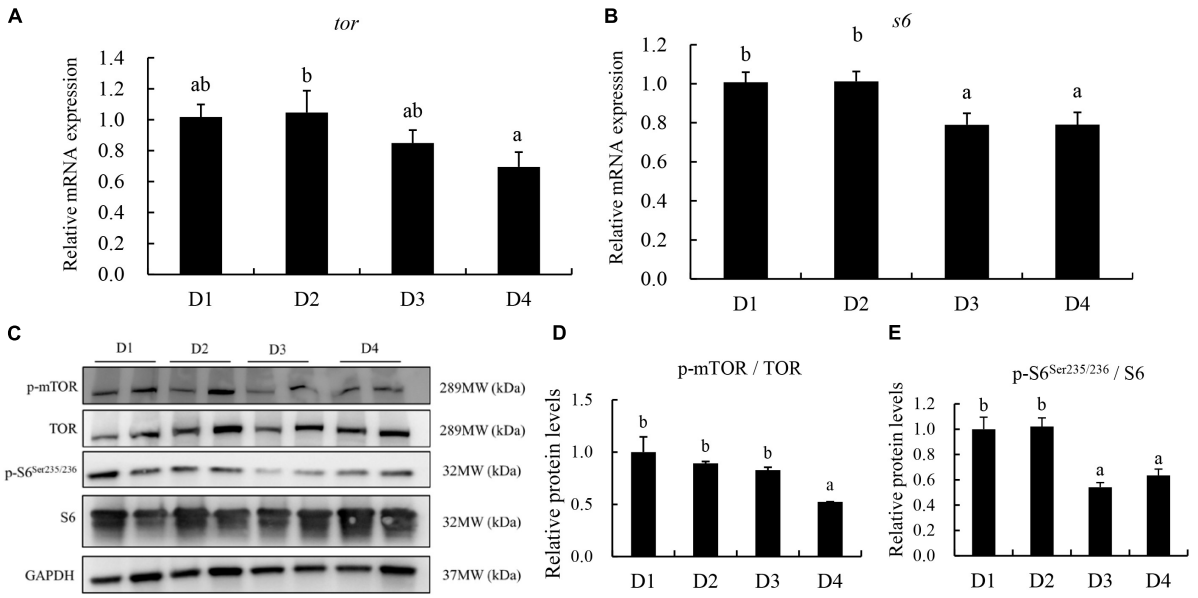


FIGURE 8 | The transcriptional and protein level of TOR and S6 in the liver of largemouth bass fed different cottonseed protein concentrate diets for 8 weeks. **(A)** Relative mRNA expression of target of rapamycin (*tor*). **(B)** Relative mRNA expression of ribosomal protein (*s6*). **(C–E)** Western blot analysis for protein levels of *tor* and *s6*. Bars assigned with different letters were significantly different ($P < 0.05$). D1–D4: 0, 25, 50, and 75% of the fish meal was replaced by cottonseed protein concentrate.

one important reason accounting for liver lipid deposition in largemouth bass. pparα is one of the important transcriptional factors in regulating fatty acids oxidation and lipolysis (Goto et al., 2011). Similarly, cpt1 plays an important role in mediating long-chain fatty acids oxidation (Kerner and Hoppel, 2000). In the present study, the gene expression of *pparα* and *cpt1* were lower in the D4 group than the D1 group. A similar result was reported that juvenile hybrid grouper fed a high level of dietary mixture plant protein downregulated pparα and cpt1 mRNA expression (Ye et al., 2019). fasn is an important enzyme involved in lipid synthesis, the mRNA level of *fasn* was not affected by different CPC inclusion level. The current results indicated that high-level dietary CPC inclusion caused lipid deposition in the liver of largemouth bass.

The organism antioxidant enzyme activity is an important indicator in reflecting the status of oxidative stress in fish response to external stimuli (Deng et al., 2015). T-SOD and CAT activities can reflect the ability of the body against oxidative stress (Yuan et al., 2019). In the present study, T-SOD and CAT were significantly increased in largemouth bass fed D4 diet, indicating that largemouth bass had suffered from oxidative stress caused by high lever CPC replacement, increased T-SOD and CAT activities moderated the damage caused by oxidative stress. Similar results were obtained in hybrid grouper (Yin et al., 2018). MDA is one of the important indicators of lipid peroxidation, it has a strong biotoxicity to cells (Parvez and Raisuddin, 2005). In the present study, there was no significant difference in MDA content among groups, which indicated that increased T-SOD and CAT mitigated the lipid peroxidation caused by oxidative stress. Similar results were observed in juvenile *Trachinotus ovatus* and *Penaeus monodon*, the MDA content in liver was not influenced by different CPC replacement level (Shen et al., 2020; Jiang et al., 2021). AKP is an important enzyme reflecting the health of organism, has a potential protective effect on fish (Ghehdarijani et al., 2016). In the current study, D2 group showed the highest AKP activity, but the D4 group had the lowest AKP activity among all groups. The present results indicated that oxidative stress occurred when 75% of dietary fishmeal was replaced by CPC, which may be one of the reasons for the negative effect on growth performance in largemouth bass fed high-level CPC diets.

CONCLUSION

In the present study, CPC can replace up to 50% of dietary fishmeal without any adverse influence on the growth, body composition, antioxidant indices, and intestinal health of largemouth bass. However, high dietary inclusion of CPC (75% replacement of fishmeal) would cause significant negative effects on the growth performance, liver, and intestine health of the fish. The present results indicated that suitable substitution of fishmeal by less than 222g CPC/kg diet had a positive effect on growth performance of fish, but an excessive substitution of 75% fishmeal by CPC led to the suppressed growth, liver, and intestinal damage of largemouth bass.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Institute of Hydrobiology, Chinese Academy of Sciences.

AUTHOR CONTRIBUTIONS

DH and YL designed the study. YL performed the experimental work and wrote the manuscript under the direction of DH. QL and LX contributed to perform the experiment. YY helped with biochemical analysis. JS, YG, ZZ, and HL gave helps for analyzing data. JJ, XZ, and SX provided suggestions on experimental design and manuscript writing. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by the National Key R&D Program of China (2019YFD0900200), China Agriculture Research System of MOF and MARA (CARS-46), National Natural Science Foundation of China (31972771, 31672670, and 31972805), Fund Project in State Key Laboratory of Freshwater Ecology and Biotechnology (2019FBZ02 and 2019FBZ05), Hubei High-Tech Innovation and Business Incubation Center (2019-02-055), and Science and Technology Project of Wuhan (2019020701011459).

ACKNOWLEDGMENTS

The authors are grateful to Guanghan Nie for his technical help.

REFERENCES

Abbasi, A., Oujifard, A., Torfi, M. M., Habibi, H., and Nafisi, B. M. (2020). Dietary simultaneous replacement of fish meal and fish oil with blends of plant proteins and vegetable oils in yellowfin seabream (*Acanthopagrus latus*) fry: Growth, digestive enzymes, antioxidant status and skin mucosal immunity. *Aquacult. Nutr.* 26, 1131–1111. doi: 10.1111/anu.13070

Alam, M. S., Watanabe, W. O., Carroll, P. M., Gabel, J. E., Corum, M. A., et al. (2018). Evaluation of genetically-improved (glandless) and genetically-modified low-gossypol cottonseed meal as alternative protein sources in the diet of juvenile southern flounder *Paralichthys lethostigma* reared in a recirculating aquaculture system. *Aquaculture* 489, 36–45.

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.

Association of Official Analytical Chemists [AOAC] (2003). *Official methods of analysis*. Rockville, MD: AOAC.

Bian, F., Zhou, H., He, G., and Wang, C. (2016). 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

Bian, F. Y., Jiang, H. W., Man, M. S., Mai, K. S., Zhou, H. H., Xu, W., et al. (2017). Dietary gossypol suppressed postprandial TOR signaling and elevated ER stress pathways in turbot (*Scophthalmus maximus* L.). *Am. J. Physiol.: Endocrinol. Metab.* 312, E37–E47. doi: 10.1152/ajpendo.00285.2016

Chen, G. F., Yin, B., Liu, H. Y., and Tan, B. P. (2020). Effects of fishmeal replacement with cottonseed protein concentrate on growth, digestive proteinase, intestinal morphology and microflora in pearl gentian grouper (*Epinephelus fuscoguttatus* ♀× *Epinephelus lanceolatu* ♂). *Aquacult. Res.* 51, 2870–2884.

Chen, Y. J., Liu, Y. J., Yang, H. J., Yuan, Y., Liu, F. J., Tian, L. X., et al. (2012). Effect of dietary oxidized fish oil on growth performance, body composition, antioxidant defense mechanism and liver histology of juvenile largemouth bass *Micropterus salmoides*. *Aquacult. Nutr.* 18, 321–331. doi: 10.1111/j.1365-2095.2011.00900.x

Cheng, Z. Y., Ai, Q. H., Mai, K. S., Xu, W., Ma, H. M., Li, Y., et al. (2010). Effects of dietary canola meal on growth performance, digestion and metabolism of Japanese seabass. Lateolabrax japonicus. *Aquaculture*. 305, 102–108. doi: 10.1016/j.aquaculture.2010.03.031

China Fishery Statistical Yearbook (2021). *Fishery Bureau*. Beijing: Ministry of Agriculture, China Agriculture Press.

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

Elangovan, A., and Shim, K. F. (2000). The influence of replacing fish meal partially in the diet with soybean meal on growth and body composition of juvenile tin foil barb (*Barbodes altus*). *Aquaculture* 189, 133–144. doi: 10.1016/S0044-8486(00)00365-3

FAO (2018). *The State of World Fisheries and Aquaculture 2018: Meeting the Sustainable Development Goals*. Rome: FAO.

Ghehdarijani, M. S., Hajimoradloo, A., Ghorbani, R., and Roohi, Z. (2016). The effects of garlic-supplemented diets on skin mucosal immune responses, stress resistance and growth performance of the caspian roach (*Rutilus rutilus*) fry. *Fish Shellfish Immunol.* 49, 79–83. doi: 10.1016/j.fsi.2015.12.021

Glencross, B. D., Baily, J., Berntssen, M. H., Hardy, R., MacKenzie, S., and Tocher, D. R. (2020). Risk assessment of the use of alternative animal and plant raw material resources in aquaculture feeds. *Rev. Aquacult.* 12, 703–758. doi: 10.1111/raq.12347

Goto, T., Lee, J., Teraminami, A., Kim, Y., Hirai, S., Uemura, T., et al. (2011). Activation of peroxisome proliferator-activated receptor-alpha stimulates both differentiation and fatty acid oxidation in adipocytes. *J. Lipid Res.* 52, 873–884. doi: 10.1194/jlr.M011320

Hardy, R. W. (2010). Utilization of plant proteins in fish diets: Effects of global demand and supplies of fishmeal. *Aquacult. Res.* 41, 770–776. doi: 10.1111/j.1365-2109.2009.02349.x

He, M., Li, X. Q., Poolsawat, L., Guo, Z. H., Yao, W. X., Zhang, C. Y., et al. (2020). Effects of fish meal replaced by fermented soybean meal on growth performance, intestinal histology and microbiota of largemouth bass (*Micropterus salmoides*). *Aquacult. Nutr.* 26, 1058–1071. doi: 10.1111/anu.13064

He, Y. F., Guo, X. W., Tan, B. P., Dong, X. H., and Chi, S. Y. (2021). Replacing fishmeal with cottonseed protein concentrate in feed for pearl gentian groupers (*Epinephelus fuscoguttatus* ♀× *E. lanceolatus* ♂): Effects on growth and expressions of key genes involved in appetite and hepatic glucose and lipid metabolism. *Aquacult. Rep.* 20:100710. doi: 10.1016/j.aqrep.2021.100710

Hoseinifar, S. H., Zou, H. K., Doan, H. V., Miandare, H. K., and Hoseini, S. M. (2018). Evaluation of some intestinal cytokines genes expression and serum innate immune parameters in common carp (*Cyprinus carpio*) fed dietary loquat (*Eriobotrya japonica*) leaf extract. *Aquacult. Res.* 49, 120–127. doi: 10.1111/are.13440

Huang, D., Wu, Y. B., Lin, Y. Y., Chen, J. M., Karrow, N., Ren, X., et al. (2017). Dietary protein and lipid requirements for juvenile largemouth bass, *Micropterus salmoides*. *J. World Aquacult. Soc.* 48, 782–790. doi: 10.1111/jwas.12417

Jiang, S., Chen, Z. B., Zhou, F. L., Yang, Q. B., Huang, J. H., Yang, L. S., et al. (2021). Study on partial replacement of fish meal with concentrated dephenolized cottonseed protein in feed of *Penaeus monodon*. *Aquacult. Res.* 52, 3871–3881. doi: 10.1111/are.15231

Kerner, J., and Hoppel, C. (2000). Fatty acid import into mitochondria. *BBA-Mol Cell Biol L.* 1486, 1–17. doi: 10.1016/S1388-1981(00)00044-5

Lansard, M., Panserat, S., Plagnes-Juan, E., Seiliez, I., and Skiba-Cassy, S. (2010). Integration of insulin and amino acid signals that regulate hepatic metabolism-related gene expression in rainbow trout: role of tor. *Amino Acids* 39, 801–810. doi: 10.1007/s00726-010-0533-3

Li, S. L., Dai, M., Qiu, H. J., and Chen, N. S. (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, X. Y., Zheng, S. X., Ma, X. K., Cheng, K. M., and Wu, G. Y. (2020). Effects of dietary protein and lipid levels on the growth performance, feed utilization, and liver histology of largemouth bass (*Micropterus salmoides*). *Amino Acids* 52, 1043–1061. doi: 10.1007/s00726-020-02874-9

Lim, C. (2010). Substitution of cottonseed meal for marine animal protein in diets for *Penaeus vannamei*. *J. World Aquacult. Soc*. 27, 402–409.

Lim, C., and Dominy, W. (1990). Evaluation of soybean meal as a replacement for marine animal protein in diets for shrimp (*Penaeus vannamei*). *Aquaculture* 87, 53–63. doi: 10.1016/0044-8486(90)90210-E

Liu, H., Dong, X. H., Tan, B. P., Du, T., Zhang, S., Yang, Y. Z., et al. (2020). Effects of fish meal replacement by low-gossypol cottonseed meal on growth performance, digestive enzyme activity, intestine histology and inflammatory gene expression of silver sillago (*Sillago sihama* Forssk'al) (1775). *Aquacult. Nutr*. 26, 1724–1735. doi: 10.1111/anu.13123

Liu, X. H., Ye, J. D., Wang, K., Kong, J. H., Yang, W., and Zhou, L. (2012). Partial replacement of fish meal with peanut meal in practical diets for the Pacific white shrimp, *Litopenaeus vannamei*. *Aquacult. Res*. 43, 745–755. doi: 10.1111/j.1365-2109.2011.02883.x

Naylor, R. L., Hardy, R. W., Bureau, D. P., Chiu, A., Elliott, M., Farrell, A. P., et al. (2009). Feeding aquaculture in an era of finite resources. *Proc. Natl. Acad. Sci*. 106, 15103–15110. doi: 10.1073/pnas.0905235106

NRC (2011). *Nutrient Requirements of Fish*. Washington, DC: National Academy Press.

Parvez, S., and Raisuddin, S. (2005). Protein carbonyls: novel biomarkers of exposure to oxidative stress-inducing pesticides in freshwater fish *Channa punctata* (Bloch). *Environ. Toxicol. Phar*. 20, 112–117.

Roux, P. P., and Topisirovic, I. (2012). Regulation of mRNA translation by signaling pathways. *Cold Spring Harb. Perspect. Biol*. 4, 843–853. doi: 10.1128/MCB.00070-18

Shen, J. F., Liu, H. Y., Tan, B. P., Dong, X. H., Yang, Q. H., Chi, S. Y., et al. (2020). Effects of replacement of fishmeal with cottonseed protein concentrate on the growth, intestinal microflora, haematological and antioxidant indices of juvenile golden pompano (*Trachinotus ovatus*). *Aquacult. Nutr*. 26, 1119–1130. doi: 10.1111/anu.13069

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

Su, J. Z., Gong, Y. L., Mei, L. Y., Xi, L. W., Chi, S. Y., Yang, Y. X., et al. (2020). The characteristics of glucose homeostasis in grass carp and chinese longsnout catfish after oral starch administration: A comparative study between herbivorous and carnivorous species of fish. *Br. J. Nutr*. 123, 627–641. doi: 10.1017/S0007114519003234

Tamura, S., and Shimomura, L. (2005). Contribution of adipose tissue and de novo lipogenesis to nonalcoholic fatty liver disease. *J. Clin. Invest*. 115, 1139–1142. doi: 10.1172/JCI24930

Tu, Y. Q., Xie, S. Q., Han, D., Yang, Y. X., Jin, J. Y., and Zhu, X. M. (2015). Dietary arginine requirement for gibel carp (*Carassis auratus gibelio* var. CAS III) reduces with fish size from 50 g to 150 g associated with modulation of genes involved in TOR signaling pathway. *Aquaculture* 449, 37–47. doi: 10.1016/j.aquaculture.2015.02.031

Turchini, G. M., Trushenski, J. T., and Glencross, B. D. (2018). Thoughts for the Future of Aquaculture Nutrition: Realigning Perspectives to Reflect Contemporary Issues Related to Judicious Use of Marine Resources in Aquafeeds. *N. Am. J. Aquacult*. 81, 13–39. doi: 10.1002/naaq.10067

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*). *Aquacult. Nutr*. 24, 1845–1854. doi: 10.1111/anu.12823

Wang, J., Tao, Q. Y., Wang, Z., Mai, K. S., Xu, W., Zhang, Y. J., et al. (2016). Effects of fish meal replacement by soybean meal with supplementation of functional compound additives on intestinal morphology and microbiome of Japanese seabass (*Lateolabrax japonicus*). *Aquacult. Res*. 48, 2186–2197. doi: 10.1111/are.13055

Wang, L., Cui, Z. H., Ren, X., Li, P., and Wang, Y. (2021). Growth performance, feed cost and environmental impact of largemouth bass *Micropterus salmoides* fed low fish meal diets. *Aquacult. Rep*. 20:100757. doi: 10.1016/j.aqrep.2021.100757

Yaghoubi, M., Torfi, M. M., Ghafle, M. J., Safari, O., Hekmatpour, F., and Gisbert, E. (2020). Lysine and methionine supplementation in high soy protein content diets for silvery-black porgy (*Sparidentex hasta*) juveniles. *Iran. J. Fish. Sci*. 19, 1329–1343. doi: 10.22092/IJFS.2019.119235

Yang, B. Y., Zhai, G., Gong, Y. L., Han, D., Yin, Z., Xie, S. Q., et al. (2018). Different physiological roles of insulin receptors in mediating nutrient metabolism in zebrafish. *Am. J. Physiol.: Endocrinol. Metab*. 315, E38–E51. doi: 10.1152/ajpendo.00227.2017

Ye, H. Q., Xu, M. L., Chen, L. L., Tan, X. H., and Wang, A. L. (2019). Effects of dietary plant protein sources influencing hepatic lipid metabolism and hepatocyte apoptosis in hybrid grouper (*Epinephelus lanceolatus*♀× *Epinephelus fuscoguttatus*♂). *Aquaculture* 506, 437–444. doi: 10.1016/j.aquaculture.2019.03.075

Yin, B., Liu, H. Y., Tan, B. P., Dong, X. H., Chi, S. Y., Yang, Q. H., et al. (2018). Cottonseed protein concentrate (CPC) suppresses immune function in different intestinal segments of hybrid grouper ♀*Epinephelus fuscoguttatus*×♂*Epinephelus lanceolatu via* TLR-2/Myd88 signaling pathways. *Fish. Shellfish Immunol*. 81, 318–328. doi: 10.1016/j.fsi.2018.07.038

Yu, H. H., Zhang, L. L., Chen, P., Liang, X. F., Cao, A. Z., Han, J., et al. (2019). Dietary bile acids enhance growth, and alleviate hepatic fibrosis induced by a high starch diet *via* AKT/FOXO1 and cAMP/AMPK/SREBP1 pathway in *Micropterus salmoides*. *Front. Physiol*. 10:1430–1430.

Yuan, X. Y., Jiang, G. Z., Cheng, H. H., Cao, X. F., Shi, H. J., and Liu, W. B. (2019). An evaluation of replacing fish meal with cottonseed meal protein hydrolysate in diet for juvenile blunt snout bream (*Megalobrama amblycephala*): growth, antioxidant, innate immunity and disease resistance. *Aquacult. Nutr*. 25, 1334–1344. doi: 10.1111/anu.12954

Zhang, Y., Chen, P., Liang, X. F., Han, J., Wu, X. F., Yang, Y. H., et al. (2019). Metabolic disorder induces fatty liver in japanese seabass, *Lateolabrax japonicas* fed a full plant protein diet and regulated by cAMP-JNK/NF-kB-caspase signal pathway. *Fish Shellfish Immunol*. 90, 223–234. doi: 10.1016/j.fsi.2019.04.060

Zhang, Y., Xie, S., Wei, H., Zheng, L., Liu, Z., Fang, H., et al. (2020). High dietary starch impaired growth performance, liver histology and hepatic glucose metabolism of juvenile largemouth bass, *Micropterus salmoides*. *Aquacult. Nutr*. 26, 1083–1095. doi: 10.1111/anu.13066

Zhao, W., Liu, Z. L., and Niu, J. (2021). Growth performance, intestinal histomorphology, body composition, hematological and antioxidant parameters of *Oncorhynchus mykiss* were not detrimentally affected by replacement of fish meal with concentrated dephenolization cottonseed protein. *Aquacult. Rep*. 19:100557. doi: 10.1016/j.aqrep.2020.100557

Zhou, Q. L., Habte-Tsion, H. M., Ge, X., Xie, J., Ren, M., Liu, B., et al. (2018). Graded replacing fishmeal with canola meal in diets affects growth and target of rapamycin pathway gene expression of juvenile blunt snout bream, *Megalobrama amblycephala*. *Aquacult. Nutr*. 24, 300–309. doi: 10.1111/anu.12560

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: 24 January 2022
doi: 10.3389/fmars.2021.759086

Complete Replacement of Fishmeal With Plant Protein Ingredients in Gibel Carp (*Carassius auratus gibelio*) Diets by Supplementation With Essential Amino Acids Without Negative Impact on Growth Performance and Muscle Growth-Related Biomarkers

Wanjie Cai[1,2], Haokun Liu[1], Dong Han[1,2,3], Xiaoming Zhu[1,3], Junyan Jin[1], Yunxia Yang[1] and Shouqi Xie[1,2,3,4]*

[1] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China, [2] College of Advanced Agricultural Sciences, University of Chinese Academy of Sciences, Beijing, China, [3] Hubei Engineering Research Center for Aquatic Animal Nutrition and Feed, Wuhan, China, [4] The Innovative Academy of Seed Design, Chinese Academy of Sciences, Wuhan, China

OPEN ACCESS

Edited by:
Jin Niu,
Sun Yat-sen University, China

Reviewed by:
Zhigang Zhou,
Feed Research Institute, Chinese Academy of Agricultural Sciences (CAAS), China
Ümit Acar,
Çanakkale Onsekiz Mart University, Turkey

***Correspondence:**
Haokun Liu
liuhaokun@ihb.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: 15 August 2021
Accepted: 24 September 2021
Published: 24 January 2022

Citation:
Cai W, Liu H, Han D, Zhu X, Jin J, Yang Y and Xie S (2022) Complete Replacement of Fishmeal With Plant Protein Ingredients in Gibel Carp (Carassius auratus gibelio) Diets by Supplementation With Essential Amino Acids Without Negative Impact on Growth Performance and Muscle Growth-Related Biomarkers. Front. Mar. Sci. 8:759086. doi: 10.3389/fmars.2021.759086

This study was conducted to investigate the effects of substitution of dietary fishmeal (FM) by compound plant protein supplemented with essential amino acids on growth performance, plasma physiology, and muscle growth-related genes of gibel carp (*Carassius auratus gibelio*). Four diets with equal digestible protein were prepared, where 30FM (control feed) contained 30% FM and land animal protein as a protein source, 10FM contained 10% FM, PMAa contained full plant meal (PM) supplemented with crystalline amino acid, and PM contained full PM feed. There was no significant difference in the specific growth rate (SGR) with 30FM, 10FM, and PMAa diets ($p > 0.05$); however, the SGR of PM group was the lowest with significant difference ($p < 0.05$). Feed efficiency of the PM group was the lowest with significant difference ($p < 0.05$). The whole-body crude protein content of fish in PMAa group was significantly higher than that in each group with additional FM ($p < 0.05$). There were no significant differences in plasma total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) or low-density lipoprotein cholesterol (LDL-C), and free amino acid profile of 30FM, 10FM, and PMAa groups ($p > 0.05$); however, they were significantly higher than that in PM group ($p < 0.05$). The expression of key genes in the TOR signaling pathway (*tor*, *s6k1*, *4e-bp2,* and *eif4e*), autophagy (*ulk1* and *atg13*), and ubiquitin–proteasome (*trim63* and *znrf2*) system of the PMAa group was similar to that of the FM group ($p > 0.05$), indicating that the muscle growth-related biomarker genes were positively regulated by the appropriate dietary amino acid composition

at the transcriptional level. These results suggest that FM in gibel carp diet can be totally replaced by PM without negative impact on growth performance and muscle growth-related biomarkers at the transcriptional level, which provided the requirement of digestible protein and balanced amino acid profile is satisfied.

Keywords: plant protein, fishmeal, digestible protein, amino acid balance, TOR pathway

INTRODUCTION

Over the past decades, aquaculture has been growing rapidly (Verhoeven et al., 2016) and has been a large contributor of animal protein for humans. Fishmeal (FM), which is not only a superior protein source in aquaculture but also a limited resource, plays an important role in aquaculture feed industry (Bu et al., 2018). The long-term sustainability of FM is a serious concern in the aquaculture industry. For sustainable development of aquaculture, more attention has been focused on replacing FM with plant feedstuffs (Benedito-Palos et al., 2007).

Previously, particularly over the past two decades, several studies have been conducted to determine alternative protein sources for substituting FM (Dossou et al., 2018; Inanan and Acar, 2019). It has been confirmed that the content of dietary FM could be reduced using plant ingredients in many cultured species. The addition of FM is low in the commercial feed formulation for omnivorous species. However, studies shown that FM was still the most optimal protein source in the diet and leads to the best growth performance for omnivorous species, such as tilapia (*Oreochromis niloticus*, Ahmad et al., 2020; Ismail et al., 2020) and carp (*Cyprinus carpio*, Wang et al., 2020), even herbivorous species, such as grass carp (*Ctenopharyngodon idella*, Jiang et al., 2016). Few reports are available to guide the total replacement of FM by plant ingredients in Cyprinidae species, which are widely cultured in China. The use of plant protein sources has been reported to induce intestinal inflammation in gibel carp (Liu et al., 2017) and grass carp (Duan et al., 2019), and abnormal cholesterol metabolism in blunt snout bream (*Megalobrama amblycephala*) (Xu et al., 2017). There are several factors restricting the usage of plant materials in aquatic feeds, such as amino acid imbalances, antinutritional factors (Tibbetts et al., 2017), and low mineral and high cellulose content (Hansen et al., 2007).

Several technologies have been developed to further increase the plant content in feeds, such as the use of mixed plant ingredients, supply of amino acids, steam modulation, and extrusion technology. Multiple usages of several techniques can ensure the supply of the essential amino acid, increase palatability of the diets, and decrease the levels of antinutritional factors (Gao et al., 2019). Albrektsen et al. (2006) used a complex protein source substitution (a mixture of soybean meal and corn gluten) and found that it has no effect on the growth and feed intake of farmed fish. Some studies have shown that by supplementing feed with exogenous essential amino acids, a few plant protein sources can partially or completely replace dietary FM (Kaushik et al., 1995, 2004; Jiang et al., 2016) and can promote growth performance by improving palatability and protein synthesis (Hansen et al., 2007). Several studies have revealed the importance of feed processing in aquaculture, and that the content of antinutritional factors in feed can be reduced by some processing techniques such as dry, wet heating, and extrusion (Li et al., 2018).

Feed is the main source of amino acids, which are important substrates for protein synthesis and other nitrogenous compounds, which are also considered as signaling molecules that participate in the regulation of major metabolic pathways (Gomes et al., 1993; National Research Council [NRC], 2011). Many studies have demonstrated that the target of rapamycin (TOR) signaling pathway plays a key role in nutrient perception and energy and protein metabolism (Wullschleger et al., 2006; Crovetto, 2010; Sun et al., 2019). Amino acids can activate the TOR complex 1 (*tor*) (Jobgen et al., 2006; Xia et al., 2016), then upregulate the downstream ribosomal protein S6 kinase 1 (*s6k1*), and suppress the expression level of eukaryotic translation factor 4E-binding protein 2 (*4e-bp2*) and eukaryotic translation initiation factor 4E (*eif4e*) (Zhang et al., 2018).

In addition, imbalanced amino acid concentrations can result in protein degradation (Hansen et al., 2007), which helps to redistribute nutrients from unnecessary processes to critical processes to maintain the quality of proteins (Akiko et al., 2004; Yuan et al., 2019). There are two main protein degradation pathways in living organisms: autophagy and ubiquitin–proteasome system. Autophagy-related protein 13-like (*atg13*) and serine/threonine-protein kinase ULK1 (*ulk1*) play important roles in the autophagy pathway (Chan et al., 2009; Uddin et al., 2012). E3 ubiquitin-protein ligase TRIM63 (*trim63*) and E3 ubiquitin-protein ligase ZNRF2 (*znrf2*) genes are the important biomarkers in the ubiquitin–proteasome system.

China produced about 2.7 million tons of *Carassius* spp. each year, about 2 million tons of them are gibel carp (Fisheries Bureau, Department of Agriculture of China, 2020). Gibel carp is an economical freshwater fish species that is known for its high yield, fast growth, and stronger resistibility and is widely cultivated in China (Gui and Zhou, 2010). Although gibel carp is a typical omnivorous fish, Xie et al. (2001) and Liu et al. (2016) have revealed that the best growth performance of gibel carp is obtained with diets containing high FM because of balanced amino acid profile and the absence of terrestrial, plant-derived, antinutritional factors. Thus, the purpose of this study is to reduce the amount of FM in gibel carp diets based on amino acid requirement and investigate the effects of low- or no-FM diets on growth performance, plasma physiology, the content of free amino acids in dorsal muscles and blood, and the expression of some genes involved in muscle protein synthesis and degradation.

MATERIALS AND METHODS

Experimental Diets

Four diets with equal digestible protein and crude lipid were prepared. FM, chicken meal, bone meal, and blood meal were used as the animal protein sources. Seven plant proteins [soybean meal, rapeseed meal, cottonseed meal, distillers dried grains with soluble (DDGS), wheat gluten, soy protein concentrates, and corn gluten meal] were used as the plant protein sources. The chemical compositions and crude protein digestibility of the protein sources are shown in **Table 1**.

Diet formulations are shown in **Table 2**. Diet 30FM contained 30% FM and 22% terrestrial animal protein. Diet 10FM contained 10% FM and 45.4% vegetable protein. Diet PMAa and PM both contained 53.9% vegetable protein. Each vegetable protein source was added restrictedly to reduce antinutritional factors. Blood meal was added to low-FM diets to compensate for low amino acid content. Crystalline amino acids (L-lysine, L-methionine, and L-threonine) were added to PMAa to meet the requirements of the fish. Yeast hydrolyzate and spirulina (*Arthrospira platensis*) were added to 10FM, PMAa, and PM as immunopotentiators.

All ingredients of each diet were mixed, ground through a particle size of 100 mesh, completely mixed again, and then extruded using a twin-screwed extruder (Jinan Dingrun Machinery Co., Ltd., Jinan, Shandong, China). The pellets were stored at 4°C after drying in an oven at 60°C. The amino acid compositions of the diets are shown in **Table 3**.

TABLE 1 | Chemical composition and the protein digestibility of raw material in diet.

Raw material	Crude protein (g/kg)	Crude lipid (g/kg)	Energy (KJ/g)	Protein digestibility (%)
Fishmeal[a]	720	100	229.4	92.00
Chicken meal[b]	570	140	211.2	71.99
Meat and bone meal[c]	500	85	164.4	78.18
Soybean meal[d]	450	5	190.0	73.03
Rapeseed meal[c]	380	26	167.0	86.30
Cottonseed meal[c]	410	39	197.3	81.73
Distillers dried grains with soluble (DDGS)[c]	290	84	203.8	73.70
Wheat gluten[e]	710	10	180.0	95.00
Soy protein concentrate[f]	652	10	200.0	85.00
Corn gluten meal[g]	600	20	213.0	79.52
Blood meal[c]	800	10	218.3	91.40
Yeast hydrolyzate[h]	580	23	192.8	NA
Spirulina[i]	420	70	194.5	NA

[a] *Fishmeal: White fishmeal, American Seafoods Company, Seattle, United States.*
[b] *Chicken meal: Legend Trade Co., Ltd., Dalian, China.*
[c] *Meat and bone meal, rapeseed meal, cottonseed meal, DDGS, Blood meal: Wuhan Dabeinong Feed Co., Ltd., Wuhan, China.*
[d] *Soybean meal: Yihai Oils & Grains Industries Co., Ltd., Lianyungang, China.*
[e] *Wheat gluten: Shandong Qufeng Food Tech Co., Ltd., Anqiu, China.*
[f] *Soy protein concentrate: Yihai Kerry Group, Taizhou, China.*
[g] *Corn gluten meal: Henan Julong Biological Engineering Co., Ltd., Ruzhou, China.*
[h] *Yeast hydrolyzate: Angel Yeast Co., Ltd., Yichang, China.*
[i] *Spirulina: Lvfuyuan Biotechnology Co., Ltd., Erdos, China.*

TABLE 2 | Formulation of experimental diets.

Ingredients (g.kg^{-1})	30FM	10FM	PMAa	PM
Fishmeal	307.0	100.0	0.0	0.0
Chicken meal	110.0	0.0	0.0	0.0
Meat and bone meal	110.0	0.0	0.0	0.0
Soybean meal	0.0	110.0	110.0	110.0
Rapeseed meal	0.0	110.0	110.0	110.0
Cottonseed meal	0.0	110.0	110.0	110.0
DDGS[a]	0.0	50.0	50.0	50.0
Wheat gluten	0.0	25.0	53.0	53.0
Soy protein concentrate	0.0	25.0	53.0	53.0
Corn gluten meal	0.0	25.0	53.0	53.0
Blood meal	0.0	30.0	30.0	45.0
Yeast hydrolyzate	0.0	52.0	67.0	77.0
Spirulina	0.0	52.0	52.0	62.0
Tapioca starch	290.0	150.0	100.0	100.0
Corn starch	45.0	20.0	55.0	45.0
L-lysine	0.0	0.0	14.0	0.0
L-methionine	0.0	0.0	5.0	0.0
L-threonine	0.0	0.0	4.0	0.0
Fish oil	20.0	27.5	32.5	32.5
Soybean oil	20.0	27.0	32.5	32.5
Mineral premix[b]	50.0	50.0	50.0	50.0
Vitamin premix[c]	3.9	3.9	3.9	3.9
Choline chloride[d]	1.1	1.1	1.1	1.1
Ethoxy quinoline	0.5	0.5	0.5	0.5
Cellulose	42.5	31.0	13.5	11.5

[a] *DDGS, Distillers dried grains with soluble.*
[b] *Mineral premix,* [c] *vitamin premix, and* [d] *choline chloride: the compositions were the same as previous study (Zhang et al., 2019).*

Fish and Feeding Trial

All experimental animal care protocols were approved by the Ethics Committee of the Institute of Hydrobiology, Chinese Academy of Sciences. The feeding trial was conducted at Shishou Original Seed Stock Farm of Four Major Carps (Shishou, China). The gibel carp was obtained from Guanqiao hatchery of Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, Hubei, China). The experimental fishes were acclimated for 4 weeks before the feeding trial. All fish were fed with commercial feed two times a day during acclimation. At the beginning of the trial, fish were fasted for 24 h. Fish with apparent healthy and similar size (initial body weight: 14.98 ± 0.04 g) were randomly distributed into 12 floating net cages (2.0 m × 2.0 m × 2.0 m, water depth: 1.7 m) in the center of pond (70 m × 30 m × 3.5 m, water depth: 3 m), and 60 fishes were allocated in each cage. The diets were randomly assigned to triplicate floating net cages. During the feeding experiment, fish were hand-fed to apparent satiation two times a day at 8:30 and 16:30 for 45 days. The water temperature was recorded daily and maintained from 27 to 32°C during the trial. A part of the pond water was renewed every day. The dissolved oxygen was 5.0–7.5 mg/L and PH was 7.2–7.3,

TABLE 3 | Amino acid content of the experimental diets (g/100 g dry matter).

Amino acids	30FM	10FM	PMAa	PM	EAAs requirement of gibel carp
Lysine	2.76	2.08	3.08	1.55	3.30 (Zhou et al., 2006)
Methionine	0.83	0.71	0.96	0.63	0.89 (Jia et al., 2013)
Threonine	1.64	1.58	1.69	1.30	1.70 (Li, 2009)
Arginine	2.31	2.39	2.16	2.16	1.50 (Tu et al., 2015)
Leucine	2.76	2.95	2.98	3.04	1.80 (Li, 2009)
Histidine	2.33	2.14	2.01	2.15	0.80 (Ma, 2009)
Isoleucine	1.47	1.26	1.44	1.30	1.30 (Li, 2009)
Phenylalanine	1.51	1.71	1.75	1.76	1.10 (Ma et al., 2010)
Valine	1.73	1.69	1.87	1.73	1.70 (Li et al., 2010)
Aspartic acid	3.59	3.00	3.52	3.04	
Serine	1.68	1.43	1.56	1.48	
Glycine	2.96	2.29	1.97	1.53	
Glutamic acid	5.47	6.66	6.49	7.31	
Alanine	2.34	2.04	2.11	1.96	
Cystine	0.42	0.43	0.49	0.49	
Tyrosine	1.06	1.21	1.16	1.21	
Proline	2.11	2.50	1.98	2.50	
EAA[a]	17.34	16.51	17.94	15.62	
NEAA[b]	19.63	19.56	19.28	19.52	

[a]*EAA: Essential amino acids (lysine, methionine, threonine, arginine, leucine, histidine, isoleucine, phenylalanine, and valine).*
[b]*NEAA: Non-essential amino acids (aspartic acid, serine, glycine, glutamic acid, alanine, cystine, tyrosine, and proline).*

ammonia nitrogen kept under 0.1 mg/L throughout the feeding trial. The photoperiod was subjected to nature.

Sample Collection

At the beginning of the trial, three fishes of each net cage were sampled randomly and stored at −20°C for initial body composition analysis. At the end of the experiment, all fish from each cage were weighed after depriving of food for 1 day. A total of two fishes of each cage were sampled randomly and also stored at −20°C for final body composition analysis. Another two individuals were anesthetized with MS-222 (100 mg L^{-1} tricaine methane sulfonate, Argent Chemical Laboratories Inc., Redmond, WA, United States). The tail venous blood of two fishes was drew from a 2-ml heparinized injector, and the blood samples were centrifuged at 3,500 *g* for 10 min. Plasma samples were frozen and stored at −80°C for further analysis. After blood sampling, samples of liver and dorsal muscles were dissected from those fish on ice, and these samples also kept at −80°C until analysis.

Biochemical Composition

The sample of experimental fish and diets was conducted for proximate composition following the method of the Association of Official Analytical Chemists (AOAC, 2003). The moisture content was determined by oven drying at 105°C until a constant weight. The content of ash was quantified by incineration in a muffle furnace at 550°C for 12 h. A Soxtec system (Soxtec^{TM} 2055, FOSS Tecator, Höganäs, Sweden) was used to analyze the content of crude lipid. Crude protein was tested through a Kjeltec Analyzer Unit (FOSS Tecator 8400, Höganäs, Sweden). Automatic biochemical analyzer (Mindray BS-460, Shenzhen, China) was used to determine the content of plasma total protein (TP), glucose (GLU), total triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C), using standard kits according to the instructions [TP (P/N:105-000451-00), GLU (P/N:105-000949-00), TG (P/N:105-000449-00), TC (P/N:105-000448-00), LDL-C (P/N:105-000464-00), and HDL-C (P/N:105-000463-00)]. The hydrolyzation method of dietary amino acids was according to Liu et al. (2016). The separation method of free amino acids from muscle and plasma was according to Xu et al. (2015). The total amino acid levels of the experimental diets and the free amino acid levels in the dorsal muscle and plasma were with an amino acid analyzer (A300, MembraPure GmbH, Germany).

Real-Time Quantitative Polymerase Chain Reaction Analysis

The primers for RT-qPCR were designed based on the National Center for Biotechnology Information (NCBI) primer BLAST service and from Tu et al. (2015; **Table 4**). According to the manufacturer's instructions, total RNA in the dorsal muscle of gibel carp was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, United States). The NanoDrop® ND-2000 UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, United States) was used to evaluate the concentration and quantity. Meanwhile, the integrity of the RNA sample was tested by a 1% denaturing agarose gel. The RNA was reverse-transcribed into cDNA through an M-MLV First-Strand Synthesis Kit (Invitrogen, Shanghai, China) following the protocol of the manufacturer. RT-qPCR was conducted using LightCycler 480 II system (Roche, Basel, Switzerland). Every sample with three biological duplicates *ef1α* and β-*actin* was selected as the internal reference gene. The relative expression levels of target genes were calculated following with Vandesompele et al. (2002).

Statistical Analysis

All data of different treatments were analyzed using SPSS (IBM SPSS Statistics 20.0, IBM, United States). The homogeneity of variance was analyzed by Levene's test. Then, one-way ANOVA and Duncan's multiple range test were conducted to analyze the differences among treatments. The differences were considered significant at $p < 0.05$. The results were presented as the mean ± standard error (SE).

RESULTS

Chemical Composition of the Fishmeal and Other Protein Sources and the Experimental Diets

Chemical compositions of the protein sources used in the experimental diet are shown in **Table 1**. Protein apparent digestibility coefficient of the ingredients of gibel carp was measured in our laboratory, previously, according to the method

TABLE 4 | Primers used for quantitative RT-qPCR.

Accession no.	Gene name	Primer sequence (5′-3′)	Annealing temperature (°C)	Product length (bp)
KF772613	*tor*[1]	F: TTGATGGCACGGTGTTTCCTAA R: GCCCTGGTTCTGGTGCTTGTAG	60	195
KF880601	*s6k1*[2]	F: CGAGCTGGAGTTAATAGGGTT R: AGGTGACATGCACCATCTATG	57	243
KF900277	*4e-bp2*[3]	F: CACTTTATTCTCCACCACCC R: GATGTTGTTAGCCTCATTCCT	60	175
MF461722	*eif4e*[4]	F: AAAATCTGCGTCTCATCTCC R: TATTCCTGTCATCCTCCCAC	58	150
XM026267678	*atg13*[5]	F: AGTGAGCCTATGCTTACCTGC R: AGGTCCAGCTCCTAACGACT	60	172
XM026247240.1	*ulk1*[6]	F: TGAGTAACAGCTCGAATGCTT R: TTAGCAACGGTTCGGTGTCC	60	213
XM026290505.1	*trim63*[7]	F: GATGCGCGTGGGTTTAGACT R: TGCTGGCACGGTAGTATCAC	60	128
XM026283393.1	*znrf2*[8]	F: TGACGGCTACGTTTCGATGG R: TCCAGATGCATGGGACGTTA	60	174
AB039726.2	β-*actin*	F: TTGAGCAGGAGATGGGAACCG R: AGAGCCTCAGGGCAACGGAAA	60	115
AB056104	*ef1α*[9]	F: GTTGGAGTCAACAAGATGGACTCCAC R: CTTCCATCCCTTGAACCAGCCCAT	60	198

[1]*tor: Target of rapamycin.* [2]*s6k1: Ribosomal protein S6 kinase 1.* [3]*4e-bp2: Eukaryotic translation factor 4E-binding protein 2.* [4]*elf4e: Eukaryotic translation initiation factor 4E.* [5]*atg13: Autophagy-related protein 13.* [6]*ulk1: Serine/threonine-protein kinase ULK1.* [7]*trim63: E3 ubiquitin-protein ligase trim63.* [8]*znrf2: E3 ubiquitin-protein ligase znrf2.* [9]*ef1α: Translation elongation factor 1-alpha.*

described by Dong et al. (2012). The chemical composition and amino acid content of the experimental diets are shown in **Tables 3**, **5**, respectively. The crude protein content of PMAa and PM diets was higher than that of 30FM and 10FM diets. The digestible protein content was almost the same in all diets (**Table 5**). The limiting amino acids in PM diet were lysine, methionine, and threonine. The essential amino acid requirements of gibel carp were in accordance with our previous work (Zhou et al., 2006; Li, 2009; Li et al., 2010; Ma, 2009; Ma et al., 2010; Jia et al., 2013; Tu et al., 2015). The total contents of essential amino acids were the highest in PMAa and the lowest in PM (**Table 3**).

Growth Performance and Feed Utilization

The survival rate of fish in all the dietary treatments was 100%. The results of growth performance of gibel carp are shown in **Table 6**. The final body weight and specific growth rate (SGR) of fish fed 30FM, 10FM, and PMAa diets were not significantly different ($p > 0.05$), but were significantly higher than those of fish fed PM diet ($p < 0.05$). Feeding rate in PM group was higher than others though there were no significant differences. However, it was observed that feeding efficiency in PMAa was significantly higher than PM group ($p < 0.05$) and no significant difference with other groups ($p > 0.05$). There was no significant difference in protein retention efficiency (PRE) and protein efficiency ratio (PER) among all treatment groups except for PM group ($p > 0.05$). The PRE and PER of the PM group were significantly lower than those of the other groups ($p < 0.05$).

TABLE 5 | Chemical composition of experimental diets.

Proximate composition	30FM	10FM	PMAa	PM
Moisture (%)	5.6	4.9	5.0	4.8
Crude protein (%, DM)	37.0	38.3	39.5	39.6
Crude lipid (%, DM)	6.1	6.5	6.7	6.1
Ash (%, DM)	10.5	8.9	7.3	7.0
Digestible protein (%, DM)[a]	30.2	30.2	30.1	30.2

[a]*Calculated based on the data in* ***Table 1****.*

Body Composition

Whole-body compositions of gibel carp are presented in **Table 7**. The moisture and protein contents in the whole body of gibel carp were not significantly different among all groups ($p > 0.05$). The fish in the PM group had lower whole-body lipid content than that in the other groups ($p < 0.05$). There were no significant differences in whole-body ash content among all groups ($p > 0.05$).

Physiological and Biochemical Indices in Plasma

There were no significant differences in TP and GLU in the plasma among all diet groups (**Table 8**). However, PM group had the lowest TG, TC, LDL-C, and HDL-C levels. Significant differences ($p < 0.05$) were observed in TG between PM and 30FM groups, in TC and HDL-C between PM and the other three groups, and in LDL-C between PM and the two groups containing dietary FM. The levels of TG, TC, LDL-C, and HDL-C in PMAa group were similar to those in 30FM and 10FM groups;

TABLE 6 | Growth performance, feed utilization of gibel carp fed with different experimental diets.

	30FM	10FM	PMAa	PM
FBW, g[1]	35.48 ± 1.09[a]	35.67 ± 1.86[a]	35.36 ± 0.54[a]	30.33 ± 0.67[b]
SGR, %/d[2]	1.93 ± 0.08[a]	1.94 ± 0.10[a]	1.92 ± 0.02[a]	1.56 ± 0.05[b]
FR, bw % d[3]	2.88 ± 0.05	2.83 ± 0.17	2.86 ± 0.02	3.10 ± 0.02
FE, %[4]	63.05 ± 3.20[a]	65.06 ± 6.71[a]	63.42 ± 0.90[a]	48.37 ± 1.11[b]
PRE, %[5]	26.02 ± 1.84[a]	25.7 ± 3.00[a]	26.49 ± 0.58[a]	18.35 ± 0.57[b]
PER, %[6]	170.40 ± 8.65[a]	169.87 ± 17.53[a]	160.55 ± 2.27[ab]	127.64 ± 5.07[b]

Data are presented as the means ± SE (n = 3). Values within the same row with different letters are significantly different ($p < 0.05$).
[1]FBW, final body weight.
[2]SGR, specific growth rate (% d^{-1}) = 100 × [ln (final body weight) − ln (initial body weight)]/days.
[3]FR, feeding rate (% body weight day^{-1}) = 100 × (feed intake in dry matter)/[days × (initial body weight + final body weight)/2].
[4]FE, feed efficiency (%) = 100 × (final body weight-initial body weight)/feed intake in dry matter.
[5]PRE, protein retention efficiency = 100 × protein retained in fish body/protein intake.
[6]PER, protein efficiency ratio = 100 × weight gain/protein intake.

TC and HDL-C concentrations in PMAa group were higher than in PM group ($p < 0.05$).

Free Amino Acid Content in Dorsal Muscle and Blood of Gibel Carp

Free amino acid composition of plasma of gibel carp after the feeding trial is presented in **Table 9**. The fish from PMAa treatment had similar levels of all free amino acid concentrations with 30FM and 10FM treatments ($p > 0.05$). The essential free amino acids in the plasma of the PM treatment were also similar to those in other treatments and showed a downward trend ($p > 0.05$). However, the plasma aspartic acid, serine, alanine, and tyrosine concentrations in the PM group were significantly lower than those in the 30FM group ($p < 0.05$). The PM group also had the lowest plasma total essential amino acids compared to other groups ($p > 0.05$).

Results of dorsal muscle-free amino acid concentrations of gibel carp are listed in **Table 10**. There was a remarkable difference in the levels of free amino acids such as arginine, histidine, and glycine; total essential amino acid; total non-essential amino acid; and sum of flavor amino acids among the dietary groups. Even though there was no significant difference in lysine, methionine, and threonine among the four treatments,

TABLE 7 | Whole-body compositions (fresh weight) of gibel carp fed different experimental diets.

	30FM	10FM	PMAa	PM
Moisture %	72.44 ± 0.28	72.62 ± 0.56	72.28 ± 0.30	74.13 ± 0.49
Protein %	15.48 ± 0.17	15.4 ± 0.21	16.2 ± 0.13	15.08 ± 0.40
Lipid %	6.61 ± 0.14[a]	5.49 ± 0.36[b]	5.67 ± 0.23[b]	4.62 ± 0.23[c]
Ash %	2.99 ± 0.06	3.25 ± 0.01	3.28 ± 0.04	3.51 ± 0.22

Values are mean of three replicates ± SEM (standard error). Means in the same row with different superscripts are significantly different ($p < 0.05$).

TABLE 8 | Plasma biochemical composition of gibel carp fed different experimental diets.

	30FM	10FM	PMAa	PM
TP ($g{\cdot}L^{-1}$)	34.70 ± 1.93	33.60 ± 0.8	33.65 ± 0.35	29.73 ± 2.17
GLU ($mmol{\cdot}L^{-1}$)	5.02 ± 0.78	3.48 ± 0.54	4.13 ± 0.77	4.00 ± 0.84
TG ($mmol{\cdot}L^{-1}$)	5.80 ± 0.08[b]	5.20 ± 0.90[ab]	3.92 ± 1.00[ab]	3.03 ± 0.12[a]
TC ($mmol{\cdot}L^{-1}$)	8.23 ± 0.49[b]	7.74 ± 0.31[b]	7.28 ± 0.10[b]	5.68 ± 0.54[a]
LDL-C ($mmol{\cdot}L^{-1}$)	2.02 ± 0.18[b]	1.86 ± 0.03[b]	1.71 ± 0.07[ab]	1.33 ± 0.13[a]
HDL-C ($mmol{\cdot}L^{-1}$)	3.72 ± 0.10[b]	3.53 ± 0.16[b]	3.60 ± 0.01[b]	2.82 ± 0.24[a]

Values are mean of three replicates ± SEM (standard error). Means in the same row with different superscripts are significantly different ($p < 0.05$). TP, total protein; GLU, glucose; TG, triglyceride; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.

TABLE 9 | Plasma free amino acid concentrations (mg/100 ml) in gibel carp fed dietary with different amino acid pattern about 45 days.

	30FM	10FM	PMAa	PM
Lysine	4.07 ± 0.59	4.67 ± 0.52	5.14 ± 0.98	3.27 ± 0.28
Methionine	0.73 ± 0.10	0.85 ± 0.07	0.72 ± 0.04	0.55 ± 0.02
Threonine	11.17 ± 2.17	12.30 ± 1.97	9.95 ± 2.54	7.15 ± 1.08
Arginine	3.29 ± 0.34	4.27 ± 0.24	3.77 ± 0.81	2.80 ± 0.10
Leucine	5.05 ± 1.19	4.97 ± 0.71	4.87 ± 0.99	3.45 ± 0.39
Histidine	4.71 ± 0.67	4.67 ± 0.79	4.37 ± 0.03	3.75 ± 0.08
Isoleucine	2.74 ± 0.55	2.98 ± 0.36	2.75 ± 0.57	1.89 ± 0.30
Phenylalanine	2.49 ± 0.45	2.86 ± 0.26	2.39 ± 0.16	1.88 ± 0.28
Valine	4.01 ± 0.65	3.94 ± 0.45	4.14 ± 0.89	2.85 ± 0.30
Aspartic acid	1.19 ± 0.09[b]	0.95 ± 0.26[ab]	0.93 ± 0.10[ab]	0.49 ± 0.07[a]
Serine	1.56 ± 0.08[b]	1.51 ± 0.15[b]	1.27 ± 0.20[ab]	0.93 ± 0.29[a]
Glycine	1.88 ± 0.29	1.78 ± 0.14	1.69 ± 0.36	1.31 ± 0.24
Glutamic acid	0.33 ± 0.04	0.16 ± 0.03	0.43 ± 0.24	0.35 ± 0.10
Alanine	3.41 ± 0.03[b]	2.54 ± 0.09[b]	2.78 ± 0.06[ab]	2.47 ± 0.07[a]
Cystine	0.36 ± 0.05	0.39 ± 0.07	0.45 ± 0.00	0.34 ± 0.02
Tyrosine	1.41 ± 0.32[b]	1.71 ± 0.33[b]	1.56 ± 0.52[b]	0.65 ± 0.28[a]
Proline	12.10 ± 3.07	9.92 ± 2.39	9.81 ± 3.45	14.30 ± 2.44
ΣEAA	38.28 ± 5.34	41.55 ± 3.57	38.10 ± 6.87	27.60 ± 2.81
ΣNEAA	22.32 ± 2.87	18.98 ± 3.13	20.19 ± 5.42	19.45 ± 4.37

Values are mean of three replicates ± SEM (standard error). Means in the same row with different superscripts are significantly different ($p < 0.05$).
ΣEAA: The sum of essential amino acids (Lys, Met, Thr, Arg, Leu, His, Ile, Phe, and Val).
ΣNEAA: The sum of non-essential amino acids (Asp, Ser, Glu, Ala, Cys, Gly, Tyr, and Pro).

TABLE 10 | Dorsal muscle free amino acid concentrations (μg/g) in gibel carp fed dietary with different amino acid pattern about 45 days.

	30FM	10FM	PMAa	PM
Lysine	242.3 ± 0.19	237.5 ± 0.35	189.5 ± 0.37	158.5 ± 0.04
Methionine	21.2 ± 0.09	29.4 ± 0.09	23.6 ± 0.01	23.8 ± 0.11
Threonine	380.8 ± 0.46	302.9 ± 0.08	270.8 ± 0.35	290.7 ± 0.33
Arginine	182.1 ± 0.01^{b}	96.5 ± 0.21^{a}	90.8 ± 0.01^{a}	100.9 ± 0.21^{a}
Leucine	190.0 ± 0.28	206.2 ± 0.20	177.6 ± 0.28	200.0 ± 0.30
Histidine	116.0 ± 0.04^{ab}	126.7 ± 0.25^{b}	83.4 ± 0.01^{ab}	74.9 ± 0.12^{a}
Isoleucine	95.8 ± 0.26	109.7 ± 0.13	106.2 ± 0.01	97.1 ± 0.29
Phenylalanine	119.5 ± 0.12	127.1 ± 0.09	113.4 ± 0.08	108.2 ± 0.13
Valine	135.9 ± 0.22	150.1 ± 0.19	123.4 ± 0.17	134.0 ± 0.29
Aspartic acid	20.8 ± 0.03	17.3 ± 0.02	16.2 ± 0.02	13.4 ± 0.04
Serine	106.0 ± 0.05	91.8 ± 0.16	72.8 ± 0.22	76.3 ± 0.08
Glycine	108.4 ± 0.17^{b}	77.2 ± 0.20^{ab}	63.4 ± 0.20^{ab}	47.0 ± 0.07^{a}
Glutamic acid	25.4 ± 0.06^{b}	12.3 ± 0.01^{a}	11.2 ± 0.03^{a}	13.9 ± 0.00^{a}
Alanine	250.8 ± 0.44	213.5 ± 0.27	176.9 ± 0.43	159.8 ± 0.17
Cystine	19.0 ± 0.05^{b}	10.4 ± 0.02^{ab}	08.5 ± 0.02^{a}	14.6 ± 0.02^{ab}
Tyrosine	63.3 ± 0.03^{ab}	75.5 ± 0.05^{b}	48.8 ± 0.05^{a}	68.7 ± 0.12^{ab}
Proline	138.3 ± 0.02^{ab}	66.3 ± 0.06^{a}	229.3 ± 0.63^{b}	247.2 ± 0.55^{b}
ΣEAA	1577.3 ± 0.81^{b}	1386.1 ± 0.26^{ab}	1179.5 ± 0.56^{a}	1188.0 ± 0.73^{a}
ΣNEAA	771.8 ± 0.68^{b}	501.9 ± 0.28^{a}	627.3 ± 0.18^{ab}	641.1 ± 0.33^{ab}
ΣFAA	352.1 ± 0.29^{b}	320.5 ± 0.77^{b}	234.1 ± 0.22^{ab}	206.2 ± 0.24^{a}

Values are mean of three replicates ± SEM (standard error). Means in the same row with different superscripts are significantly different ($p < 0.05$).
ΣEAA: The sum of essential amino acids (Lys, Met, Thr, Arg, Leu, His, Ile, Phe, and Val).
ΣNEAA: The sum of non-essential amino acids (Asp, Ser, Glu, Ala, Cys, Gly, Tyr, and Pro).
ΣFAA: The sum of flavor amino acids (Asp, Glu, Gly, and Ala).

PMAa and PM treatments had lower levels of lysine and threonine than 30FM and 10FM treatments ($p > 0.05$). The arginine concentrations of 10FM, PM, and PMAa groups were significantly lower than that of 30FM group ($p < 0.05$). As for the contents of non-essential free amino acid in gibel carp dorsal muscle, the contents of glycine and glutamic acid of PM group were significantly lower than that of 30FM group ($p < 0.05$). However, PMAa and PM groups' proline content was the highest among all groups. Notably, the total flavor amino acid levels of 30PM and 10FM were significantly higher than that of PM ($p < 0.05$) and was similar to that of PMAa ($p > 0.05$).

Relative mRNA Expression Levels of Muscle Protein Synthesis and Degradation Genes

The relative transcriptional levels of genes involved in muscle protein synthesis are presented in **Figure 1**. There were no significant differences observed in *tor* and *eif4e* expression among all experimental groups. Meanwhile, the expression of *s6k1* in fish muscles in PMAa group was notably higher than that in PM group ($p < 0.05$); *s6k1* expression in PM group was the lowest. The transcriptional levels of *4e-bp2* in PM group were the highest ($p < 0.05$) and were not significantly different among the remaining groups ($p > 0.05$).

The relative transcriptional expression of genes involved in muscle protein degradation is illustrated in **Figure 2**. The relative expression of *atg13* in PM group was significantly higher than that in 10FM group ($p < 0.05$). The relative expression level of *ulk1* in the PM group was also significantly higher than that in 30FM and 10FM groups ($p < 0.05$). However, the relative expression of *trim63* in 10FM group was the highest value among all groups ($p < 0.05$). There were no significant differences in the expression of *znrf2* with all treatments ($p > 0.05$).

DISCUSSION

In this study, the possibility of replacing FM with mixed plant protein in the diet of gibel carp was investigated. The results showed that 20% of dietary FM and 22% of terrestrial animal protein could be replaced by mixed plant protein if digestible protein requirement in the diet was satisfied, the antinutritional factors from oil seed meals (soybean meal, rapeseed meal, and cottonseed meal) was low, and single-cell protein (blood meal, yeast hydrolyzate, spirulina) was supplement. All FM and terrestrial animal protein can be replaced by mixed plant protein if essential amino acid requirements are satisfied. Blood meal and spirulina were added to the plant-supplied diet because of their high lysine and threonine contents. Yeast hydrolyzate and spirulina were used as attractants and immunopotentiators. However, without the supplementation of essential amino acids, growth performance decreased significantly.

Many previous studies have revealed that poor growth performance of cultured fishes could result from the high level replacement of FM with single-plant ingredients (Watanabe et al., 1998; Gomez-Requeni et al., 2004; Kaushik et al., 2004; Panserat et al., 2008), such as soybean meal (Alexis and Nengas, 2001), rapeseed meal (Burel et al., 2000), and cottonseed meal (Luo et al., 2012), whether in carnivorous (*Trachinotus ovatus*, Wu et al., 2015; *Lates calcarifer*, Ma et al., 2019) or in herbivorous (*Ctenopharyngodon Idella*, Jiang et al., 2016) fish. Therefore, using a combination of protein ingredients with various nutritive properties can improve amino acid profile, dilute or even mitigate antinutrition factors, and consequently allow higher inclusion of alternative protein sources in the diet of cultured fish. However, in this study, PM diet, in which the dietary FM content was totally replaced by plant protein combination, showed lower growth performance in fish than 30FM, 10FM, and PMAa diet. The ability of fish to utilize crystalline amino acids is well reported (Kaushik and Seiliez, 2010), and limiting amino acid supplementation in plant-based diets is necessary to complement the low amino acid profile. We found that the growth performance and feed utilization of fish receiving PMAa treatment, in which plant protein combination was supplemented with limiting amino acids, were not significantly different from those of fish receiving 30FM and 10FM treatments and were much higher than those of fish receiving PM treatment. Similar observations have been reported in rainbow trout (Kaushik et al., 1995; Watanabe et al., 1998) and sea bass (*Dicentrarchus labrax*) (Tibaldi et al., 1999), where FM was replaced by a mixture of vegetable protein and adequate supplement of limiting

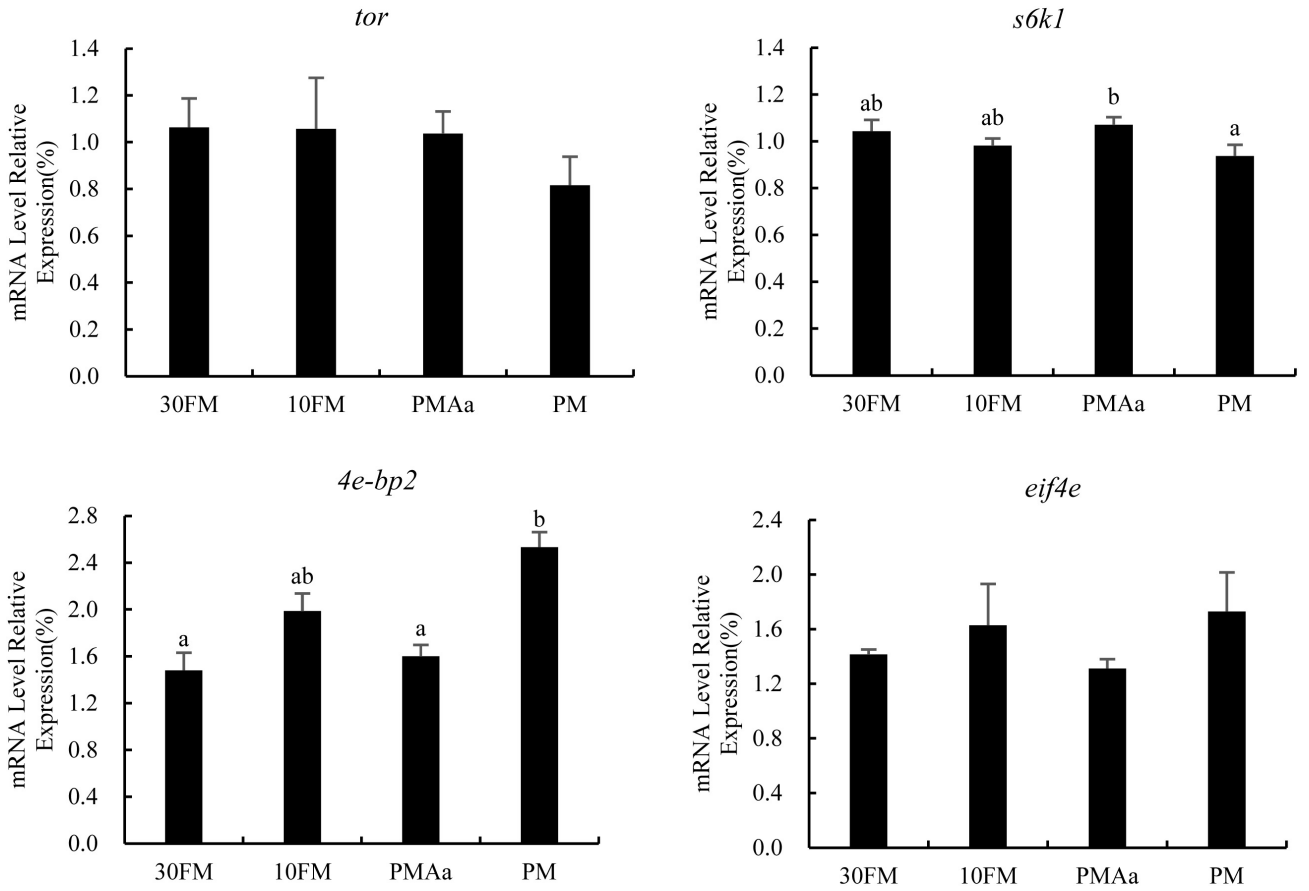


FIGURE 1 | Relative expression of protein synthesis genes (*tor*, *s6k1*, *4e-bp2*, and *eif4e*) in the muscle of gibel carp β-*actin* and *ef1α* was used as reference gene for the assessment of expression. Columns represent the mean ± SEM ($n = 6$). For each index, bars without sharing a common letter indicate significant differences ($p < 0.05$).

amino acids without negative effects on growth. There have been several experiments in which the replacement of FM with high proportions or complete plant proteins in diets did not only result in decreased feed intake and growth performance but also caused oxidative stress or subhealth status (Sitjà-Bobadilla et al., 2005; Hansen et al., 2007). Single-cell proteins, such as spirulina and yeast, contain various water-soluble fractions, such as free amino acids, small peptides, nucleotides, vitamins, and minerals. In this study, spirulina and yeast hydrolyzate, which were used as attractants and immunopotentiators to cover for the shortage of FM replacement (Burgents et al., 2004; Sheikhzadeh et al., 2019), were supplied in the low-FM diet. Based on the results, total replacement of FM with compound plant proteins in the diet of gibel carp is a possibility, and it provides a promising approach for the sustainable development of aquaculture.

The whole-body proximate composition of gibel carp did not show considerable variability with the replacement of FM, except for the treatment containing PM only and without amino acid supplement (PM), with which fish had reduced protein and lipid contents. High proportion of dietary FM (200 g kg^{-1} diet) can be replaced with compound plant proteins without having significant effect on body composition. Similar results have been reported in trout (Santigosa et al., 2008), silvery-black porgy (*Sparidentex hasta*) (Yaghoubi et al., 2016), and gilthead sea bream (Sitjà-Bobadilla et al., 2005). PRE also decreased in the PM group. This phenomenon could probably be attributed to the unbalanced amino acid composition in the diet, which leads to increased protein degradation (Langar et al., 1993). Besides, when dietary amino acids that are absorbed into the body cannot match the profile needed for protein synthesis, the excess amino acids would result in increased oxidation and catabolism of amino acids (Kim et al., 1983; Fauconneau et al., 1992). The body protein levels and PRE of fish fed the PMAa diet supplemented with limiting amino acids were similar to those of fish fed 30FM diet (**Table 7**). A similar phenomenon was reported for other cultured fish, such as hybrid grouper (*Epinephelus*) (Wu et al., 2017) and large yellow croaker (*Larimichthys crocea*) (Li et al., 2013). Decreased lipid content of fish associated with very high dietary inclusion levels of PM has also been reported for Indian major carp (*Cirrhinus mrigala*) (Hasan et al., 1997) and Japanese flounder (*Paralichthys olivaceus*) (Kikuchi, 1999).

Serum parameters reflect the physiological stress response of fish to nutritional changes (Dawood et al., 2015) and have been used to assess the health of fish (Hossain et al., 2016;

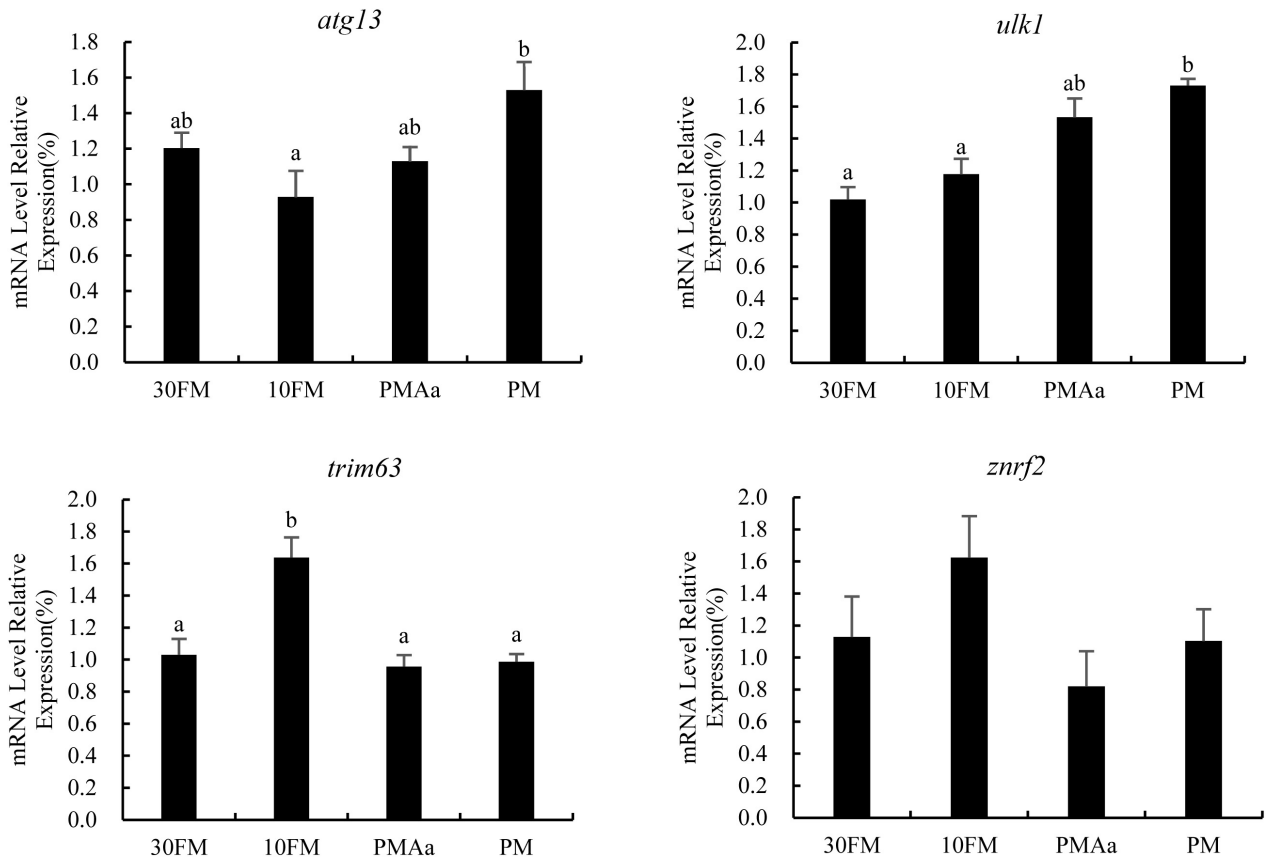


FIGURE 2 | Relative expression of protein degradation genes (*atg13*, *ulk1*, *trim63*, and *znrf2*) in the muscle of gibel carp β-*actin* and *ef1α* was used as reference gene for the assessment of expression. Columns represent the mean ± SEM (n = 6). For each index, bars without sharing a common letter indicate significant differences ($p < 0.05$).

Dawood et al., 2017; Inanan et al., 2021). There are many kinds of literature that have reported the possible physiological effects of dietary plant protein intake on fish (Dias, 1999; Regost, 1999; Kaushik et al., 2004). In this study, plasma TG, TC, LDL-C, and HDL-C concentrations decreased with an increase in dietary PM. A previous study also showed that high level of dietary plant content causes hypocholesterolemic symptoms in rat, which presented low level of plasma cholesterol (de Schrijver, 1990). A significant hypocholesterolemic effect of soybean meal diet was also observed in rainbow trout by Kaushik et al. (1995), who speculated that the problem was in response to the withdrawal of FM rather than the addition of plant protein (Kaushik et al., 2004). However, Sitjà-Bobadilla et al. (2005) inferred that fish hypocholesterolemia may result from increased excretion of bile salts caused by dietary plant protein supply, which inhibits the absorption of cholesterol in the intestine. Plasma TC and HDL-C concentrations of fish fed PMAa diet were slightly lower than those of fish fed 30FM diet ($p > 0.05$) and were significantly higher than those of fish fed PM diet ($p > 0.05$). This phenomenon confirmed that lysine, methionine, and threonine supplement could relieve hypocholesterolemic symptoms (Hirche et al., 2006). The specific mechanism of plant proteins involved in the synthesis and metabolism of cholesterol needs further study (Kaushik et al., 2004; Sitjà-Bobadilla et al., 2005).

The content of amino acids in diets could affect plasma amino acid pool, which could also be used to judge the adequacy of dietary amino acids and reflects nitrogen balance (Mente et al., 2003; Hansen et al., 2007). In this study, we also found that the free amino acid concentrations in the dorsal muscles reflected the dietary amino acid profile. The concentrations of free essential amino acids and non-essential amino acids in the plasma were high in 30FM, 10FM, and PMAa diets but were low in PM diet. Accordingly, in the dorsal muscle, free essential amino acids and flavor amino acids also decreased when a high proportion of dietary FM was replaced with mixed PM. Amino acids in muscles are associated with the synthesis of muscle proteins (Hansen et al., 2007). It is worth mentioning that the concentration of dorsal-free lysine in PMAa group was higher than that in PM group. Lower lysine content in the dietary treatments also resulted in lower free lysine content in the dorsal muscle of the fish; this outcome agrees with that reported by Gomez-Requeni et al. (2004). Carter et al. (2000) suggested that the efficiency of protein synthesis and retention was limited by the lowest relative concentration of an essential amino acid pool. Lysine, methionine, and threonine are recognized as limiting amino acids

for fish (Hansen et al., 2007). In addition to other results of this study, the positive growth performance in the PMAa group was achieved by satisfying the requirement of limiting amino acids.

Target of rapamycin signaling system pathway senses amino acid levels in cells and plays a key role in nutrient perception and energy regulation (Wullschleger et al., 2006). It also plays a key role in protein synthesis both in mammals (Dodd and Tee, 2012; Hernandez-García et al., 2016) and in fishes (Liang et al., 2016). Generally, the replacement of a high proportion of FM with PM may result in TOR signaling system pathway not being effectively activated, and the response of sensing stress pathway being activated (Xu et al., 2015; Song et al., 2016). The suppression effect of antinutritional factors in the TOR pathway was also reported by He and Klionsky (2017), who found that gossypol and soybean saponins suppress *tor* and promote catabolism, and that concanavalin-A causes inhibited insulin/IGF1/Akt signaling that consequently leads to apoptosis. In this study, compound plant ingredients were used to reduce the content of antinutritional substances. This may be the reason why the expressions of *tor* and *eif4e* genes were not significantly different among all treatments. This phenomenon was also reported for juvenile cobia (*Rachycentron canadum*) and large yellow croaker larvae (Luo et al., 2012; Li et al., 2013). The supplementation of essential amino acids can activate TOR pathway (Chen G. F. et al., 2012; Zhao et al., 2012). In the TOR signaling pathway, there are two downstream sites (4E-BPs and s6k1) for *tor*. The PMAa treatment had a significantly higher expression level of *s6k1* than that in the PM treatment, which gene was positively regulated by the TOR complex. Furthermore, the reverse was found in the *4e-bp2* mRNA levels of the muscle of gibel carp in the PM treatments. These results were justifiable as *tor* promotes cap-dependent translation initiation through the inactivation of its downstream effector (namely 4E-BPs) or increases the translation of 5′-TOP mRNAs *via* the activation of S6K1 (Wullschleger et al., 2006). Similar results were reported that dietary supplement of essential amino acids, such as lysine (Cai et al., 2018), threonine (Habte-Tsion et al., 2015), and methionine (Pan et al., 2016), could promote the expression of *s6k1* and depress the expression of *4e-bp2* in cultured fish species.

Growth performance can be partly reflected in the dynamic balance of muscle protein synthesis and degradation (Zeitz et al., 2019). Skeletal muscle protein turnover is the ratio between protein synthesis and protein breakdown rates (Burd et al., 2009). When positive, protein synthesis is higher than breakdown, and results in muscle mass gain. Autophagy and ubiquitin–proteasome system are the main pathways of protein degradation in the skeletal muscle of vertebrates (Sandri, 2010). This study found that the *atg13* and *ulk1* genes in PM group were highly expressed and indicated that the dorsal protein degradation rate in the PM treatment was higher than those in the other treatments. Ulk1 is a fundamental protein activated by AMP-activated protein kinase (AMPK) for survival under conditions of reduced nutrient availability (Bach et al., 2011). The high protein breakdown rate in the dorsal muscle of PM treatment explains the low growth performance. TRIM63-encoding muscle ring finger 1 maintains muscle protein homeostasis by tagging the sarcomere proteins with ubiquitin for subsequent degradation by the ubiquitin–proteasome system (Chen S. N. et al., 2012). In this study, the *trim63* was highly expressed in 10FM treatment, which diet was not supplied with crystal amino acids. These phenomena suggest that the nutritional requirement of gibel carp could not be satisfied by compound plant ingredients without limiting amino acid supplements.

In summary, this study comprehensively evaluated the possibility of totally replacing FM with PM in the feed of gibel carp. The growth performance, feed utilization, and plasma biochemical composition in PMAa treatment were not different from those of the 30FM treatment. The key genes in the TOR signaling pathway and autophagy and ubiquitin–proteasome system corresponded with the dietary amino acid profile. We conclude that replacing FM with total plant protein is feasible and provides a reference for cost optimization design of feed formulation for gibel carp.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of the Institute of Hydrobiology, Chinese Academy of Sciences.

AUTHOR CONTRIBUTIONS

WC and HL made major contributions to this work. WC was analysis of the data and draft of the manuscript. HL reviewed and corrected the manuscript. SX, XZ, YY, DH, and JJ participated in experiment design and sample collection. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Key R&D Program of China (2019YFD0900200 and 2018YFD0900400), China Agriculture Research System (CARS-45-09), Science and Technology Project of Wuhan (2019020701011459), and Poverty Alleviation through Agricultural Projects from the Agricultural Office of Chinese Academy of Sciences and the Fund Project of the State Key Laboratory of Freshwater Ecology and Biotechnology (2019FBZ02 and 2019FBZ05).

ACKNOWLEDGMENTS

The authors thank Guanghan Nie for his technical assistance.

REFERENCES

Ahmad, N., Siddiqui, P. J. A., Khan, K. M., Ali, A., Tahir, M., Akbar, N. U., et al. (2020). Effects of partial substitution of fishmeal by soybean meal in *Nile tilapia* (*Oreochromis niloticus*). *J. Anim. Plant Sci.* 30, 364–370. doi: 10.36899/JAPS.2020.2.0058

Akiko, K., Masahiko, H., Makoto, M., Akitsugu, Y., Haruaki, N., Tamotsu, Y., et al. (2004). The role of autophagy during the early neonatal starvation period. *Nature* 432, 1032–1036. doi: 10.1038/nature03029

Albrektsen, S., Mundheim, H., and Aksnes, A. (2006). Growth, feed efficiency, digestibility and nutrient distribution in Atlantic cod (*Gadus morhua*) fed two different fish meal qualities at three dietary levels of vegetable protein sources. *Aquaculture* 261, 626–640. doi: 10.1016/j.aquaculture.2006.08.031

Alexis, M. N., and Nengas, I. (2001). *Current State of Knowledge Concerning the Use of Soy Products in Diets for Feeding Sea Bass and Seabream*. Brussels: American Soybean Association, 32.

AOAC (2003). *Official Methods of Analysis of the Association of Official Analytical Chemists*, 17th Edn. Arlington, VA: Association of Official Analytical Chemists.

Bach, M., Larance, M., James, D. E., and Ramm, G. (2011). The serine/threonine kinase ULK1 is a target of multiple phosphorylation events. *Biochem. J.* 440, 283–291. doi: 10.1042/BJ20101894

Benedito-Palos, L., Saera-Vila, A., and Calduch-Giner, J. A. (2007). Combined replacement of fish meal and oil in practical diets for fast growing juveniles of gilthead sea bream (*Sparus aurata* L.): networking of systemic and local components of GH/IGF axis. *Aquaculture* 267, 199–212. doi: 10.1016/j.aquaculture.2007.01.011

Bu, X. Y., Wang, Y. Y., Chen, F. Y., Tang, B. B., Luo, C. Z., Wang, Y., et al. (2018). An evaluation of replacing fishmeal with rapeseed meal in the diet of *Pseudobagrus ussuriensis*: growth, feed utilization, nonspecific immunity, and growth-related gene expression. *J. World Aquac. Soc.* 49, 1068–1080. doi: 10.1111/jwas.12470

Burd, N. A., Tang, J. E., Moore, D. R., and Phillips, S. M. (2009). Exercise training and protein metabolism: influences of contraction, protein intake, and sex-based differences. *J. Appl. Physiol.* 106, 1692–1701. doi: 10.1152/japplphysiol.91351.2008

Burel, C., Boujard, T., Escaffre, A. M., Kaushik, S. J., Boeuf, G., Mol, K. A., et al. (2000). Dietary low-glucosinolate rapeseed meal affects thyroid status and nutrient utilization in rainbow trout (*Oncorhynchus mykiss*). *Br. J. Nutr.* 83, 653–664. doi: 10.1017/S0007114500000830

Burgents, J. E., Burnett, K. G., and Burnett, L. E. (2004). Disease resistance of Pacific white shrimp, *Litopenaeus vannamei*, following the dietary administration of a yeast culture food supplement. *Aquaculture* 231, 1–8. doi: 10.1016/j.aquaculture.2003.09.003

Cai, W. C., Liu, W. B., Jiang, G. Z., Wang, K. Z., Sun, C. X., and Li, X. F. (2018). Lysine supplement benefits the growth performance, protein synthesis, and muscle development of *Megalobrama amblycephala* fed diets with fish meal replaced by rice protein concentrate. *Fish Physiol. Biochem.* 44, 1159–1174. doi: 10.1007/s10695-018-0503-3

Carter, C. G., Houihan, D. F., and He, Z. Y. (2000). Changes in tissue free amino acid concentrations in Atlantic salmon, *Salmo salar* L., after consumption of a low ration. *Fish Physiol. Biochem.* 23, 295–306. doi: 10.1023/a:1011187026997

Chan, E. Y. W., Longatti, A., McKnight, N. C., and Tooze, S. A. (2009). Kinase-inactivated ULK proteins inhibit autophagy via their conserved C-terminal domains using an Atg13-independent mechanism. *Mol. Cell. Biol.* 29, 157–171. doi: 10.1128/MCB.01082-08

Chen, G. F., Feng, L., Kuang, S. Y., Liu, Y., Jiang, J., Hu, K., et al. (2012). Effect of dietary arginine on growth, intestinal enzyme activities and gene expression in muscle, hepatopancreas and intestine of juvenile Jian carp (*Cyprinus carpio* var. Jian). *Br. J. Nutr.* 108, 195–207. doi: 10.1017/s0007114511005459

Chen, S. N., Czernuszewicz, G., Tan, Y., Lombardi, R., Jin, J., Willerson, J. T., et al. (2012). Human molecular genetic and functional studies identify TRIM63, encoding muscle RING finger protein 1, as a novel gene for human hypertrophic cardiomyopathy. *Circ. Res.* 111, 907–919. doi: 10.1161/circresaha.112.270207

Crovetto, G. M. (2010). *Energy and Protein Metabolism and Nutrition*. Wageningen: Wageningen Academic Publishers. doi: 10.3920/978-90-8686-781-3

Dawood, M. A. O., Koshio, S., Ishikawa, M., El-Sabagh, M., Yokoyama, S., Wang, W. L., et al. (2017). Physiological response, blood chemistry profile and mucus secretion of red sea bream (*Pagrus major*) fed diets supplemented with *Lactobacillus rhamnosus* under low salinity stress. *Fish Physiol. Biochem.* 43, 179–192. doi: 10.1007/s10695-016-0277-4

Dawood, M. A. O., Koshio, S., Ishikawa, M., and Yokoyama, S. (2015). Effects of partial substitution of fishmeal by soybean meal with or without heat-killed *Lactobacillus plantarum* (lp20) on growth performance, digestibility, and immune response of amberjack, *Seriola dumerili* juveniles. *Biomed Res. Int.* 2015:514196. doi: 10.1155/2015/514196

de Schrijver, R. (1990). Cholesterol metabolism in mature and immature rats fed animal or plant protein. *J. Nutr.* 120, 1624–1632. doi: 10.1093/jn/120.12.1624

Dias, J. (1999). *Lipid Deposition in Rainbow Trout (Oncorhynchus mykiss) and European seabass (Dicentrarchus labrax L.): Nutritional Regulation of Hepatic Lipogenesis*. Doctoral thesis. Porto: University of Porto, 190.

Dodd, K. M., and Tee, A. R. (2012). Leucine and MTORC1: a complex relationship. *Am. J. Physiol. Endocrinol. Metab.* 302, 1329–1342. doi: 10.1152/ajpendo.00525.2011

Dong, X. L., Xie, S. Q., Lei, W., Zhu, X. M., Han, D., and Yang, Y. X. (2012). Effect of faeces collection methods on estimation of apparent digestibility coefficient in gibel carp (*Carassius auratus gibelio*). *Acta Hydrobiol. Sin.* 36, 450–456. doi: 10.1007/s11783-011-0280-z

Dossou, S., Koshio, S., Ishikawa, M., Yokoyama, S., Dawood, M. A., El Basuini, M. F., 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. doi: 10.1016/j.aquaculture.2018.02.010

Duan, X. D., Jiang, W. D., Wu, P., Liu, Y., Jiang, J., Tan, B. P., et al. (2019). Soybean βconglycinin caused intestinal inflammation and oxidative damage in association with NF-κB, TOR and Nrf2 in juvenile grass carp (*Ctenopharyngodon idella*): varying among different intestinal segments. *Fish Shellfish Immunol.* 95, 105–116. doi: 10.1016/j.fsi.2019.10.021

Fauconneau, B., Basseres, A., and Kaushik, S. J. (1992). Oxidation of phenylalanine and threonine in response to dietary arginine supply in rainbow trout (*Salmo gairdneri* R.). *Comp. Biochem. Physiol.* 101A, 395–401. doi: 10.1016/0300-9629(92)90552-2

Fisheries Bureau, Department of Agriculture of China (2020). *China Fishery Statistical Yearbook*. Beijing: China Agriculture Press, 24.

Gao, S. Y., Jin, J. Y., Liu, H. K., Han, D., Zhu, X. M., Yang, Y. X., et al. (2019). Effects of pelleted and extruded feed of different ingredients particle sizes on feed quality and growth performance of gibel carp (*Carassius gibelio* var. CAS V). *Aquaculture* 511:734236. doi: 10.1016/j.aquaculture.2019.734236

Gomes, E. F., Corraze, G., and Kaushik, S. (1993). Effects of dietary incorporation of a coextruded plant protein (rapeseed and peas) on growth, nutrient utilization and muscle fatty-acid composition of rainbow-trout (*Oncorhynchus-Mykiss*). *Aquaculture* 113, 339–353. doi: 10.1016/0044-8486(93)90404-M

Gomez-Requeni, P., Mingarro, M., Calduch-Giner, J. A., Medale, F., Martin, S. A. M., Houlihan, D. F., et al. (2004). Protein growth performance, amino acid utilisation and somatotropic axis responsiveness to fish meal replacement by plant protein sources in gilthead sea bream (*Sparus aurata*). *Aquaculture* 232, 493–510. doi: 10.1016/S0044-8486(03)00532-5

Gui, J. F., and Zhou, L. (2010). Genetic basis and breeding application of clonal diversity and dual reproduction modes in polyploid *Carassius auratus gibelio*. *Sci. China Life Sci.* 53, 409–415. doi: 10.1007/s11427-010-0092-6

Habte-Tsion, H. M., Ge, X. P., Liu, B., Xie, J., Ren, M. C., Zhou, Q. L., et al. (2015). A deficiency or an excess of dietary threonine level affects weight gain, enzyme activity, immune response and immune-related gene expression in juvenile blunt snout bream (*Megalobrama amblycephala*). *Fish Shellfish Immunol.* 42, 439–446. doi: 10.1016/j.fsi.2014.11.021

Hansen, A. C., Rosenlund, G., Karlsen, Ø, Koppe, W., and Hemre, G. I. (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

Hasan, M. R., Haq, M. S., Das, P. M., and Mowlah, G. (1997). Evaluation of poultry-feather meal as a dietary protein source for Indian major carp, *Labeo rohita* fry. *Aquaculture* 151, 47–54. doi: 10.1016/S0044-8486(96)01498-6

He, C., and Klionsky, D. J. (2017). Regulation mechanisms and signaling pathways of autophagy. *Annu. Rev. Genet.* 43, 67–93. doi: 10.1146/annurev-genet-102808-114910

Hernandez-García, A. D., Columbus, D. A., Manjarín, R., Nguyen, H. V., Suryawan, A., Orellana, R. A., et al. (2016). Leucine supplementation stimulates protein synthesis and reduces degradation signal activation in muscle of newborn pigs during acute endotoxemia. *Am. J. Physiol. Endocrinol. Metab.* 311, 791–801. doi: 10.1007/s00726-015-2078-y

Hirche, F., Schroder, A., Knoth, B., Stangl, G. I., and Eder, K. (2006). Effect of dietary methionine on plasma and liver cholesterol concentrations in rats and expression of hepatic genes involved in cholesterol metabolism. *Br. J. Nutr.* 95, 879–888. doi: 10.1079/BJN20061729

Hossain, M. S., Koshio, S., Ishikawa, M., Yokoyama, S., Sony, N. M., Ono, S., et al. (2016). Comparison of the effects of inosine and inosine monophosphate on growth, immune response, stress resistance and gut morphology of juvenile red sea bream, *Pagrus major*. *Aquaculture* 57, 96–106. doi: 10.1016/j.aquaculture.2016.02.032

Inanan, B. E., and Acar, U. (2019). Evaluation of sugar beet leave extracts in goldfish (*Carassius auratus*) diets: effects on blood and semen parameters. *Acta Aquat. Turcica* 4, 458–468. doi: 10.22392/actaquatr.556299

Inanan, B. E., Acar, U., and Inanan, T. (2021). Effects of dietary *Ferula elaeochytris* root powder concentrations on haematology, serum biochemical parameters, spermatozoa parameters, and oxidative status in tissues of male goldfish (*Carassius auratus*). *Aquaculture* 544:737087. doi: 10.1016/j.aquaculture.2021.737087

Ismail, T., Hegazi, E., Dawood, M. A. O., Nassef, E., Bakr, A., Paray, B. A., et al. (2020). Using of betaine to replace fish meal with soybean or/and corn gluten meal in *Nile tilapia* (*Oreochromis niloticus*) diets: histomorphology, growth, fatty acid, and glucose-related gene expression traits. *Aquac. Rep.* 17:100376. doi: 10.1016/j.aqrep.2020.100376

Jia, P., Xue, M., Zhu, X., Liu, H. Y., Wu, X. F., Wang, J., et al. (2013). Effects of dietary methionine levels on the growth performance of juvenile gibel carp (*Carassius auratus gibelio*). *Acta Hydrobiol. Sin.* 37, 217–226.

Jiang, J., Shi, D., Zhou, X. Q., Feng, L., Liu, Y., Jiang, W. D., et al. (2016). Effects of lysine and methionine supplementation on growth, body composition and digestive function of grass carp (*Ctenopharyngodon idella*) fed plant protein diets using high-level canola meal. *Aquac. Nutr.* 22, 1126–1133. doi: 10.1111/anu.12339

Jobgen, W. S., Fried, S. K., Fu, W. J., Meininger, C. J., and Wu, G. (2006). Regulatory role for the arginine-nitric oxide pathway in metabolism of energy substrates. *J. Nutr. Biochem.* 17, 571–588. doi: 10.1016/j.jnutbio.2005.12.001

Kaushik, S. J., Covès, D., Dutto, G., and Blanc, D. (2004). Almost total replacement of fish meal by plant protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchus labrax*. *Aquaculture* 230, 391–404. doi: 10.1016/S0044-8486(03)00422-8

Kaushik, S. J., Cravedi, J. P., Lalles, J. P., Sumpter, J., Fauconneau, B., and Laroche, M. (1995). Partial or total replacement of fish meal by soybean protein on growth, protein utilization, potential estrogenic or antigenic effects, cholesterolemia and flesh quality in rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* 133, 257–274. doi: 10.1016/0044-8486(94)00403-b

Kaushik, S. J., and Seiliez, I. (2010). Protein and amino acid nutrition and metabolism in fish: current knowledge and future needs. *Aquac. Res.* 41, 322–332. doi: 10.1111/j.1365-2109.2009.02174.x

Kikuchi, K. (1999). Use of defatted soybean meal as a substitute for fish meal in diets of Japanese flounder (*Paralichthys olivaceus*). *Aquaculture* 179, 3–11. doi: 10.1016/s0044-8486(99)00147-7

Kim, K., McMillan, I., and Bayley, H. S. (1983). Determination of amino acid requirements of young pigs using an indicator amino acid. *Br. J. Nutr.* 50, 369–382. doi: 10.1079/bjn19830104

Langar, H., Guillaume, J., Metailler, R., and Fauconneau, B. (1993). Augmentation of protein synthesis and degradation by poor dietary amino acid balance in European sea bass (*Dicentrarchus labrax*). *J. Nutr.* 123, 1754–1761. doi: 10.1093/jn/123.10.1754

Li, G. M. (2009). *Studies on Dietary Requirements of Threonine,Leucine, Valine and Isoleucine for Juvenile Gibel Carp (Carassius auratus gibelio)*. Ph.D. thesis. Wuhan: Institute of Hydrobiology, Chinese Academy of Sciences.(In Chinese with English abstract).

Li, G. M., Xie, S. Q., Lei, W., Zhu, X. M., Han, D., and Yang, Y. X. (2010). Dietary valine requirement for juvenile gibel carp (*Carassius auratus gibelio*). *Acta Hydrobiol. Sin.* 34, 1157–1165. doi: 10.3724/SP.J.1035.2010.01157

Li, W. J., Ai, Q. H., Mai, K. S., Xu, W., Luo, Y. W., and Zhang, Y. J. (2013). Effects of dietary amino acid patterns on growth and protein metabolism of large yellow croaker (*Larimichthys crocea*) larvae. *Aquaculture* 406–407, 1–8. doi: 10.1016/j.aquaculture.2013.04.029

Li, X. Q., Xu, H. B., Sun, W. T., Xu, X. Y., Xu, Z., and Leng, X. J. (2018). Grass carp fed a fishmeal-free extruded diet showed higher weight gain and nutrient utilization than those fed a pelleted diet at various feeding rates. *Aquaculture* 493, 283–288. doi: 10.1016/j.aquaculture.2018.04.058

Liang, H. L., Ren, M. C., Habte-Tsion, H. M., Ge, X. P., Xie, J., Mi, H. F., et al. (2016). Dietary arginine affects growth performance, plasma amino acid contents and gene expressions of the TOR signaling pathway in juvenile blunt snout bream, *Megalobrama amblycephala*. *Aquaculture* 461, 1–8. doi: 10.1016/j.aquaculture.2016.04.009

Liu, H. K., Jin, J. Y., Zhu, X. M., Han, D., Yang, Y. X., and Xie, S. Q. (2017). Effect of substitution of dietary fishmeal by soybean meal on different sizes of gibel carp (*Carassius auratus gibelio*): digestive enzyme gene expressions and activities, and intestinal and hepatic histology. *Aquac. Nutr.* 23, 129–147. doi: 10.1111/anu.12375

Liu, H. K., Zhu, X. M., Yang, Y. X., Han, D., Jin, J., and Xie, S. Q. (2016). Effect of substitution of dietary fishmeal by soya bean meal on different sizes of gibel carp (*Carassius auratus gibelio*): nutrient digestibility, growth performance, body composition and morphometry. *Aquac. Nutr.* 22, 142–157. doi: 10.1111/anu.12239

Luo, Y. W., Ai, Q. H., Mai, K. S., Zhang, W. B., Xu, W., and Zhang, Y. J. (2012). Effects of dietary rapeseed meal on growth performance, digestion and protein metabolism in relation to gene expression of juvenile cobia (*Rachycentron canadum*). *Aquaculture* 368, 109–116. doi: 10.1016/j.aquaculture.2012.09.013

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*. *Comp. Biochem. Phys. C* 216, 29–37. doi: 10.1016/j.cbpc.2018.10.006

Ma, Z. Y. (2009). *Studies on Dietary Requirements of Arginine, Histidine, Phenylalanine, Tryptophan and Taurine for Carassius auratus gibelio*. Ph.D. thesis. Wuhan: Institute of Hydrobiology, Chinese Academy of Sciences. (In Chinese with English abstract)

Ma, Z. Y., Zhu, X. M., Xie, S. Q., Yang, Y. X., and Han, D. (2010). Dietary phenylalanine requirement of juvenile gibel carp. *Acta Hydrobiol. Sin.* 34, 1012–1021. doi: 10.3724/SP.J.1035.2010.01012

Mente, E., Deguara, S., Santos, M. B., and Houlihan, D. F. (2003). White muscle free amino acid concentrations following feeding a maize gluten dietary protein in Atlantic salmon (*Salmo salar* L.). *Aquaculture* 225, 133–147. doi: 10.1016/s0044-8486(03)00285-0

National Research Council [NRC] (2011). *Nutrient Requirements of Fish*. Washington, DC: National Academy of Sciences, 218.

Pan, F. Y., Feng, L., Jiang, W. D., Jiang, J., Wu, P., Kuang, S. Y., et al. (2016). Methionine hydroxy analogue enhanced fish immunity via modulation of NF-κB, TOR, MLCK, MAPKs and Nrf2 signaling in young grass carp (*Ctenopharyngodon idella*). *Fish Shellfish Immunol.* 56, 208–228. doi: 10.1016/j.fsi.2016.07.020

Panserat, S., Kolditz, C., Richard, N., Plagnes-Juan, E., Piumi, F., Esquerré, D., et al. (2008). Hepatic gene expression profiles in rainbow trout (*Oncorhynchus mykiss*) fed fish meal or fish oil-free diets. *Br. J. Nutr.* 100, 953–967. doi: 10.1017/S0007114508981411

Regost, C. (1999). Partial or total replacement of fish meal by corn gluten meal in diet for turbot (*Psetta maxima*). *Aquaculture.* 180, 99–117. doi: 10.1016/s0044-8486(99)00026-5

Sandri, M. (2010). Autophagy in skeletal muscle. *FEBS Lett.* 584, 1411–1416. doi: 10.1016/j.febslet.2010.01.056

Santigosa, E. J., Sánchez, F., Médale, F., Kaushik, S., Perez-Sanchez, J., and Gallardo, M. A. (2008). Modifications of digestive enzymes in trout (*Oncorhynchus mykiss*) and sea bream (*Sparus aurata*) in response to dietary fish meal replacement by plant protein sources. *Aquaculture* 252, 68–74. doi: 10.1016/j.aquaculture.2008.06.007

Sheikhzadeh, N., Mousavi, S., Hamidian, G., Firouzamandi, M., Khani Oushani, A., and Mardani, K. (2019). Role of dietary *Spirulina platensis* in improving

mucosal immune responses and disease resistance of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 510, 1–8. doi: 10.1016/j.aquaculture.2019.05.009

Sitjà-Bobadilla, A., Peña-Llopis, S., Gómez-Requeni, P., Médale, F., Kaushik, S., and Pérez-Sánchez, J. (2005). Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*). *Aquaculture* 249, 387–400. doi: 10.1016/j.aquaculture.2005.03.031

Song, Z. D., Li, H. Y., Wang, J. Y., Li, P. Y., and Zhang, L. M. (2016). Effects of replacement fishmeal with soy protein hydrolysates on growth, blood biochemistry, body composition of juvenile starry flounder *Platichthys stellatus pallas*. *Acta Hydrobiol. Sin.* 40, 165–172. doi: 10.7541/2016.23

Sun, H., Jiang, W. D., Wu, P., Liu, Y., Jiang, J., Yang, Q. H., et al. (2019). Betaine supplementations enhance the intestinal immunity of on-growing grass carp (*Ctenopharyngodon idella*): partly related to TOR and NF-κB signaling pathways. *Aquaculture* 2019:734846. doi: 10.1016/j.aquaculture.2019.734846

Tibaldi, E., Tulli, F., and Amerio, M. (1999). "Feed intake and growth responses of sea bass (*D. labrax*) fed different plant-protein sources are not affected by supplementation with a feeding stimulant," in *Recent Progress in Animal Production Science: I. Proceedings of the A.S.P.A. XIII Congress, Piacenza, Italy, 21–24 June 1999*, eds G. Piva, G. Bertoni, S. Satoh, P. Bani, and L. Calamari (Piacenza: Assn. Sci. Anim. Production), 752–754.

Tibbetts, S. M., Mann, J., and Dumas, A. (2017). Apparent digestibility of nutrients, energy, essential amino acids and fatty acids of juvenile Atlantic salmon (*Salmo salar* L.) diets containing whole-cell or cell-ruptured *Chlorella vulgaris* meals at five dietary inclusion levels. *Aquaculture* 481, 25–39. doi: 10.1016/j.aquaculture.2017.08.018

Tu, Y. Q., Xie, S. Q., Han, D., Yang, Y. X., Jin, J., and Zhu, X. M. (2015). Dietary arginine requirement for gibel carp (*Carassis auratus gibelio* var. CAS III) reduces with fish size from 50 g to 150 g associated with modulation of genes involved in TOR signaling pathway. *Aquaculture* 449, 37–47. doi: 10.1016/j.aquaculture.2015.02.031

Uddin, M. N., Nishio, N., Ito, S., Suzuki, H., and Isobe, K. (2012). Autophagic activity in thymus and liver during aging. *Age* 34, 75–85. doi: 10.1007/s11357-011-9221-9

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., et al. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3:research0034.1. doi: 10.1186/gb-2002-3-7-research0034

Verhoeven, J., Salvo, F., Hamoutene, D., and Dufour, S. (2016). Bacterial community composition of flocculent matter under a salmonid aquaculture site in Newfoundland, Canada. *Aquac. Environ. Interact.* 8, 637–646. doi: 10.3354/aei00204

Wang, T. T., Xu, M. M., Wang, J. T., Wan, W. J., Guan, D. Y., Han, H. J., et al. (2020). A combination of rapeseed, cottonseed and peanut meal as a substitute of soybean meal in diets of Yellow River carp *Cyprinus carpio* var. *Aquac. Nutr.* 26, 1520–1532. doi: 10.1111/anu.13099

Watanabe, T., Verakunpiriya, V., Watanabe, K., Viswanath, K., and Satoh, S. (1998). Feeding of rainbow trout with non-fish meal diets. *Fish. Sci.* 63, 258–266. doi: 10.2331/fishsci.63.258

Wu, M. J., Lu, S. D., Wu, X. Y., Jiang, S. T., Luo, Y., and Yao, W. (2017). Effects of dietary amino acid patterns on growth, feed utilization and hepatic IGF-I, TOR gene expression levels of hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂) juveniles. *Aquaculture* 468, 508–514. doi: 10.1016/j.aquaculture.2016.11.019

Wu, Y., Han, H., Qin, J., and Wang, Y. (2015). Replacement of fish meal by soy protein concentrate with taurine supplementation in diets for golden pompano (*Trachinotus ovatus*). *Aquac. Nutr.* 21, 214–222. doi: 10.1111/anu.2015.21.issue-2

Wullschleger, S., Loewith, R., and Hall, M. N. (2006). TOR signaling in growth and metabolism. *Cell* 124, 471–484. doi: 10.1016/j.cell.2006.01.016

Xia, J., Wang, R., Zhang, T. L., and Ding, J. P. (2016). Structural insight into the arginine-binding specificity of CASTOR1 in amino acid-dependent mTORC1 signaling. *Cell Discov.* 2:16035. doi: 10.1038/celldisc.2016.35

Xie, S. Q., Zhu, X. M., Cui, Y. B., and Yang, Y. X. (2001). Utilization of several plant proteins by gibel carp (*Carassius auratus gibelio*). *J. Appl. Ichthyol.* 17, 70–76. doi: 10.1046/j.1439-0426.2001.00275.x

Xu, D. D., He, G., Mai, K. S., Zhou, H. H., Xu, W., and Song, F. (2015). 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/S0007114514535

Xu, W. N., Qian, Y., Li, X. F., Li, J. Y., Li, P. F., Cai, D. S., et al. (2017). Effects of dietary biotin on growth performance and fatty acids metabolism in blunt snout bream, *Megalobrama amblycephala* fed with different lipid levels diets. *Aquaculture* 479, 790–797. doi: 10.1016/j.aquaculture.2017.07.018

Yaghoubi, M., Mozanzadeh, M. T., Marammazi, J. G., Safari, O., and Gisbert, E. (2016). Dietary replacement of fish meal by soy products (soybean meal and isolated soy protein) in silvery-black porgy juveniles (*Sparidentex hasta*). *Aquaculture* 464, 50–59. doi: 10.1016/j.aquaculture.2016.06.002

Yuan, X. Y., Liu, M. Y., Cheng, H. H., Huang, Y. Y., Dai, Y. J., Liu, W. B., et al. (2019). Replacing fish meal with cottonseed meal protein hydrolysate affects amino acid metabolism via AMPK/SIRT1 and TOR signaling pathway of *Megalobrama amblycephala*. *Aquaculture* 510, 225–233. doi: 10.1016/j.aquaculture.2019.05.056

Zeitz, J. O., Käding, S. K., Niewald, I. R., Most, E., Dorigam, J. C., de, P., et al. (2019). The influence of dietary leucine above recommendations and fixed ratios to isoleucine and valine on muscle protein synthesis and degradation pathways in broilers. *Poult. Sci.* 98, 6772–6786. doi: 10.3382/ps/pez396

Zhang, P., Fu, L., Liu, H., Huda, N. U, Zhu, X., Han, D., et al. (2019). Effects of inosine 5′-monophosphate supplementation in high fishmeal and high soybean diets on growth, immune-related gene expression in gibel carp (*Carassius auratus gibelio* var. CAS III), and its challenge against *Aeromonas hydrophila* infection. *Fish Shellfish Immunol.* 86, 913–921. doi: 10.1016/j.fsi.2018.12.016

Zhang, Y. L., Wang, P., Lin, S., Mercier, Y., Yin, H. J., Song, Y. M., et al. (2018). mTORC1 signaling-associated protein synthesis in porcine mammary glands was regulated by the local available methionine depending on methionine sources. *Amino Acids* 50, 105–115. doi: 10.1007/s00726-017-2496-0

Zhao, J., Liu, Y., Jiang, J., Wu, P., Chen, G. F., Jiang, W. D., et al. (2012). Effects of dietary isoleucine on growth, the digestion and absorption capacity and gene expression in hepatopancreas and intestine of juvenile Jian carp (*Cyprinus carpio* var. Jian). *Aquaculture* 368-369, 117–128. doi: 10.1016/j.aquaculture.2012.09.019

Zhou, X. J., Xie, S. Q., Xie, C. X., Lei, W., and Zhu, X. M. (2006). The utilization and requirement of dietary lysine for juvenile gibel carp. *Acta Hydrobiol. Sin.* 30, 247–255. doi: 10.3321/j.issn:1000-3207.2006.03.001

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

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

frontiers in Physiology

ORIGINAL RESEARCH
published: 11 February 2022
doi: 10.3389/fphys.2022.850470

Effects of Dietary Inositol Supplementation on Growth, Digestive Performance, Antioxidant Capacity, and Body Composition of Golden Pompano (*Trachinotus ovatus*)

Xu Chen[1,2], *Jun Wang*[1,3]* *and Wei Zhao*[4]*

[1]*Key Laboratory of Aquatic Product Processing, Ministry of Agriculture and Rural Affairs, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou, China,* [2]*Tropical Fisheries Research and Development Center, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences/Sanya Tropical Fisheries Research Institute, Sanya, China,* [3]*Southern Marine Science and Engineering Guangdong Laboratory, Zhanjiang, China,* [4]*Guangdong Provincial Key Laboratory of Improved Variety Reproduction in Aquatic Economic Animals, School of Life Sciences, Institute of Aquatic Economic Animals, Sun Yat-sen University, Guangzhou, China*

OPEN ACCESS

Edited by:
Shiwei Xie,
Guangdong Ocean University, China

Reviewed by:
Xiaodan Wang,
East China Normal University, China
Yu Hong Liu,
Guangdong Ocean University, China

***Correspondence:**
Wei Zhao
zwtjnxysc@163.com
Jun Wang
junnywang@163.com

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

Received: *07 January 2022*
Accepted: *24 January 2022*
Published: *11 February 2022*

Citation:
Chen X, Wang J and Zhao W (2022) Effects of Dietary Inositol Supplementation on Growth, Digestive Performance, Antioxidant Capacity, and Body Composition of Golden Pompano (Trachinotus ovatus). Front. Physiol. 13:850470. doi: 10.3389/fphys.2022.850470

A 56-day culture experiment was performed to evaluate effects of inositol supplementation on growth, digestive performance, antioxidant capacity, and body composition of golden pompano (*Trachinotus ovatus*). Five experimental diets (D1, D2, D3, D4, and D5) supplemented with 0, 150, 300, 600, and 1,200 mg kg^{-1} inositol were formulated, respectively. Triplicate groups of 300 fish with an initial weight of (18.78 ± 0.21 g) and 15 seawater cages were used in the feeding experiment. Results indicated that the final body weight (FBW), weight gain rate (WGR), specific growth ratio (SGR), and condition factor (CF) in fish fed with D3–D5 diets were significantly higher than those fed the D1 and D2 diets, and the highest values were detected in D3 diet treatment. The whole-body composition was not significantly affected by different experimental diets. Fish fed with D3-D5 diets showed higher activities of amylase (AMS), lipase (LPS), and superoxide dismutase (SOD), and significantly higher than those fed with D1 and D2 diets. In contrast, fish fed with D3–D5 diets showed lower MDA content and significantly lower than those fed with D1 and D2 diets. The mRNA level of glutathione reductase (GR) in fish fed with D3 and D4 diets was significantly higher than those fed with D1, D2, and D5 diets. Likewise, the mRNA level of catalase (CAT) significantly increased in the dietary inositol groups compared with the D1 group. In conclusion, the supplement of inositol not less than 300 mg kg^{-1} in the diet is indispensable to maintain the rapid growth and promote antioxidative capacity of *T. ovatus*.

Keywords: *Trachinotus ovatus*, inositol, growth, digestive performance, antioxidant capacity

INTRODUCTION

Inositol is classified as a B-complex vitamin and is widely distributed in plants and animals in the form of phospholipids as the main structural component of biological membranes (Peres et al., 2004; Cui and Ma, 2020). Biochemical functions of inositol include transmembrane signal transfer, regulation of enzyme activity, mediation of lipid transport and metabolism, and protection of the liver (Wang et al., 2018; Cui and Ma, 2020). The intestinal microbial flora of some species of fish also have a certain ability to synthesize inositol, including Atlantic salmon (*Salmo salar*; Waagbø et al., 1998) and sunshine bass (*Morone chrysops* female × *Morone saxatilis* male; Deng et al., 2002), which do not need dietary inositol supplementation for growth and development. However, for most fish species, their ability to synthesize inositol is not enough to meet the metabolic needs, so dietary inositol supplementation is indispensable for growth and development (Jiang et al., 2010). Moreover, adding enough inositol to the feed is beneficial to the digestion and antioxidant performance of fish (Zhang et al., 2014; Wang et al., 2020). Therefore, inositol is widely supplemented to fish diets as a nutrient. So far, the importance of dietary inositol for maximum growth of fish have been widely reported, such as jian carp (*Cyprinus carpio*; Jiang et al., 2009), gibel carp (*Carassius auratus gibelio*; Gong et al., 2015), parrot fish (*Oplegnathus fasciatus*; Khosravi et al., 2015), Amur sturgeon (*Acipenser schrenckii*; Wang et al., 2018), and taimen (*Hucho taimen* fry; Wang et al., 2020), with the requirement level varying from 100 to 517 mg kg^{-1} diet. In contrast, insufficient supply of dietary inositol can lead to deficiency symptoms, including growth retardation, liver lipid deposition, decreased feed intake, decreased transaminase activity, fin erosion, reduction of skin mucosa and anemia (National Research Council, NRC, 2011).

Trachinotus ovatus is widely distributed in southern China, Southeast Asia, Japan, and Australia (Zhao et al., 2021). It has the characteristics of fast growth, high nutritional value, and delicious meat. Due to the increasing market demand, *T. ovatus* has become a very important economically cultured marine fish in the southern coast of China. In order to improve the growth performance and ensure its sustainable supply, it is necessary to optimize feed formula for *T. ovatus*. Up to now, however, no studies have been conducted to investigate the effects of inositol on the growth, digestive performance, antioxidant capacity, and body composition of *T. ovatus*. As the requirement of inositol differs from species to species, the purpose of this study is to determine the requirement for inositol of *T. ovatus*, mainly based on growth, digestive capacity, and antioxidant performance parameters.

MATERIALS AND METHODS

Experimental Diets

Five isonitrogenous and isolipidic diets supplemented with 0 (D1), 150 (D2), 300 (D3), 600 (D4), and 1,200 (D5) mg kg^{-1} inositol (Sigma-Aldrich, Purity ≥ 99%) were prepared for the fish. The formulation and proximate composition analysis of the experimental diets were shown in **Table 1**. The experimental diets were manufactured following the procedure described by Zhao et al. (2020), and then stored at −20°C until feeding.

Fish and Feeding Experiment

Juvenile *T. ovatus* were purchased from a commercial farm (Lingshui, Hainan, China). Fifteen seawater cages (1.0 m × 1.0 m × 1.5 m) were placed in the Bay (Lingshui, Hainan, China) and used for feeding experiment. Before the feeding experiment, all fish were acclimatized to the experimental conditions and facilities and fed the experimental control feed for 2 weeks. At the beginning of the feeding experiment, the fish were starved for 24 h, and then healthy fish (average body weight 18.78 ± 0.21 g) were randomly stocked to 15 seawater cages at 20 fish per cage. Each experimental feed was assigned three replicates. The feeding experiment lasted for 56 days, during which fish were slowly hand-fed to apparent satiation

TABLE 1 | Composition and nutrient levels of the experimental diets.

Items	D1	D2	D3	D4	D5
Ingredients (g/kg)					
Fish meal	300.00	300.00	300.00	300.00	300.00
Soybean meal	320.00	320.00	320.00	320.00	320.00
Wheat flour	180.00	179.85	179.70	179.40	178.80
Shrimp head meal	30.00	30.00	30.00	30.00	30.00
Chicken meal	30.00	30.00	30.00	30.00	30.00
Fish oil	65.00	65.00	65.00	65.00	65.00
Soybean lecithin	20.00	20.00	20.00	20.00	20.00
$Ca(H_2PO_4)_2$	20.00	20.00	20.00	20.00	20.00
Vitamin premix[a]	10.00	10.00	10.00	10.00	10.00
Mineral premix[b]	10.00	10.00	10.00	10.00	10.00
Choline	5.00	5.00	5.00	5.00	5.00
Vc	5.00	5.00	5.00	5.00	5.00
DL-Met	2.50	2.50	2.50	2.50	2.50
Lys-HCL	2.50	2.50	2.50	2.50	2.50
Inositol	0.00	0.15	0.3	0.6	1.2
Total	100.00	100.00	100.00	100.00	100.00
Nutrient levels[c] (g/kg)					
Moisture	101.50	96.10	100.00	94.00	98.60
Crude protein	473.80	472.90	476.50	483.70	480.20
Crude lipid	113.20	115.80	111.70	116.10	114.30
Ash	110.00	112.70	110.00	110.60	114.30
Inositol	0.28	0.40	0.53	0.80	1.39

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

[b]*Mineral premix provides the following per kg of diet: NaF 2 mg, KI 0.8 mg, $CoCl_2$•$6H_2O$ (1%) 50 mg, $CuSO_4$•$5H_2O$ 10 mg, $FeSO_4$•H_2O 80 mg, $ZnSO_4$•H_2O 50 mg, $MnSO_4$•H_2O 60 mg, $MgSO_4$•$7H_2O$ 1,200 mg, $ZnSO_4$•H_2O 50 mg, $Ca(H_2PO_4)_2$•H_2O 3,000 mg, NaCl 100 mg, and zeolite powder 15.447 g.*

[c]*Measured values.*

twice daily (8:00 and 16:00). At the end of the feeding experiment, all survival fish were starved for 24 h, and then weighed in batches after anesthesia. The final body weight (FBW) and length were gauged to calculate the growth performance.

Sample Collection

After the feeding experiment, four fish were randomly selected from each cage and frozen in liquid nitrogen, and then stored at −80°C for whole body composition analysis. The liver and mid-gut were stripped from five fish per cage, and frozen in liquid nitrogen and stored at −80°C for enzyme activity analysis. Meantime, the liver was sampled from another three fish per cage, placed in RNAlater™ Stabilization Solution (ThermoFisher Scientific, Shanghai, China) and then stored at −20°C for gene expression analysis.

Proximate Composition Analysis of the Experimental Diets and Whole Body

The crude lipid, crude protein, moisture, and ash of experimental diets and whole body were determined and analyzed according to the standard procedures of AOAC (1995).

Enzyme Activity Analysis

Liver and mid-gut samples were homogenized and centrifuged to collect supernatant for enzyme activity analysis using reagent kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Superoxide dismutase (SOD; A001-1) activities and malondialdehyde (MDA; A003-1) contents in liver were determined following the instructions of the kit. Similarly, the activities of amylase (AMS; C016) and lipase (LPS; A054-2) in the mid-gut were measured according to the procedure described by instructions of the kit. The specific operation process is carried out according to the previous description of Zhang et al. (2017).

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

Gene name	Primer sequence (5′-3′)	References
GR	F-GTGTGTGTGGGCAAGGAGGA	Zhao et al., 2020
	R-AGATGAGGTGGGGTGAATGG	
CAT	F-AGTTTTACACCGAGGAGGGC	Tan et al., 2018
	R-TGTGGGTTTGGGGATTGC	
β-actin	F-TACGAGCTGCCTGACGGACA	Zhao et al., 2020
	R-GGCTGTGATCTCCTTCTGC	

Total RNA Extraction and Gene Expression Analysis

Total RNA extraction and real-time quantitative PCR analysis were performed following our previously published methods (Zhao et al., 2020). Briefly, the livers from each cage were pooled for the extraction of total RNA using a Reagent kit (Takara, Dalian, China). Around 10 g/L agarose gel electrophoresis and spectrophotometer (NanoDrop 2000, Thermo Fisher, United States) were used to evaluate the quality and quantity of RNA. Then, cDNA was synthesized using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China) following the manufacturer's instructions. Primers for real-time quantitative PCR were presented in **Table 2**. Real-time quantitative PCR assays were performed on the Light Cycler 480 real-time fluorescence quantitative PCR System (Roche Applied Science, Basel Switzerland). The relative expression levels of target genes were quantified using $2^{-\Delta\Delta Ct}$ method.

Calculations and Statistical Analysis

The weight gain rate (WGR), survival rate (SR), feed conversion ratio (FCR), and specific growth ratio (SGR) were determined according to the equation described by Zhao et al. (2020). Condition factor (CF) = 100 × [(body weight, g)/(length, cm)^3].

All data were presented in the form of means ± SEM. All data were analyzed by one-way ANOVA and Duncan's multiple range tests in SPSS 19.0 (SPSS, Chicago, IL, United States). A value of $p < 0.05$ was deemed to be statistically significant.

RESULTS

Biological Performances

The growth performance (WGR, SGR, and *CF*), SR, and FCR of *T. ovatus* were shown in **Table 3**. The highest values of FBW, WGR, SGR, and *CF* were detected in fish fed with D3 diet, and significantly higher than those fed the D1 and D2 diets ($p < 0.05$). However, there were no significant difference in the FBW, WGR, SGR, and *CF* among D3–D5 diet treatments ($p > 0.05$). Likewise, SR and FCR were not significantly influenced by dietary treatments ($p > 0.05$).

Body Composition

Whole body composition was shown in **Table 4**. The values of crude protein, crude lipid, moisture, and ash were not significantly affected by different experimental diets ($p > 0.05$).

TABLE 3 | Effects of inositol on growth performance of *Trachinotus ovatus*.

Items	IBW/g	FBW/g	WGR/%	SGR/(%/d)	SR/%	FCR	*CF*
D1	18.72 ± 0.30	104.15 ± 7.49^a	455.32 ± 36.71^a	2.87 ± 0.19^a	91.11 ± 7.63	1.04 ± 0.07	3.34 ± 0.24^a
D2	18.91 ± 0.14	107.95 ± 3.86^a	470.78 ± 23.05^a	3.00 ± 0.07^a	93.33 ± 2.89	1.07 ± 0.10	3.34 ± 0.16^a
D3	18.68 ± 0.26	123.54 ± 7.26^b	560.12 ± 37.12^b	3.23 ± 0.11^b	90.00 ± 7.32	1.09 ± 0.08	3.66 ± 0.36^b
D4	18.72 ± 0.27	121.37 ± 7.15^b	550.02 ± 34.13^b	3.20 ± 0.16^b	88.33 ± 5.77	1.05 ± 0.07	3.54 ± 0.30^b
D5	18.83 ± 0.10	121.02 ± 5.61^b	550.69 ± 31.19^b	3.19 ± 0.07^b	95.00 ± 7.07	1.04 ± 0.08	3.58 ± 0.21^b

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

Antioxidant Capacity

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

The SOD activity in fish fed with D3–D5 diets was significantly higher than those fed with D1 and D2 diets ($p < 0.05$). In contrast, fish fed with D3–D5 diets showed lower MDA content and significantly lower than those fed with D1 and D2 diets ($p < 0.05$).

The mRNA level of glutathione reductase (*GR*) in fish fed with D3 and D4 diets were significantly higher than those fed with D1, D2, and D5 diets ($p < 0.05$), and the highest value was detected in the D3 diet treatment. Likewise, the mRNA level of catalase (*CAT*) significantly increased in the dietary inositol groups compared with the D1 group ($p < 0.05$), and the highest value was detected in the D3 diet treatment (**Figure 2**).

Digestive Enzyme Activity

The activities of AMS and LPS were shown in **Figure 3**. Fish fed with D3–D5 diets showed higher activities of AMS and LPS, and significantly higher than those fed with D1 and D2 diets ($p < 0.05$).

DISCUSSION

A sufficient amount of inositol in the diet to meet the various metabolic needs is extremely important for the fish to obtain the maximum growth. In this study, the growth response of *T. ovatus* to dietary inositol clearly proved the importance of inositol for maximum growth. The growth performance of *T. ovatus* was significantly improved by feeding a diet containing not less than 300 mg kg^{-1} inositol. The similar

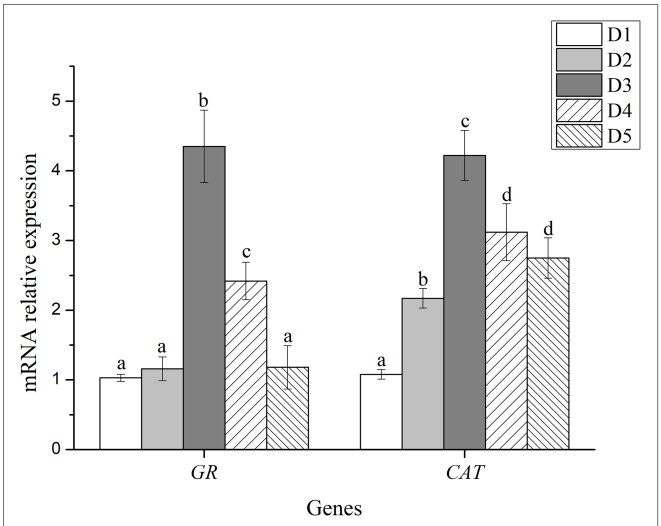


FIGURE 2 | Relative mRNA expression of antioxidant-related genes in liver of fish fed with experimental diets. $n = 3$. The small letters indicated significant differences at $p<0.05$.

TABLE 4 | Effects of inositol on whole body composition (g/kg dry matter) of *Trachinotus ovatus*.

Items	D1	D2	D3	D4	D5
Moisture	710.4 ± 10.6	713.0 ± 8.9	711.6 ± 14.4	714.3 ± 17.2	703.6 ± 14.3
Crude protein	607.2 ± 16.1	609.5 ± 19.9	623.4 ± 7.3	621.9 ± 16.5	593.0 ± 15.9
Crude lipid	238.4 ± 15.3	246.7 ± 10.7	228.4 ± 14.4	237.3 ± 10.1	251.1 ± 19.2
Ash	156.2 ± 6.1	149.9 ± 4.5	152.3 ± 13.4	155.5 ± 11.4	144.4 ± 12.0

Values are means ± SE of three replicates.

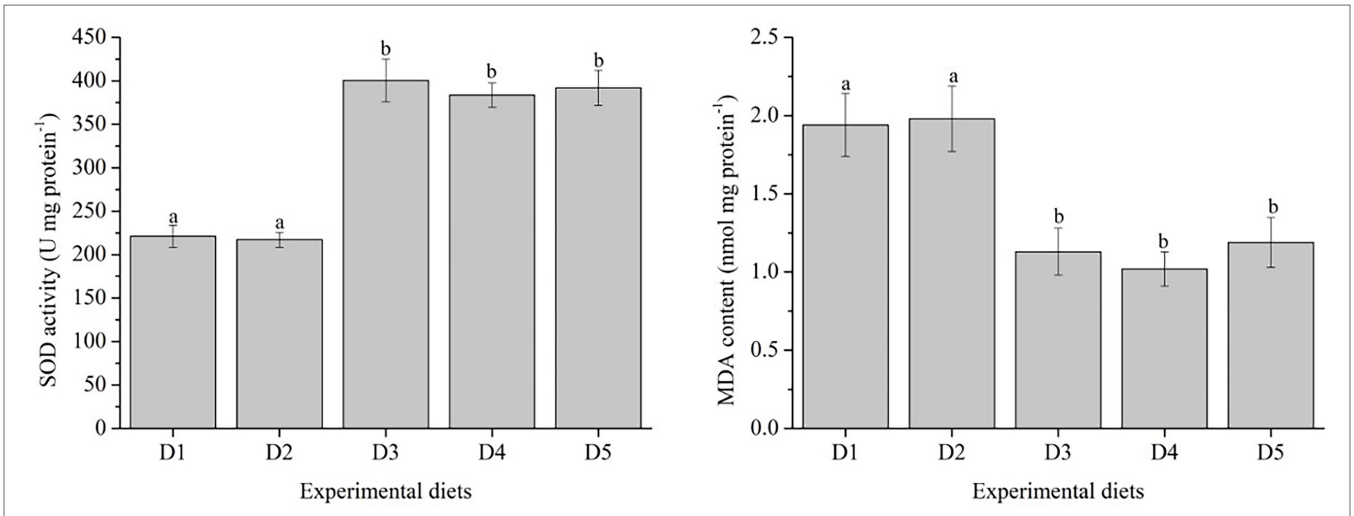


FIGURE 1 | Effects of inositol levels on antioxidant parameters of *Trachinotus ovatus* during the 8 weeks rearing period. $n = 3$. The small letters indicated significant differences at $p<0.05$.

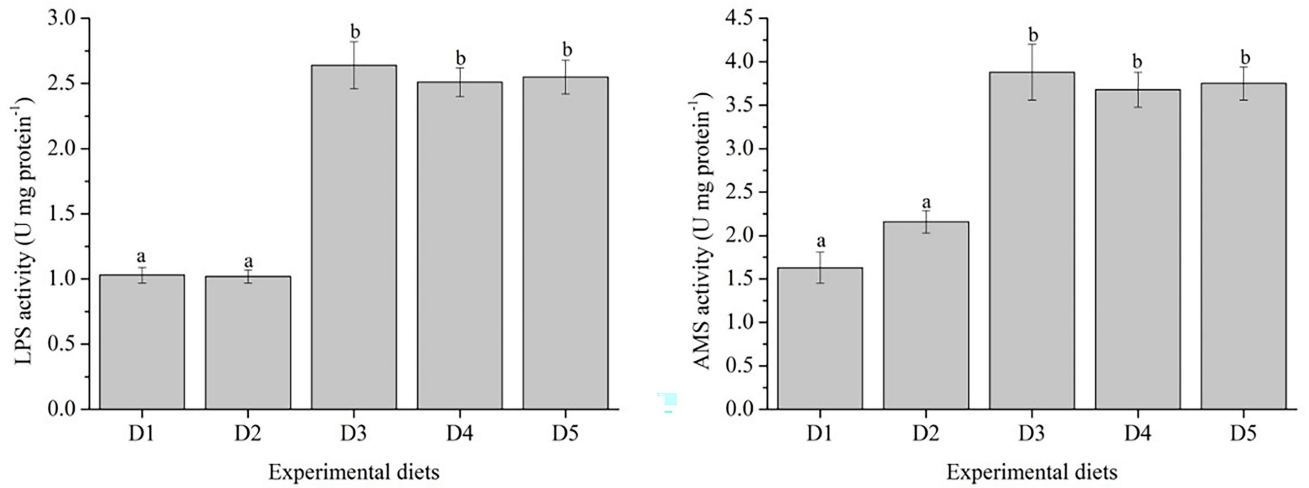


FIGURE 3 | Specific activities of mid-gut digestive enzymes of *Trachinotus ovatus* fed with experimental diets. $n=3$. The small letters indicated significant differences at $p<0.05$.

results were found in studies on *C. carpio* (Jiang et al., 2009), *Lates calcarifer* Bloch (Diao et al., 2010), *C. auratus gibelio* (Gong et al., 2015), *O. fasciatus* (Khosravi et al., 2015), *A. schrenckii* (Wang et al., 2018), and *H. taimen* fry (Wang et al., 2020), which showed that the addition of inositol in the feed could ensure maximum growth and prevent deficiency symptoms. However, Waagbø et al. (1998) and Deng et al. (2002) found that dietary supplementation with inositol did not promote the growth of *S. salar* and sunshine bass (*M. chrysops* × *M. saxatilis*), which indicated that these fish species may be able to synthesize enough inositol for growth without additional supplementation. Current research results demonstrated that the requirement level for inositol in fish diets is related to species differences. In addition, the difference in fish requirement for inositol is also related to growth stage, physiological and nutritional status, source and level of carbohydrate and lipid (Khosravi et al., 2015).

The fluctuation of digestive enzyme activity is a direct response of fish to food source and nutrient concentration (Carneiro et al., 2020). High activity of digestive enzyme helps to improve the digestion and absorption of nutrients, which contributes to the improvement of fish growth performance (Sun et al., 2020; Zhao et al., 2020). Dietary supplementation of inositol improved digestive enzyme activity and promoted growth, which has been reported in studies on *H. taimen* and grass carp (*Ctenopharyngodon idellus*; Zhang et al., 2014; Lin, 2018). Zhang et al. (2014) indicated that the addition of inositol to feed significantly increased the activities of protease and LPS in the intestine of *H. taimen*, which helped to improve the digestion and absorption of feed by fish. Similarly, Lin (2018) also found that inositol supplementation significantly increased the activities of protease, AMS, and LPS in the intestine of *C. idellus*, which was consistent with the results of growth performance. As a structural element of cell membranes, inositol can bind to phospholipids on cell membranes to form inositol phosphate, which helps to maintain the structure and function of the intestine. Therefore, inositol supplementation in feed can promote intestinal development and the secretion of digestive enzymes (Lin, 2018). Similar results were also obtained in this study, the present results indicated that dietary supplementation of inositol not less than 300 mg kg^{-1} significantly increased the activities of AMS and LPS in the intestine of *T. ovatus*. The change trend of growth performance is consistent with that of digestive enzyme activity. Dietary inositol supplementation improved the activity of digestive enzymes to improve the digestion and absorption of nutrients by fish, and finally promote the growth performance of *T. ovatus*.

Regarding body composition, no significant differences were observed in the crude protein, crude lipid, moisture, and ash. The present results indicated that the whole-body composition of *T. ovatus* was not affected by dietary inositol supplementation. The similar results were reported in studies on *S. salar* (Waagbø et al., 1998), *C. idellus* (Wen et al., 2007), and *L. calcarifer* Bloch (Diao et al., 2010). In contrast to our results, Khosravi et al. (2015) found that dietary inositol supplementation significantly reduced the whole-body protein level of *O. fasciatus*. However, dietary inositol supplementation significantly increased the whole-body protein level of *C. auratus gibelio* (Gong et al., 2015). The current results showed that the effect of inositol on whole body composition is species-specific.

Superoxide dismutase, CAT, and GR play important roles in removing oxidative damage and maintaining intracellular homeostasis (Chen et al., 2021). The activation of these antioxidant enzymes is an important protective mechanism for cells to reduce oxidative damage (Zhao et al., 2020). The present results found that dietary supplementation of inositol not less than 300 mg kg^{-1} significantly increased the activity and mRNA level of antioxidant enzyme and decreased MDA content in the liver of *T. ovatus*. Our results demonstrated that dietary inositol supplementation could improve the antioxidant capacity of *T. ovatus* by increasing the activity and mRNA level of antioxidant enzymes. Similarly,

Wang et al. (2020) also reported that dietary inositol supplementation significantly increased the SOD activity and decreased MDA content in the skin mucus of *H. taimen*. Dietary supplementation of inositol not less than 232.7 mg kg^{-1} significantly increased the activities of SOD, CAT, and GR in the serum of *C. carpio* (Jiang et al., 2009). Inositol can improve the antioxidant capacity of fish may be related to the structure of polyols.

The polyol structure of inositol can transfer H^+ to free radicals, scavenge OH·produced by Feton reaction system, and chelate high-valent chromium compounds, thereby playing an antioxidant effect (Hu et al., 1995; Santoro et al., 2007).

CONCLUSION

In conclusion, dietary supplementation of inositol not less than 300 mg kg^{-1} increases the growth performance (WGR, SGR, and *CF*) and digestive enzyme activities (AMS and LPS) of *T. ovatus*, and exerts antioxidant effects by boosting the activities and mRNA levels of antioxidant enzymes. The present results indicated that *T. ovatus* lacks the ability to biosynthesize inositol or the synthesis level of inositol is insufficient to supply the requirements for growth and metabolism. Therefore, the supplement of inositol not less than 300 mg kg^{-1} in the diet is indispensable to maintain the rapid growth and promote antioxidative capacity of *T. ovatus*.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in Sun Yat-sen University, Guangzhou, China, and conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85–23, revised 1985).

AUTHOR CONTRIBUTIONS

WZ and JW designed the study, analyzed the results, and wrote the paper with contributions from the other authors. XC carried out the rearing work and measured experimental parameters. All authors contributed to the article and approved the submitted version.

FUNDING

The study was supported by National Natural Science Foundation of China (32172984); the Southern Marine Science and Engineering Guangdong Laboratory funds (ZJW-2019-06); Central Public-interest Scientific Institution Basal Research Fund, CAFS (2020TD55); and Central Public-interest Scientific Institution Basal Research Fund, South China Sea Fisheries Research Institute, CAFS (2020XK02, 2021SD09).

ACKNOWLEDGMENTS

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

REFERENCES

AOAC (1995). Official Methods of Analysis of AOAC, International. Association of Official Analytical Chemist International Washington, DC.

Carneiro, W. F., Castro, T. F. D., Orlando, T. M., Meurer, F., Paula, D. A. D. J., Virote, B. D. C. R., et al. (2020). Replacing fish meal by *chlorella* sp. meal: effects on zebrafish growth, reproductive performance, biochemical parameters and digestive enzymes. *Aquaculture* 528:735612. doi: 10.1016/j.aquaculture.2020.735612

Chen, Y., Chi, S., Zhang, S., Dong, X., Yang, Q., Liu, H., et al. (2021). Replacement of fish meal with methanotroph (*Methylococcus capsulatus*, Bath) bacteria meal in the diets of Pacific white shrimp (*Litopenaeus vannamei*). *Aquaculture* 541:736801. doi: 10.1016/j.aquaculture.2021.736801

Cui, W., and Ma, A. (2020). Transcriptome analysis provides insights into the effects of myo-inositol on the turbot *Scophthalmus maximus*. *Fish Shellfish Immunol.* 106, 691–704. doi: 10.1016/j.fsi.2020.07.019

Deng, D. F., Hemre, G. I., and Wilson, R. P. (2002). Juvenile sunshine bass (*Morone chrysops*♀ × *Morone saxatilis*♂) do not require dietary myo-inositol. *Aquaculture* 213, 387–393. doi: 10.1016/S0044-8486(02)00119-9

Diao, S. Q., Huang, Z., Chen, S. S., Niu, J., Li, Z., Ding, X., et al. (2010). Effect of dietary inositol on growth, feed utilization and blood biochemical parameters for juvenile barramundi (*Lates calcarifer* Bloch). *Am. J. Agric. Biol. Sci.* 5, 370–375. doi: 10.3844/ajabssp.2010.370.375

Gong, W., Lei, W., Zhu, X., Yang, Y., Han, D., and Xie, S. (2015). Dietary myo-inositol requirement for juvenile gibel carp (*Carassius auratus gibelio*). *Aquac. Nutr.* 20, 514–519. doi: 10.1111/anu.12104

Hu, M. L., Chen, Y. K., and Lin, Y. F. (1995). The antioxidant and prooxidant activity of some B vitamins and vitamin-like compounds. *Chem. Biol. Interact.* 97, 63–73. doi: 10.1016/0009-2797(95)03608-8

Jiang, W. D., Feng, L., Liu, Y., Jiang, J., Hu, K., Li, S. H., et al. (2010). Lipid peroxidation, protein oxidant and antioxidant status of muscle, intestine and hepatopancreas for juvenile Jian carp (*Cyprinus carpio* var Jian) fed graded levels of myo-inositol. *Food Chem.* 120, 692–697. doi: 10.1016/j.foodchem.2009.10.062

Jiang, W., Feng, L., Liu, Y., Jiang, J., and Zhou, X. (2009). Growth, digestive capacity and intestinal microflora of juvenile jian carp (*Cyprinus carpio* var. jian) fed graded levels of dietary inositol. *Aquac. Res.* 40, 955–962. doi: 10.1111/j.1365-2109.2009.02191.x

Khosravi, S., Lim, S. J., Rahimnejad, S., Kim, S. S., Lee, B. J., Kim, K. W., et al. (2015). Dietary myo-inositol requirement of parrot fish, *Oplegnathus fasciatus*. *Aquaculture* 436, 1–7. doi: 10.1016/j.aquaculture.2014.10.034

Lin, K. (2018). Effect of Inositol Supplementation on Growth Performance, Glycolipid Metabolism, Digestion and Antioxidant Activity of Grass Carp (*Ctenopharyngodon idellus*). Master thesis. Chongqing: Southwest University.

National Research Council, NRC (2011). *Nutrient Requirements of Fish and Shrimp*. Washington DC: National Academies Press, 206–207.

Peres, H., Lim, C., and Klesius, P. H. (2004). Growth, chemical composition and resistance to *Streptococcus iniae* challenge of juvenile Nile tilapia (*Oreochromis niloticus*) fed graded levels of dietary inositol. *Aquaculture* 235, 423–432. doi: 10.1016/j.aquaculture.2003.09.021

Santoro, M., Caffaratti, E., Salas-Peregrin, J. M., Korecz, L., Rockenbauer, A., Sala, L. F., et al. (2007). Kinetics and mechanism of the chromic

oxidation of myo-inositol. *Polyhedron* 26, 169–177. doi: 10.1016/j.poly.2006.08.003

Sun, Y., Wang, X., Zhou, H., Mai, K., and He, G. (2020). Dietary *Astragalus* polysaccharides ameliorates the growth performance, antioxidant capacity and immune responses in turbot (*Scophthalmus maximus* L.). *Fish Shellfish Immunol.* 99, 603–608. doi: 10.1016/j.fsi.2020.02.056

Tan, X., Sun, Z., Zhou, C., Huang, Z., Tan, L., Xun, P., et al. (2018). Effects of dietary dandelion extract on intestinal morphology, antioxidant status, immune function and physical barrier function of juvenile golden pompano *Trachinotus ovatus*. *Fish Shellfish Immunol.* 73, 197–206. doi: 10.1016/j.fsi.2017.12.020

Waagbø, R., Sandnes, K., and Lie, O. (1998). Effects of inositol supplementation on growth, chemical composition and blood chemistry in Atlantic salmon, *Salmo salar* L., fry. *Aquac. Nutr.* 4, 53–59. doi: 10.1046/j.1365-2095.1998.00043.x

Wang, C., Liu, H., Li, J., Wang, L., Zhao, Z., Luo, L., et al. (2018). Effects of dietary myo-inositol on growth, chemical composition and plasma chemistry of Amur sturgeon *Acipenser schrenckii*. *Aquac. Int.* 26, 1481–1492. doi: 10.1007/s10499-018-0299-2

Wang, C., Lu, S., Li, J., Wang, L., Jiang, H., Liu, Y., et al. (2020). Effects of dietary myo-inositol on growth, antioxidative capacity, and nonspecific immunity in skin mucus of taimen *Hucho taimen* fry. *Fish Physiol. Biochem.* 46, 1011–1018. doi: 10.1007/s10695-020-00766-z

Wen, H., Zhao, Z. Y., Jiang, M., Liu, A. L., Wu, F., and Liu, W. (2007). Dietary myo-inositol requirement for grass carp, *Ctenopharyngodon idella* fingerling. *J. Fish. Sci. China* 14, 794–800.

Zhang, M., Wang, C., and Xu, Q. (2014). Effects of myo-inositol on growth performance, body composition, and digestive enzyme activity in *Hucho taimen*. *J. Fish. Sci. China* 21, 560–566. doi: 10.3724/SP.J.1118.2014.00560

Zhang, C. N., Zhang, J. L., Ren, H. T., Zhou, B. H., Wu, Q. J., and Sun, P. (2017). Effect of tributyltin on antioxidant ability and immune responses of zebrafish (*Danio rerio*). *Ecotoxicol. Environ. Saf.* 138, 1–8. doi: 10.1016/j.ecoenv.2016.12.016

Zhao, W., Fang, H. H., Gao, B. Y., Dai, C. M., Liu, Z. Z., Zhang, C. W., et al. (2020). Dietary *Tribonema* sp. supplementation increased growth performance, antioxidant capacity, immunity and improved hepatic health in golden pompano (*Trachinotus ovatus*). *Aquaculture* 529:735667. doi: 10.1016/j.aquaculture.2020.735667

Zhao, W., Fang, H., Liu, Z., Chen, J., Zhang, C., Gao, B., et al. (2021). Responses in growth performance, enzymatic activity, immune function and liver health after dietary supplementation of *Porphyridium* sp. in juvenile golden pompano (*Trachinotus ovatus*). *Aquac. Nutr.* 27, 679–690. doi: 10.1111/anu.13214

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: 23 February 2022
doi: 10.3389/fmars.2021.815323

A Challenge to Conventional Fish Meal: Effects of Soy Protein Peptides on Growth, Histomorphology, Lipid Metabolism and Intestinal Health for Juvenile Pompano *Trachinotus ovatus*

Huaxing Lin[1,2,3†], Beiping Tan[1,2,3], Gyan Watson Ray[1,2], Min Zeng[4], Mei Li[4], Shuyan Chi[1,2] and Qihui Yang[1,2,3†]*

[1] College of Fisheries, Guangdong Ocean University, Zhanjiang, China, [2] Aquatic Animals Precision Nutrition and High Efficiency Feed Engineering Research Center of Guangdong Province, Zhanjiang, China, [3] Guangdong Provincial Key Laboratory of Pathogenic Biology and Epidemiology for Aquatic Economic Animals, Zhanjiang, China, [4] Yisheng (Yangjiang) Biotechnology Company Limited, Yangjiang, China

This experiment was conducted to assess the possibility of replacing fish meal (FM) with soy protein peptide (SPP) at different levels—0% (FM), 14.29% (S5), 28.57% (S10), 57.14% (S20), 71.42%(S25)—and its effects on growth, histology, gene expression related to liver lipid metabolism and intestinal immunity in juvenile pompano *Trachinotus ovatus* (initial mean weight = 39.88 ± 0.15 g). 600 healthy and uniformed-size fish were distributed to five groups of three replicates, each with 40 fish in each floating cage and fed twice daily for 8 weeks. Results showed that no significant difference in the growth was observed with SPP replacing FM ($P > 0.05$). Serum glutathione peroxidase activity in the S10 group was significantly higher than that in the FM group, and serum malondialdehyde content significantly decreased ($P < 0.05$). SPP significantly improved intestinal immunity by increasing alkaline phosphatase and lysozyme activities and up-regulating interleukin 10 and complement 4 mRNA levels while simultaneously decreasing triglyceride and total cholesterol content and down-regulating interleukin 1β mRNA expression. Villus length and muscle thickness in the S10 group were significantly higher than those in the FM group ($P < 0.05$). SPP significantly improved liver fat metabolism by increasing carnitine palmitoyl transferase I mRNA levels, and down-regulating fatty acid synthesis mRNA expression ($P < 0.05$). In summary, SPP substitution for FM promoted intestinal health, liver lipid metabolism and reduced liver fat accumulation for juvenile pompano *T. ovatus*, with no significant effect on growth performance. Based on the second-order polynomial analysis model of LYZ activity, the optimal replacement SPP level for juvenile pompano *T. ovatus* was 11.82%.

Keywords: *Trachinotus ovatus*, soy protein peptide, fish meal, histological morphology, lipid metabolism

OPEN ACCESS

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

Reviewed by:
Kang-le Lu, Jimei University, China
Chunxiao Zhang, Jimei University, China

****Correspondence:***
Qihui Yang
qihuiyang03@163.com

†ORCID:
Qihui Yang
orcid.org/0000-0001-9107-2541
Huaxing Lin
orcid.org/0000-0003-2333-6327

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

Received: *15 November 2021*
Accepted: *30 December 2021*
Published: *23 February 2022*

Citation:
Lin H, Tan B, Ray GW, Zeng M, Li M, Chi S and Yang Q (2022) A Challenge to Conventional Fish Meal: Effects of Soy Protein Peptides on Growth, Histomorphology, Lipid Metabolism and Intestinal Health for Juvenile Pompano Trachinotus ovatus. Front. Mar. Sci. 8:815323. doi: 10.3389/fmars.2021.815323

HIGHLIGHTS

- The replacement of FM with partial SPP can significantly improve disease resistance without affecting growth performance for juvenile pompano *Trachinotusovatus*under experimental conditions.
- By analyzing serum immune indexes, intestinal histology and intestinal immune-related genes, the replacement of 10%FM with SPP significantly promotes the intestinal healthforjuvenile pompano *Trachinotusovatus*.
- Based on the broken-line regression analysis model of LYZ activity in serum, the SPP optimal replacement level for juvenile pompano *Trachinotusovatus* is 12.80%.

INTRODUCTION

Fish meal (FM) has high amounts of proteins, essential amino acids, N-3 polyunsaturated fatty acids, and vitamins and has been the most used protein source in the compound feed of pompano *Trachinotus ovatus* (Zhang et al., 2018). However, the declining world fish meal production and the rising prices lead to an increasingly prominent conflict between supply and demand (Tacon and Metian, 2008). The increasing cost of feed has severely limited the sustainable development of the aquafeed industry. Therefore, nutrient-rich, efficient, and environmentally friendly plant protein sources as replacements for FM has become a major topic in the aquatic feed industry (Food and Agriculture Organization [FAO], 2014).

Alternative protein sources are essential to the growth of the aquaculture industry (Tacon, 2003) and can be used to reduce protein sources from FM (Naylor et al., 2000). The final products of protein digestion in the digestive tract mainly originate from small peptides rather than from free amino acids, and enter the blood circulation in the form of two or three peptides (Liu et al., 2007; Zhou et al., 2011). Peptides as feeding materials have been widely studied. Peptides can strengthen the immune system, reduce feed coefficient rate, and accelerate protein synthesis (Liang et al., 2020a; Wang J.X. et al., 2020). In *Gadus morhua* (Aksnes et al., 2006a), *Salmo salar* (BØGwald et al., 1996), *Dicentrarchus labrax* (Kotzamanis et al., 2007), and *Litopenaeus vannamei* (Gyan et al., 2020), similar improvements in weight gain rate, feed efficiency, and survival were observed (Savoie et al., 2006). In addition, small peptides preferentially act as energy substrates for the intestinal mucosa and can effectively promote the development of intestinal mucosal tissue (Zheng et al., 2006). Soybean protein peptide (SPP) is a mixed oligopeptide prepared through protease hydrolysis or microbial fermentation (Zhang J. et al., 2020). It has no anti-nutritional factors and is rich in amino acids, low-molecular-weight small peptide and special nutrients (Puchalska et al., 2014). In addition, it is easy to digest and absorb, and has physiologically active substances that promote fat metabolism (Huang, 2015). Previous studies showed that SPP had many advantages, including antioxidant (Ma et al., 2016), cholesterol-lowering and immune-enhancing activities (Zhao Z. X. et al., 2016; Cheng et al., 2017; Zhang Y. J. et al., 2020).

Pompano *T. ovatus*, a valuable food fish with tender flesh and delicious flavor, is mainly distributed in the warm tropical waters of the Pacific Ocean, Indian Ocean, and Atlantic Ocean (Tutman et al., 2004; Li M. M. et al., 2020). This fish thrives in tropical and subtropical climates due to its delicious flesh, rapid growth, simple feeding habits and high survival rate (Qu et al., 2014; Zhang et al., 2019). The annual production of *T. ovatus* in China has exceeded 100,000 tons in 2020, promising candidates for intensive aquaculture in Southern China with annual production of around 120,000 tons (Li M. et al., 2020). Plant protein sources in Pompano *T. ovatus* feed, including soybean meal (Wu et al., 2010; Niu et al., 2016), fermented soybean meal (Lin et al., 2012), rapeseed meal (Kou et al., 2015), and cottonseed protein concentrate (Shen et al., 2019), have attracted considerable interest. Meanwhile, SPP has not been reported. In this experiment, juvenile Pompano *T. ovatus* was used as a research target, and different proportions of FM were substituted with SPP to prepare iso-nitrogenous and iso-lipid test feeds. This study aimed to investigate the effects of the replacement of FM with SPP on growth, histomorphology, liver fat metabolism, and intestinal immune function for juvenile Pompano *T. ovatus*.

MATERIALS AND METHODS

Diet Formulation and Preparation

The experimental diet was formulated to provide 41.00% of crude protein (**Table 1**), and the dietary amino acid composition was shown (**Table 2**). The proximate compositions and amino acid composition of FM and SPP used in this study were presented in **Table 3**. The proportion of SPP with a relative molecular mass of less than 1,000 Da was 100%, provided by Yisheng Biotechnology Company (Yangjiang, Guangdong, China). Five iso-nitrogenous and iso-lipidic experiment diets were formulated to contain 0, 5.45, 10.90, 21.78, and 27.23% of SPP by replacing 0, 14.29, 28.57, 57.14, and 71.42% of FM, respectively. Reduction in feed by 0% (FM, control group), 5% (S5), 10% (S10), 20% (S20), and 25% (S25) FM, respectively. FM and soybean meal were the main sources of protein in the diet. Fish oil, soybean oil, and soybean lecithin oil were the lipid sources.

All raw material was crushed through a 60-mesh sieve, mixed thorough a V-type vertical mixer (JS-14S; Zhejiang Zhengtai Electric Co., Ltd.), followed by adding with oil and water, and then pelleted (2.5 mm diameter) making use of a double screw extruder (F-75; South China University of Technology). After the prepared experimental feed was naturally dried to about 10% moisture, it was sealed in a vacuum-packed bag and stored at −20°C until it was fed (He et al., 2021; Lin et al., 2021).

Experimental Animals and Breeding Management

The experiment was conducted at an experimental site in Zhanjiang, Guangdong, China. Juvenile *T. ovatus* was procured from a seedling farm in Hainan Province for this investigation. Live fish were transported from Hainan to Guangdong using live fish transport techniques and cultured in a floating cage

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

Ingredients	Diets				
	FM	S5	S10	S20	S25
Brown fish meal	35.00	30.00	25.00	15.00	10.00
Soybean protein peptide	0.00	5.45	10.90	21.78	27.23
Soybean meal	20.00	20.00	20.00	20.00	20.00
Peanut meal	4.00	4.00	4.00	4.00	4.00
Corn gluten meal	4.00	4.00	4.00	4.00	4.00
Wheat flour	20.00	20.00	20.00	20.00	20.00
Calcium dihydrogen phosphate	1.50	1.50	1.50	1.50	1.50
Vitamin C	0.05	0.05	0.05	0.05	0.05
Choline chloride	0.30	0.30	0.30	0.30	0.30
Soybean lecithin oil	1.50	1.50	1.50	1.50	1.50
Soybean oil + fish oil (1:1)	4.18	4.35	4.53	4.89	5.07
Vitamin premix[a]	0.50	0.50	0.50	0.50	0.50
Mineral premix[b]	0.50	0.50	0.50	0.50	0.50
Methionine	0.41	0.47	0.53	0.65	0.71
Lysine	0.24	0.34	0.44	0.64	0.74
Threonine	0.00	0.03	0.06	0.13	0.16
Arginine	0.13	0.12	0.12	0.11	0.10
Microcrystalline cellulose	7.69	6.89	6.07	4.46	3.64
Total	100.00	100.00	100.00	100.00	100.00
Nutrient levels					
Crude protein[c]	40.48	40.72	40.43	40.71	40.21
Crude lipid[c]	7.97	8.27	8.26	8.16	8.02
Crude ash[c]	8.76	8.37	7.84	6.97	6.41

[a]*The vitamin premix: vitamin A 500,000 IU/kg; vitamin D_3 100,000 IU/kg; vitamin E 4,000 mg/kg; vitamin K_3 1,000 mg/kg; vitamin B1 500 mg/kg; vitamin B_2 1,000 mg/kg; vitamin B_6 1,000 mg/kg; vitamin B_{12} 2.0 mg/kg; nicotinic acid 4,000 mg/kg; D-calcium pantothenate 2,000 mg/kg; folic acid 100 mg/kg; biotin 10.0 mg/kg; vitamin C 15,000 mg/kg.*

[b]*Mineral mixture: Fe 10,000 mg/kg; Cu 300 mg/kg; Zn 5,000 mg/kg; Mn 1,200 mg/kg; I 80 mg/kg; Se 30 mg/kg; Co 20 mg/kg.*

[c]*Crude protein, crude lipid and ash contents were measured values.*

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

	Experimental diets				
	FM	S5	S10	S20	S25
EAA[a]					
Threonine	1.48	1.51	1.50	1.56	1.52
Valine	1.82	1.80	1.79	1.72	1.74
Methionine	1.05	1.03	0.98	0.81	0.82
Leucine	3.04	3.07	3.09	3.06	3.08
Phenylalanine	1.76	1.78	1.79	1.87	1.84
Lysine	2.59	2.62	2.65	2.64	2.63
Histidine	1.11	1.10	1.07	1.08	1.02
NEEA[b]					
Aspartic acid	3.49	3.57	3.61	3.75	3.74
Serine	1.59	1.66	1.64	1.78	1.68
Glutamic acid	6.68	6.85	7.01	7.37	7.39
Glycine	1.98	1.93	1.86	1.75	1.70
Alanine	2.19	2.14	2.03	1.96	1.84
Isoleucine	1.59	1.59	1.62	1.58	1.64
Tyrosine	1.18	1.20	1.10	1.18	1.16
Proline	2.41	2.44	2.44	2.51	2.52
Arginine	2.03	2.08	2.07	2.14	2.07
Cystine	0.41	0.45	0.46	0.45	0.45

[a]*EAA, essential amino acids;* [b]*NEAA, non-essential amino acids.*

(length: 5 m; width: 5 m; and height: 5 m) (Harmon, 2009; Zhang et al., 2021). Before the experiment, all fish were fed commercial diets (Zhanjiang Yuehai Feed Co. Ltd., Guangdong, China; crude protein 42%, crude lipid 8%) for a week to acclimatize to the conditions. At this experiment, the total number of the experimental fish was 600, and there were 40 fish (initial body weight: 39.88 ± 0.15 g) in each floating cage (length: 1 m; width: 1 m; and height: 2 m), respectively. The 15 groups were randomly assigned to the five test diets, with three replicates each. All fishes were fed twice daily (7:00 and 17:00) to visual satiety for 8 weeks. During the experiment, water temperature ranged from 29.0 to 31.0°C, the salinity was 24–26‰ and the dissolved oxygen was not less than 6 mg/L.

All procedures involving live animals were approved by the Guangdong Ocean University Institutional Animal Care and Use Committee.

Sample Collection

Fish samples were kept fast for 24 h before collection at the end of trial and anesthetized with MS-222 (1:10,000; Zhou et al., 2019). The fish samples of each experimental group were calculated and weighed, and it was determined that weight gain ratio, the survival rate, specific growth rate and feed conversion ratio. Afterward, three fish samples of each floating cage were randomly used to test the body length and body weight to make a calculation of the hepatosomatic index, condition factor and viscerosomatic index, and then stored at −20°C to detect the whole-body composition (Wang et al., 2018; Wu et al., 2021). Blood samples were gotten from the tail veins of seven randomly selected fish samples from each floating cage, and then stored at 4°C for 12 h (Chen et al., 2016; Cai et al., 2020). After centrifuged (4,000 × *g*, 4°C, 10 min), the serum of fish samples was obtained and immediately preserved at −80°C for analyzing serum biochemical (Wang J. et al., 2020). Furthermore, the fish liver and intestine samples were quickly removed, one part was stored in 4% paraformaldehyde solution for histological analysis (Cai et al., 2021), whereas the others were stored in RNA-later at −80°C before RNA isolation.

Formula for Calculations

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

$$\text{Feed coefficient rate (FCR)} = \textit{feed intake}\ (g)/[\text{final body weight (g)} - \text{initial body weight (g)}];$$

TABLE 3 | Proximate composition of the fishmeal and soy protein peptide (% dry matter).

Ingredient	FM	SPP
Nutrients		
Moisture	8.15	8.00
Crude protein	70.03	60.87
Crude lipid	7.57	3.68
Amino acids		
Aspartate	5.80	4.80
Threonine	2.76	1.54
Serine	2.51	2.01
Glutamic	8.73	7.75
Glycine	3.99	1.68
Alanine	4.22	1.82
Cystine	0.57	0.00
Valine	3.04	1.74
Methionine	1.94	0.15
Isoleucine	2.73	1.67
Leucine	4.82	2.99
Tyrosine	2.24	1.30
Phenylalanine	2.66	1.97
Lysine	5.18	2.43
Histidine	2.28	1.21
Arginine	3.75	2.54
Proline	2.51	1.96

$$\text{Specific growth rate (SGR, \%)} = 100 \times [\ln(\text{final body weight(g)}) - \ln(\text{initial body weight (g)})] / \text{days};$$

$$\text{Survival rate (SR, \%)} = 100 \times (\text{final fish number} / \text{initial fish number});$$

$$\text{Hepatic somatic indices (HSI, \%)} = 100 \times \text{hepatic weight (g)} / \text{body weight (g)};$$

$$\text{Condition factor (CF, g/cm}^3\text{)} = 100 \times [\text{body weight (g)}] / [\text{body length (cm)}]^3;$$

$$\text{Daily feed intake (DFI, \%/days)} = 100 \times \text{feed intake} / [(\text{initial body weight} + \text{final body weight})/2 \times \text{experimental period}].$$

Chemical Analyses

The ingredients of the experimental diets and fish samples (crude protein, crude lipid, moisture, and ash) were measured by using standard methods AOAC (Association of Official Analytical Chemists [AOAC], 2005).

The total protein (TP), triglyceride (TG), total cholesterol (T-CHO), and glucose (GLU) in serum were assayed using an automatic blood analyzer (Hitachi 7020, Hitachi Science Systems, Japan), following a previously described method of Gyan et al. (2020).

The immune enzyme activities were determined by using a detection kit (Nanjing Jian Cheng Bioengineering Institute, China). The total superoxide dismutase (T-SOD) was measured by the xanthine oxidase method according to Wang et al. (2011). The activity of glutathione peroxidase (GSH-Px) was determined using the xanthine oxidase method (Ma et al., 2014). The total antioxidant capacity (T-AOC) was measured by Yang et al. (2017). The malondialdehyde (MDA) content was determined as described by Liang et al. (2020a) and Lin et al. (2020). The acid phosphatase (ACP) and alkaline phosphatase (AKP) were determined following Zhu et al. (2012) and Zhu et al. (2021). The lysozyme (LYZ) activity was assayed according to Liu et al. (2021).

Real-Time PCR Analysis of the Organization

Total RNA from the liver and hind intestines was extracted with an RNA extraction kit (TransZol Up Plus RNA Kit, Beijing, China). PrimeScript^{TM} RT-PCR Kit (TaKaRa, Kusatsu, Japan) was used to synthesize complementary DNA (cDNA) according to the manufactures' instructions. The PCR primers were listed in **Table 4**. The PCR cycling protocol by Liang et al. (2020b) was used, and all the real-time PCR reactions were performed on a Roche LightCycler480II (Switzerland) using an SYBR @ Premix Ex Taq^{TM}Kit (Takara). The relative mRNA expressions were calculated using the $2^{-\Delta\Delta CT}$ method.

Histological Morphology

The intestinal tracts and livers of the fish were quickly removed, and one part was stored in 4% paraformaldehyde solution for histological analysis using hematoxylin-eosin (H&E) (Martínez-Llorens et al., 2012). The tissue sections were observed and photographed under an electron microscope scanner (VS 120-S6, Olympus, Norway) with villi length and muscle thickness measured according to the method of Wang J.X. et al. (2020). Sections from each floating cage were randomly measured for villi lengths and muscle layer thicknesses (Lin et al., 2019).

Statistical Analysis

All data were subjected to ANOVA using SPSS 21.0 (SPSS Inc., Chicago, IL, United States). Tukey's HSD multiple comparisons were performed in the case of a significant overall difference between the experimental group and the control ($P < 0.05$). The results were presented as the mean ± *SEM* (standard error of the mean).

TABLE 4 | Sequences of primers used for real-time quantitative PCR.

Gene name	Primer sequence (5′-3′)	References
c4	F-TGGAGAAAAAGTTAAAGGGGC R-CAGGAAGGAAGTATGAGCGAGT	Tan et al., 2018
nf-κb	F-TGCGACAAAGTCCAGAAAGAT R-CTGAGGGTGGTAGGTGAAGGG	Zhou et al., 2020
il-8	F-GAGAAGCCTGGGAATGGA R-GAGCCTCAGGGTCTAAGCA	Zhou et al., 2020
il-10	F-CTCCAGACAGAAGACTCCAGCA R-GGAATCCCTCCACAAAACGAC	Tan et al., 2017
il-1β	F-CGGACTCGAACGTGGTCACATTC R-AATATGGAAGGCAACCGTGCTCAG	Xie et al., 2020
*cpt*I	F-CTTTAGCCAAGCCCTTCATC R-CACGGTTACCTGTTCCCTCT	Liu et al., 2018
fsan	F-GAAGGAGAGGGGGTGGAGTC R-GTGTGAAGGTGGAGGGTGTG	Liu et al., 2018
apob100	F-AAAAGCCACAAGACGAAAGCA R-GAAGCAGCAAAAGGCAGAGC	Liu et al., 2018
srebp-I	F-GAGCCAAGACAGAGGAGTGT R-GTCCTCTTGTCTCCCAGCTT	Li et al., 2020
*fabp*I	F-AGTCATTGTCTGGGGAGGG R-GTCAAGGCGGTGGTTCA	Liu et al., 2018
β-actin	F-TACGAGCTGCCTGACGGACA R-GGCTGTGATCTCCTTCTGC	Xie et al., 2019

*c4, complement 4; nf-κb, nuclear factor kappa B; il-8, interleukin 8; il-10, interleukin 10; il-1β, interleukin 1β; cpt*I*, carnitine palmitoyl transferase* I*; fsan, fatty acid synthesis; apob100, apolipoprotein B-100; srebp-*I*, sterol-regulatory element binding protein-*I*; fabp*I*, fatty acid binding protein* I.

RESULTS

Growth Performance and Whole-Body Composition

No significant differences in IBW, WGR, SGR, SR, FCR, CF, and DFI were found among the groups ($P > 0.05$; **Table 5**). As SPP increased, VSI firstly decreased and then increased, and the S10 group was significantly lower than the FM group ($P < 0.05$). As SPP level increased, HSI gradually decreased and was significantly lower in the S10, S20, and S25 groups than in the FM group ($P < 0.05$).

The results of the whole-body composition for juvenile pompano *T. ovatus* are shown in **Table 6**. No significant differences were found in the whole-body composition among juvenile pompano *T. ovatus* fed with different amounts of dietary SPP ($P > 0.05$).

Serum Antioxidant and Immune Enzyme Activities

As SPP level increased, AKP activity showed an increasing trend followed by a decreasing trend (**Table 7**; $P < 0.05$). AKP activity in the S10 group was significantly higher than that in the control group ($P < 0.05$). ACP activity showed an increasing tendency followed by a decreasing trend, but the difference among the groups was not significant ($P > 0.05$). LYZ activity increased and then decreased, showing a trend change in line with AKP activity. LYZ activity in the S10 group was significantly higher than that in the control group ($P < 0.05$). According to the second-order polynomial analysis model of LYZ activity, the SPP replacement level of juvenile pompano *T. ovatus* was 11.82%, corresponding to SPP replacement level (**Figure 1**).

T-SOD activity in the S5, S10, and S20 groups was not significantly different from that of the control group, or the high substitution group (S25) had significantly lower T-SOD activity than that in the control group. The GSH-Px activity showed an increase followed by a decrease. GSH-Px activity in the S10 and S20 groups was significantly higher than in the FM group ($P < 0.05$). As SPP increased, MDA content showed a gradually decreasing trend, and the groups in S10, S20, and S25 were significantly lower than that in the FM group ($P < 0.05$). No significant difference in T-AOC activity was found among the groups ($P > 0.05$), but T-AOC activity was higher in the substitution groups than in the FM group.

Serum Biochemical Indices

TP content in the S5, S10, and S20 groups were significantly higher than that in the FM group (**Table 8**, $P < 0.05$), but difference in TP content between the S25 and FM groups was not significant ($P > 0.05$). As SPP increased, TG levels gradually decreased, and the FM groups had the highest TG levels ($P < 0.05$). T-CHO and TG levels in the experimental group gradually decreased and were significantly lower than those in the FM group ($P < 0.05$). No significant differences in glucose content were found among the groups ($P > 0.05$).

Liver and Intestinal Morphology

Observation of liver tissue (**Figure 2**) showed that serious vacuolation occurred in the FM group, but the cell boundaries of FM, S5, and S10 groups were obvious, and the rate of intracellular vacuolization decreased. The nuclei of hepatocytes in the S20 group began to gradually lyse or disappear, and the nuclei of hepatocytes increased in spacing and showed blurred outlines after cell disintegration.

We stained the liver with oil red to observe fat deposition in the liver (the fat was stained red, **Figure 3**). Fat deposition tended to decrease with increasing SPP level. The fat droplets in the FM group were large and dense, whereas the fat droplets were significantly smaller and the deposition sites were more dispersed in the experimental groups.

The hind-gut structure data of juvenile *T. ovatus* are provided (**Figures 4**, **5**). As SPP level increased, villus length (VL) increased first and then decreased and was significantly higher in the S10 group than in the FM group ($P < 0.05$). The trend of muscle thickness (MT) was in line with that of VL. All substitution groups were significantly higher than the FM group ($P < 0.05$), and the S10 group was higher than all other groups.

Expression of Lipid Metabolism-Related Genes in the Liver

In the present study, the expression of lipid metabolism-related genes in the liver were analyzed (**Figure 6**). The hepatic carnitine palmitoyl transferase I (*cpt*I) mRNA levels in the S5 and S10 groups were significantly higher than those in the other groups ($P < 0.05$). The fatty acid synthesis (*fasn*) mRNA

TABLE 5 | Growth performance and biometry for juvenile *Trachinotus ovatus* fed the experimental diet.

Parameters	Experimental diets				
	FM	**S5**	**S10**	**S20**	**S25**
IBW (g)	39.97 ± 0.21	39.94 ± 0.27	39.91 ± 0.08	39.75 ± 0.14	39.88 ± 0.18
WGR (%)	117.67 ± 3.45	118.10 ± 4.09	117.70 ± 3.24	111.82 ± 0.79	114.76 ± 1.04
SGR (%/d)	1.39 ± 0.03	1.39 ± 0.03	1.38 ± 0.04	1.34 ± 0.01	1.39 ± 0.04
FCR	2.43 ± 0.10	2.37 ± 0.10	2.39 ± 0.15	2.56 ± 0.05	2.53 ± 0.07
SR (%)	99.17 ± 1.44	100.00 ± 0.00	99.17 ± 1.44	98.33 ± 2.89	99.17 ± 1.44
VSI (%)	4.70 ± 0.29^{b}	4.21 ± 0.23^{ab}	4.06 ± 0.04^{a}	4.39 ± 0.12^{ab}	4.58 ± 0.18^{b}
HSI (%)	0.99 ± 0.12^{b}	0.98 ± 0.07^{b}	0.65 ± 0.03^{a}	0.54 ± 0.05^{a}	0.57 ± 0.03^{a}
CF (g/cm^3)	4.31 ± 0.04	4.35 ± 0.07	4.32 ± 0.14	4.55 ± 0.25	4.33 ± 0.06
DFI (%/days)	3.15 ± 0.07	3.14 ± 0.06	3.16 ± 0.10	3.23 ± 0.02	3.21 ± 0.04

Data are mean ± S.E.M. (n = 3). Values in the same row with different superscripts represent significant difference ($P < 0.05$).
IBW, initial mean body weight; WGR, weight gain rate; SGR, specific growth rate; FCR, feed coefficient rate; SR, survival rate; VSI, viscerosomatic index; HSI, hepatic somatic indices; CF, condition factor; DFI, daily feed intake.

TABLE 6 | The composition of whole body for juvenile *Trachinotus ovatus* fed the experimental diet (% dry matter).

Parameters	Experimental diets				
	FM	**S5**	**S10**	**S20**	**S25**
Moisture	68.91 ± 0.94	69.53 ± 0.56	69.28 ± 0.85	69.75 ± 0.34	67.37 ± 1.44
Crude protein	59.04 ± 0.64	60.16 ± 0.50	61.31 ± 0.39	61.06 ± 0.79	59.05 ± 1.77
Crude lipid	27.56 ± 0.47	27.15 ± 1.38	26.96 ± 2.92	25.79 ± 1.32	27.53 ± 2.98
Crude ash	13.32 ± 0.21	13.30 ± 0.44	12.69 ± 0.10	14.16 ± 0.34	12.85 ± 1.52

Data are mean ± S.E.M. (n = 3). Values in the same row with different superscripts represent significant difference ($P < 0.05$).

level was significantly down-regulated after the replacement of FM with SPP in compound feed ($P < 0.05$). The S25 group had significantly lower fatty acid-binding protein I (*fabp*I) and apolipoprotein B-100 (*apob100*) mRNA levels than the FM group ($P < 0.05$). No significant difference in sterol-regulatory element-binding protein-I (*srebp*-I) mRNA level was found between the FM and replacement groups ($P > 0.05$).

Expression of Intestinal Immunity-Related Genes

The expression of intestinal immunity-related genes of juvenile *T. ovatus* is shown (**Figure 7**). The expression of genes involved in intestinal immunity-related genes, including interleukin 10 (*il-10*) and complement 4 (*c4*), was significantly up-regulated after FM was replaced with SPP ($P < 0.05$). As SPP level increased, interleukin 1β (*il-1*β) mRNA level was significantly down-regulated ($P < 0.05$). No significant difference in nuclear factor kappa B (*nf-*κ*b*) mRNA level was observed between the FM group and the replacement groups ($P > 0.05$).

DISCUSSION

Soya bean meal has a high crude protein content and is rich in essential amino acids and widely available. The presence of anti-nutritional factors limits the use of soybean meal compared with FM (Yildirim et al., 2009). Indeed, excessive levels of plant proteins in feed can inhibit animal growth (Floreto et al., 2000). However, fermented soy peptides are rich in organic acids, bacterial active proteins, folic acid, and B vitamins and have increased palatability (Cao et al., 2007). In the present study, the replacement of FM with SPP resulted in no significant differences in the WGR and SGR of juvenile *T. ovatus* cultured in floating cages under experimental conditions. We recommend that it is feasible to replace FM with SPP in compound feeds, and the studies on which is consistent with *Epinephelus akaara* (Zhao S. Y. et al., 2016) and *Acipenser baerii* (Wang et al., 2010). This may be related to the nutritional profile of SPP, which contains nutrients, such as low-molecular-weight peptides, vitamins, and other special nutrients, making up for the shortcomings of many plant proteins (Puchalska et al., 2014). Moreover, small peptides are preferentially used as energy substrates for the structural and functional development of intestinal mucosal epithelial cells, effectively promoting the development of intestinal mucosal tissues (Wang et al., 2003). In the present study, the replacement of FM with SPP significantly promoted intestinal growth. Therefore, the high content of low molecular weight peptides (Puchalska et al., 2014) and intestinal growth are responsible for the unaffected growth rate for juvenile *T. ovatus*.

Body composition can directly reflect animal growth and indirectly reflect feed quality (Li et al., 2009). In the present study, no significant differences in moisture content and crude protein content were found among the groups. This study is in line with the studies on *Pelteobagrus fulvidraco* (Jing et al., 2021) and *Heterotis niloticus* (Monentcham et al., 2010). As SPP increased, the body fat content of fish decreased gradually, and all groups

TABLE 7 | Serum enzyme activity indices for juvenile *Trachinotus ovatus* fed the experimental diet.

Parameters	Experimental diets				
	FM	S5	S10	S20	S25
Serum immune ability					
AKP (U/L)	34.01 ± 9.19^{a}	41.40 ± 6.37^{a}	51.61 ± 6.54^{b}	41.08 ± 3.31^{a}	30.24 ± 1.96^{a}
ACP (U/L)	41.37 ± 0.71	41.83 ± 1.47	41.59 ± 3.19	40.41 ± 2.48	39.94 ± 0.71
LYZ (U/mL)	54.17 ± 11.53^{ab}	64.71 ± 15.55^{ab}	117.01 ± 4.97^{c}	68.92 ± 5.29^{b}	37.30 ± 11.47^{a}
Serum oxidation resistance					
T-SOD (U/ml prot)	35.41 ± 4.16^{bc}	42.81 ± 2.01^{c}	30.95 ± 6.85^{abc}	25.37 ± 3.62^{ab}	22.31 ± 5.57^{a}
GSH-Px (U/g prot)	25.26 ± 6.32^{a}	48.42 ± 3.65^{ab}	56.84 ± 10.94^{b}	56.84 ± 10.94^{b}	44.21 ± 12.63^{ab}
T-AOC (mM)	0.80 ± 0.01	0.82 ± 0.04	0.85 ± 0.02	0.81 ± 0.01	0.81 ± 0.01
MDA (nmol/mL)	6.36 ± 0.61^{b}	6.06 ± 0.30^{ab}	4.85 ± 0.30^{a}	4.85 ± 0.80^{a}	4.75 ± 0.35^{a}

Data are mean ± S.E.M. (n = 3). Values in the same row with different superscripts represent significant difference ($P < 0.05$).
AKP, alkaline phosphatase; ACP, acid phosphatase; LYZ, lysozyme; T-SOD, total superoxide dismutase; GSH-Px, glutathione peroxidase; T-AOC, total antioxidant capacity; MDA, malondialdehyde.

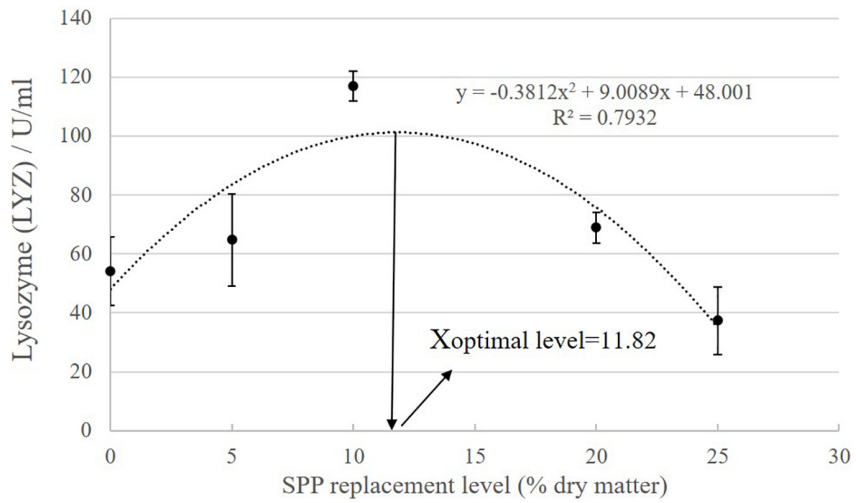


FIGURE 1 | The second-order polynomial analysis between LYZ activity and SPP replacement level, where X represents SPP replacement level for the maximum LYZ activity of juvenile pompano *Trachinotus ovatus*.

had lower fat content than the control group. In *Platichthys stellatus* (Jiang, 2013), small peptides could significantly reduce the amount of crude fat in the body. Moreover, Hou (2012) showed that SPP promoted fat and energy metabolism and inhibited fat deposition in the body. These effects are due to small peptides, which improve protein synthesis, reduce the deposition of free amino acids into fat (Boza et al., 2000), impede the absorption of fat and promote lipid metabolism (Christian, 2005). These results indicated that the replacement of FM with SPP can increase protein synthesis, promote the consumption of excess fat, and reduce fat deposition.

Condition factor, HSI, and VSI are important indices that reflect the body fat and lean status and growth status of fish (Aksnes et al., 2006a,b). In the present study, as SPP levels increased, the HSI and VSI significantly decreased, consistent with those of a hybrid grouper (*Epinephelus lanceolatus* ♂ × *Epinephelus fuscoguttatus* ♀; Jiang et al., 2015). This result may be related to the absorption mechanism of small peptides that enter the bloodstream quickly and directly without degradation. Moreover, in *E. akaara*, reduction in HSI was associated with increased rates of small peptide and free amino acid transport and absorption and enhanced lipid metabolism (Zhao S. Y. et al., 2016). In the present study, a significant reduction in liver fat content was observed with oil red staining. Therefore, SPP may reduce liver fat accumulation and improve liver condition.

Animal blood is an important carrier of nutrients and metabolites, and elevated levels of TG and T-CHO in the blood can be detrimental to animal health (Kim et al., 2005). Increase or decrease in TG levels can be used as an indicator of fat metabolism and liver function (He et al., 2021). Nagasawa et al. (2003) reported that SPP significantly reduces triglyceride content and fatty acid synthase mRNA levels in adipose tissues, suggesting that soy isolate controlled gene expression in adipose tissues and effectively regulated adipocyte differentiation. In the present study, T-CHO and TG levels were significantly lower

TABLE 8 | Serum biochemical indices for juvenile *Trachinotus ovatus* fed the experimental diet.

Parameters	Experimental diets				
	FM	**S5**	**S10**	**S20**	**S25**
TP (g/L)	6.08 ± 1.80^{a}	28.64 ± 2.01^{c}	16.27 ± 1.74^{b}	12.37 ± 3.00^{b}	5.05 ± 1.60^{a}
TG (mmol/L)	2.02 ± 0.11^{c}	1.38 ± 0.14^{b}	1.24 ± 0.06^{ab}	1.09 ± 0.12^{a}	1.10 ± 0.10^{ab}
T-CHO (mmol/L)	23.78 ± 2.99^{b}	16.50 ± 0.31^{a}	16.35 ± 2.00^{a}	15.02 ± 1.78^{a}	12.65 ± 1.49^{a}
GLU (mmol/L)	4.36 ± 0.24	4.32 ± 0.31	3.95 ± 0.15	4.11 ± 0.34	3.78 ± 0.17

Data are mean ± S.E.M. (n = 3). Values in the same row with different superscripts represent significant difference ($P < 0.05$).
TP, total protein; TG, triglyceride; T-CHO, total cholesterol; GLU, glucose.

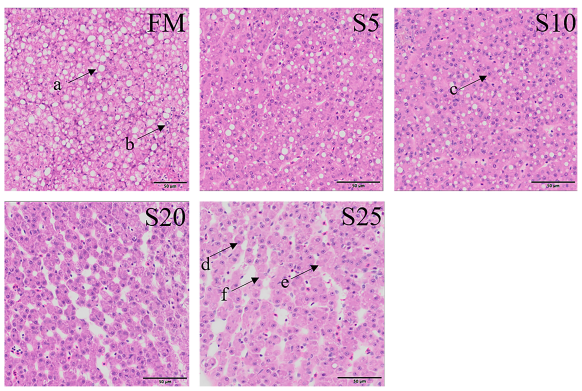


FIGURE 2 | Electron microscope scanner (VS 120-S6, Olympus, Norway) of the liver tissue morphology for juvenile Pompano *T. ovatus* fed diets with FM replacement by SPP from 0 to 25% (H&E staining). a, fat cavitation; b, hepatic sinusoid; c, nucleus; d, nucleus excursion; e, cytoplasmic loss; f, cell breakdown.

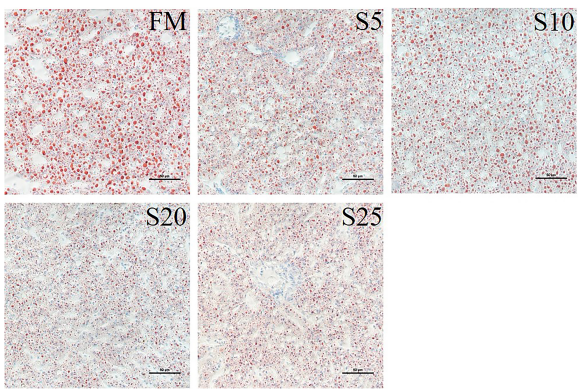


FIGURE 3 | Light microscopy of the liver tissue morphology for juvenile Pompano *T. ovatus* fed diets with FM replacement by SPP from 0 to 25% (oil red staining).

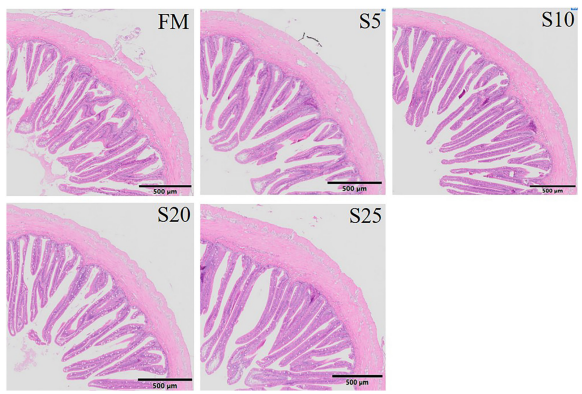


FIGURE 4 | Electron microscope scanner (VS 120-S6, Olympus, Norway) of the proximal intestine morphology for juvenile Pompano *T. ovatus* fed diets with FM replacement by SPP from 0 to 25% (H&E staining).

than those in the control group (FM), indicating that SPP can effectively regulate TG and T-CHO metabolism. LPYPR (Leu-Pro-Tyr-Pro-Arg) and VK (Val-Lys) derived from glycine in soy are important components that lower cholesterol peptides and triglycerides, respectively (Inoue et al., 2011, 2015). SPP are more beneficial to the balanced absorption of amino acids in fish than FM.

Fish fat metabolism mainly occurs in the liver, including synthesis, catabolism, and transport, and is regulated by a multitude of factors with synergistic activities (Hu, 2004; Weng et al., 2012). The *fasn* is the key enzyme for the *de novo* fatty acid biosynthesis, catalyzing the synthesis of malonyl-CoA and acetyl coenzyme A into long-chain saturated fatty acids (He et al., 2021). The *cpt*I is a key and rate-limiting enzyme that regulates the beta-oxidation of fatty acids, the main catabolic process in the body (Nilsson-Ehle et al., 1980). In the present study, the FM group had the highest liver *fasn* mRNA level, and *cpt*I expression in the S5 and S10 groups was significantly up-regulated. These findings were consistent with those of Nagasawa et al. (2003). In addition, fat deposition is related to fat transport rate. Increase in very-low-density lipoprotein (VLDL) secretion rate can reduce hepatic lipid deposition (Nagayoshi et al., 1995). Hussain et al. (2008) reported that *apob100* is an essential component in the assembly of VLDL particles. The *fabp*I can bind long-chain fatty acids for oxidation and storage through the cell membrane (Yan et al., 2015). In the present study, the S25 group had significantly lower in *apob100* and *fabp*I expression levels than that the other groups. The morphology of the liver cells in the FM, S5, and S10 groups was normal, and fat accumulation in the liver gradually decreased. These results were in line with those of the HSI and VSI analyses. With FM decreased, liver

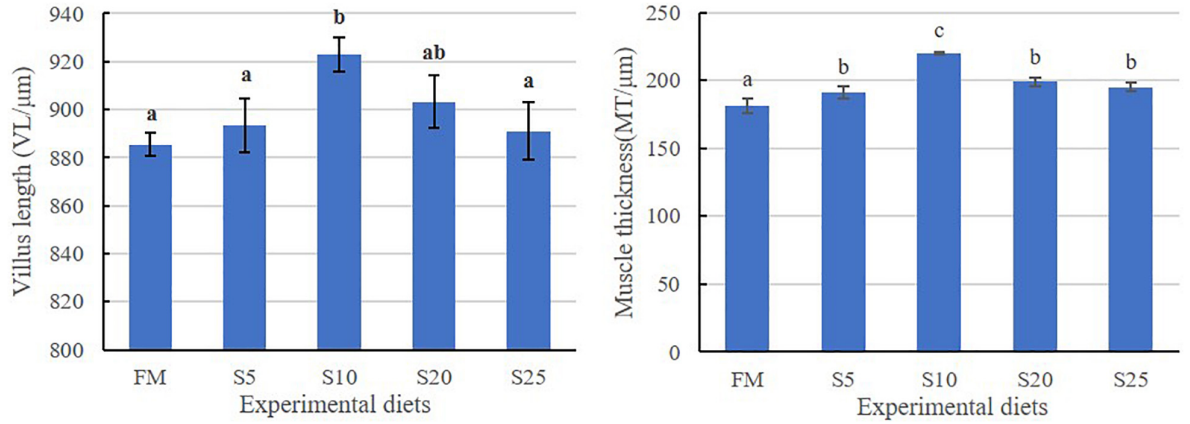


FIGURE 5 | Hindgut intestine for juvenile *T. ovatus* fed the experimental diet. Data are mean ± S.E.M. ($n = 3$). Values in the same row with different superscripts represent a significant difference ($P < 0.05$).

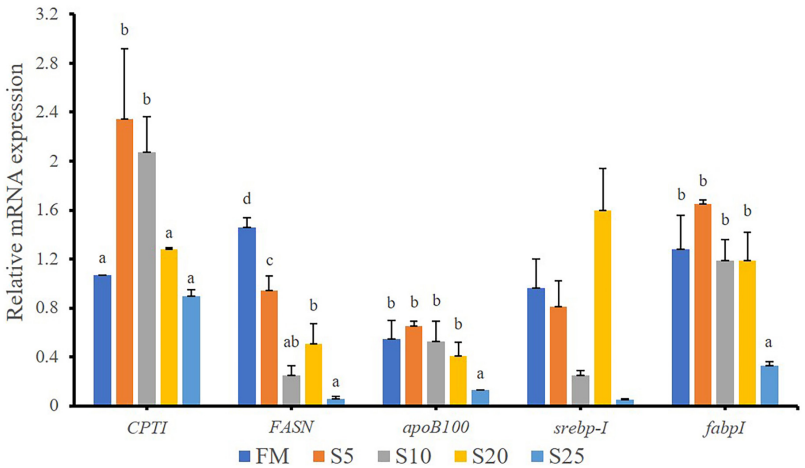


FIGURE 6 | Relative expression levels of lipid metabolism-related genes in liver for juvenile *T. ovatus*. Data are expressed as means ± *SEM.* ($n = 3$). Different letters above a bar are statistically significant different among treatments ($P < 0.05$).

cells were seriously damaged, the cells collapsed, and the nuclei disappeared. These effects were in line with the observation on *Oncorhynchus mykiss* (Feng et al., 2016), *Carassius auratus* (Shi et al., 2015), and *Lateolabrax japonicus* (Hu et al., 2013). We considered that excessive soy protein peptides can damage the liver, leading to dysfunction (Zhang et al., 2002). Therefore, our observations indicated that the replacement of 10% FM with SPP significantly promotes liver fat metabolism in grouper, reduces liver fat deposition, and improves liver condition.

The defense system can protect cells and cell membranes from oxidative damage and maintain normal physiological function (Bu et al., 2017). MDA is the oxidative end-product of free radicals acting on the peroxidation reaction of fat, and its content indirectly reflects the content of oxygen free radicals in the cells and the severity of free radical attacks (Cheng et al., 2017). In the present study, MDA content significantly decreased with increasing SPP supplementation. This result was in line with the results of Gyan et al. (2020). Deng et al. (2006) reported that soybean peptides had strong DPPH (1,1-Diphenyl-2-picrylhydrazyl) radical-scavenging ability and most functional peptides with antioxidant properties were mainly concentrated in small peptides with molecular weights of lower than 5,000 Da. The molecular weights of SPP used in this experiment were mainly lower than 1,000 Da, providing a good molecular basis for antioxidant function. We suggest that SPP can inhibit free radicals and reduce the content of lipid peroxides during stress. GSH-Px is important antioxidant enzymes in fish (Cheng et al., 2017). GSH-Px catalyzes the reduction of glutathione and removes hydrogen peroxide and lipid peroxides produced during metabolism. In the present study, GSH-Px activity reached a maximum in the S10 and S20 groups significantly higher than that in the other groups. Similar results were *Cyprinus carpio* (Wang et al., 2014), *Ctenopharyngodon idella* (Zheng et al., 2012), and *P. fulvidraco* (Jing et al., 2021), indicating that SPP could increase antioxidant activity, and it is related to functional peptides with antioxidant properties in soy peptides

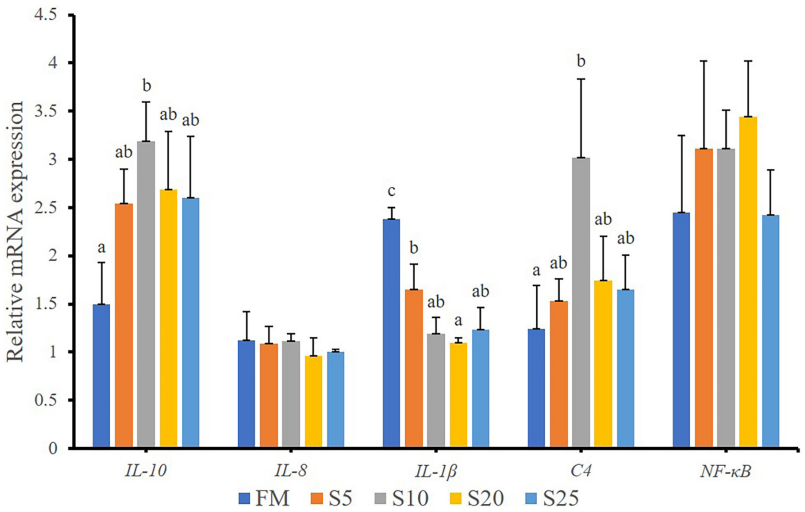


FIGURE 7 | Relative expression levels of immunity-related gene in intestine for juvenile *T. ovatus*. Data are expressed as means ± *SEM*. Different letters above a bar are statistically significant different among treatments ($P < 0.05$).

(Deng et al., 2006). The above antioxidant indices indicate that soy protein peptides can increase the antioxidant activity for juvenile *T. ovatus*.

Active small peptides can participate in immune system regulation, can improve immunity, and exerts immunomodulatory effects, specifically stimulating macrophage phagocytosis and inhibiting lymphocyte proliferation (Kong et al., 2008). In serum, increases in LYZ, ACP, and AKP contribute to the immune system of aquatic organisms (Tseng et al., 2009). LYZ can hydrolyze mucopolysaccharides and catalyze the hydrolysis of glycosidic bonds in bacterial cell walls, thus causing bacterial cell walls to rupture (Xie et al., 2013). In the present study, serum LYZ activity significantly increased. The same effect was observed in *L. Vannamei*, indicating that the addition of small peptides significantly increases antimicrobial activity and lysozyme specific activity in sera (Lin et al., 2010). Based on the present results, the optimal SPP level that supported the most powerful immune function of juvenile *T. ovatus* was 11.82%, as predicted by the LYZ activity models. ACP and AKP catalyze phosphate monostearate hydrolysis and play important roles in the immune response of the body against pathogens (Liu et al., 2004). In the present study, serum AKP activity increased first and then decreased with as the amount of replacement SPP increased in *T. ovatus* diet. This result was in line with the results obtained in *Sardine* (Ben Khaled et al., 2012) and *P. fulvidraco* (Zhao Z. X. et al., 2016). In addition, soy peptides help eliminate anti-nutritional factors and act as immunomodulators, inducing defense genes involved in pathogenic attacks (Pearce et al., 2010).

As an important immune organ in the animal body, the intestine provides protection against external pathogens. As reported by Zhang et al. (2020), SPP can reduce inflammation and enhance immune function by regulating the expression of pro-inflammatory cytokines, such as *il-1*β, which in turn affects the expression and secretion levels of T cells. The *il-1*β plays an important role in inflammatory responses (Dinarello, 2000). In the present study, the mRNA expression level of *il-1*β in the intestine was significantly down-regulated as SPP replacement level increased. Moreover, the anti-inflammatory factor *il-10* was significantly up-regulated in the S10 group. Complement is the main humoral component of the innate immune response and plays an essential role in the killing of microorganisms, phagocytosis, inflammatory response, immune complexes, and antibody production (Holland and Lambris, 2002). Fish can recognize and neutralize a variety of harmful microorganisms by specifically binding different forms of complement (Zarkadis et al., 2001). In the present study, the replacement of 10% FM with SPP significantly up-regulated gene expression of *c4*. The possible reasons are that small peptides are efficiently absorbed, and the amount of undigested protein in the intestine are reduced (Zheng et al., 2006). The exact mechanisms of the effects have not been explained and further studies are needed.

Among the internal organs of fish, the gut has the largest area of contact with the internal environment. The intestine is the main part of the fish that digests and absorbs nutrients (Zhou, 2012). The shape of the fold is positively correlated with nutrient absorption area (Wu et al., 2010). A decrease in the height of the intestinal fold means that the intestinal tract has a smaller absorptive area and absorb lower amounts of nutrients (Wu et al., 2014). The results of the present study showed that the replacement of FM with SPP can promote the growth of intestinal villi and muscle layer. Thus, the area of intestine absorption was enlarged. The present study observed a trend in line with the study of Jiang et al. (2010), indicating that increase in intestinal absorption area and muscle layer thickness can facilitate the absorption of SPP and improve feed utilization. In addition, the replacement of FM with a high proportion of SPP can inhibit villi growth. A high proportion of FM replacement reduces the protein metabolism of fish and damages the intestinal tissue structure (Wang et al., 2017). If the proportion of a plant-based protein used to replace FM is extremely high, the

intestinal tract of aquatic animals will be affected to some extent. This observation showed that under the present experimental conditions, the replacement of 10% FM with SPP could promote intestinal muscle thickening and wrinkled wall growth, thus improving intestinal absorption.

CONCLUSION

The replacement of FM with SPP can promote intestinal growth for nutrient absorption without affecting the growth performance of juvenile pompano *T. ovatus* under experimental conditions. In addition, SPP can reduce liver fat accumulation, improve liver condition, and enhance antioxidant capacity and immunity. Based on the second-order polynomial analysis model of LYZ activity, the optimal replacement SPP level for juvenile pompano *T. ovatus* is 11.82%.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Guangdong Ocean University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

HL and QY designed the experiments. HL carried out the experiments and drafted the manuscript. BT, MZ, ML, and SC were accountable for some aspects (such as ingredients and sites) of the work in ensuring that experiments can be carried out properly. QY and GR reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the National Natural Science Foundation (31802316). The experimental funding was offered with the National Key Research and Development Program (2019YFD0900200) and Postgraduate Education Innovation Project of Guangdong Ocean University (202155).

REFERENCES

Aksnes, A., Hope, B., Høstmark, Ø, and Albrektsen, S. (2006a). Inclusion of Size Fractionated Fish Hydrolysate in High Plant Protein Diets for Atlantic Cod, *Gadus Morhua*. *Aquaculture* 261, 1102–1110. doi: 10.1016/j.aquaculture.2006.07.038

Aksnes, A., Hope, B., Jönsson, E., Björnsson, B. T., and Albrektsen, S. (2006b). Size-fractionated fish hydrolysate as feed ingredient for rainbow trout (*Oncorhynchus Mykiss*) fed high plant protein diets. I: growth, growth regulation and feed utilization. *Aquaculture* 261, 305–317. doi: 10.1016/j.aquaculture.2006.07.025

Association of Official Analytical Chemists [AOAC] (2005). *Official methods of analysis of the association of official analytical chemists international*, 16th Edn. Washington: Association of Official Analytical Chemists.

Ben Khaled, H., Ghlissi, Z., Chtourou, Y., Hakim, A., Ktari, N., Fatma, M. A., et al. (2012). Effect of protein hydrolysates from sardinelle (*Sardinella aurita*) on the oxidative status and blood lipid profile of cholesterol-fed rats. *Food Res. Int.* 45, 60–68. doi: 10.1016/j.foodres.2011.10.003

BØGwald, J., Dalmo, R. O. Y., McQueen Leifson, R., Stenberg, E., and Gildberg, A. (1996). The Stimulatory Effect of a Muscle Protein Hydrolysate from Atlantic Cod, Gadus Morhual., on Atlantic Salmon, *Salmo Salarl.*, Head Kidney Leucocytes. *Fish Shellfish Immunol.* 6, 3–16. doi: 10.1006/fsim.1996.0002

Boza, J. J., Moënnoz, D., Vuichoud, J., Jarret, A. R., Gaudard-de-Weck, D., and Ballèvre, O. (2000). Protein hydrolysate vs free amino acid-based diets on the nutritional recovery of the starved rat. *Eur. J. Nutr.* 39, 237–243. doi: 10.1007/s003940070001

Bu, X., Chen, A., Lian, X., Chen, F., 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

Cai, R. J., Zhang, J., Huang, J. S., Chen, G., Zhang, J. D., Pan, C. H., et al. (2021). Effects of Low Temperature Stress on Physiology and Biochemistry of Lipid Metabolism of Juvenile Cobia, *Rachycentron canadum*. *J. Guangdong Ocean Univ.* 41, 123–130.

Cao, L., Wang, W. M., and Yang, C. T. (2007). Advances in replacement of fish meal by soy protein in aquiculture feed. *Feed Industry* 4, 57–61.

Chen, Q., Liu, H. Y., Tan, B. P., Dong, X. H., Chi, S. Y., Yang, Q. H., et al. (2016). Effects of Dietary Cholesterol Level on Growth Performance, Blood Biochemical Parameters and Lipid Metabolism of Juvenile Cobia (*Rachycentron canadum*). *J. Guangdong Ocean Univ.* 36, 35–43.

Cheng, Y. B., Zhang, Y. X., Dong, Z. Y., Lu, B. Y., and Wang, Y. C. (2017). Effects of Dietary Replacement of Fish Meal and Soy Protein by Wheat Gluten on Plasma Biochemical Indices and Liver Anti-Oxidative Indices of Nibea japonica and Sparus macrocephalus. *Prog. Fish. Sci.* 38, 106–114. doi: 10.11758/yykxjz.20160426001

Christian, A. D. (2005). Fatty acids and expression of adipokines. *Biochim. Biophy. Acta Mol. Dis.* 1740, 287–292. doi: 10.1016/j.bbadis.2004.11.019

Deng, C. P., Xue, W. T., Sun, X. L., and Quan, M. H. (2006). Functional Properties of Different Molecular Weight Segments of Soybean Peptides. *Food Sci.* 27, 109–112.

Dinarello, C. A. (2000). Proinflammatory cytokines. *Chest* 118, 503–508.

Food and Agriculture Organization [FAO] (2014). *The State of World Fisheries and Aquaculture 2014*. Rome: FAO, 40–41.

Feng, J., Wang, P., He, J. J., Lou, Y., Dang, H., and Deng, R. (2016). Effects of Replacement of Fish Meal by Fermented Soybean Meal on Growth Performance, Body Composition, Serum Biochemical Indices and Liver Tissue Morphology of Juvenile Large Yellow Croaker (*Larimichthys crocea*). *J. Anim. Nutr.* 28, 3493–3502. doi: 10.3969/j.issn.1006-267x.2016.11.016

Floreto, E. A. T., Bayer, R. C., and Brown, P. B. (2000). The effects of soybean-based diets, with and without amino acid supplementation, on growth and biochemical composition of juvenile American lobster, *Homarus americanus*. *Aquaculture* 189, 211–235. doi: 10.1016/s0044-8486(00)00363-x

Gyan, W. R., Yang, Q., Tan, B., Jan, S. S., Jiang, L., Chi, S., et al. (2020). Effects of antimicrobial peptides on growth, feed utilization, serum biochemical indices and disease resistance of juvenile shrimp, *Litopenaeus vannamei*. *Aquac. Res.* 51, 1222–1231. doi: 10.1111/are.14473

Harmon, T. S. (2009). Methods for reducing stressors and maintaining water quality associated with live fish transport in tanks: a review of the basics. *Rev. Aquac.* 1, 58–66. doi: 10.1111/j.1753-5131.2008.01003.x

He, S., Ding, M., Watson Ray, G., Yang, Q., Tan, B., Dong, X., et al. (2021). Effect of Dietary Vitamin D Levels on Growth, Serum Biochemical Parameters, Lipid Metabolism Enzyme Activities, Fatty Acid Synthase and Hepatic Lipase Mrna Expression for Orange-Spotted Grouper (*Epinephelus Coioides*) in Growth Mid-Stage. *Aquac. Nutr.* 27, 655–665. doi: 10.1111/anu.13212

Holland, M. C., and Lambris, J. D. (2002). The complement system in teleosts. *Fish Shellfish Immunol.* 12, 399–420. doi: 10.1006/fsim.2001.0408

Hou, L. (2012). Research progress of soybean peptides and sports medicine. *Gansu Med.* 31, 907–909. doi: 10.15975/j.cnki.gsyy.2012.12.042

Hu, J. C. (2004). Control of fatty liver disease in factory farmed ovine pomfret. *Sci. Fish Farm.* 1:42.

Hu, L., Yun, B., Xue, M., Wang, J., Wu, X., Zheng, Y., et al. (2013). Effects of Fish Meal Quality and Fish Meal Substitution by Animal Protein Blend on Growth Performance, Flesh Quality and Liver Histology of Japanese Seabass (*Lateolabrax Japonicus*). *Aquaculture* 37, 52–61. doi: 10.1016/j.aquaculture.2012.10.025

Huang, W. (2015). Research Progress on the Application in Aquaculture of Soybean Peptide Protein Feeds. *Taiwan Agric. Exploration* 2, 67–70.

Hussain, M. M., Rava, P., Pan, X., Dai, K., Dougan, S. K., Iqbal, J., et al. (2008). Microsomal triglyceride transfer protein in plasma and cellular lipid metabolism. *Curr. Opin. Lipidol.* 19, 277–284. doi: 10.1097/MOL.0b013e3282feea85

Inoue, N., Fujiwara, Y., Kato, M., Funayama, A., Ogawa, N., Tachibana, N., et al. (2015). Soybean beta-conglycinin improves carbohydrate and lipid metabolism in Wistar rats. *Biosci. Biotechnol. Biochem.* 79, 1528–1534. doi: 10.1080/09168451.2015.1034650

Inoue, N., Nagao, K., Sakata, K., Yamano, N., Gunawardena, P. E., Han, S. Y., et al. (2011). Screening of soy protein-derived hypotriglyceridemic di-peptides in vitro and in vivo. *Lipids Health Dis.* 10:85. doi: 10.1186/1476-511X-10-85

Jiang, K. J. (2013). *Effects of small peptides on growth, muscle quality and physiological and biochemical parameters of juvenile Platichthys stellatus. (M.S.).* Shanghai: Shanghai Ocean University.

Jiang, Y. B., Yin, Q. Q., and Yang, Y. R. (2010). Effect of soybean peptides on growth performance, intestinal structure and mucosal immunity of broilers. *J. Anim. Physiol. Anim. Nutr.* 93, 754–760. doi: 10.1111/j.1439-0396.2008.00864.x

Jing, T. S., Zhou, M. R., Li, Z., Li, Y. L., Sun, W. B., Liang, J. T., et al. (2021). Effects of replacing fish meal with soybean small peptide protein on growth performance, digestive enzyme activity and antioxidant function of juvenile *Pelteobagrus fulvidraco*. *Prog. Fish. Sci.* 1–9.

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

Kim, K. N., Joo, E. S., Kim, K. I., Kim, S.-K., Yang, H. P., and Jeon, Y.-J. (2005). Effect of Chitosan Oligosaccharides on Cholesterol Level and Antioxidant Enzyme Activities in Hypercholesterolemic Rat. *J. Kor. Soc. Food Sci. Nutr.* 34, 36–41 doi: 10.3746/jkfn.2005.34.1.036

Kong, X., Guo, M., Hua, Y., Cao, D., and Zhang, C. (2008). Enzymatic Preparation of Immunomodulating Hydrolysates from Soy Proteins. *Bioresour. Technol.* 99, 8873–8879. doi: 10.1016/j.biortech.2008.04.056

Kotzamanis, Y. P., Gisbert, E., Gatesoupe, F. J., Zambonino Infante, J., and Cahu, C. (2007). Effects of Different Dietary Levels of Fish Protein Hydrolysates on Growth, Digestive Enzymes, Gut Microbiota, and Resistance to Vibrio Anguillarum in European Sea Bass (*Dicentrarchus Labrax*) Larvae. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 147, 205–214. doi: 10.1016/j.cbpa.2006.12.037

Kou, H. Y., Xu, S. Y., and Wang, A. L. (2015). Effect of replacing canola meal for fish meal on the growth, digestive enzyme activity, and amino acids, of ovate pompano, *Trachinotus ovatus*. *Isr. J. Aquac. Bamidgeh* 67, 1–10. doi: 10.46989/001c.20720

Li, E. C., Chen, L. Q., Gu, S. Z., and Cai, Y. Y. (2009). Evaluation method of nutritional value of aquatic feed protein source. *Mar. Sci.* 113–117.

Li, M., Xu, C., Ma, Y., Ye, R., Chen, H., Xie, D., et al. (2020). Effects of dietary n-3 highly unsaturated fatty acids levels on growth, lipid metabolism and innate immunity in juvenile golden pompano (*Trachinotus ovatus*). *Fish Shellfish Immunol.* 105, 177–185. doi: 10.1016/j.fsi.2020.06.060

Li, M. M., Zhang, M., Ma, Y. C., Ye, R. K., Wang, M., Chen, H. Y., et al. (2020). Dietary supplementation with n-3 high unsaturated fatty acids decreases serum lipid levels and improves flesh quality in the marine teleost golden pompano *Trachinotus ovatus*. *Aquaculture* 516:734632. doi: 10.1016/j.aquaculture.2019.734632

Liang, D., Wang, J., Warson Ray, G., Yang, Q., Tan, B., Dong, X., et al. (2020a). Effects of Different Dietary Levels of Soybean Protein Hydrolysates on the Growth Performance, Antioxidant Capacity and Relative Mrna Expression Levels of Juvenile Hybrid Grouper (*Epinephelus Fuscoguttatus* ♀× *Epinephelus Lanceolatus* ♂). *Aquac. Nutr.* 26, 1857–1870. doi: 10.1111/anu.13134

Liang, D., Yang, Q., Tan, B., Dong, X., Chi, S., Liu, H., et al. (2020b). Dietary vitamin A deficiency reduces growth performance, immune function of intestine, and alters tight junction proteins of intestine for juvenile hybrid grouper (*Epinephelus fuscoguttatus* ♀× *Epinephelus lanceolatus* ♂). *Fish Shellfish Immunol.* 107, 346–356. doi: 10.1016/j.fsi.2020.10.016

Lin, A. Y., Wang, W. Z., Chen, G., Zhang, J. D., Huang, J. S., Pan, C. H., et al. (2020). Effects of Two Lactic Acid Bacteria on Growth Performance and Activities of Digestive and Non-specific Immune Enzymes of Juvenile Cobia (*Rachycentroncanadum*). *J. Guangdong Ocean Univ.* 40, 112–117. doi: 10.3969/j.issn.1673-9159.2020.05.014

Lin, H., Chen, X., Chen, S., Zhuo, J. L., Huang, Z., Niu, J., et al. (2012). Replacement of Fish Meal with Fermented Soybean Meal in Practical Diets for Pompano *Trachinotus Ovatus*. *Aquac. Res.* 44, 151–156.

Lin, H., Yang, Q., Wang, A., Wang, J., Tan, B., Ray, G. W., et al. (2021). Effects of Fish Meal under Different Storage Conditions on Growth, Serum Biochemical Indices and Antioxidant Capacity for Juvenile Grouper *Epinephelus Coioides*. *Aquac. Nutr.* 27, 723–733. doi: 10.1111/anu.13218

Lin, Q. C., Fang, C. F., Zhong, G. F., Feng, Y., and Zhu, B. Y. (2010). Effect of dietary small peptides on growth and non-specific immunity of *Penaeus vannamei larvae*. *Zhejiang J. Agric.* 22, 590–595.

Lin, Y. F., Shao, X. P., Jin, Y., Zhang, Y. X., Wu, C. L., Wang, C. L., et al. (2019). Effects of Fish Meal Replacement by Animal and Plant Protein Mixture on Morphology Indexes, Tissue Fatty Acid Composition, Serum Biochemical Indexes and Hepatic Histology of Juvenile Black Carp (*Mylopharyngodon piceus*). *J. Anim. Nutr.* 31, 732–745. doi: 10.3969/j.issn.1006-267x.2019.02.030

Liu, H. Y., Li, L. X., Ayiku, A., Tang, Z., Fan, W., Tan, B. P., et al. (2021). Effects of Dietary Yeast Culture Supplementation on Growth, Intestinal morphology, Immune, and Disease Resistance in *Epinephelus fuscoguttatus* ♀× *Epinephelus lanceolatu* ♂. . *J. Guangdong Ocean Univ.* 41, 1–11.

Liu, K., Liu, H. Y., Chi, S. Y., Dong, X. H., Yang, Q. H., and Tan, B. P. (2018). Effects of different dietary lipid sources on growth performance, body composition and lipid metabolism-related enzymes and genes of juvenile golden pompano, *Trachinotus ovatus*. *Aquac. Res.* 49, 717–725. doi: 10.1111/are.13502

Liu, W., Qian, D., Yang, G. L., Zhang, Y. F., and Wang, J. Y. (2004). A Study on the Pathogen of Luminous Bacterial Disease of Penaeus vannamei Post-Larvae During Desalinization. *J. Jimei Univ.* 4, 300–304. doi: 10.19715/j.jmuzr.2004.04.003

Liu, Y. B., Wang, F., Tian, C. Y., Dalai, and Rong, W. H. (2007). Utilizing the advantages of animal husbandry resources to accelerate the development of sheep farming in Inner Mongolia. *Chin. Herbivore* 4, 22–25.

Ma, H., Liu, R., Zhao, Z., Zhang, Z., Cao, Y., Ma, Y., et al. (2016). A Novel Peptide from Soybean Protein Isolate Significantly Enhances Resistance of the Organism under Oxidative Stress. *PLoS One* 11:e0159938. doi: 10.1371/journal.pone.0159938

Ma, H. Y., Yang, Q. H., Dong, X. H., Tan, B. P., Chi, S. Y., and Liu, H. Y. (2014). Dietary Requirement of Juvenile Grouper. Epinephelus coioides for Hydroxy Methionine Copper. *J. Guangdong Ocean Univ.* 34, 19–26.

Martínez-Llorens, S., Baeza-Ariño, R., Nogales-Mérida, S., Jover-Cerdá, M., and Tomás-Vidal, A. (2012). Carob seed germ meal as a partial substitute in gilthead sea bream (*Sparus aurata*) diets: amino acid retention, digestibility, gut and liver histology. *Aquaculture* 338–341, 124–133. doi: 10.1016/j.aquaculture.2012.01.029

Monentcham, S. E., Pouomogne, V., and Kestemont, P. (2010). Influence of Dietary Protein Levels on Growth Performance and Body Composition of African Bonytongue Fingerlings, Heterotis Niloticus (Cuvier, 1829). *Aquac. Nutr.* 16, 144–152. doi: 10.1111/j.1365-2095.2008.00646.x

Nagasawa, A., Fukui, K., Kojima, M., Kishida, K., Maeda, N., Nagaretani, H., et al. (2003). Divergent effects of soy protein diet on the expression of adipocytokines. *Biochem. Biophys. Res. Commun.* 311, 909–914. doi: 10.1016/j.bbrc.2003.10.087

Nagayoshi, A., Matsuki, N., Saito, H., Tsukamoto, K., Kaneko, K., Wakashima, M., et al. (1995). Defect in Assembly Process of Very-Low-Density Lipoprotein in Suncus Liver: an Animal Model of Fatty Liver. *J. Biochem.* 117, 787–93. doi: 10.1093/oxfordjournals.jbchem.a124777

Naylor, R. L., Goldburg, R. J., Primavera, J. H., Kautsky, N., Beveridge, M. C., Clay, J., et al. (2000). Effect of Aquaculture on World Fish Supplies. *Nature* 405, 1017–1024. doi: 10.1038/35016500

Nilsson-Ehle, P., Garfinkel, A. S., and Schotz, M. C. (1980). Lipolytic enzymes and plasma lipoprotein metabolism. *Annu. Rev. Biochem.* 49, 667–693. doi: 10.1146/annurev.bi.49.070180.003315

Niu, J., Figueiredo-Silva, C., Dong, Y., Yue, Y. R., Lin, H. Z., Wang, J., et al. (2016). Effect of Replacing Fish Meal with Soybean Meal and of Dl-Methionine or Lysine Supplementation in Pelleted Diets on Growth and Nutrient Utilization of Juvenile Golden Pompano (*Trachinotus Ovatus*). *Aquac. Nutr.* 22, 606–614. doi: 10.1111/anu.12284

Pearce, G., Munske, G., Yamaguchi, Y., and Ryan, C. A. (2010). Structure–activity studies of GmSubPep, a soybean peptide defense signal derived from an extracellular protease. *Peptides* 31, 2159–2164. doi: 10.1016/j.peptides.2010.09.004

Puchalska, P., García, M. C., and Marina, M. L. (2014). Development of a Capillary High Performance Liquid Chromatography–Ion Trap-Mass Spectrometry Method for the Determination of Vlivp Antihypertensive Peptide in Soybean Crops. *J. Chromatogr. A* 1338, 85–91. doi: 10.1016/j.chroma.2014.02.059

Qu, Y. J., Fan, C. Y., Li, J. E., and Yu, N. (2014). Effects of salinity on the osmoregulation and weight loss from starvation in Trachinotus ovatus juvenile. *J. Ecol.* 24, 293–300. doi: 10.5846/stxb201303190450

Savoie, A., Le François, N. R., Cahu, C., Blier, P. U., and Andreassen, I. (2006). Do Protein Hydrolysates Improve Survival and Growth of Newly-Hatched Spotted Wolffish (*Anarhichas Minor*), a Non-Metamorphic Aquaculture Fish Species?. *Aquaculture* 261, 782–788. doi: 10.1016/j.aquaculture.2006.08.047

Shen, J. F., Chen, M. C., Liu, H. Y., Tan, B. P., Dong, X. H., Yang, Q. H., et al. (2019). Effects of Fish Meal Replacement by Concentrated Cottonseed Protein on Growth Performance, Serum Biochemical Indices, Liver Antioxidant Indices and Gastrointestinal Tract Protease Activities of Juvenile *Trachinotus ovatus*. *J. Anim. Nutr.* 31, 746–756.

Shi, X., Luo, Z., Huang, C., Zhu, X. M., and Liu, X. (2015). Effects of substituting chlorella sp. For regular fish meal on growth, body composition, hepatic lipid metabolism and histology in crucian carp Carassius curatus. *J. Aquat. Biol.* 39, 498–506.

Tacon, A. G. J., and Metian, M. (2008). Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: trends and future prospects. *Aquaculture* 285, 146–158. doi: 10.1016/j.aquaculture.2008.08.015

Tacon, A. J. (2003). Aquaculture Production Trends Analysis. *Prog. Theor. Phys.* 46, 1–14. doi: 10.1143/PTP.46.1

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 Immunol.* 70, 656–664. doi: 10.1016/j.fsi.2017.09.041

Tan, X., Sun, Z., Zhou, C., Huang, Z., Tan, L., Xun, P., et al. (2018). Effects of dietary dandelion extract on intestinal morphology, antioxidant status, immune function and physical barrier function of juvenile golden pompano *Trachinotus ovatus*. *Fish Shellfish Immunol.* 73, 197–206. doi: 10.1016/j.fsi.2017.12.020

Tseng, D. Y., Ho, P. L., Huang, S. Y., Cheng, S. C., Shiu, Y. L., Chiu, C. S., et al. (2009). Enhancement of immunity and disease resistance in the white shrimp, *Litopenaeus vannamei*, by the probiotic, Bacillus subtilis E20. *Fish Shellfish Immunol.* 26, 339–344. doi: 10.1016/j.fsi.2008.12.003

Tutman, P., Glavić, N., Kožul, V., Skaramuca, B., and Glamuzina, B. (2004). Preliminary Information on Feeding and Growth of Pompano, Trachinotus Ovatus (Linnaeus, 1758) (Pisces; Carangidae) in Captivity. *Aquac. Int.* 12, 387–393. doi: 10.1023/B:AQUI.0000042135.88381.f4

Wang, A. J., Yang, Q. H., Tan, B. P., Xiao, W. W., Jia, J., Dong, X. H., et al. (2018). Effects of Enzymolytic Soybean Meal on Growth Performance, Serum Biochemical Indices, Non-specific Immunity and Disease Resistance of Juvenile *Litopenaeus vannamei*. *J. Guangdong Ocean Univ.* 38, 14–21. doi: 10.3969/j.issn.1673-9159.2018.01.003

Wang, J. X., Liang, D. Z., Yang, Q. H., Tan, B. P., Dong, X. H., Chi, S. Y., et al. (2020). The effect of partial replacement of fish meal by soy protein concentrate on growth performance, immune responses, gut morphology and intestinal inflammation for juvenile hybrid grouper (*Epinephelus fuscoguttatus*♀× *Epinephelus lanceolatus*♂). *Fish Shellfish Immunol.* 98, 619–631. doi: 10.1016/j.fsi.2019.10.025

Wang, J., Zhang, H., Yang, Q., Tan, B., Dong, X., Chi, S., et al. (2020). Effects of replacing soybean meal with cottonseed meal on growth, feed utilization and non-specific immune enzyme activities for juvenile white shrimp, *Litopenaeus vannamei*. *Aquac. Rep.* 16:100255. doi: 10.1016/j.aqrep.2019.100255

Wang, S. J., Zhang, H. F., Zhao, J., Yang, Y. Q., and Yang, S. S. (2011). Effects of Different Salinities on the Growth and Physiology of Orange-Spotted Grouper, *Epinephelus coioides*. *J. Guangdong Ocean Univ.* 31, 39–44.

Wang, T., Fu, Y. M., Lu, J. L., Jiang, H. S., Li, Y. P., Chen, C. Y., et al. (2003). Effect of mini-peptide on the growth performance and the development of small intestines in weaning piglets. *Livestock Vet. Med.* 35, 4–8.

Wang, X. F., Li, X. Q., Leng, X. J., Shan, L. L., Zhao, J. X., and Wang, Y. T. (2014). Effects of Dietary Cottonseed Meal Level on the Growth, Hematological Indices, Liver and Gonad Histology of Juvenile Common Carp (*Cyprinus Carpio*). *Aquaculture* 428–429, 79–87. doi: 10.1016/j.aquaculture.2014.02.040

Wang, Y. R., Wang, L., Zhang, C. X., and Song, K. (2017). Effects of Substituting Fish meal with Soybean Meal on Growth Performance and Intestinal Morphology in Orange-Spotted Grouper (*Epinephelus coioides*). *Aquac. Rep.* 5, 52–57. doi: 10.1016/j.aqrep.2016.12.005

Wang, C. A., Xu, Q., Xu, H., Chang, Y., Zhu, Q., and Sun, D. (2010). Effects of replacing fish meal with small peptides on growth performance, body composition and serum indices of *Acipenser baerii* Brandt. *J. Chin. Cereals Oils Assoc.* 25, 55–58+64.

Weng, X. J., Wu, X. F., Tian, J., Li, X. Q., Guan, L., and Weng, D. C. (2012). Molecular cloning of fatty acid synthase from grass carp (*Ctenopharyngodon idella*) and the regulation of its expression by dietary fat level. *Aquac. Nutr.* 18, 551–558. doi: 10.1111/j.1365-2095.2011.00917.x

Wu, L. F., Qin, G. X., Sun, Z. W., Wang, H. H., Zhang, D. M., Sun, L., et al. (2010). Effect of Dietary Dehulled Soyabean Meal Replacing Fish Meal on the Activity of Digestive Enzyme and the Intestinal Tissue of Clarias lazera. *J. Zhongshan Univ.* 49, 99–105.

Wu, L. F., Xing, X. P., Lai, H. E., Yang, H. H., Qin, G. X., and Yang, H. (2014). Effects of soybean antigen protein Glycinin on intestinal tissues of larval and juvenile Common carps. *J. Northwest Agric. For. Univ. Sci. Technol.* 42, 7–14. doi: 10.13207/j.cnki.jnwafu.2014.10.065

Wu, Y. C., Li, R. M., Shin, G. R., Huang, F., Yang, Q. H., Tan, B. P., et al. (2021). Effects of dietary small peptides on growth, antioxidant capacity, nonspecific immunity and ingut microflora structure of *Litopenaeus vannamei*. *J. Guangdong Ocean Univ.* 41, 1–9. doi: 10.3969/j.issn.1673-9159.2021.05.001

Xie, J., Fang, H., He, X., Liao, S., Liu, Y., Tian, L., et al. (2020). Study on mechanism of synthetic astaxanthin and Haematococcus pluvialis improving the growth performance and antioxidant capacity under acute hypoxia stress of golden pompano (*Trachinotus ovatus*) and enhancing anti-inflammatory by activating Nrf2-ARE pathway to antagonize the NF-κB pathway. *Aquaculture* 518:734657. doi: 10.1016/j.aquaculture.2019.734657

Xie, J., Fang, H., Liao, S., Guo, T., Yin, P., Liu, Y., et al. (2019). Study on Schizochytrium sp. improving the growth performance and non-specific immunity of golden pompano (*Trachinotus ovatus*) while not affecting the antioxidant capacity. *Fish Shellfish Immunol.* 95, 617–623. doi: 10.1016/j.fsi.2019.10.028

Xie, J. H., Qiu, D. Q., Liu, C. X., Zhu, W. W., and Zeng, L. (2013). Effcets of Vibrio alginolyticus Peptidoglycan on Astaxanthin Level, Immune Indicators and Protection in *Litopenaeus vannamei*. *J. Guangdong Ocean Univ.* 33, 50–55.

Yan, J., Liao, K., Wang, T., Mai, K., Xu, W., and Ai, Q. (2015). Dietary Lipid Levels Influence Lipid Deposition in the Liver of Large Yellow Croaker (*Larimichthys crocea*) by Regulating Lipoprotein Receptors, Fatty Acid Uptake and Triacylglycerol Synthesis and Catabolism at the Transcriptional Level. *PLoS One* 10:e0129937. doi: 10.1371/journal.pone.0129937

Yang, Q., Ding, M., Tan, B., Dong, X., Chi, S., Zhang, S., et al. (2017). Effects of dietary vitamin A on growth, feed utilization, lipid metabolism enzyme

activities, and fatty acid synthase and hepatic lipase mRNA expression levels in the liver of juvenile orange spotted grouper, *Epinephelus coioides*. *Aquaculture* 479, 501–507. doi: 10.1016/j.aquaculture.2017.06.024

Yildirim, O., Ergun, S., Yaman, S., and Turker, A. (2009). Effects of Two Seaweeds (*Ulva lactuca and Enteromorpha linza*) as a Feed Additive in Diets on Growth Performance, Feed Utilization, and Body Composition of Rainbow Trout (*Oncorhynchus mykiss*). *Kafkas Universitesi Veteriner Fakultesi Dergisi* 15, 455–460.

Zarkadis, I. K., Mastellos, D., and Lambris, J. D. (2001). Phylogenetic aspects of the complement system. *Dev. Comp. Immunol.* 25, 745–762. doi: 10.1016/s0145-305x(01)00034-9

Zhang, C. X., Rahimnejad, S., Wang, Y. R., Lu, K. L., Song, K., Wang, L., et al. (2018). Substituting Fish Meal with Soybean Meal in Diets for Japanese Seabass (*Lateolabrax Japonicus*): effects on Growth, Digestive Enzymes Activity, Gut Histology, and Expression of Gut Inflammatory and Transporter Genes. *Aquaculture* 483, 173–182. doi: 10.1016/j.aquaculture.2017.10.029

Zhang, J., Li, W., Ying, Z., Zhao, D., Yi, G., Li, H., et al. (2020). Soybean protein-derived peptide nutriment increases negative nitrogen balance in burn injury-induced inflammatory stress response in aged rats through the modulation of white blood cells and immune factors. *Food Nutr. Res.* 64:3677. doi: 10.29219/fnr.v64.3677

Zhang, K., Liu, S. C., Fan, X. P., Wei, S., Sun, Q. X., Xia, Q. Y., et al. (2021). Review on Strategies and Key Technologies of Live Fish Transportation. *J. Guangdong Ocean Univ.* 41, 137–144. doi: 10.3969/j.issn.1673-9159.2021.05.018

Zhang, M., Chen, C., You, C., Chen, B., Wang, S., and Li, Y. (2019). Effects of different dietary ratios of docosahexaenoic to eicosapentaenoic acid (DHA/EPA) on the growth, non-specific immune indices, tissue fatty acid compositions and expression of genes related to LC-PUFA biosynthesis in juvenile golden pompano *Trachinotus ovatus*. *Aquaculture* 505, 488–495. doi: 10.1016/j.aquaculture.2019.01.061

Zhang, Y. C., Li, D. F., Fan, S. J., Piao, X. S., Wang, J. T., and Han, I. K. (2002). Effects of casein and protein-free diets on endogenous amino acid losses in pigs. *Asian Austral. J. Anim.* 15, 1634–1638. doi: 10.5713/ajas.2002.1634

Zhang, Y. J., Shi, L., Xie, T., and Zhong, G. F. (2020). Effects of Soybean Peptide Replacement with Fish Meal on Intestinal Microflora Structure of Pacific White Shrimp, *Litopenaeus vannamei*. *Genomics Appl. Biol.* 39, 5084–5090. doi: 10.13417/j.gab.039.005084

Zhao, S. Y., Lin, H. Z., Huang, Z., Zhou, C. P., Wang, J., Wang, Y., et al. (2016). Effect of small peptide supplementation at different protein levels growth performance, digestive enzymes activities, serum biochemical indices and antioxidant abilities of grouper (*Epinephelus akaara*). *Southern Fish. Sci.* 12, 15–23.

Zhao, Z. X., Song, C. Y., Xie, J., Ge, X. P., Liu, B., Xia, S. L., et al. (2016). Effects of Fish Meal Replacement by Soybean Peptide on Growth Performance, Digestive Enzyme Activities, and Immune Responses of Yellow Catfish *Pelteobagrus Fulvidraco*. *Fish. Sci.* 82, 665–673. doi: 10.1007/s12562-016-0996-6

Zheng, Q., Wen, X., Han, C., Li, H., and Xie, X. (2012). Effect of Replacing Soybean Meal with Cottonseed Meal on Growth, Hematology, Antioxidant Enzymes Activity and Expression for Juvenile Grass Carp, *Ctenopharyngodon Idellus*. *Fish. Physiol. Biochem.* 38, 1059–1069. doi: 10.1007/s10695-011-9590-0

Zheng, Y. F., Xu, Y. Y., and Xu, Y. J. (2006). New advances in the study of small peptides for protein nutrition. *Feed Industry* 27, 16–18.

Zhou, C., Lin, H., Huang, Z., Wang, J., Wang, Y., and Yu, W. (2020). Effects of dietary leucine levels on intestinal antioxidant status and immune response for juvenile golden pompano (*Trachinotus ovatus*) involved in Nrf2 and NF-kappaB signaling pathway. *Fish Shellfish Immunol.* 107, 336–345. doi: 10.1016/j.fsi.2020.10.012

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

Zhou, X. G., Tang, G. H., Deng, F. C., Shen, J. M., Liang, Y., Liu, K., et al. (2011). Effect of small peptide replacing plasma protein powder on production performance of weaned piglets. *Foreign Anim. Husbandry* 30, 64–65.

Zhou, X. Q. (2012). Relationship between nutrients and intestinal health of fish. Chinese Society of Animal Husbandry and Veterinary Medicine, Animal Nutrition Branch. *Adv. Anim. Nutr. Res.* 253–267.

Zhu, Q. G., Lin, J. B., Huang, C. J., Chen, D. H., Liang, P., Qin, C. Q., et al. (2012). Effects of Dietary n-3 Highly Unsaturated Fatty Acids on Growth and Muscle Fatty Acid Composition of Juvenile Grouper (*Epinephlus coioides*). *J. Guangdong Ocean Univ.* 32, 20–27.

Zhu, Z., Yang, Q., Tan, B., Zhou, X., Dong, X., Chi, S., et al. (2021). Effects of replacing fish meal with soybean protein concentrate (SPC) on growth, blood biochemical indexes, non-specific immune enzyme activity, and nutrient apparent digestibility for juvenile *Litopenaeus vannamei*. *Aquac. Int.* 29, 2535–2554. doi: 10.1007/s10499-021-00765-8

Conflict of Interest: MZ and ML were employed by the company Yisheng (Yangjiang) Biotechnology Company Limited.

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 March 2022
doi: 10.3389/fmars.2022.830398

Effects of Lower Fishmeal With Hydrolyzed Fish Protein Powder on the Growth Performance and Intestinal Development of Juvenile Pearl Gentian Grouper (*Epinephelus fuscoguttatus* ♀ and *Epinephelus lanceolatus* ♂)

Vivian Hlordzi[1,2†], Junqing Wang[3†], Tianbi Li[3], Zhiying Cui[3], Beiping Tan[1,2], Hongyu Liu[1,2], Qihui Yang[1,2], Xiaohui Dong[1,2], Shuang Zhang[1,2] and Shuyan Chi[1,2]*

[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 Center of Guangdong Province, Zhanjiang, China, [3] Guangdong XIPU Biotechnology Co., Ltd., Guangzhou, China

The effect of hydrolyzed fish protein powder (HFP) on the growth, intestinal development, gene mRNA expression, and enzyme activity in the intestine and liver of juvenile hybrid grouper (*Epinephelus fuscoguttatus* ♀ and *Epinephelus lanceolatus* ♂) was assessed after an 8-week feeding trial. Seven isonitrogenous (50%) and isolipidic (9%) diets were fed to hybrid grouper with 0% (CT), 1% (H1), 1.5% (H2), 2% (H3), 2.5% (H4), 3% (H5), and 4% (H6) HFP. No significant difference ($p > 0.05$) in weight gain rate (WGR), specific growth rate (SGR), feed conversion ratio (FCR), and survival rate (SR) was observed in all the groups. The crude protein content in the H6 group was significantly higher than in the other groups ($p < 0.05$). Intestinal lipase and trypsin activity were significantly higher in H3 and H5 groups ($p < 0.05$). In the serum, superoxide dismutase (SOD) activity was significantly higher in H5 and H6 groups, while malondialdehyde (MDA) activity was lower ($p < 0.05$) compared to other treatments. Insulin-like growth factor (*IGF-I*) and target of rapamycin (*TOR*) mRNA expression levels in the intestine and muscle were significantly higher in the H2 group and H1 group ($p < 0.05$), respectively. The most abundant intestinal bacteria found at the genus level are *Acinetobacter*, *Vibrio*, and *Flavobacteriaceae.* The villus was significantly longer in hybrid grouper fed with different levels of HFP compared to the control, and fish in the H2 group had thicker intestinal muscle compared to the other groups ($p < 0.05$). In conclusion, the addition of HFP to the low fishmeal (FM) diets of juvenile grouper improved the intestinal development and increased the levels of intestinal digestive enzymes.

Keywords: hydrolyzed fish protein, pearl gentian hybrid grouper, small peptide, intestinal development, fishmeal

OPEN ACCESS

Edited by:
Jin Niu,
Sun Yat-sen University, China

Reviewed by:
Kang-le Lu,
Jimei University, China
Omid Safari,
Ferdowsi University of Mashhad, Iran

****Correspondence:***
Shuyan Chi
chishuyan77@163.com

†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: *07 December 2021*
Accepted: *27 January 2022*
Published: *14 March 2022*

Citation:
Hlordzi V, Wang J, Li T, Cui Z, Tan B, Liu H, Yang Q, Dong X, Zhang S and Chi S (2022) Effects of Lower Fishmeal With Hydrolyzed Fish Protein Powder on the Growth Performance and Intestinal Development of Juvenile Pearl Gentian Grouper (Epinephelus fuscoguttatus ♀ and Epinephelus lanceolatus ♂). Front. Mar. Sci. 9:830398. doi: 10.3389/fmars.2022.830398

INTRODUCTION

Aquaculture is anticipated to fulfill the global request for aquatic animals due to the reduction in capture fisheries since the 1990s (FAO, 2020). In aquaculture practice, feed accounts for over 50% of production cost, which is mainly composed of protein (Tacon and Metian, 2008). About 22 million tonnes of the world's fish production in 2018 was used for non-food purposes, of which 18 million tonnes were used to make fish oil and fishmeal (FM) (FAO, 2020). Traditionally, the most preferred dietary source of protein, which is the most expensive and important nutrient influencing fish growth and feed cost, especially in carnivorous fish, is obtained from FM due to its high digestibility, well-balanced amino acid, and rich source of essential n-3 fatty acids (Tacon and Metian, 2008; Olsen and Hasan, 2012). Environmental and ecological distress on the use of marine pelagic fish, limited supply, and increasing demand for FM has resulted in the intensive study of identifying viable alternative protein sources in aquafeed (FAO, 2020).

Processing fish produces a substantial amount of waste, which includes skin/scales, bones, swim bladders, roes, intestines, blood, and liver, representing about 57% of total weight. Large portions of these by-products, which contain a large amount of bioactive-rich materials, are wasted, discarded, or underutilized (Meeker, 2009; Kumar et al., 2018). Karayannakidis and Zotos (2016) stated that recycling these by-products into profitable goods for agriculture can be a waste management scheme. Quality protein in animal by-products can be hydrolyzed to obtain small molecular peptides which can act as a flavoring and good source of amino acids (Choi et al., 2012; Kumar et al., 2012).

Groupers are among the most common fish and extremely merchandized seafood in the Asia-Pacific region (FAO, 2020). Due to its rapid growth rate, hardiness to environmental conditions, high disease resistance, and high nutritional value, it is an ideal species for intensive aquaculture (Ch'ng and Senoo, 2008; Jiang et al., 2015; Arrokhman et al., 2017; Bunlipatanon and U-taynapun, 2017). With regard to marine fish culture output in China, groupers are ranked third (Yang et al., 2021). Hybrid grouper with a higher disease resistance, faster growth rate, and better feed conversion ratio (FCR) was produced at the University of Malaysia Sabah from brown-marbled grouper (*Epinephelus fuscoguttatus* ♀) and giant grouper (*Epinephelus lanceolatus* ♂) (Rahimnejad et al., 2015; Firdaus et al., 2016). The hybrid produced grows to 1 kg in a period of 6–7 months, while the parents require 8 months to 1 year to attain a similar weight (Arrokhman et al., 2017).

Nutrient utilization and growth performance are the conditions mostly used to assess substitute protein sources in aquafeeds, whereas intestinal health and immunity are often overlooked (Ye et al., 2019). Using hydrolyzed fish protein powder (HFP) to replace FM has been studied by Nguyen et al. (2012); Egerton et al. (2020), and Rimoldi et al. (2020) in gilthead sea bream (*Sparus aurata*), Pacific white shrimp (*Litopenaeus vannamei*), and Atlantic salmon (*Salmo salar*), respectively, but they focused mainly on growth and intestinal microbiota. This study aims to evaluate the effect of HFP on the growth, survival, whole-body composition, serum and liver physiological and biochemical indexes, intestinal morphology, digestive enzymes, gene mRNA expression, and intestinal microbiota in juvenile hybrid grouper.

MATERIALS AND METHODS

Experimental Diets

The HFP with 90% crude protein and 2% crude lipid (obtained from Maoming Xipu Biotechnology Co., Ltd.) was used as a substitute for FM in this experiment (**Table 1**). Seven fish diets containing crude protein (50%) and crude lipid (9%) were formulated, as shown in **Table 2**. The protein level of the formulated feed was balanced using cottonseed protein, which is in abundance and a good ingredient for fish feed, as observed by Yin et al. (2018). The group CT was fed with a diet that had no HFP replacing FM. Due to the high small peptides level, the experimental groups, namely, H1, H2, H3, H4, H5, and H6 were fed with diets containing 1, 1.5, 2, 2.5, 3, and 4% of HFP replacing the corresponding amount of FM, respectively. The raw materials were crushed after visual inspection and sieved using the 60 mm mesh screen. The raw ingredients were then weighed and mixed thoroughly using the V-mixer-type machine (JS-14S, Zhejiang Zhengtai Electrical Appliance Co., Ltd., China). Using a Hobart-type mixer (Food Mixer B60, Guangdong Henglian Food Machinery Co., Ltd., China), the ingredients were mixed with water and choline chloride to form a moist dough. The feed was pelletized into 2 and 2.5 mm granules, air-dried for 48 h, and the

TABLE 1 | Proximate composition of fishmeal (FM) and hydrolyzed fish protein powder (HFP).

	Ingredients	FM/%	HFP/%
Nutrient	Moisture	8.15	4.04
	Crude protein	64.32	90.02
	Crude lipid	7.57	2.00
Amino acid	Aspartate	5.76	7.14
	Threonine	2.79	3.14
	Serine	2.55	2.82
	Glutamine	8.53	12.14
	Glycine	3.89	11.36
	Alanine	4.21	6.71
	Cystine	0.63	0.04
	Valine	3.20	3.37
	Methionine	1.83	1.55
	Isoleucine	2.68	2.62
	Leucine	4.73	4.57
	Tyrosine	2.23	1.55
	Phenylalanine	2.76	2.44
	Lysine	4.97	5.04
	Histidine	1.98	1.58
	Arginine	3.70	4.92
	Proline	2.49	7.00
	*Tryptophan	0.00	0.44

**Tryptophan in FM was not determined (manufacturer's data).*

TABLE 2 | Formulation and nutrient composition of the experimental diets (% dry matter).

Diets and groups	CT	H1	H2	H3	H4	H5	H6
Fishmeal replacement ratio (%)	0	5	10	15	20	25	35
Poultry by-product meal	6.00	6.00	6.00	6.00	6.00	6.00	6.00
Fishmeal[b]	40.00	38.00	36.00	34.00	32.00	30.00	26.00
Hydrolyzed fish protein powder[a]	0.00	1.00	1.50	2.00	2.50	3.00	4.00
Cottonseed protein[b]	4.10	4.90	6.27	7.66	9.05	10.45	13.25
Soybean meal[b]	13.50	13.50	13.50	13.50	13.50	13.50	13.50
Wheat gluten[b]	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Wheat flour[b]	18.20	18.20	18.20	18.20	18.20	18.20	18.20
Soybean oil	1.80	1.80	1.80	1.80	1.80	1.80	1.80
Fish oil	1.35	1.55	1.68	1.79	1.90	2.00	2.20
Soy lecithin	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Choline Chloride	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Calcium monophosphate	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Vitamin C	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Premix (Vitamin + Mineral)[c]	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Attractant	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Total (%)	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Proximate composition[d]							
Crude protein	49.56	49.97	50.19	50.03	50.06	50.63	50.39
Crude lipid	9.11	8.47	9.36	9.80	8.84	8.61	8.88
Moisture	8.36	8.05	8.10	8.34	9.13	8.65	8.89
Ash	8.16	8.72	5.26	5.69	6.15	5.88	6.24

[a]Hydrolyzed fish protein powder was provided by Maoming Xipu Biotechnology Co., Ltd. [b]Ingredients purchased from Zhanjiang HaiBao Feed Factory, Zhanjiang, Guangdong, China. [c]Premix (vitamin + mineral) supplied the following per kilogram of the diet: vitamin A, 500,000 IU; vitamin D3, 100,000 IU; vitamin E, 4,000 mg; vitamin K3, 1,000 mg; vitamin B1, 500 mg; vitamin B2, 1,000 mg; vitamin B6, 1,000 mg; vitamin B12, 2; niacin, 4,000 mg; calcium pantothenate, 2,000 mg; folic acid, 100; biotin, 10; vitamin C, 15,000 mg; iron, 10,000 mg; copper, 300 mg; zinc, 5,000 mg; manganese, 1,200 mg; iodine, 80 mg; selenium, 30 mg; cobalt 20 mg (obtained from Zhanjiang Yuehai Feed Co., Ltd., Guangdong, China). [d]Proximate composition was measured values.

dried feed was stored in sealed plastic bags at −20°C until the experiment started.

Experimental Fish and Feeding Trial

Healthy pearl gentian groupers purchased from Hongyun seedling farm were used for the study. They were kept in aerated cement tanks for an acclimatization period of 3 weeks and hand-fed commercial diets (Dongwan No. 5 feed, China). This study was conducted using indoor fiberglass tanks (0.3 m^3) at the Marine Biological Research Base of Guangdong Ocean University with a natural photoperiod (12 h light/12 h dark) regime. A total of 630 hybrid groupers with no signs of disease were starved for 24 h, batch-weighed to determine an initial average weight (31.56 ± 0.04 g), and randomly distributed at a stocking density of 30 fish per tank. Each treatment was assigned to three replicate tanks. The experimental feed was fed manually twice a day (08:00 and 16:00) until a visually apparent satisfied state was reached, thus as much as they consume during feeding for 56 days. The amount of feed consumed for the period was recorded to check feed intake. Using single-air stones, aeration was provided, and the water temperature was 27.2 ± 1.32°C. Culture water was maintained by changing about 70% in the tanks daily. Daily, mortalities were checked, weighed, and recorded.

Sample Collection

Before the feeding trial, about 20 fish were randomly sampled and stored at −20°C for the initial chemical proximate composition analysis. To obtain the optimum levels of body metabolism, fish were starved for 24 h before the cessation of the experiment. The final number of fish and body weight were checked and recorded. Survival rate (SR), weight gain rate (WGR), FCR, and specific growth rate (SGR) were calculated based on the recordings. The weight and length of five fish per replicate were checked after which their viscera were harvested and weighed to establish their viscerosomatic index (VSI) and condition factor (CF).

$$\textit{Weight gain}\ (\%) = 100 \times \frac{\text{average final individual weight} - \text{average initial individual weight}}{\text{average initial individual weight}}$$

$$\textit{Specific growth rate}\ (\%/\textit{day}) = 100 \times \frac{\log_e \text{average final weight} - \log_e \text{average initial weight}}{\text{days of feeding}}$$

$$\textit{Feed conversion ratio} = \frac{\text{Feed consumed}}{\text{weight gain}}$$

$$\textit{Survival rate}\ (\%) = 100 \times \frac{\text{Final fish number}}{\text{Initial fish number}}$$

$$\text{Protein production value (PPV) (\%)} = 100 \times \frac{(\textit{Final weight} \times \textit{Crude protein}) - (\textit{Initial weight} \times \textit{Crude protein})}{\textit{Feed given} \times \textit{Crude protein}}$$

$$CF\ (\%) = 100 \times \left(\frac{\textit{final weight}}{(\textit{fish length})^3}\right)$$

$$VSI\ (\%) = 100 \times \frac{\textit{viscera weight}}{\textit{body weight}}$$

Nutrient Composition

Nutrient composition (e.g., moisture, crude protein, crude lipid, and ash) in feed and fish was determined using the standard methodology of the Association of Official Analytical Chemists (Baur and Ensminger, 1977).

Intestinal Enzyme Evaluation

Intestinal samples of 3 fish per replicate were weighed and homogenized in 0.9% aseptic saline as described by the specific operation method provided by the commercial kits obtained from the Nanjing Jiancheng Institute of Biological Engineering, China. Using the specific operation method and calculation method provided by the kit, amylase and lipase in the intestine were determined, and absorbance was measured using the microplate reader. Chymotrypsin, trypsin, and total protease activity in the intestine were checked by Shanghai Enzyme-Linked Biotechnology Co., Ltd., China.

Determination of Enzyme Activities

Blood was pooled from 3 fish per replicate using a 1-ml sterile syringe. Blood obtained was transferred into 1.5 ml Eppendorf tubes and stored at 4°C overnight. The blood samples were centrifuged at 4°C at 4,000 rpm for 15 min. The supernatant (serum) was collected and stored at −80°C to check the levels of total protein (TP), albumin (ALB), superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione peroxidase (GHS-PX) using the test kits (Nanjing Jiancheng Institute of Biological Engineering, China). The liver was harvested from the fish and stored at −80°C to check SOD and MDA activities using the test kits (Nanjing Jiancheng Institute of Biological Engineering, China). The absorbance was read with a microplate reader (Thermo Fisher Scientific, United States). The specific operation methods and calculation formulas were used referring to the test kit instructions. Using the isotope method, growth hormone (GH) and insulin (INS) in the serum were checked by Beijing North Biotechnology Research Co., Ltd.

RNA Extraction and Real-Time Quantitative Reverse Transcriptase PCR

The samples of the muscle, liver, and intestine were taken from 3 fish per replicate, and total RNA was extracted using the specific operation method of TRIzol (Invitrogen, United States) reagent. Using 1% agarose gel electrophoresis, the integrity of the extracted RNA was verified, and a spectrophotometer [NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific, United States)] was used to measure the concentration and purity of RNA. Reverse transcription was performed using the PrimeScript^{TM} kit (TaKaRa, China) and its method. The β-*actin* gene was used as the housekeeping gene, and real-time fluorescent quantitative PCR assays were conducted to detect gene expression levels for genes shown in **Table 3** using a quantitative thermal cycler (Bio-Rad CFX96; Bio-Rad Labs, Hercules, CA, United States). Relative expression levels of genes were calculated by $2^{-\Delta\Delta CT}$.

Intestinal Microbiota Analysis

The intestinal samples were sent to Beijing Biomarker Technologies Co., Ltd., for DNA extraction and PCR amplification using Illumina MiSep sequencing analysis. Using NucleoSpin Soil kit, the total genome DNA in the stool of the intestinal sample was extracted. Universal primers (338F: 5′-ACTCCTACGGGAGGCAGCA-3′ and 806R: 5′-GGACTACHVGGGTW TCTAAT-3′) were used to amplify the V3-V4 region of the 16S rRNA gene for Illumina deep sequencing. The microbiota alpha diversity, community composition, and community abundance at phylum and genus levels were performed by using the mothur (Schloss et al., 2009), a free online platform.

Intestinal Morphology

A grouper per replicate was randomly selected, and its mid intestine was harvested and stored in formaldehyde solution for hematoxylin and eosin (H&E) staining using the sectioning method. Results obtained were used for histological examination of the villus length (VL), villus width (VW), and muscle thickness (MT) using ImageJ software.

Statistical Analysis

All original data were subjected to statistical verification using one-way analysis of variance (ANOVA) after consistency and

TABLE 3 | Primer sequence used for real-time quantitative PCR analysis.

Gene	Primer sequence	Source
β-*Actin*	F:GGCTACTCCTTCACCACCACAG R: TCTGGGCAACGGAACCTCT	Liu et al., 2020
TOR	F: CCACTCTTTCTTTGCGGCTT R: GGGTTCTCGTCCCTCACTTG	Ren et al., 2020
IGF-1	F:TATTTCAGTAAACCAACAGGCTATG R:TGAATGACTATGTCCAGGTAAAGG	Wu et al., 2017
IFN-γ	F: TCCGTCAGGATTGAAACAGT R: CCTCCATCTTGGTGGTCAGTG	Liu et al., 2020
IL-β	F:ATGGCAACTGTTCCTGAACTCAACT R:TTTCCTTTCTTAGATATGGACAGGAC	Liu et al., 2020
FAS	F: CGGGTGTCTACATTGGGGTG R: GAATAGCGTGGAAGGCGTTT	Zou et al., 2019
LPL	F: TTCAACAGCACCTCCAAAACC R: GTGAGCCAGTCCACCACGAT	Zou et al., 2019

β-Actin-Beta Actin, TOR-Target of rapamycin, IGF-1-Insulin-like growth factor-I, IFN-γ-Interferon-gamma, IL-β-Interleukin beta, FAS-Fatty acid synthase, LPL-Lipoprotein lipase.

TABLE 4 | Effect of hydrolyzed fish protein powder on the growth and survival of juvenile pearl gentian hybrid grouper.

Group	WGR %	SGR %/d	FCR	SR %	CF (%)	VSI (%)
CT	265.28 ± 0.31	2.34 ± 0.01	0.89 ± 0.04	100.00 ± 0.00	3.25 ± 0.17^{b}	8.76 ± 0.17
H1	264.09 ± 13.13	2.35 ± 0.07	0.87 ± 0.01	100.00 ± 0.00	3.12 ± 0.12^{ab}	8.27 ± 0.12
H2	231.92 ± 4.20	2.17 ± 0.07	0.95 ± 0.03	97.78 ± 2.22	3.03 ± 0.16^{ab}	8.59 ± 0.16
H3	245.15 ± 4.26	2.24 ± 0.02	0.93 ± 0.01	100.00 ± 0.00	2.82 ± 0.02^{ab}	8.31 ± 0.02
H4	238.01 ± 1.51	2.24 ± 0.03	0.93 ± 0.01	100.00 ± 0.00	2.78 ± 0.46^{a}	8.58 ± 0.05
H5	251.31 ± 8.54	2.17 ± 0.03	0.85 ± 0.05	97.78 ± 2.22	2.81 ± 0.04^{ab}	8.29 ± 0.04
H6	236.36 ± 0.53	2.17 ± 0.03	0.95 ± 0.04	100.00 ± 0.00	2.74 ± 0.03^{a}	8.15 ± 0.03

Values are mean values of each group of hybrid grouper (three replicates) ± SE. Means in each row without superscript do not differ significantly ($p > 0.05$), while those with superscript differ significantly ($p < 0.05$). WGR, weight gain rate; SGR, specific growth rate; FCR, feed conversion ratio; SR, survival rate; CF, condition factor; VSI, viscerosomatic index.

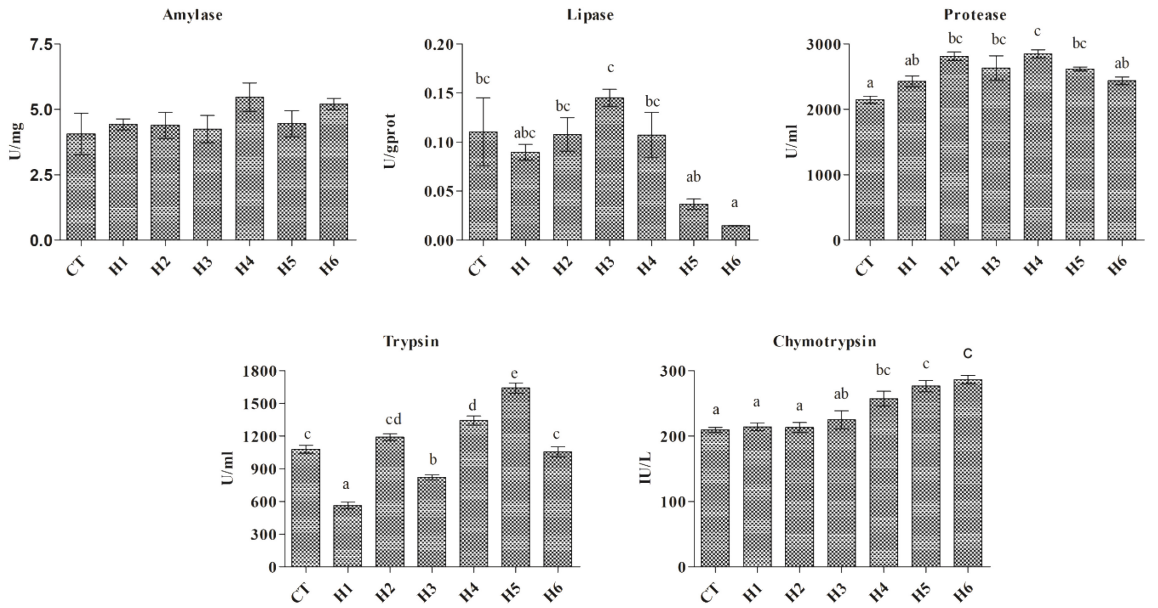


FIGURE 1 | Effect of HFP on digestive enzymes activity in the intestine of juvenile pearl gentian hybrid grouper. Data are presented as Means ± SE. Means without superscript do not differ significantly (P < 0.05), whiles those with superscript differ significantly (P < 0.05).

normality of data variance was checked. All statistical analyses were performed using the SPSS 22.0 for Windows and general differences were found to be significant at $p < 0.05$. Tukey's honest significant difference (HSD) test was used to compare the mean values between individual treatments. Data are represented as mean values of each group of hybrid grouper (three replicates) ± standard error (SE).

RESULTS

Growth Performance, Survival, and Body Composition

The hybrid grouper did not have a significant difference in WGR, SGR, FCR, SR, and VSI ($p > 0.05$) in all groups. CF of hybrid grouper in the CT group was not significantly different with H1, H2, H3, and H5 groups ($p > 0.05$) as summarized in **Table 3**.

In **Table 4**, it was observed that body moisture, crude lipid, and PPV did not show significant differences ($p > 0.05$) in all groups. The crude protein content in the H6 group was significantly higher than CT and H1 groups ($p < 0.05$). Ash content in H1, H2, H4, and H5 groups was significantly higher than the CT group but significantly lower than in the H3 group ($p < 0.05$).

Intestinal Digestive Enzyme Activity

No significant difference in intestinal amylase activity was observed among all the groups ($p > 0.05$), while lipase activity was significantly higher in the H3 group compared to H5 and H6 groups ($p < 0.05$). Intestinal trypsin activity was significantly lower in the H1 group ($p < 0.05$). Intestinal chymotrypsin activity was significantly lower in CT, H1, and H2 groups ($p < 0.05$). The highest intestinal protease activity was observed in the H4 group which did not differ significantly from H2, H3, and H5 groups (**Figure 1**).

Enzyme Activities in the Serum and Liver

In **Table 5**, the serum TP level in the H3 group was not significantly different from that of the H2, H4, H5, and H6

TABLE 5 | Effect of hydrolyzed fish protein powder on the enzyme activities in the serum and liver of juvenile pearl gentian hybrid grouper.

	CT	H1	H2	H3	H4	H5	H6
Serum							
TP	103.46 ± 2.78^{a}	104.15 ± 3.51^{a}	111.22 ± 6.59^{ab}	135.36 ± 3.87^{b}	116.75 ± 5.92^{ab}	108.29 ± 4.41^{ab}	127.59 ± 10.5^{ab}
ALB	7.52 ± 0.71	6.57 ± 0.40	7.58 ± 0.56	7.40 ± 0.38	7.17 ± 0.17	5.30 ± 0.66	7.88 ± 0.63
SOD	90.64 ± 4.01^{ab}	97.58 ± 2.31^{ab}	86.78 ± 1.39^{a}	81.70 ± 1.23^{a}	100.66 ± 6.63^{ab}	110.68 ± 5.24^{b}	109.44 ± 3.96^{b}
GSH-PX	372.86 ± 23.9	404.69 ± 4.28	325.71 ± 22.04	339.18 ± 44.44	351.83 ± 28.71	398.57 ± 22.65	354.69 ± 29.70
MDA	8.60 ± 0.27^{c}	5.17 ± 0.61^{ab}	7.36 ± 0.52^{bc}	8.77 ± 0.52^{c}	6.93 ± 0.26^{bc}	5.35 ± 0.61^{ab}	4.03 ± 0.17^{a}
GH	3.47 ± 0.46^{a}	5.09 ± 0.19^{abc}	6.02 ± 0.76^{bc}	5.82 ± 0.48^{bc}	4.95 ± 0.08^{abc}	6.44 ± 0.054^{c}	3.94 ± 0.36^{ab}
INS	16.39 ± 1.14^{a}	24.70 ± 0.79^{ab}	29.02 ± 1.95^{b}	25.91 ± 2.02^{ab}	25.44 ± 3.53^{ab}	28.22 ± 2.07^{b}	30.33 ± 1.75^{b}
Liver							
MDA	1.45 ± 0.09	1.80 ± 0.37	1.95 ± 0.36	2.04 ± 0.43	2.11 ± 0.32	2.52 ± 0.50	2.66 ± 0.10
SOD	3.76 ± 0.34	3.91 ± 0.43	4.60 ± 0.43	3.96 ± 0.33	3.92 ± 0.34	4.35 ± 0.18	4.00 ± 0.13

Values are mean values of each group of hybrid grouper (three replicates) ± SE. Means in each row without superscript do not differ significantly ($p > 0.05$), while those with superscript differ significantly ($p < 0.05$). GH, growth hormone; INS, insulin; TP, total protein; ALB, albumin; SOD, superoxide dismutase; MDA, malondialdehyde; GHS-PX, glutathione peroxidase.

TABLE 6 | Effect of hydrolyzed fish protein powder on mRNA *IGF-I* and *TOR* expression in the intestine, muscle, and liver of juvenile pearl gentian hybrid grouper.

	CT	H1	H2	H3	H4	H5	H6
Intestine							
IGF-I	0.37 ± 0.08^{a}	1.08 ± 0.08^{bc}	2.12 ± 0.18^{c}	1.60 ± 0.16^{bc}	1.08 ± 0.17^{bc}	0.96 ± 0.07^{ab}	1.42 ± 0.13^{bc}
TOR	1.02 ± 0.11^{a}	1.62 ± 0.20^{ab}	2.66 ± 0.18^{c}	2.23 ± 0.25^{bc}	1.48 ± 0.04^{a}	1.05 ± 0.07^{a}	1.67 ± 0.18^{ab}
Muscle							
IGF-I	0.79 ± 0.09^{ab}	1.37 ± 0.55^{b}	1.00 ± 0.04^{ab}	0.64 ± 0.04^{ab}	0.36 ± 0.05^{a}	0.59 ± 0.09^{ab}	1.09 ± 0.11^{ab}
TOR	0.99 ± 0.03^{abc}	1.47 ± 0.18^{c}	0.99 ± 0.18^{abc}	0.85 ± 0.06^{ab}	0.48 ± 0.06^{a}	0.54 ± 0.03^{a}	1.26 ± 0.19^{bc}
Liver							
IGF-I	1.49 ± 0.08^{b}	1.19 ± 0.10^{ab}	1.02 ± 0.09^{a}	1.02 ± 0.08^{a}	0.98 ± 0.12^{a}	1.08 ± 0.09^{ab}	1.11 ± 0.13^{ab}
TOR	1.39 ± 0.19	1.19 ± 0.13	0.92 ± 0.08	0.94 ± 0.07	0.92 ± 0.12	0.95 ± 0.06	1.04 ± 0.07

Values are mean values of each group of hybrid grouper (three replicates) ± SE. Means in each row without superscript do not differ significantly ($p > 0.05$), while those with superscript differ significantly ($p < 0.05$). IGF-I, insulin-like growth factor; TOR, target of rapamycin.

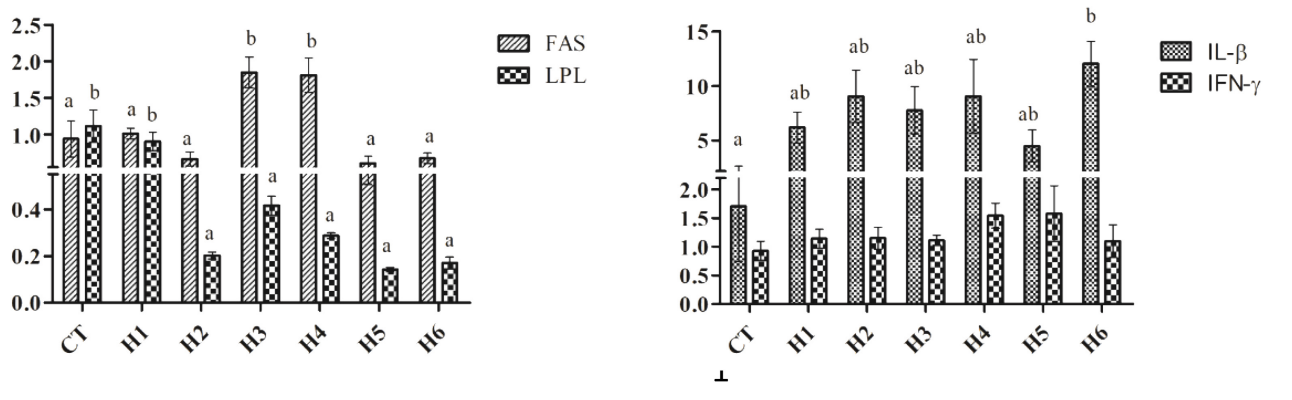


FIGURE 2 | Effect of HFP on the expression of *FAS*, *LPL*, *IL*-β, and *IFN*-γ in the hepatopancreas of juvenile pearl gentian hybrid grouper. *FAS*, fatty acid synthase; *LPL*, lipoprotein lipase; *IL*-β, interleukin beta; *IFN*-γ, interferon-gamma.

groups ($p > 0.05$) but significantly higher than CT and H1 groups ($p < 0.05$). There were no significant differences in the level of ALB and GSH-PX activities in the serum in all the groups ($p > 0.05$). Serum SOD was significantly higher in H5 and H6 groups in comparison with groups H2 and H3 ($p < 0.05$) but similar to the remaining groups ($p > 0.05$). Serum MDA in the H6 group was significantly lower but highest in H3 and CT groups ($p < 0.05$). No significant difference in MDA and SOD activity in the liver was observed for all groups ($p > 0.05$).

The highest level of GH was observed in the H5 group compared to CT and H6 groups ($p < 0.05$) but did not differ significantly from H1, H2, and H3 groups ($p > 0.05$). For serum INS levels, the CT group was significantly lower than H2, H5, and

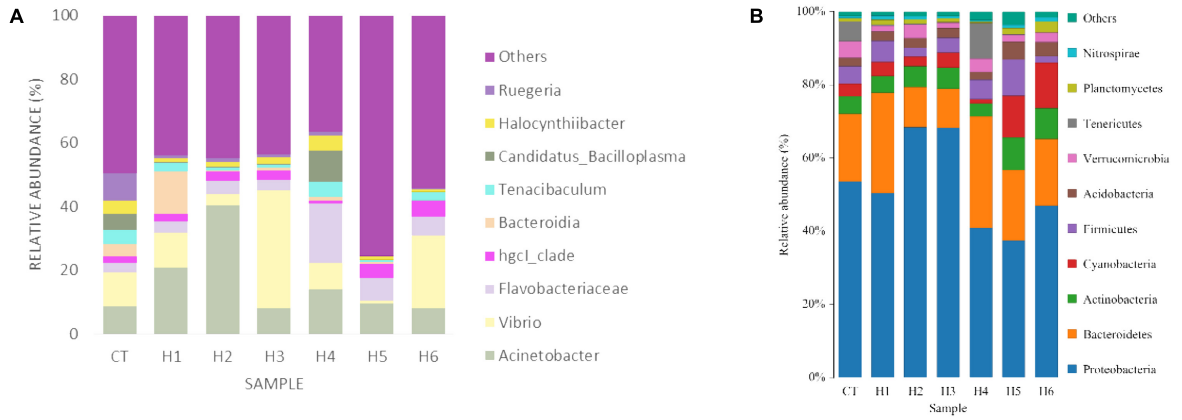


FIGURE 3 | Effect of HFP on genus **(A)** and phylum **(B)** diversity in the intestine of juvenile pearl gentian hybrid grouper.

H6 groups ($p < 0.05$) but did not have any significant difference with H1, H3, and H4 groups ($p > 0.05$).

mRNA Gene Expression in the Intestine, Muscle, and Liver

In the fish intestine, insulin-like growth factor (*IGF-I*) expression in H1, H2, H3, H4, and H6 groups was significantly higher than that in the CT and H5 groups ($p < 0.05$). The target of rapamycin (*TOR*) expression level in the intestine was significantly higher in the H2 group ($p < 0.05$), which was not significantly different from the H3 group ($p > 0.05$).

The *IGF-I* expression level in the muscle was significantly lower in the H4 group than the H1 group ($p < 0.05$) but was not significantly different from CT, H2, H3, H5, and H6 groups ($p > 0.05$). *TOR* expression level in the muscle was significantly higher in the H1 group ($p < 0.05$) but was not significantly different when compared to CT, H2, and H6 groups ($p > 0.05$).

The *IGF-I* expression level in the liver was significantly higher in the CT group compared to H2, H3, and H4 groups ($p < 0.05$), but no significant difference in liver *TOR* expression was observed ($p > 0.05$), as shown in **Table 6**. As shown in **Figure 2**, fatty acid synthase (*FAS*) expression levels in the liver were significantly higher in H3 and H4 groups than those in other groups ($p < 0.05$). The higher lipoprotein lipase (*LPL*) levels were observed in CT and H1 groups compared to the remaining groups ($p < 0.05$). In the liver, the interleukin beta (*IL*-β) expression level in the H6 group was significantly higher than that in CT group ($p < 0.05$). No significant difference in interferon-gamma (*IFN*-γ) expression levels was observed in all the groups ($p > 0.05$).

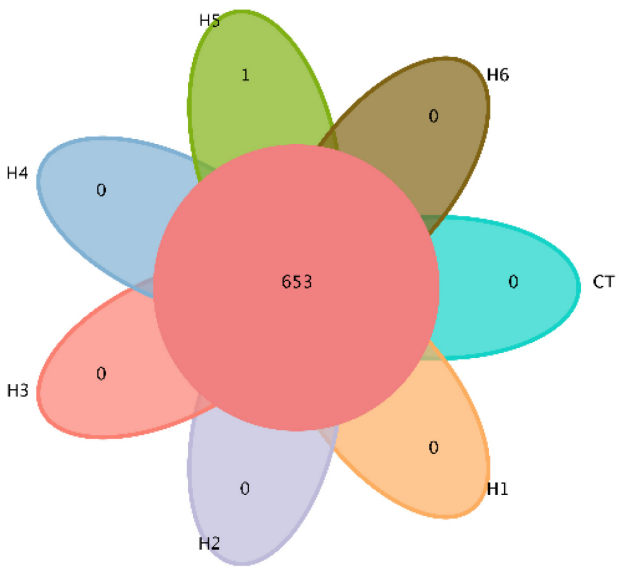


FIGURE 4 | Venn diagram demonstrating the distribution of operational taxonomic units (OTUs) shared by juvenile pearl gentian hybrid grouper fed with different levels of HFP.

Intestinal Microbiota

In the gut of juvenile hybrid grouper, the most abundant bacteria found at the genus level are *Acinetobacter*, *Vibrio,* and *Flavobacteriaceae* (**Figure 3A**). *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* were observed to be the most abundant bacteria at the phylum level, as shown in **Figure 3B**. As shown in **Figure 4**, a total of 653 operational taxonomic units (OTUs) were shared among all the treatments, and only H5 group had 1 unique OTU. *Vibrio*, *Flavobacteriaceae*, and *Acinetobacter* were significantly higher in H3, H4, and H2 groups, respectively, according to the heatmap (**Figure 5**). From the beta diversity distance matrix presented in **Figure 6**, the unweighted pair group method with arithmetic mean (UPGMA) tree showed a distinct dissociation from the control group. This indicates that HFP modified the overall structure of intestinal microbiota in hybrid grouper.

Intestinal Morphology

The VL was significantly longer in the H2 group compared with the CT group ($p < 0.05$). Compared to the other groups, the villus was significantly wider in H5 and H6 groups ($p < 0.05$), and the least VW was recorded in the H4 group. The intestinal muscle layer in CT and H1 groups was significantly thicker than that of

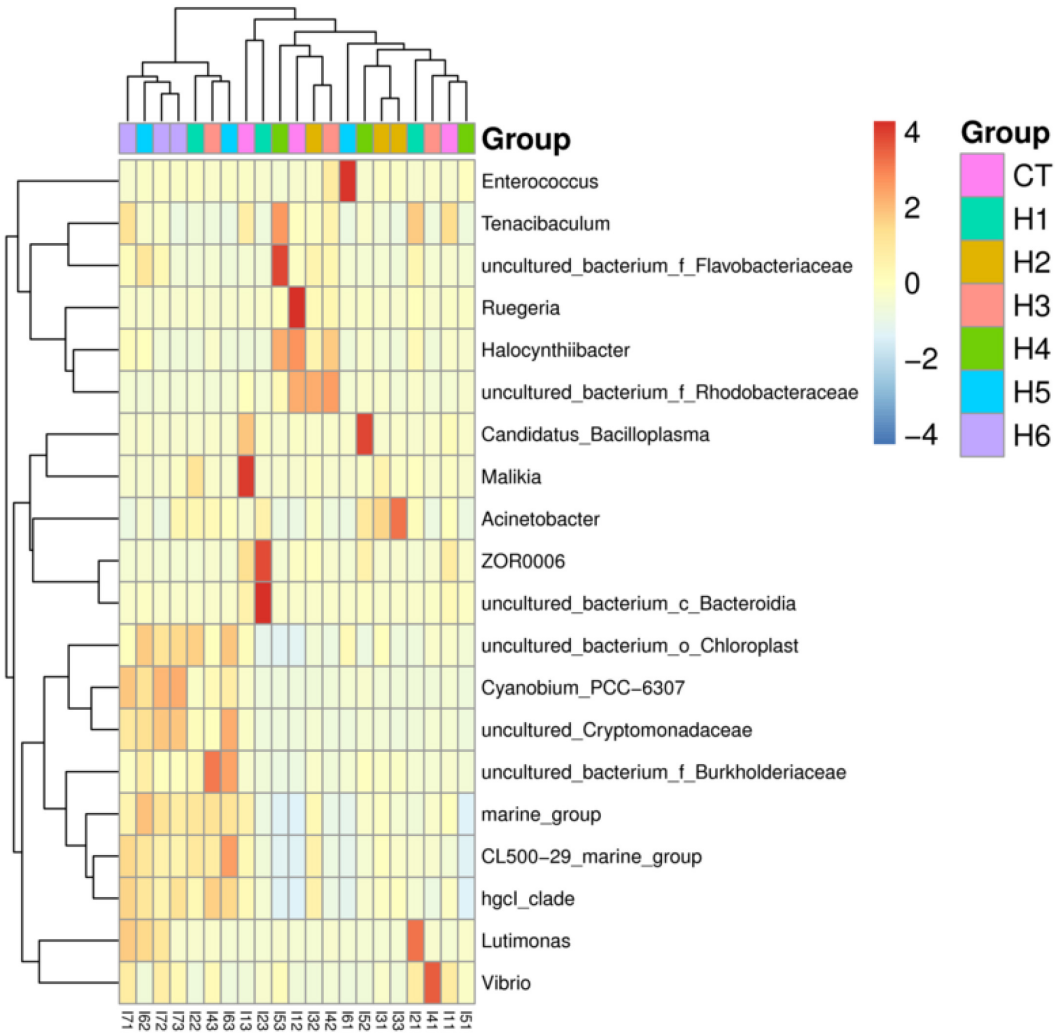


FIGURE 5 | Heatmap of the abundance of intestinal bacteria at the genus level of juvenile pearl gentian hybrid grouper fed with different levels of HFP. Color intensity indicates the relative enrichment of OTUs.

the H2 group but significantly thinner than that of the three other groups ($p < 0.05$), as summarized in **Table 7** and **Figure 7**.

DISCUSSION

The HFP is a promising ingredient used in the diet of aquatic animals due to its potential function to improve growth as well as immune status (Siddik et al., 2019b). HFP used to partially replace FM in the experimental diets in this study contains about 78.5% of small peptides less than 1,000 Da (**Figure 8**). No significant difference and high SR were observed in this study, which showed that all nutritional requirements were met in all the diets and experimental conditions were suitable for hybrid grouper in this study (Wei et al., 2016). The growth performance of hybrid grouper fed with different levels of HFP was slightly reduced, but there was no significant difference to the control group. This reduction in growth performance was also observed by Oliva-Teles et al. (1999) and Khieokhajonkhet and Surapon (2020) when HFP was added to the diet of Nile tilapia (*Oreochromis niloticus*), and juvenile turbot (*Scophthalmus maximus*), respectively. However, results observed in this study were not in accordance with the study conducted in juvenile barramundi (*Lates calcarifer*) fed with different levels of HFP which contained peptides with a molecular size of <3,000 Da (Siddik et al., 2019b). It might be a result of an increase in the catabolism of small peptides and amino acids *via* the gut wall due to the limited availability of peptide transporters (Bakke-McKellep et al., 2000). This can be related to reduce growth rate performance in this study. It is also possible that the macronutrients' requirements by hybrid grouper were met by FM, hence, masking the profitable effect of HFP (Wei et al., 2016).

At various inclusion levels of HFP, whole-body ash and crude protein contents were altered similar to the results observed by Zheng et al. (2012) in Japanese flounder (*Paralichthys*

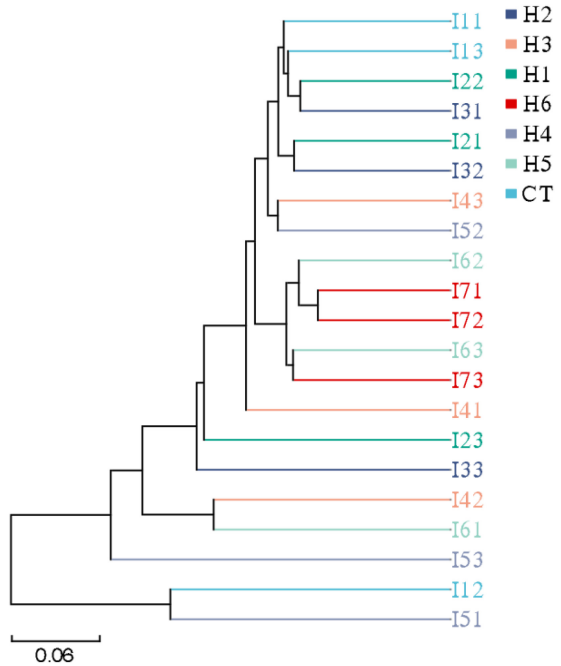


FIGURE 6 | Unweighted pair group method with arithmetic mean-clustering tree of juvenile pearl gentian hybrid grouper fed with different levels of HFP.

olivaceus) when fed with feed containing HFP with about 66.4% small peptides with a size between 100 and 1,000 Da. Sources of hydrolysates and small peptide-sized molecules may have influenced these results. Fish fed with different levels of HFP obtained significantly higher crude protein levels compared to those fed the control diet. In aquatic animal nutrition, levels of TP have been regarded as an indicator of the health and physiological condition of an aquatic organism (Harikrishnan et al., 2003).

The *IGF-I* and *TOR* levels have been evaluated to correlate with growth in different aquatic animals, such as European sea bass (*Dicentrarchus labrax*) (Carnevali et al., 2006), tilapia (*O. niloticus*) (Yan et al., 2013), juvenile turbot (*S. maximus*) (Wang et al., 2016), and pacific white shrimp (Liu et al., 2018). In this study, it was observed that hybrid grouper fed with different levels of HFP obtained a significantly higher total crude protein level compared to the control. A higher level of *IGF-I* and *TOR* mRNA gene expression in the intestine of hybrid grouper was also observed in fish fed with different levels of HFP compared to the control. Wei et al. (2020) also observed a higher *IGF-I* mRNA gene expression when FM was replaced with HFP in the diet of turbot. This could be due to better digestion and absorption of hydrolysate protein and signifies an improvement in general fish health (Khosravi et al., 2015).

The addition of suitable amounts of ingredients with small-sized peptides in feed increased digestive enzyme activity in the intestine of hybrid grouper (Yang et al., 2021), turbot (Jia et al., 2019), and sea bass (Zambonino Infante et al., 1997). Small-size peptide in HFP may play essential roles in regulating enzyme activity by promoting the secretion of digestive enzymes in aquatic animals (Madeira and Paula-Barbosa, 1999). In this study, intestinal trypsin and chymotrypsin were significantly high in the H5 group. These results show that 3% HFP in the diet of hybrid grouper could efficiently increase trypsin and chymotrypsin activity; hence, the presence of rich small-sized peptides could be linked to high levels of digestive enzymes.

The immunity and health of fish are greatly linked to the antioxidant defense system (Ahmadifar et al., 2019). Small-sized molecular peptides can improve and stimulate the capacity and activity of antioxidants in fish (Moure et al., 2006; Wu et al., 2018), and resistance in oxide damage is performed by antioxidant capacity (Lopes et al., 2001). The highest SOD activity in the serum was observed in the H6 group which contained 4% HFP. This shows that a 35% replacement ratio of dietary FM with HFP can increase the level of SOD in the serum (Moure et al., 2006).

Cytokines, which are small glycoprotein messengers, help in intercellular communication to support adaptive and innate immune responses against parasites, bacteria, and viruses (Bruce and Brown, 2017). Kotzamanis et al. (2007) stated that bioactive peptides with antibacterial and immunostimulating properties are produced during the procedure of hydrolysis. HFP used in this study is assumed to contain these peptides. *IL*-β is a pro-inflammatory cytokine in fish, which enhances lysozyme synthesis and defense mechanism with regard to bacterial colonization (Kim and Austin, 2006; Giri et al., 2015). *IL*-β was significantly higher in groups fed with different levels of HFP compared to the control. This was in agreement with Siddik et al. (2019a) who observed an upregulation of *IL*-β when HFP was included in the diet of juvenile barramundi. Results observed

TABLE 7 | Effect of hydrolyzed fish protein powder on the body composition of juvenile pearl gentian hybrid grouper.

Group	Moisture	Crude protein	Crude lipid	Ash	PPV (%)
CT	56.21 ± 0.94	66.17 ± 0.78^{ab}	21.03 ± 0.71	9.95 ± 0.74^{a}	35.26 ± 0.29
H1	56.75 ± 1.56	64.91 ± 0.39^{a}	20.19 ± 1.03	12.98 ± 0.58^{b}	37.29 ± 3.23
H2	53.11 ± 2.26	66.55 ± 1.01^{abc}	20.45 ± 0.36	12.96 ± 1.21^{b}	34.50 ± 0.12
H3	57.60 ± 1.65	68.50 ± 1.38^{abc}	21.96 ± 0.74	18.60 ± 0.38^{c}	35.15 ± 0.85
H4	56.92 ± 1.83	71.12 ± 1.03^{abc}	22.83 ± 0.99	13.60 ± 0.44^{b}	31.89 ± 0.91
H5	57.25 ± 1.16	72.51 ± 1.68^{bc}	20.90 ± 1.18	13.33 ± 0.29^{b}	27.82 ± 6.73
H6	55.25 ± 0.49	72.88 ± 3.03^{c}	20.38 ± 1.31	12.18 ± 0.06^{ab}	32.77 ± 0.51

Values are mean values of each group of hybrid grouper (three replicates) ± SE. Means in each row without superscript do not differ significantly ($p > 0.05$), while those with superscript differ significantly ($p < 0.05$). PPV, protein production value.

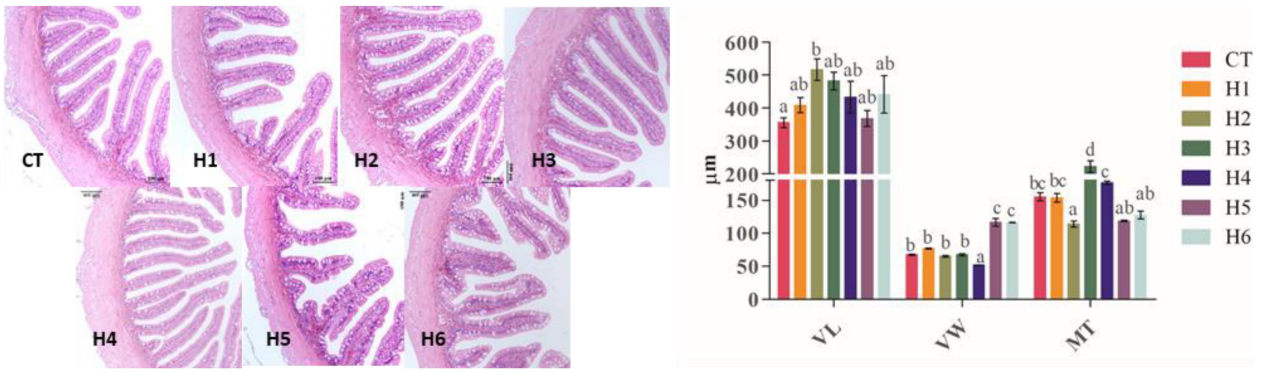


FIGURE 7 | Effect of HFP on the intestinal morphology of juvenile pearl gentian hybrid grouper (H&E, 20×). VL, villus length; VW, villus width; MT, muscle thickness.

in this study could be due to the sufficient content of bioactive peptides in HFP used (Tang et al., 2008; Bui et al., 2014).

Fish gut comprises different groups of microbial communities, including virus, protists, fungi, and bacteria. The bacterial community, which is the dominant group found in the intestine, affects the immune system, metabolism, and health (Wardwell et al., 2011; Tran et al., 2018). The intestinal bacteria community can be influenced by feed composition (Merrifield et al., 2009). The results of this study were not entirely different from Kuebutornye et al. (2020) and Amoah et al. (2021) in tilapia (*O. niloticus*) and northern whiting fish (*Sillago sihama*), respectively. They noted that prevalent bacteria phyla found in the gut of fishes include *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria*. At this level, *Proteobacteria* were the most abundant bacteria which increased and later decreased with increasing HFP inclusion in this study.

In this study, VL was significantly affected by HFP with the highest value observed when 1.5% HFP was used to replace FM. Improvement in the intestinal morphology, thus VL, is a positive indication of the fish's ability to digest feed and absorb nutrients in the digestive canal (Dimitroglou et al., 2009; Tan et al., 2018). Villi growth and intestinal digestive enzymes are effectively stimulated by small peptides (Zhang et al., 2017; Jia et al., 2019). The intestinal surface available for nutrient absorption can be expanded by VL and VW, while the efficiency of nutrient absorption is determined by MT in the intestine (Geda et al., 2012; Lauriano et al., 2016).

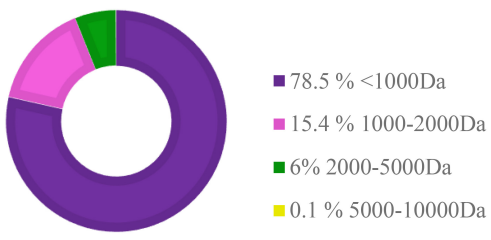


FIGURE 8 | The molecular weight distribution of peptides in hydrolyzed fish protein powder (HFP). Da, Dalton.

CONCLUSION

The addition of HFP to a low FM diet of hybrid grouper will increase the activity of intestinal trypsin and chymotrypsin and the deposition of crude protein and ash in hybrid groupers. An improvement in antioxidant capacity and the development of the intestine in hybrid grouper fed with different levels of HFP was observed. A 5% replacement ratio using 1% HFP is suggested in the diet of hybrid grouper due to a higher WGR compared to the other groups containing HFP.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Review Board of Guangdong Ocean University.

AUTHOR CONTRIBUTIONS

SC aided in the experimental design, fund acquisition, supervision of the project, and review and editing of the manuscript. VH conducted the study, analyzed the data, and drafted the original manuscript. BT and TL aided in funding acquisition. HL, QY, XD, SZ, JW, and ZC aided in the experimental design. All authors contributed to the article and approved the submitted version.

FUNDING

This study was financially supported by the National Key R&D Program of China (2019YFD0900200), the Special Project on Key Fields of Guangdong Universities (Rural Revitalization) (2020ZDZX1034), and the Science-Technology Plan of Zhanjiang (2020A02001).

REFERENCES

Ahmadifar, E., Dawood, M. A. O., Moghadam, M. S., Sheikhzadeh, N., Hoseinifar, S. H., and Musthafa, M. S. (2019). Modulation of immune parameters and antioxidant defense in zebrafish (*Danio rerio*) using dietary apple cider vinegar. *Aquaculture* 513:734412. doi: 10.1016/j.aquaculture.2019.734412

Amoah, K., Dong, X., Tan, B., Zhang, S., Chi, S., Yang, Q., et al. (2021). Effects of three probiotic strains (*Bacillus coagulans*, *B. licheniformis* and *Paenibacillus polymyxa*) on growth, immune response, gut morphology and microbiota, and resistance against *Vibrio harveyi* of northern whitings, sillago sihama forsskál (1775). *Animal Feed Sci. Technol.* 277:114958. doi: 10.1016/j.anifeedsci.2021.114958

Arrokhman, S., Wijayanti, N., and Soegianto, A. (2017). Survival and osmoregulation of juvenile of hybrid grouper (*Epinephelus fuscoguttatus*× *Epinephelus lanceolatus*) during acclimation in calcium-supplemented freshwater. *Aquacu. Int.* 25, 693–704. doi: 10.1007/s10499-016-0069-y

Bakke-McKellep, A. M., Nordrum, S., Krogdahl, Å, and Buddington, R. K. (2000). Absorption of glucose, amino acids, and dipeptides by the intestines of Atlantic salmon (*Salmo salar* L.). *Fish Physiol. Biochem.* 22, 33–44. doi: 10.1023/A:1007872929847

Baur, F. J., and Ensminger, L. G. (1977). The association of official analytical chemists (AOAC). *J. Am. Oil Chem. Soc.* 54, 171–172. doi: 10.1007/BF02670789

Bruce, T. J., and Brown, M. L. (2017). A review of immune system components, cytokines, and immunostimulants in cultured finfish species. *Open J. Animal Sci.* 7:267. doi: 10.4236/ojas.2017.73021

Bui, H. T. D., Khosravi, S., Fournier, V., Herault, M., and Lee, K.-J. (2014). Growth performance, feed utilization, innate immunity, digestibility and disease resistance of juvenile red seabream (*Pagrus major*) fed diets supplemented with protein hydrolysates. *Aquaculture* 418, 11–16. doi: 10.1016/j.aquaculture.2013.09.046

Bunlipatanon, P., and U-taynapun, K. (2017). Growth performance and disease resistance against V ibrio vulnificus infection of novel hybrid grouper (E pinephelus lanceolatus× E pinephelus fuscoguttatus). *Aquaculture Research* 48, 1711–1723. doi: 10.1111/are.13008

Carnevali, O., de Vivo, L., Sulpizio, R., Gioacchini, G., Olivotto, I., Silvi, S., et al. (2006). Growth improvement by probiotic in European sea bass juveniles (*Dicentrarchus labrax*, L.), with particular attention to IGF-1, myostatin and cortisol gene expression. *Aquaculture* 258, 430–438.

Ch'ng, C. L., and Senoo, S. (2008). Egg and larval development of a new hybrid grouper, tiger grouper *Epinephelus fuscoguttatus*× giant grouper E. lanceolatus. *Aquacul. Sci.* 56, 505–512.

Choi, J., Sabikhi, L., Hassan, A., and Anand, S. (2012). Bioactive peptides in dairy products. *Int. J. Dairy Technol.* 65, 1–12. doi: 10.1111/j.1471-0307.2011.00725.x

Dimitroglou, A., Merrifield, D. L., Moate, R., Davies, S. J., Spring, P., Sweetman, J., et al. (2009). Dietary mannan oligosaccharide supplementation modulates intestinal microbial ecology and improves gut morphology of rainbow trout, *Oncorhynchus mykiss* (walbaum). *J. Animal Sci.* 87, 3226–3234. doi: 10.2527/jas.2008-1428

Egerton, S., Wan, A., Murphy, K., Collins, F., Ahern, G., Sugrue, I., et al. (2020). Replacing fishmeal with plant protein in Atlantic salmon (*Salmo salar*) diets by supplementation with fish protein hydrolysate. *Sci. Rep.* 10, 1–16. doi: 10.1038/s41598-020-60325-7

FAO (2020). *The State of World Fisheries and Aquaculture 2020. Sustainability in Action*. Rome: FAO.

Firdaus, R. F., Lim, L.-S., Kawamura, G., and Shapawi, R. (2016). Assessment on the acceptability of hybrid grouper, *Epinephelus fuscoguttatus*♀× *Epinephelus lanceolatus*♂ to soybean meal-based diets. *Aquacul. Aquar. Conserv. Legis. -Int. J. Bioflux Soc. (AACL Bioflux)* 9, 284–290.

Geda, F., Rekecki, A., Decostere, A., Bossier, P., Wuyts, B., Kalmar, I. D., et al. (2012). Changes in intestinal morphology and amino acid catabolism in common carp at mildly elevated temperature as affected by dietary mannanoligosaccharides. *Animal Feed Sci. Technol.* 178, 95–102. doi: 10.1016/j.anifeedsci.2012.09.008

Giri, S. S., Sen, S. S., Chi, C., Kim, H. J., Yun, S., Park, S. C., et al. (2015). Effect of cellular products of potential probiotic bacteria on the immune response of Labeo rohita and susceptibility to *Aeromonas hydrophila* infection. *Fish Shellfish Immunol.* 46, 716–722. doi: 10.1016/j.fsi.2015.08.012

Harikrishnan, R., Rani, M. N., and Balasundaram, C. (2003). Hematological and biochemical parameters in common carp, *Cyprinus carpio*, following herbal treatment for *Aeromonas hydrophila* infection. *Aquaculture* 221, 41–50. doi: 10.1016/S0044-8486(03)00023-1

Jia, G., Guo, R., Zhang, Y., Li, X., Xia, H., Gong, C., et al. (2019). Effects of partial replacement of fish meal by peptides hydrolyzed from poultry by-products on growth performance, digestive indices and specific immune indices of turbot. *Chin. J. Animal Nutr.* 31, 2201–2211.

Jiang, S., Wu, X., Li, W., Wu, M., Luo, Y., Lu, S., et al. (2015). Effects of dietary protein and lipid levels on growth, feed utilization, body and plasma biochemical compositions of hybrid grouper (*Epinephelus lanceolatus* ♀× *Epinephelus fuscoguttatus* ♂) juveniles. *Aquaculture* 446, 148–155. doi: 10.1016/j.aquaculture.2015.04.034

Karayannakidis, P. D., and Zotos, A. (2016). Fish processing by-products as a potential source of gelatin: a review. *J. Aquat. Food Prod. Technol.* 25, 65–92. doi: 10.1080/10498850.2013.827767

Khieokhajonkhet, A., and Surapon, K. (2020). Effects of fish protein hydrolysate on the growth performance, feed and protein utilization of *Nile tilapia* (*Oreochromis niloticus*). *Int. J. Agricul. Technol.* 16, 641–654.

Khosravi, S., Bui, H. T. D., Rahimnejad, S., Herault, M., Fournier, V., Jeong, J. B., et al. (2015). Effect of dietary hydrolysate supplementation on growth performance, non-specific immune response and disease resistance of olive flounder (*Paralichthys olivaceus*) challenged with edwardsiella tarda. *Aquacul. Nutr.* 21, 321–331. doi: 10.1111/anu.12157

Kim, D.-H., and Austin, B. (2006). Cytokine expression in leucocytes and gut cells of rainbow trout, *Oncorhynchus mykiss* walbaum, induced by probiotics. *Vet. Immunol. Immunopathol.* 114, 297–304. doi: 10.1016/j.vetimm.2006.08.015

Kotzamanis, Y. P., Gisbert, E., Gatesoupe, F. J., Zambonino Infante, J., and Cahu, C. (2007). Effects of different dietary levels of fish protein hydrolysates on growth, digestive enzymes, gut microbiota, and resistance to *Vibrio anguillarum* in European sea bass (*Dicentrarchus labrax*) larvae. *Comparat. Biochem. Physiol. Mol. Integrat. Physiol.* 147, 205–214. doi: 10.1016/j.cbpa.2006.12.037

Kuebutornye, F. K. A., Wang, Z., Lu, Y., Abarike, E. D., Sakyi, M. E., Li, Y., et al. (2020). Effects of three host-associated *Bacillus* species on mucosal immunity and gut health of *Nile tilapia*, *Oreochromis niloticus* and its resistance against *Aeromonas hydrophila* infection. *Fish Shellfish Immunol.* 97:46. doi: 10.1016/j.fsi.2019.12.046

Kumar, N. S. S., Nazeer, R. A., and Ganesh, R. J. (2012). Functional properties of protein hydrolysates from different body parts of horse mackerel (*Magalaspis cordyla*) and croaker (*Otolithes ruber*). *Med. J. Nutr. Metab.* 5, 105–110. doi: 10.1007/s12349-011-0078-3

Kumar, V., Muzaddadi, A. U., Mann, S., Balakrishnan, R., Bembem, K., and Kalnar, Y. (2018). *Utilization of Fish Processing Waste: A Waste to Wealth Approach*. Ludhiana: ICAR-CIPHET.

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-cholesterol diet. *Fish Shellfish Immunol.* 59, 250–255. doi: 10.1016/j.fsi.2016.11.003

Liu, X., Shi, H., He, Q., Lin, F., Wang, Q., Xiao, S., et al. (2020). Effect of starvation and refeeding on growth, gut microbiota and non-specific immunity in hybrid grouper (*Epinephelus fuscoguttatus* ♀×*E. lanceolatus* ♂). *Fish Shellfish Immunol.* 97, 182–193. doi: 10.1016/j.fsi.2019.11.055

Liu, X., Wang, M., Sun, G., Wang, B., Jiang, K., Shao, J., et al. (2018). Mechanistic target of rapamycin inhibition with rapamycin induces autophagy and correlative regulation in white shrimp (*Litopenaeus vannamei*). *Aquac. Nutr.* 24, 1509–1520.

Lopes, P. A., Pinheiro, T., Santos, M. C., da Luz Mathias, M., Collares-Pereira, M. J., and Viegas-Crespo, A. M. (2001). Response of antioxidant enzymes in freshwater fish populations (*Leuciscus alburnoides* complex) to inorganic pollutants exposure. *Sci. Total Environ.* 280, 153–163. doi: 10.1016/S0048-9697(01)00822-1

Madeira, M. D., and Paula-Barbosa, M. M. (1999). Effects of alcohol on the synthesis and expression of hypothalamic peptides. *Brain Res. Bull.* 48, 3–22. doi: 10.1016/S0361-9230(98)00131-2

Meeker, D. L. (2009). North American Rendering: processing high quality protein and fats for feed. *Rev. Brasileira De Zoot.* 38, 432–440. doi: 10.1590/S1516-35982009001300043

Merrifield, D. L., Burnard, D., Bradley, G., Davies, S. J., and Baker, R. T. M. (2009). Microbial community diversity associated with the intestinal mucosa of farmed rainbow trout (*Oncoryhnchus mykiss* walbaum). *Aquacul. Res.* 40, 1064–1072. doi: 10.1111/j.1365-2109.2009.02200.x

Moure, A., Domínguez, H., and Parajó, J. C. (2006). Antioxidant properties of ultrafiltration-recovered soy protein fractions from industrial effluents and their hydrolysates. *Proc. Biochem.* 41, 447–456. doi: 10.1016/j.procbio.2005.07.014

Nguyen, H. T. M., Pérez-Gálvez, R., and Bergé, J. P. (2012). Effect of diets containing tuna head hydrolysates on the survival and growth of shrimp *Penaeus vannamei*. *Aquaculture* 324–325, 127–134. doi: 10.1016/j.aquaculture.2011.11.014

Oliva-Teles, A., Cerqueira, A. L., and Gonçalves, P. (1999). The utilization of diets containing high levels of fish protein hydrolysate by turbot (*Scophthalmus maximus*) juveniles. *Aquaculture* 195:201. doi: 10.1016/S0044-8486(99)00162-3

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

Rahimnejad, S., Bang, I. C., Park, J.-Y., Sade, A., Choi, J., and Lee, S.-M. (2015). Effects of dietary protein and lipid levels on growth performance, feed utilization and body composition of juvenile hybrid grouper, *Epinephelus fuscoguttatus*× *E. lanceolatus*. *Aquaculture* 446, 283–289. doi: 10.1016/j.aquaculture.2015.05.019

Ren, Z., Wang, S., Cai, Y., Wu, Y., Tian, L., Wang, S., et al. (2020). Effects of dietary mannan oligosaccharide supplementation on growth performance, antioxidant capacity, non-specific immunity and immune-related gene expression of juvenile hybrid grouper (*Epinephelus lanceolatus*♀× *Epinephelus fuscoguttatus*♂). *Aquaculture* 523:735195. doi: 10.1016/j.aquaculture.2020.735195

Rimoldi, S., Gini, E., Koch, J. F. A., Iannini, F., Brambilla, F., and Terova, G. (2020). Effects of hydrolyzed fish protein and autolyzed yeast as substitutes of fishmeal in the gilthead sea bream (*Sparus aurata*) diet, on fish intestinal microbiome. *BMC Vet. Res.* 16:1–13. doi: 10.1186/s12917-020-02335-1

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/AEM.01541-09

Siddik, M. A. B., Howieson, J., and Fotedar, R. (2019b). Beneficial effects of tuna hydrolysate in poultry by-product meal diets on growth, immune response, intestinal health and disease resistance to *Vibrio harveyi* in juvenile barramundi, *Lates calcarifer*. *Fish Shellfish Immunol.* 89, 61–70. doi: 10.1016/j.fsi.2019.03.042

Siddik, M. A. B., Chaklader, M. R., Foysal, M. J., Howieson, J., Fotedar, R., and Gupta, S. K. (2019a). Influence of fish protein hydrolysate produced from industrial residues on antioxidant activity, cytokine expression and gut microbial communities in juvenile barramundi *Lates calcarifer*. *Fish Shellfish Immunol.* 97, 465–473. doi: 10.1016/j.fsi.2019.12.057

Tacon, A. G. J., and Metian, M. (2008). Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: trends and future prospects. *Aquaculture* 285, 146–158. doi: 10.1016/j.aquaculture.2008.08.015

Tan, X., Sun, Z., Zhou, C., Huang, Z., Tan, L., Xun, P., et al. (2018). Effects of dietary dandelion extract on intestinal morphology, antioxidant status, immune function and physical barrier function of juvenile golden pompano *Trachinotus ovatus*. *Fish Shellfish Immunol.* 73, 197–206. doi: 10.1016/j.fsi.2017.12.020

Tang, H. G., Wu, T. X., Zhao, Z. Y., and Pan, X. D. (2008). Effects of fish protein hydrolysate on growth performance and humoral immune response in large yellow croaker (*Pseudosciaena crocea* R.). *J. Zhejiang Univ. Sci. B* 9, 684–690. doi: 10.1631/jzus.B0820088

Tran, T. N., Xiong, F., Hao, Y.-T., Zhang, J., Wu, S.-G., and Wang, G.-T. (2018). Starvation influences the microbiota assembly and expression of immunity-related genes in the intestine of grass carp (*Ctenopharyngodon idellus*). *Aquaculture* 489, 121–129. doi: 10.1016/j.aquaculture.2018.02.016

Wang, Q., He, G., Mai, K., Xu, W., and Zhou, H. (2016). Fishmeal replacement by mixed plant proteins and maggot meal on growth performance, target of rapamycin signalling and metabolism in juvenile turbot (*Scophthalmus maximus* L.). *Aquac. Nutr.* 22, 752–758.

Wardwell, L. H., Huttenhower, C., and Garrett, W. S. (2011). Current concepts of the intestinal microbiota and the pathogenesis of infection. *Curr. Infect. Dis. Rep.* 13, 28–34. doi: 10.1007/s11908-010-0147-7

Wei, Y., Liang, M., Mu, Y., Zheng, K., and Xu, H. (2016). The effect of ultrafiltered fish protein hydrolysate level on growth performance, protein digestibility and m RNA expression of P ep T 1 in juvenile turbot (S cophthalmus maximus L.). *Aquacul. Nutr.* 22, 1006–1017. doi: 10.1111/anu.12319

Wei, Y., Liang, M., and Xu, H. (2020). Fish protein hydrolysate affected amino acid absorption and related gene expressions of IGF-1/AKT pathways in turbot (*Scophthalmus maximus*). *Aquacul. Nutr.* 26, 145–155. doi: 10.1111/anu.12976

Wu, D., Zhou, L., Gao, M., Wang, M., Wang, B., He, J., et al. (2018). Effects of stickwater hydrolysates on growth performance for yellow catfish (*Pelteobagrus fulvidraco*). *Aquaculture* 488, 161–173. doi: 10.1016/j.aquaculture.2018.01.031

Wu, M., Lu, S., Wu, X., Jiang, S., Luo, Y., Yao, W., et al. (2017). Effects of dietary amino acid patterns on growth, feed utilization and hepatic IGF-I, TOR gene expression levels of hybrid grouper (*Epinephelus fuscoguttatus*♀× *Epinephelus lanceolatus*♂) juveniles. *Aquaculture* 468, 508–514. doi: 10.1016/j.aquaculture.2016.11.019

Yan, B., Zhu, C.-D., Guo, J.-T., Zhao, L.-H., and Zhao, J.-L. (2013). miR-206 regulates the growth of the teleost tilapia (*Oreochromis niloticus*) through the modulation of IGF-1 gene expression. *J. Exp. Biol.* 216, 1265–1269.

Yang, X., Wang, G., Zhao, X., Dong, X., Chi, S., and Tan, B. (2021). Addition of hydrolysed porcine mucosa to low-fishmeal feed improves intestinal morphology and the expressions of intestinal amino acids and small peptide transporters in hybrid groupers (*Epinephelus fuscoguttatus*♀× *E. lanceolatus*♂). *Aquaculture* 535:736389. doi: 10.1016/j.aquaculture.2021.736389

Ye, H., Zhou, Y., Su, N., Wang, A., Tan, X., Sun, Z., et al. (2019). Effects of replacing fish meal with rendered animal protein blend on growth performance, hepatic steatosis and immune status in hybrid grouper (*Epinephelus fuscoguttatus*♀× *Epinephelus lanceolatus*♂). *Aquaculture* 511:734203. doi: 10.1016/j.aquaculture.2019.734203

Yin, B., Liu, H., Tan, B., Dong, X., Chi, S., Yang, Q., et al. (2018). Cottonseed protein concentrate (CPC) suppresses immune function in different intestinal segments of hybrid grouper♀ *Epinephelus fuscoguttatus*×♂ *Epinephelus lanceolatu* via TLR-2/MyD88 signaling pathways. *Fish Shellfish Immunol.* 81, 318–328. doi: 10.1016/j.fsi.2018.07.038

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

Zhang, D., Zhang, Y., Liu, B., Jiang, Y., Zhou, Q., Wang, J., et al. (2017). Effect of replacing fish meal with fermented mushroom bran hydrolysate on the growth, digestive enzyme activity, and antioxidant capacity of allogynogenetic crucian carp (*Carassius auratus* gibelio). *Turkish J. Fish. Aquatic Sci.* 17, 1039–1048.

Zheng, K., Liang, M., Yao, H., Wang, J., and Chang, Q. (2012). Effect of dietary fish protein hydrolysate on growth, feed utilization and IGF-I levels of Japanese flounder (*Paralichthys olivaceus*). *Aquacul. Nutr.* 18, 297–303. doi: 10.1111/j.1365-2095.2011.00896.x

Zou, C., Xu, M., Chen, L., Liu, Q., Zhou, Y., Sun, Z., et al. (2019). Xiaochaihu decoction reduces hepatic steatosis and improves D-GalN/LPS-induced liver injury in hybrid grouper (*Epinephelus lanceolatus*♀× *Epinephelus fuscoguttatus*♂). *Fish Shellfish Immunol.* 91, 293–305. doi: 10.1016/j.fsi.2019.05.025

Conflict of Interest: JW, TL, and ZC were employed by Guangdong XIPU Biotechnology Co., Ltd.

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

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

frontiers | Frontiers in Marine Science

ORIGINAL RESEARCH
published: 07 April 2022
doi: 10.3389/fmars.2022.857592

Brewer's Spent Dry Yeast Modulates Immunity in Gilthead Sea Bream (*Sparus aurata*)

*Ioannis Konstantinidis[1], Joana P. Firmino[2], Alberto Ruiz[2], Bruno Iñarra[3], David San Martin[3], Alicia Estévez[2], Jorge M. O. Fernandes[1] and Enric Gisbert[2]**

[1] Faculty of Biosciences and Aquaculture, Nord University, Bodø, Norway, [2] Aquaculture Program, Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Centre de la Ràpita (IRTA-SCR), La Ràpita, Spain, [3] AZTI, Food Research, Basque Research and Technology Alliance (BRTA), Bizkaia, Spain

In this study, we evaluated the replacement of dietary protein sources like fishmeal (FM) and plant proteins (PP) by Brewer's spent dry yeast (SDY) on the transcriptomic response (RNA-seq, NextSeq500 platform Illumina) in the liver, anterior-mid intestine, and head kidney in juveniles of gilthead sea bream (*Sparus aurata*). The inclusion of SDY at 30% in the experimental diet (40% crude protein, 16% crude lipid) resulted in a reduction in FM (10%) and PP (31.4%) contents. Using RNA-seq, a total of 19.4 million raw reads per library were obtained, from whose 99.8% of the sequenced data were retained. The alignment efficiency of uniquely mapped reads was 90.3, 89.5, and 89.8% for head kidney, liver, and anterior-mid intestine, respectively. In total, 218 differentially expressed genes (DEGs) were identified among all tissues, out of which, 141 were up- and 77 down-regluated. The enrichment analysis of DEGs revealed that SDY had a modulatory effect on several processes related to host's immunity, oxygen's carrier capacity, steroidogenesis, metabolism, and digestion. It is of special relevance the immunomodulatory effects of the tested ingredient as data from RNA-seq showed from the three target tissues analyzed. These results indicated that this ingredient in addition to being considered as a sustainable raw material for replacing conventional protein sources in aquafeeds may also be considered as a functional ingredient due to its content in β-glucans. The overall results of this study coupled with previous nutritional studies on this ingredient indicated the suitability of brewery's by-products like SDY in aquafeeds for carnivorous species like gilthead seabream, as well as supporting a circular bioeconomy model that reuses, recovers, and recycles resources instead of producing wastes.

Keywords: gilthead sea bream, functional feed, alternative dietary protein, RNA-seq, immunity, circular feeds, aquafeed

OPEN ACCESS

Edited by:
Ikram Belghit, Norwegian Institute of Marine Research (IMR), Norway

Reviewed by:
Gonzalo Martinez-Rodriguez, Department of Marine Biology and Aquacuture, Institute of Marine Sciences of Andalusia (CSIC), Spain
Yulema Valero Valero, University of Murcia, Spain

****Correspondence:***
Enric Gisbert
enric.gisbert@irta.es

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

Received: *18 January 2022*
Accepted: *28 February 2022*
Published: *07 April 2022*

Citation:
Konstantinidis I, Firmino JP, Ruiz A, Iñarra B, San Martin D, Estévez A, Fernandes JMO and Gisbert E (2022) Brewer's Spent Dry Yeast Modulates Immunity in Gilthead Sea Bream (Sparus aurata). Front. Mar. Sci. 9:857592. doi: 10.3389/fmars.2022.857592

INTRODUCTION

Although it is generally accepted that aquaculture can relieve pressure on wild fisheries by producing alternative fish for human consumption, the production of those fish often requires inputs from wild fish stocks in the form of feed ingredients. The paradox stems from the diversity of farmed species and husbandry systems (Stevens et al., 2018). Regardless of the remarkable

advances in aquafeed formulation, most feeds used in aquaculture still rely on fishmeal (FM) and fish oil derived from fisheries. However, there is increasing pressure on these raw materials and conventional alternative plant protein (PP) sources due to growing demands from a variety of users. For instance, the use of soy protein and other plant products in aquaculture reduces their availability for direct human consumption (Ytrestøyl et al., 2015). Under this scenario, the sustainability and competitiveness of aquaculture may depend on their replacement with alternative ingredients ranging from plant-based meal and plant oil to terrestrial by-products and microbial ingredients (Naylor et al., 2021). Thus, the mantra about the economic and environmental sustainability of the aquaculture industry based on the identification and use of alternative raw ingredients to the classical ones is still valid.

Microbial products, particularly yeast, are potential sustainable ingredients in aquafeeds due to the ability to convert low-value non-food biomass from forestry, and agricultural and food technology industries into high-value feed ingredients with limited dependence on arable land, water, and changing climatic conditions (Øverland and Skrede, 2017; Agboola et al., 2021). Among them, Brewer's spent yeast (SDY), a by-product of the brewing industry, is a rich source of B vitamins, proteins (45–60%) and other compounds like β-glucans and mannoproteins (Jaeger et al., 2020; Agboola et al., 2021). Therefore, BSY is mainly utilized in animal feed formulations as a low-cost source of protein (Agboola et al., 2021; Estévez et al., 2021; Nazzaro et al., 2021) even though it may be also used as an ingredient in functional feeds due to its potential health benefits to the host (Jaeger et al., 2020). Under this scenario, Estévez et al. (2021) have shown that the replacement of FM and plant protein sources by SDY in the feed of a carnivorous marine teleost fish species, the gilthead sea bream (*Sparus aurata*), resulted in a better growth, as well as a higher protein digestibility and feed efficiency indexes than in their congeners fed a reference diet. The above-mentioned results showed that this by-product from the brewing industry is a promising feed ingredient in aquafeeds, especially when considering its use in terms of an alternative plant protein source, but also when considering its associated sustainable and environmental benefits. Those benefits can be achieved by means of a circular economy strategy, which helps in providing added value to the brewing industry through wastes' reuse and, consequently, reducing the industry carbon footprint (Kerby and Vriesekoop, 2017; Jaeger et al., 2020).

Once validated the use of SDY as an alternative protein source in aquafeeds (Estévez et al., 2021), the present study aimed to evaluate at the transcriptomic level the impact of SDY in the head kidney, intestine, and liver of *S. aurata*. This marine species is the most important Mediterranean aquaculture fish species in terms of volume and economic value (FAO, 2020). Regarding the selected target tissues, the head kidney was chosen as it is one of the most important organs in fish due to its role in endocrine and hematopoietic functions, as well as a major secondary lymphoid organ (Tort, 2011). Furthermore, the liver was selected as the central metabolic organ of the body with a predominant role in the intermediary metabolism, with important functions in lipid storage and digestive and detoxification functions (Bruslé and González, 1996). Finally, the intestine was chosen due to its involvement in important physiological digestive functions, being the primary site of food digestion and nutrient uptake, whereas it is also reputed for providing support to gut microbiota and maintaining an effective barrier defense against pathogens and tolerance toward dietary antigens (Salinas and Parra, 2015; Wang et al., 2018). Thus, a proper understanding of the effects of this new alternative ingredient at systemic and local levels as well as its mode of action upon the selected target tissues would support its proper use as an ingredient for sustainable aquafeeds.

MATERIALS AND METHODS

Experimental Diets

Two experimental diets (**Table 1**) were designed in order to evaluate the impact of brewer's SDY on gilthead sea bream growth and feed efficiency performance (Estévez et al., 2021), as well as evaluate global transcriptome changes in selected tissues (head kidney, anterior-mid intestine, and liver). A control diet was formulated containing 15% of FM and 46.7% of a blend of PP sources, whereas lipids were mainly provided by fish oil (7.9%), soybean oil (6.7%), and lecithin (2.0%). The inclusion of Brewer's SDY in the experimental diet resulted in a reduction in FM (10%) and PP (31.4%) contents (**Table 1**). This alternative protein source and level of dietary inclusion were selected according to previous results (Estévez et al., 2021).

Experimental diets (pellet size: 4 mm) were manufactured by IRTA (Mas de Bover, El Morell, Spain). The main ingredients were ground in a hammer mill (Rosal VRE-40, Barcelona, Spain).

TABLE 1 | List of ingredients and proximal composition of experimental diets used for evaluating the inclusion of Brewer's spent dry yeast (SDY) in compound diets for gilthead sea bream (*Sparus aurata*).

Ingredient (%)	Control diet	SDY diet
Fishmeal (Super prime 70 LT)	15.00	10.00
Wheat gluten	25.00	17.84
Soybean meal (Soycomeal P)	16.70	8.54
Soybean meal low protein	5.00	5.00
Wheat starch	19.44	11.26
Fish oil	7.92	7.31
Soybean oil	6.72	6.40
Soybean lecithin	2.00	2.00
Phosphate	1.09	0.92
DL-methionine	0.07	0.07
Choline	0.23	0.26
Lysine HCl	0.43	-
Vitamin premix	0.10	0.10
Mineral mix	0.10	0.10
Brewer's spent dry yeast (SDY)	-	30.0
Proximate composition		
Crude protein,% dry weight	43.0	40.4
Crude fat,% dry weight	16.9	16.4

Powder ingredients and oils were then mixed according to the target formulation in a 500 L Rosal Mixer, with a double horizontal ribbon, for 6 min. Both diets were manufactured by temperature-controlled extrusion (90°C at the conditioner and 120°C at the screw) by means of an extruder press (Rosal RS-50). Upon extrusion, feed batches were dried and cooled by a hot air aspiration system (MABRIK S.A., Barbera del Vallés, Spain).

Fish and Experimental Design

Samples of target tissues used for transcriptomic analyses were taken from a previous trial in which we evaluated different by-products from the brewer's industry as potencial feed ingredients in gilthead sea bream (Estévez et al., 2021). In brief, juveniles (age1 +) were obtained from a commercial fish farm (Albadalejo, San Pedro del Pinatar, Murcia, Spain) and transported by road to IRTA-Sant Carles de la Ràpita research facilities (REGA number: ES431360036277; Sant Carles de la Ràpita, Tarragona, Spain). Fish were kept under quarantine conditions and fed a commercial diet for 2 weeks. Then, fish (N = 90) were gently anesthetized (tricaine methanesulfonate, MS-222, 150 mg/L), individually weighed for initial body weight (BWi) and distributed among 6 experimental 200 L tanks (3 replicate tanks per dietary group; n = 15 fish per tank; initial stocking density = 13–14 kg m^{-3}) as described in Estévez et al. (2021). The mean BWi values for fish from the control and SDY diets were 114.8 ± 16.7 g and 112.7 ± 14.8 g (mean ± standard deviation), respectively. Both diets were tested in triplicate for 60 days. Fish were fed at an initial feeding ration of 1.8% of the stocked biomass by means of two daily meals (08:00 and 14:00 h). During the nutritional trial, water temperature (pH meter 507; Crison Instruments, Barcelona, Spain), salinity (MASTER-20T; ATAGO Co., Ltd., Tokyo, Japan), and dissolved oxygen (OXI330; Crison Instruments) were 20.1 ± 3.0°C, 35.1 ± 0.1 ‰, and 6.6 ± 0.6 mg L^{-1} (mean ± SD), respectively. Water flow rate in experimental tanks was maintained at approximately 9.0 L min^{-1} (open-flow system), which guaranteed one full tank's water renewal per hour. Photoperiod followed natural changes according to the season of the year (September –November; 40°37′41″ N).

At the end of the trial, all fish were netted, anaesthetized with MS-222 (150 mg/L), and individually weighted for their final body weight (BWf). In addition, three fish per tank (9 fish per dietary condition) were sacrificed with an overdose of MS222, and their head kidney, liver, and mid-anterior-mid dissected for further transcriptomic analyses. In particular, sampled tissues (200 mg) were preserved in RNAlater® (Sigma-Aldrich, Spain) overnight, and then stored at −80°C until further RNA extraction.

RNA Sequencing and Data Analysis

Total RNA from each tissue was extracted from five randomly chosen specimens using the TRIzol reagent (Invitrogen, San Diego, CA, United States) following the manufacturer's instructions; the other samples from the remaining three fish were kept as backup. Then, total RNA concentration was quantified using a NanoDrop ND-2000 (Thermo Fisher Scientific, Waltham, United States), and RNA integrity and quality checked with Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, United States). Samples were shipped in dry ice to Nord University (Bodø, Norway) for further RNA sequencing. At their arrival, RNA integrity was checked again on an Agilent 4150 TapeStation system (Agilent Technologies) in order to confirm that the RNA quality of the samples did not deteriorate during shipment.

For library preparation, RNA concentration was calculated using the Qubit RNA high-sensitivity assay on a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltman, United States). RNA-Seq libraries were prepared using the NEBNext ultra II directional RNA library preparation kit with a poly(A) mRNA magnetic isolation module (NEB #E7490) in accordance with the manufacturer's protocol (New England BioLabs Inc., Ipswich, United Kingdom). Briefly, after poly(A) enrichment of 1 μg total RNA, mRNA was fragmented to about 100–200 nt in length and used for 1st and 2nd strand cDNA synthesis. After A-tailing, end repair, and adaptor ligation, the second strand was selectively removed using uracil-specific excision reagent (USER® II Enzyme; New England BioLabs Inc.). The resulting directional RNA-seq libraries were amplified with 8 PCR cycles and posteriorly purified using AMPure XP beads (Beckman Coulter, Inc., Brea, United States). Quality and quantity of the RNA-seq libraries were assessed using the Agilent 2200 TapeStation. Unique indices were assigned to each sample and libraries were pooled at equimolar concentrations. Sequencing was performed at Nord University (Norway) using two high output flowcells (75 cycles) on the NextSeq500 (Illumina, San Diego United States) with 4% PhiX. Library sequencing yielded 764 million 75 bp single-end reads RNA-Seq libraries.

Raw sequences were adapter-trimmed and quality checked using Trim galore v0.4.4 (Babraham Bioinformatics, United Kingdom) with the following trimming parameters: -phred33, -fastqc, and -q20. Alignments were performed using the hisat2 v2.1.0 platform with default parameters and the latest *S. aurata* genome assembly (GCA_900880675.2; NCBI, Bethesda, United States). Count-based matrices were created for each sample using featureCounts v1.5.3 and they were then combined in R using the plyr and dplyr packages. Differential expression analysis was performed using DESeq2 (Anders and Huber, 2010) and the final list of differentially expressed genes (DEGs) was filtered accordingly (adjusted P-value < 0.05) and absolute $\log_2$ fold change (FC) of 1.0. Gene set enrichment analysis was performed for down- and up-regulated DEGs separately, using g:GOSt functional profiling on gProfiler (version e104_eg51_p15_3922dba) with default parameters (Raudvere et al., 2019). The selected organism was gilthead sea bream (*Sparus aurata*) and the tailor-made algorithm g:SCS was used for multiple testing correction. Heatmaps were prepared using the R package "pheatmap." Transcriptomic raw data for samples analyzed in the current study are available through the public repository Gene Expression Omnibus (GEO) at the US National Centre for Biotechnology Information (NCBI) (accession number GSE194206).

Ethics Statement

All animal experimental procedures complied with the Guiding Principles for Biomedical Research Involving Animals (EU2010/63), the guidelines of the Spanish laws (law 32/2007

and RD 1201/2015) and were authorized by the Ethical Committee of the Institute for Research and Technology in Food and Agriculture (IRTA, Spain) for the use of laboratory animals (E-10/2020).

RESULTS

Results on growth performance, feed efficiency, and diet digestibility are presented elsewhere (Estévez et al., 2021). In brief, no differences in growth performance were found among both experimental groups with BWf average values comprised between 176.1 and 179.0 g. Regarding feed efficiency variables, gilthead sea bream fed the SDY-supplemented diet showed better feed conversion ratio (FCR = 2.0 ± 0.05) and protein efficiency rate (PER = 1.1 ± 0.03) than their congeners fed the control diet (FCR = 2.1 ± 0.29; PER = 1.0 ± 0.11) ($P < 0.05$).

RNA Sequencing

On average, we obtained 19.4 million raw reads per library. After quality and adapter trimming, 99.8% of the sequenced data were retained. The alignment efficiency of uniquely mapped reads was 90.3, 89.5, and 89.8% for head kidney, liver, and intestine, respectively. The rest of the reads (∼10%) were excluded from further analysis, either as aligned reads with multiple matches or reads that failed to align to the reference transcriptome (**Figure 1** and **Supplementary Table 1**). In total, 218 DEGs were identified among all tissues, out of which, 141 were up- and 77 down-regulated ($q < 0.05$; $-1 > \log_2$ (FC) > 1; **Supplementary Table 2**). These DEGs had a tissue-specific profile and, therefore, we investigated their functionality for each tissue separately.

The Immunomodulatory and Steroidogenic Effects of Spent Dry Yeast on the Head Kidney

Head kidney was the tissue with the highest number of DEGs (99; $q < 0.05$; $-1 > \log_2$ (FC) > 1) when compared to the other target tissues analyzed (**Supplementary Table 2**). In particular, 62 DEGs (62.6%) were up-regulated and 37 DEGs (37.4%) were down-regulated in the head kidney of fish fed the SDY diet compared to the control group (**Figure 2A**). The principal component analysis (PCA) showed no distinction between groups in the first component (PC1), which explained 90% of the variation, although a slight distinction was present in the second component (PC2) that explained a rather small fraction of the variation (4%) (**Supplementary Figure 1**). Among the top 10 up-regulated genes in head kidney ($\log_2$ (FC) > 6.7), we identified 6 genes associated with steroidogenesis (*nr5a1a*, *bicc2*, *hsd3b1*, *cyp11b1*, *cyp21a2*, *cyp17a1*), and others related to chemokine activity (*ccl17*, *cxc11,* and *cxc6*). These findings were also supported by the functional enrichment analysis (**Supplementary Table 3**; $q < 0.05$). Furthermore, the gene ontology annotation of all up-regulated genes showed an enrichment of molecular functions related to chemokine activity (GO:0008009) and chemokine receptor binding (GO:0042379), as well as to biological processes linked to steroid metabolic (GO:0008202) and biosynthetic (GO:0006694) processes, and glucocorticoid metabolism (GO:0008211). Additionally, the oxidoreductase (GO:0016705) and monooxygenase activity (GO:0004497) processes were also identified.

Down-regulated genes showed an enrichment in oxygen carrier activity and at the cellular component level an enrichment in hemoglobin complex (**Supplementary Table 3**, $q < 0.05$). Genes associated with these functions coded for hemoglobin subunits alpha-A and beta (*hbaa* and *hbb*), which are involved in oxygen transport, with the latter being a potential regulator of inflammation. In addition, one of the most down-regulated genes was the lipoxygenase homology PLAT domains 1b (*loxhd1b*), which is also involved in heme binding and oxygen binding affinity. Among the down-regulated genes ($\log_2$(FC) < -5.24), we identified the carcinoembryonic antigen-related cell adhesion molecule 6 (*ceacam6*) responsible for cell–cell adhesion and neutrophil adhesion to cytokine-activated endothelial cells, as well as the *ceacam2* gene, which is involved in energy homeostasis and negative regulation of feeding behavior.

Spent Dry Yeast Affects Genes Involved in Liver Metabolism, Growth and Immunity

In the liver, we identified 75 DEGs, of which 52 were up-regulated (69.3%) and 23 down-regulated (30.7%) in fish fed the SDY diet (**Figure 2B**; $q < 0.05$; $-1 < \log_2$ (FC) < 1). The PCA revealed a distinction of the two diets within the PC1, which explained 22% of the total variance (**Supplementary Figure 2**). Up-regulated genes (*psat1*, *phgdh*, *wars1*, *hars1*; **Supplementary Table 2**, $q < 0.05$) were primarily associated with metabolic and biosynthetic processes of L-serine and cellular amino acids, as well as organic carboxylic and oxoacids. Among up-regulated genes, we also identified *socs3* and *cish* that are involved in the regulation of inflammatory responses and growth *via* the regulation of the JAK-STAT receptor signaling pathway (**Supplementary Table 4**, $q < 0.05$).

Down-regulated genes were enriched for metal ion, cation, and zinc ion binding molecular functions (**Supplementary Table 4**, $q < 0.05$). The E3 ubiquitin-protein ligase TRIM21-like gene (*trim21*) was down-regulated in fish fed the SDY diet compared to the control group [$\log_2$(FC) = −3.9]. Similarly, among the most down-regulated genes, we identified the NACHT, LRR, and PYD domains-containing protein 12-like (*nlrp12*) and protein mono-ADP-ribosyltransferase PARP14-like (*parp14*), which are also involved in immunity through the regulation of NF-kappaβ signaling and tyrosine phosphorylation of STAT proteins, respectively.

Intestinal Host Immunity and Digestion Are Affected by the Spent Dry Yeast Diet at a Molecular Level

The tissue with the least DEGs, among the studied ones, was the anterior-mid intestine. Out of 44 DEGs, 27 were up- and 17 down-regulated in fish fed the SDY diet compared to the control group, respectively (**Figure 2C**; $q < 0.05$; $-1 > \log_2$ (FC) > 1). PCA did not reveal any distinct groupings between groups across both components, which only explained 54% of the total variation

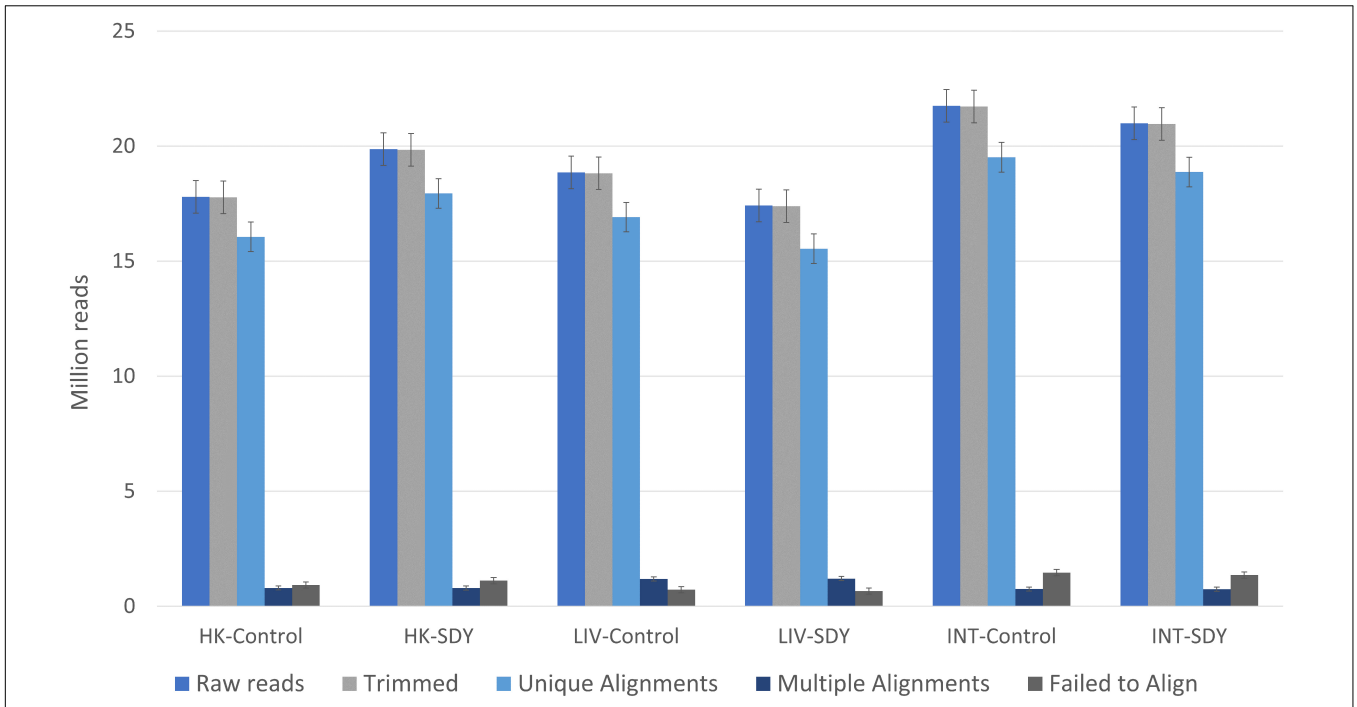


FIGURE 1 | Number of reads obtained by RNA-seq (raw reads, trimmed reads, unique alignment reads, multiple alignment reads, and reads that failed to align) for each of the targeted tissues (head kidney, liver, and anterior-mid intestine) of gilthead sea bream (*Sparus aurata*) in response to the control (Control) and the Brewer's SDY diets. The error bar represents the standard deviation of the mean values obtained for each tissue (n = 5 replicates). HK, head kidney; LIV, liver; INT, anterior-mid intestine.

in the intestine (**Supplementary Figure 3**). Interestingly, up-regulated genes were enriched for lysozyme (GO:0003796), peptidoglycan muralytic (GO:0061783), and hydrolase activities (GO:0004553; GO:0016798), all of them being highly relevant to tissue-specific functions such as intestinal host defense (**Supplementary Table 5**). Similarly, among the most down-regulated DEGs, we identified a gene linked to fatty acid oxidation (*hao2*) as well as the retinoic acid receptor beta (*rarb*) and the major histocompatibility complex class I-related gene protein (*hla*), which are involved in intestinal immunity (**Supplementary Table 5**).

DISCUSSION

In this study, we decided to explore the dietary effects of Brewer's SDY at transcriptomic level by means of RNA-seq on three different tissues selected by their relevant body functions. Regarding the head kidney, RNA-seq results showed that most of DEGs in this hematopoietic and lymphoid tissue of gilthead sea bream fed the SDY were related to increased transcription levels of genes related to chemokine activity (GO:0008009) and chemokine receptor binding (GO:0042379). This is of special relevance since chemokines are involved in the initiation and regulation of the inflammatory response. In particular, the production of pro-inflammatory chemokines (i.e., CCL2, CCL3, CCL5, CXCL1, CXCL2, and CXCL8) is generally induced to recruit immune cells to the infection site, while chemokines considered as homeostatic (i.e., CCL19 and CCL21) are involved in controlling the migration of cells during normal processes of tissue maintenance or development; thus, initiating and regulating the inflammatory response (Laing and Secombes, 2004; Sakai et al., 2021). Under present experimental conditions, we found a transcriptional regulation of *ccl17*, *cxc6, cxc11* in the head kidney of fish fed the SDY-supplemented diet. The above-mentioned chemokines are reputed for being involved in the activation (*cxc6*) and recruitment (*ccl17*, *cxc11*) of T lymphocytes. These results are in agreement with the lymphoid function of this tissue, which may be translated into an enhanced host's immune response as similar results have been observed in fish fed functional diets supplemented with zootechnical feed additives with immunomodulatory properties (Martin and Król, 2017; Hoseinifar et al., 2019; Salomón et al., 2021). The potential immunomodulatory function of the SDY diet may be attributed to its content in β-glucan (81 g/kg feed). In this sense, β-glucans are well-known in aquaculture because they promote hematopoiesis and enhance immunity (Meena et al., 2013).

Under the present experimental conditions, we also found changes in expression of genes involved in steroid synthesis and secretion (GO:0008202, GO:0006694). Among DEGs involved in steroidogenesis, *nr5a1a* was found to be up-regulated in the head kidney of fish fed the SDY-supplemented diet. This gene is expressed in the interrenal gland, gonads, and hypothalamus (Chai et al., 2003). In teleosts, the interrenal gland is embedded in the head kidney and is the major site of

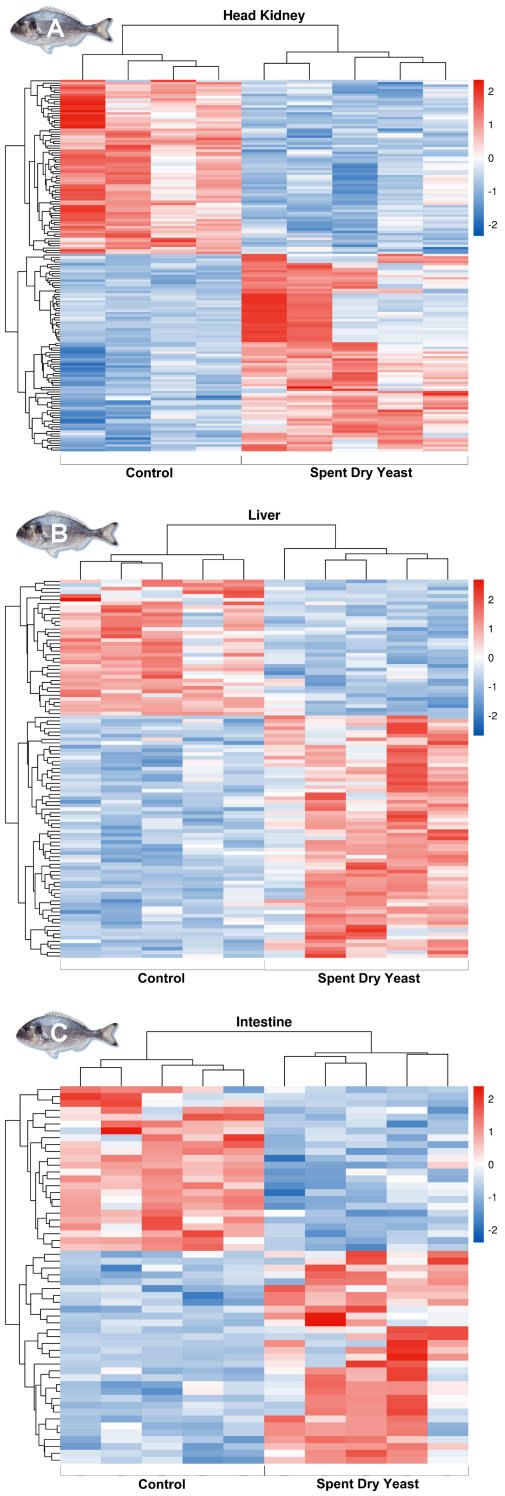


FIGURE 2 | Hierarchical clustering of transcriptomic response from the head kidney **(A)**, liver **(B)**, and anterior-mid intestine **(C)** of gilthead seabream (*Sparus aurata*) fed the control and the Brewer's SDY diets. This heat map is based on similitude patterns of the differentially expressed genes from five replicates per tissue both dietary groups with the only exception of the head kidney where one replicate from the control group was excluded from the analysis as shown in **Supplementary Figure 1** (results from the Principal Component Analysis).

steroid synthesis in response to signals from the pituitary and hypothalamus (Milano et al., 1997). Similarly, cytochrome P450 11b (*cyp11b*), steroid 21-hydroxylase (*cyp21a2*), hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase (*hsd3b1*), and steroid 17-alpha-hydroxylase/17,20 lyase-like (LOC115589985) were also differentially expressed, which is of special relevance since those genes are involved in the biosynthesis of steroid hormones like cortisol through their precursors (Mommsen et al., 1999; Li et al., 2003). These results are of special importance, since glucocorticoids are synthesized by interrenal cells of the head kidney and regulate osmolarity, metabolism, and immune responses (Vazzana et al., 2010). Furthermore, the up-regulation of bicaudal C homolog 2 (*bicc2*) that is involved in renal gluconeogenesis might show that gilthead sea bream fed the SDY-supplemented diet may have a more effective stress response, as data from genes involved in steroidogenesis also indicated.

Surprisingly, the head kidney of gilthead sea bream fed the SDY-supplemented diet showed a down-regulation of hemoglobin subunits alpha-A and beta genes (*hbaa* and *hbb*), which encoded for α and β subunits of hemoglobin. In addition, we also found a down-regulation of the lipoxygenase homology PLAT domains 1b (*loxhd1b*—LOC115592348), which is also involved in heme binding and oxygen binding affinity. Under hypoxic conditions, changes in transcription levels of *hbaa* and *hbb* in the head kidney of fish have been associated to changes in erythropoiesis and hemoglobin synthesis (Xia et al., 2016). Furthermore, changes in gene expression of *hbaa* and *hbb* are responsible for hemoglobinopathies in humans (Sabath, 2017). We found these results puzzling, since the nutritional trial was conducted under normoxic conditions (> 85% O_2 saturation) and we did not find any sign of distress in gilthead sea bream fed the SDY-supplemented diet caused by a putative anemia. Further research is needed to clarify the physiological effect of SDY on hemoglobin homeostasis and erythropoiesis in order to clarify current results.

Among the three studied tissues, the liver was the second one with a larger number of DEGs. In particular, DEGs were involved metabolic and biosynthetic processes (*psat1*, *phgdh*, *wars1*, *hars1*). These findings are in agreement with those found in the head kidney, which indicated that fish fed the SDY diet had a more biologically active organ, since the above-mentioned DEGs are involved in protein transcription and biosynthesis (Nguyen et al., 2020; Yu et al., 2021). In addition to the classical role of *wars1* in protein synthesis, this gene has been recently described in mammals to be activated upon infection, whereas its protein acts as an endogenous ligand of toll-like receptors (TLR) 2 and TLR4 (Nguyen et al., 2020).

The potential immunomodulatory properties of the SDY found in the head kidney seemed to be also supported by the up-regulation of genes involved in modulating the inflammatory responses in the liver *via* the regulation of the JAK-STAT receptor signaling pathway as transcriptomic data suggested. Specifically, by the up-regulation in the liver of cytokine-inducible SH containing protein (*cish*) and the suppressor of cytokine signaling-like (*socs*) in comparison to the control group. These genes are members of the suppressor cytokine signaling family whose proteins are involved in the control

and attenuation of cytokine-induced responses through the JAK/STAT signal transduction pathway, playing an important role in the regulation of host's immunity and the inflammatory response, as well as in cell proliferation, migration and survival (Yoshimura, 2009; Yoshimura et al., 2012; Maehr et al., 2014). Furthermore, the down-regulation of the NACHT, LRR, and PYD domains-containing protein 12-like (*nlrp12*) might suggest an enhancement of the NF-κB pathway, since these proteins have a modulatory role on the NF-κB pathway (Normand et al., 2018). This is of special relevance since the NF-kB pathway plays an important role in the regulation of several cytokines, chemokines, antimicrobial peptides, and interferon-stimulated genes, as well as being crucial in modulating the survival, activation, and differentiation of innate and adaptive immune cells (Sun, 2017). Although these results might be attributed to a potential inflammatory response derived from the inclusion of SDY in the diet, we did not find inflammatory signs in the intestinal mucosa of fish fed the SDY diet (**Supplementary Figure 4**); thus, the transcriptional regulation of the NF-kB pathway may be attributed to the immunomodulatory properties of the SDY. Furthermore, the E3 ubiquitin-protein ligase TRIM21-like gene (*trim21*) was also down-regulated in the liver of fish fed the SDY diet. This gene is generally over-expressed under prolonged activation of the immune system, being also described as a negative regulator of antioxidative stress (Lee, 2017). Similarly, protein mono-ADP-ribosyltransferase PARP14-like (*parp14*) was also among the most down-regulated genes. This gene modulates the humoral immune response through the activation of the JAK/STAT pathway (Schweiker et al., 2018), which suggests a balanced immune response in gilthead sea bream fed the SDY diet as other authors have reported when there is a sort of immune homeostasis based on an equilibrium between pro- and anti-inflammatory processes (Salomón et al., 2020, 2021; Firmino et al., 2021).

The transcriptomic response evaluated in the anterior-mid intestine was in agreement with results found in the two former tissues from gilthead sea bream fed the SDY diet. In particular, four g-type lysozyme genes were up-regulated (*LOC115585860, LOC115582400, LOC115582423,* and *LOC115585885*), which were related to the biological process linked to peptidoglycan and aminoglycan metabolic, and catabolic processes in which g lysozyme is involved (Jiménez-Cantizano et al., 2008). In this sense, lysozyme cleaves peptidoglycans of the bacterial cell wall and participates in the activation of the complement system and phagocytes. In addition, lysozymes have also been reported to have antiviral and anti-inflammatory functions, thus playing an important function at both systemic and local immune responses (Saurabh and Sahoo, 2008). Changes in the number of transcripts of the retinoic acid receptor beta (*rarb*) were not attributed to different dietary vitamin A contents between both experimental diets, since yeast does not contain this lipophilic vitamin. Thus, changes in *rarb* expression might be associated to the regulation of the intestinal immune condition in fish fed the SDY diet (Gattu et al., 2019). Similarly, we also found the major histocompatibility complex class I-related gene protein (*hla*) to be down-regulated, which may be associated with a reduction in the immunogenicity of dietary SDY and modulation of the immune response in the intestine (Groh et al., 1996). Although we did not find inflammatory signs in the intestinal mucosa of fish fed the SDY diet (**Supplementary Figure 4**), the expression profile of immune-related genes in fish fed the SDY diet indicated that further research may be focused on evaluating the effect of SDY on the mucosal-associated lymphoid tissue in the intestine and its immunocompetence under disease conditions.

Furthermore, the inclusion of SDY in the diet down-regulated the expression of hydroxyacid oxidase 2 (*hao2*) in the anterior-mid intestine of gilthead sea bream, which might be attributed to a dietary regulation of this gene. Hydroxyacid oxidase 2 is a peroxisomal 2-hydroxy acid oxidase responsible for the oxidation of 2-hydroxy fatty acids like 2-hydroxypalmitic acid, which is an intermediate in phytosphingosine metabolic pathway, an important component of sphingolipids found in yeasts and plants (Pruett et al., 2008).

CONCLUSION

Under present experimental conditions, the partial replacement of conventional dietary protein sources by spent dry yeast (SDY) obtained as a by-product of the brewer's industry had a modulatory effect on several processes related to host's immunity, oxygen's carrier capacity, steroidogenesis, metabolism, and digestion. Results related to the enhancement of the immune condition at systemic and local levels in fish fed the SDY diet are of special relevance, since they indicated that this ingredient in addition to being considered as a sustainable ingredient for replacing conventional marine and terrestrial protein sources in aquafeeds, it may also be considered as a functional one due to its immunomodulatory properties derived from its content in β-glucans. Although the results found in the present study seemed really promising in terms of the functional properties of the tested SDY, further research needs to be focused on clarifying the effect of this ingredient on the biological processes involved in oxygen transport in order to guarantee the safety of this ingredient for fish. The overall results of this study coupled with nutritional studies on this ingredient (Estévez et al., 2021; Nazzaro et al., 2021) indicated that the use of brewery's by-products like SDY in aquafeeds supports a circular bioeconomy model that reuses, recovers, and recycles resources instead of producing wastes.

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/geo/, accession ID: GSE194206.

ETHICS STATEMENT

This animal study was reviewed and approved by the Ethical Committee of the Institute for Research and Technology in Food and Agriculture (IRTA, Spain) for the use of laboratory animals (E-10/2020).

AUTHOR CONTRIBUTIONS

AE, DS, and BI: conceptualization. EG, JPF, and JMOF: methodology. JPF, IK, and AE: sample processing. IK, JPF, and JMOF: formal analysis. EG and JMOF: resources. IK and EG: writing the original draft. JPF, IK, and JMOF: data visualization and curation. EG and JMOF: supervision. EG, AE, and DS: project administration. AE and DS: funding acquisition. All authors contributed to the article and approved the submitted version.

FUNDING

This study has been conducted under the Life BREWERY project (LIFE16ENV/ES/000160) that was funded by LIFE European Environment and Resource Efficiency Program, which was the EU's financial instrument supporting environmental, nature conservation, and climate action projects throughout the EU, and partially funded by the Ministerio de Economía, Industria y Competitividad (Government of Spain) through the grant AGL2018-095653-R-I00 to IRTA. The implementation, updating, and development of EU environmental and climate policy and legislation by co-financing projects with European added value are among its main priorities. JPF has received funding from the Erasmus + Programme of the European Union, and the Autonomous University of Barcelona (UAB) Exchange Programme Traineeship 2020–2021 for conducting a research stage at Nord University (Bodø, Norway). AR was supported by a predoctoral grant (reference number: PRE2019-091259) linked to the project RTI2018-095653-RI00 (Ministerio de Ciencia e Innovación, Spain).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.857592/full#supplementary-material

REFERENCES

Agboola, J. O., Øverland, M., Skrede, A., and Hansen, J. Ø. (2021). Yeast as major protein-rich ingredient in aquafeeds: a review of the implications for aquaculture production. *Rev. Aquac.* 13, 949–970. doi: 10.1111/raq.12507

Anders, S., and Huber, W. (2010). Differential expression analysis for sequence count data. *Gen. Biol.* 10:R106. doi: 10.1186/gb-2010-11-10-r106

Bruslé, J., and González, G. (1996). "The structure and function of fish liver," in *Fish Morphology*, eds J. S. D. Munshi and H. M. Dutta (New Delhi: Science Publishers Inc).

Chai, C., Liu, Y. W., and Chan, W. K. (2003). Ff1b is required for the development of steroidogenic component of the zebrafish interrenal organ. *Dev. Biol.* 260, 226–244. doi: 10.1016/S0012-1606(03)00219-7

Estévez, A., Padrell, L., Iñarra, B., Orive, M., and Martin, D. S. (2021). Brewery by-products (yeast and spent grain) as protein sources in gilthead sea bream (*Sparus aurata*) feeds. *Aquaculture* 543:736921. doi: 10.1016/j.aquaculture.2021.736921

FAO (2020). *The State of World Fisheries and Aquaculture 2020. FAO Fisheries and Aquaculture Department*. Rome: The Food and Agriculture Organization of the United Nations.

Firmino, J. P., Vallejos-Vidal, E., Balebona, M. C., Ramayo-Caldas, Y., Cerezo, I., Salomón, R., et al. (2021). 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

Gattu, S., Bang, Y. J., Pendse, M., Dende, C., Chara, A. L., Harris, T. A., et al. (2019). Epithelial retinoic acid receptor β regulates serum amyloid a expression and vitamin a-dependent intestinal immunity. *Proc. Natl. Acad. Sci. U.S.A.* 116, 10911–10916. doi: 10.1073/pnas.1812069116

Groh, V., Bahram, S., Bauer, S., Herman, A., Beauchamp, M., and Spies, T. (1996). Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. *Proc. Natl. Acad. Sci. U.S.A.* 93, 12445–12450. doi: 10.1073/pnas.93.22.12445

Hoseinifar, S. H., Sohrabi, A., Paknejad, H., Jafari, V., Paolucci, M., and Van Doan, H. (2019). Enrichment of common carp (*Cyprinus carpio*) fingerlings diet with *Psidium guajava*: the effects on cutaneous mucosal and serum immune parameters and immune related genes expression. *Fish Shellfish Immunol.* 86, 688–694. doi: 10.1016/j.fsi.2018.12.001

Jaeger, A., Arendt, E. K., Zannini, E., and Sahin, A. W. (2020). Brewer's Spent Yeast (BSY), an underutilized brewing by-product. *Fermentation* 6:123. doi: 10.3390/fermentation6040123

Jiménez-Cantizano, R. M., Infante, C., Martin-Antonio, B., Ponce, M., Hachero, I., Navas, J. I., et al. (2008). Molecular characterization, phylogeny, and expression of c-type and g-type lysozymes in brill (*Scophthalmus rhombus*). *Fish Shellfish Immunol.* 25, 57–65. doi: 10.1016/j.fsi.2007.12.009

Kerby, C., and Vriesekoop, F. (2017). An overview of the utilisation of brewery by-products as generated by British craft breweries. *Beverages* 3:24. doi: 10.3390/beverages3020024

Laing, K. J., and Secombes, C. J. (2004). Chemokines. *Dev. Comp. Immunol.* 28, 443–460. doi: 10.1016/j.dci.2003.09.006

Lee, A. Y. (2017). A review of the role and clinical utility of anti-Ro52/TRIM21 in systemic autoimmunity. *Rheumatol. Int.* 37, 1323–1333. doi: 10.1007/s00296-017-3718-1

Li, Y. Y., Inoue, K., and Takei, Y. (2003). Interrenal steroid 21-hydroxylase in eels: primary structure, progesterone-specific activity and enhanced expression by ACTH. *J. Mol. Endocrinol.* 31, 327–340. doi: 10.1677/jme.0.0310327

Maehr, T., Vecino, J. L. G., Wadsworth, S., Wang, T., and Secombes, C. J. (2014). Four CISH paralogues are present in rainbow trout *Oncorhynchus mykiss*: differential expression and modulation during immune responses and development. *Mol. Immunol.* 62, 186–198. doi: 10.1016/j.molimm.2014.06.021

Martin, S. A., and Król, E. (2017). Nutrigenomics and immune function in fish: new insights from omics technologies. *Dev. Comp. Immunol.* 75, 86–98. doi: 10.1016/j.dci.2017.02.024

Meena, D. K., Das, P., Kumar, S., Mandal, S. C., Prusty, A. K., Singh, S. K., et al. (2013). Beta-glucan: an ideal immunostimulant in aquaculture (a review). *Fish Physiol. Biochem.* 39, 431–457. doi: 10.1007/s10695-012-9710-5

Milano, E. G., Basari, F., and Chimenti, C. (1997). Adrenocortical and adrenomedullary homologs in eight species of adult and developing teleosts: morphology, histology, and immunohistochemistry. *Gen. Comp. Endocrinol.* 108, 483–496. doi: 10.1006/gcen.1997.7005

Mommsen, T. P., Vijayan, M. M., and Moon, T. W. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Rev. Fish Biol. Fish.* 9, 211–268. doi: 10.1210/en.2004-0644

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

Nazzaro, J., San Martin, D., Perez-Vendrell, A. M., Padrell, L., Iñarra, B., Orive, M., et al. (2021). Apparent digestibility coefficients of brewer's by-products used in feeds for rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*). *Aquaculture* 530:735796. doi: 10.1016/j.aquaculture.2020.735796

Nguyen, T. T., Yoon, H. K., Kim, Y. T., Choi, Y. H., Lee, W. K., and Jin, M. (2020). Tryptophanyl-tRNA synthetase 1 signals activate TREM-1 *via* TLR2 and TLR4. *Biomolecules* 10:1283. doi: 10.3390/biom10091283

Normand, S., Waldschmitt, N., Neerincx, A., Martinez-Torres, R. J., Chauvin, C., Couturier-Maillard, A., et al. (2018). Proteasomal degradation of NOD2 by NLRP12 in monocytes promotes bacterial tolerance and colonization by enteropathogens. *Nat. Commun.* 9:5338. doi: 10.1038/s41467-018-07750-5

Øverland, M., and Skrede, A. (2017). Yeast derived from lignocellulosic biomass as a sustainable feed resource for use in aquaculture. *J. Sci. Food Agric.* 97, 733–742. doi: 10.1002/jsfa.8007

Pruett, S. T., Bushnewv, A., Hagedorn, K., Adiga, M., Haynes, C. A., Sullards, M. C., et al. (2008). Biodiversity of sphingoid bases ('sphingosines') and related amino alcohols. *J. Lipid Res.* 49, 1621–1639. doi: 10.1194/jlr.R800012-JLR200

Raudvere, U., Kolberg, L., Kuzmin, I., Arak, T., Adler, P., Peterson, H., et al. (2019). g:Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). *Nucleic Acids Res.* 47, W191–W198. doi: 10.1093/nar/gkz369

Sabath, D. E. (2017). Molecular diagnosis of thalassemias and hemoglobinopathies: an ACLPS critical review. *Am. J. Clin. Pathol.* 148, 6–15. doi: 10.1093/ajcp/aqx047

Sakai, M., Hikima, J. I., and Kono, T. (2021). Fish cytokines: current research and applications. *Fish. Sci.* 87, 1–9. doi: 10.1007/s12562-020-01476-4

Salinas, I., and Parra, D. (2015). "Fish mucosal immunity: intestine," in *Mucosal Health in Aquaculture*, eds B. J. Beck and E. Peatman (Cambridge, MA: Academic Press), 135–170. doi: 10.1016/b978-0-12-417186-2.00006-6

Salomón, R., Firmino, J. P., Reyes-López, F. E., Andree, K. B., González-Silvera, D., Esteban, M. A., 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., Furones, M. D., Reyes-López, F. E., Tort, L., Firmino, J. P., Esteban, M. A., et al. (2021). 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

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

Schweiker, S. S., Tauber, A. L., Sherry, M. E., and Levonis, S. M. (2018). Structure, function and inhibition of poly (ADP-ribose) polymerase, member 14 (PARP14). *Mini Rev. Med. Chem.* 18, 1659–1669. doi: 10.2174/1389557518666180816111749

Stevens, J. R., Newton, R. W., Tlusty, M., and Little, D. C. (2018). The rise of aquaculture by-products: increasing food production, value, and sustainability through strategic utilisation. *Mar. Policy* 90, 115–124. doi: 10.1016/j.marpol.2017.12.027

Sun, S. C. (2017). The non-canonical NF-kappaB pathway in immunity and inflammation. *Nat. Rev. Immunol.* 17, 545–558. doi: 10.1038/nri.2017.52

Tort, L. (2011). Stress and immune modulation in fish. *Dev. Comp. Immunol.* 35, 1366–1375. doi: 10.1016/j.dci.2011.07.002

Vazzana, M., Vizzini, A., Sanfratello, M. A., Celi, M., Salerno, G., and Parrinello, N. (2010). Differential expression of two glucocorticoid receptors in seabass (teleost fish) head kidney after exogeneous cortisol inoculation. *Comp. Biochem. Physiol.* 157A, 49–54. doi: 10.1016/j.cbpa.2010.05.003

Wang, A. R., Ran, C., Ringø, E., and Zhou, Z. G. (2018). Progress in fish gastrointestinal microbiota research. *Rev. Aquac.* 10, 626–640. doi: 10.1111/raq.12191

Xia, M., Chao, Y., Jia, J., Li, C., Kong, Q., Zhao, Y., et al. (2016). Changes of hemoglobin expression in response to hypoxia in a Tibetan schizothoracine fish, *Schizopygopsis pylzovi*. *J. Comp. Physiol.* 186B, 1033–1043. doi: 10.1007/s00360-016-1013-1

Yoshimura, A. (2009). Regulation of cytokine signalling by the SOCS and Spred family proteins. *Keio J. Med.* 58, 73–83. doi: 10.2302/kjm.58.73

Yoshimura, M., Suzuki, R., Sakaguchi, T., Hanada, H., and Yasukawa, H. (2012). SOCS, inflammation, and autoimmunity. *Front. Immunol.* 3:20. doi: 10.3389/fimmu.2012.00020

Ytrestøyl, T., Aas, T. S., and Asgard, T. (2015). Utilisation of feed resources in production of Atlantic salmon (*Salmo salar*) in Norway. *Aquaculture* 448, 365–374. doi: 10.1016/j.aqrep.2019.100216

Yu, Y. C., Han, J. M., and Kim, S. (2021). Aminoacyl-tRNA synthetases and amino acid signaling. *Biochim. Biophys. Acta Mol. Cell Res.* 1868:118889. doi: 10.1016/j.bbamcr.2020.118889

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

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

frontiers | Frontiers in Physiology

ORIGINAL RESEARCH
published: 26 May 2022
doi: 10.3389/fphys.2022.797625

An Eco-Friendly Conversion of Aquaculture Suspended Solid Wastes Into High-Quality Fish Food by Improving Poly-β-Hydroxybutyrate Production

Guo Qiao[1†], Xiaoxia Li[1,2†], Jun Li[3], Mingming Zhang[1], Yang Shen[1], Zhigang Zhao[4], Yichan Zhang[1], Zhitao Qi[1], Peng Chen[1], Yuyu Sun[1], Pingping Cang[1], Peng Liu[5], Eakapol Wangkahart[6] and Zisheng Wang[1]**

[1]Yancheng Institute of Technology, Yancheng, China, [2]School of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang, China, [3]Department of Biological Sciences, Biomolecular Sciences Institute, Florida International University, Miami, FL, United States, [4]Heilongjiang Provincial Key Laboratory of Cold Water Fish Germplasm Resources and Aquaculture, Heilongjiang River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Harbin, China, [5]Yantai Marine Economic Research Institute, Yantai, China, [6]Laboratory of Fish Immunology and Nutrigenomics, Applied Animal and Aquatic Sciences Research Unit, Division of Fisheries, Faculty of Technology, Mahasarakham University, Maha Sarakham, Thailand

The aquaculture industry is vital in providing a valuable protein food source for humans, but generates a huge amount of solid and dissolved wastes that pose great risks to the environment and aquaculture sustainability. Suspended solids (in short SS), one of the aquaculture wastes, are very difficult to be treated due to their high organic contents. The bioconversion from wastewater, food effluents, and activated sludge into poly-β-hydroxybutyrate (PHB) is a sustainable alternative to generate an additional income and could be highly attractive to the agricultural and environmental management firms. However, little is known about its potential application in aquaculture wastes. In the present study, we first determined that 7.2% of SS was PHB. Then, the production of PHB was increased two-fold by the optimal fermentation conditions of wheat bran and microbial cocktails at a C/N ratio of 12. Also, the PHB-enriched SS showed a higher total ammonia nitrogen removal rate. Importantly, we further demonstrated that the PHB-enriched SS as a feed could promote fish growth and up-regulate the expression of the immune-related genes. Our study developed an eco-friendly and simple approach to transforming problematic SS wastes into PHB-enriched high-quality food for omnivorous fish, which will increase the usage efficiency of SS and provide a cheaper diet for aquatic animals.

Keywords: aquaculture solid wastes, nitrogenous compounds, biopolymer, poly-β-hydroxybutyrate (PHB), accumulation optimization, fish food

OPEN ACCESS

Edited by:
Jin Niu,
Sun Yat-sen University, China

Reviewed by:
Burcu Omuzbuken,
Dokuz Eylül University, Turkey
Omid Safari,
Ferdowsi University of Mashhad, Iran
Jitendra Kumar Sundaray,
Indian Council of Agricultural Research, India

***Correspondence:**
Zhitao Qi
qizhitao@ycit.edu.cn
Zisheng Wang
wzs399@126.com

†These authors have contributed equally to this work

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

Received: *19 October 2021*
Accepted: *15 April 2022*
Published: *26 May 2022*

Citation:
Qiao G, Li X, Li J, Zhang M, Shen Y, Zhao Z, Zhang Y, Qi Z, Chen P, Sun Y, Cang P, Liu P, Wangkahart E and Wang Z (2022) An Eco-Friendly Conversion of Aquaculture Suspended Solid Wastes Into High-Quality Fish Food by Improving Poly-β-Hydroxybutyrate Production. Front. Physiol. 13:797625. doi: 10.3389/fphys.2022.797625

1 INTRODUCTION

The aquaculture industry provides human society with a nutritional food source and high proteins, vitamins, and macro minerals (Henriksson et al., 2018; Han et al., 2019; Heiderscheidt et al., 2020). During the rapid development of the aquaculture industry, two problems have become increasingly apparent: 1) feeding is expensive and 2) a large amount of waste and discharge is generated. The

aquaculture waste, containing high nitrogen and phosphorus, poses enormous environmental risks. In Japan, for example, one ton of pond-raised aqua-fish can generate 0.1 kg phosphorus and 0.8 kg nitrogen which equals to the daily waste produced by 73 people (Bangar et al., 2017). Therefore, aquaculture waste has raised the concerns of environmental scientists and challenges the sustainable development of the aquaculture (Ahmed et al., 2019; Han et al., 2019). Many countries have established standards for wastewater discharge, and strictly require that aquaculture wastes can be emitted into the environment only within the allowable range.

The byproducts of feeding and excretion, such as unconsumed food, undigested components, feces, chemicals, and untapped inputs, are the sources of aquaculture wastes (Kokou & Fountoulaki, 2018; Becke et al., 2020; Gibson et al., 2020). The wastes from aquaculture can be divided into solid wastes and suspended wastes, both contain high organic contents (Olusegun et al., 2016; Gao et al., 2020; Hesni et al., 2020; Naughton et al., 2020; Rosa et al., 2020; Sun et al., 2020). The solid wastes include the settled solids sunk to the bottom of the pond and the suspended solids (in short SS) floating in the culture water. In the traditional aquaculture system, the SS are the majority of waste products, and are challenging to be removed due to their high organic contents (Badiola et al., 2012; Becke et al., 2020). However, the SS must be properly managed to avoid the pollution to the surrounding environment and reduce the harm to aquatic animals (Holm-Nielsen et al., 2009; Kiani et al., 2020). Notably, SS can be fermented by bacteria to remove toxic organic contents for resource recycling (Ge and Champagne, 2017; Das et al., 2018; Amadu et al., 2021). These fast-growing microbes will change the toxic ammonia (NH_3) and nitrite (NO_2^-) into non-toxic nitrate (NO_3^-) (Lananan et al., 2014; Rommozzi et al., 2020). These ammonium ions and NH_3 are produced during aquaculture (Karri et al., 2018; Yun et al., 2019).

The treatments about promoting microbial growth have been successfully applied in wastewater treatment (Ge et al., 2014; Qiu et al., 2020a; Xu M. et al., 2020; Xu X. et al., 2020). It can remove nitrogen and phosphorus from the aquatic environment (Qiu et al., 2020b; Xu M. et al., 2020; Xu X. et al., 2020). It also promotes microbial growth to produce valuable energy alternatives such as biofuel, natural antioxidants, food additives, or biopolymers (Meixner et al., 2017; Ge et al., 2018; Jochum et al., 2018; Qiu et al., 2020b). Among these alternatives, popular food additives and biopolymers such as poly-β-hydroxybutyrate (PHB) or polyhydroxyalkanoates (PHAs) are the most significant compounds that have been studied (Amadu et al., 2021). PHB or PHAs are not soluble in water. As intracellular energy storage, they form "inclusion bodies" within the cytoplasm in prokaryotic organisms. The chain of PHB is shorter than that of PHAs (Carpine et al., 2018). PHB is biodegradable, biocompatible, and nontoxic (Hrabak, 1992; Arun et al., 2006; Ben Rebah et al., 2007). A considerable effort has been made to produce PHB or PHAs using various wastes, such as municipal wastewater (Chua et al., 2003), sugar cane molasses (Albuquerque et al., 2010), paper mill wastewater (Bengtsson et al., 2008), and food waste (Venkateswar Reddy et al., 2015). However, transforming aquaculture wastes into PHAs or PHB has not been well-investigated (Krasaesueb et al., 2019).

The biofloc technology is a new strategy to reduce the total ammonia nitrogen (TAN) effectively through heterotrophic microbiota by adjusting the C/N ratio in the culture water via external carbon addition (Avnimelech, 1999; Zhang et al., 2018). It was reported that SS in the biofloc system contains 15–20% PHB (Schryver & Verstraete, 2009; Ruan et al., 2011). The commercial PHB has been used as a dietary supplement to improve the growth, immunity, and disease resistance of aquatic animals (Deng et al., 2014; Defoirdt et al., 2018; Meirong et al., 2018; Qiao et al., 2018, 2020). However, the high prices for commercial PHB and water-insolubility greatly hinder its application in aquaculture.

In this study, we analyzed the PHB content in SS waste from a traditional aquaculture system, and tried to increase the PHB production by adjusting the carbon source, various C/N ratios, and bacterial strains. Furthermore, we evaluated the TAN removal during the PHB-enrichment process, analyzed the effects of PHB-enriched SS on the growth, and innate immunity of an omnivorous fish—gibel carp (*Carassius auratus gibelio*). This study will provide a good low-cost method to treat and recycle aquaculture solid wastes, and present a potential high-quality fish food and convenient method for PHB application in aquaculture.

2 MATERIALS AND METHODS

2.1 Suspended Solid Collection and Analysis

Three indoor concrete tanks cultured gibel carps (40.33 ± 5.12 g) at the Yancheng Institute of Technology were randomly selected to acquire SS. Gibel carp was fed with commercial diets (Tongwei Feeding Company, China) to satiation three times (6:30 to 7:00 a.m., 1:30 p.m. to 2:00 p.m., and 7:00 p.m. to 7:30 p.m.) each day under a 12D/12L cycle. The diets contained 32.25% crude protein, 5.90% crude lipid, 1.18% calcium, and 1.23% total phosphorus. Water temperature was maintained at 25.0 ± 2.0°C and dissolved oxygen (DO) kept higher than 5 mg L^{-1}. SS were sampled *in situ* using the Imhoff cone, that is, culture water from three tanks was withdrawn from the middle of tank into the Imhoff cone. Then, SS were collected from the bottom of the Imhoff cone (Zhang et al., 2018). The obtained SS were used to analyze the basic characterization according to the standard method (APHA, 2012; Krasaesueb et al., 2019), including pH, temperature, concentration of nitrate (NO_3^-–N), nitrite (NO_2^-–N), ammonium (NH_4^+–N), phosphate (PO_4^{3-}–P), and total phosphorus. Finally, we removed water- and acetone-soluble materials with the following steps: 1) collecting pellets by centrifugation of the SS at 8,000 rpm for 10 min, 2) resuspending the pellet in acetone (2V), 3) collecting pellets again by centrifugation of the SS at 8,000 rpm for 10 min, 4) washing with sterile distilled water, and 5) freeze-drying the pellets to check the PHB content as described below.

2.2 Poly-β-Hydroxybutyrate Content and Characterization in Suspended Solids

2.2.1 Crude Poly-β-Hydroxybutyrate Extraction

The PHB was extracted according to the procedure described by Ruan et al. (2011) with some modifications. In brief, the freeze-dried

SS were dissolved in 10% NaClO and disrupted by ultrasonic for 8 min (intermittent 5 s, working 5 s, 4°C), and the precipitate was obtained by centrifugation at 8,000 rpm for 15 min at room temperature. Then, the pellet was washed twice with acetone and sterile distilled water to remove lipids by centrifugation at 8,000 rpm for 10 min. Finally, the crude PHB was obtained after the pellet dried at 50°C for 8 h in an oven.

2.2.2 Poly-β-Hydroxybutyrate Content Analysis

The crude PHB was diluted with chloroform at 1:40 (dry weight of SS:volume of chloroform, g:mL) at 40°C for 11 h, and the pellet was collected by centrifugation, dried at 70°C for 8 h in the oven. Then, the PHB extract was diluted with chloroform at 80°C using a rotary evaporator (Eyela N-1000), and dried to a constant mass. A thin film of pure PHB was obtained and quantified using spectrometric analysis based on the standard curve (Law & Slepecky, 1961). The purity of PHB was analyzed by gas chromatography (GC–MS, Agilent 7890B/5977A) (Albuquerque et al., 2010; Alsafadi & Al-Mashaqbeh, 2017).

2.2.3 Poly-β-Hydroxybutyrate Characterization

The structural and material properties of the pure PHB were compared to a commercial PHB (Aldrich, Sigma). The surface morphology and elements of the pure PHB were observed by the scanning electron microscopy (SEM–EDS). The Fourier-transform infrared (FT-IR) spectra of the pure PHB extract were detected in the range of 400–4,000 cm^{-1} using a spectrophotometer (FT-IR Thermo is 10) (Naumann et al., 1991; Venkateswar Reddy et al., 2015).

2.3 Bacterial Community Analysis With Poly-β-Hydroxybutyrate Accumulation in Suspended Solids

2.3.1 DNA Extraction and PCR Amplification

Since PHB is synthesized by bacteria and the yield correlates with bacterial diversity closely (Crab et al., 2012; Qiao et al., 2018; Amadu et al., 2021), the bacterial community in SS was investigated by high-throughput sequencing using a MiSeq sequencing platform. In brief, the quadruple SS from each tank were sampled, immediately frozen in liquid nitrogen, and then transferred to −80°C for DNA extraction. Microbial DNA from the SS was extracted using the E.Z.N.A.® soil DNA kit (OMEGA, United States) following the manufacturer's protocol. PCR was used to amplify the V4–V5 region through primers 907R (5′-CCGTCAATTCMTTTRAGTT T-3′) and 515F (5′-barcode-GTGCCAGCMGCCGCGG-3′) (Qiao et al., 2019b). PCR products were purified using the AxyPrep DNA gel extraction kit (Axygen Biosciences, Union City, CA, United States).

2.3.2 Illumina MiSeq Sequencing

The purified PCR products were first quantified using Qubit®3.0 (Life Invitrogen). About 24 amplicons with different barcodes were mixed equally, and the pooled products were chosen to construct an Illumina Paired–End library according to the Illumina's genomic DNA library preparation procedure. Finally, the amplicon library was paired-end sequenced (2 × 250) on the MiSeq sequencing platform (Illumina, United States) according to the standard protocols (Sangon Biotech (Shanghai) Co., Ltd.).

2.3.3 Bioinformatics Analysis

The bioinformatics analysis from Illumina sequencing was constructed using the methods described previously (Qiao et al., 2019b). In brief, QIIME (version 1.17) was used to de-multiplex and quality-filter the raw FASTQ files, and any unassembled reads were discarded. Bacterial operational taxonomic units (OTUs) were generated using the uclust function in QIIME (http://qiime.org/scripts/pick.outs.html). The Shannon index calculated by mothur (version v.1.30.1) was analyzed to illustrate α-diversity. The OTUs were mapped to a gg13.5 database by QIIME's command "pick_ closed_otus" at 97% similarity. The OTU abundance was automatically normalized using 16S rRNA gene copy numbers from known bacterial genomes in Integrated Microbial Genomes.

2.4 Accumulation Optimization of Poly-β-Hydroxybutyrate in Suspended Solids

PHB as the carbon and energy reserves is stored in bacterial cells at the appropriate C/N ratio. PHB production is related to the C/N ratio, carbon sources, and bacterial community (Crab et al., 2012; Qiao et al., 2018; Amadu et al., 2021). Based on the most practical usage of carbon sources and probiotics, and the C/N ratio in aquaculture (Laranja et al., 2018), we designed various C/N ratios, carbon sources, and additional bacteria to study parameters that affect the yield of PHB. The detailed protocols are: 1) SS were collected, 2) then diluted into sterilized freshwater, 3) different C/N ratios (12, 16, and 20) using molasses as the sole carbon resource were set to illustrate the effect of the C/N ratio on PHB accumulation, 4) different carbon sources (molasses, starch, wheat bran, and corn meal) and bacteria (*Bacillus subtilis* SY01, *Pseudomonas putida* CGMCC15104 and *Lactobacillus*, and a cocktail of *Pse. putida* and *Lactobacillus*) were separately added based on the total ammonia concentration by adapting the C/N ratio of 12 each day for 7 days, and 5) PHB accumulation was evaluated. *Pse. putida* CGMCC15104 and *Lactobacillus* were obtained as described previously (Xu, 2019), and *B. subtilis* SY01 and *Pse. putida* were isolated from SS in our laboratory (Xu, 2019). During the SS treatment, the usage dose of carbon sources was calculated according to the TAN concentration and kept the C/N ratio of 12. The carbon sources were diluted with water and sprinkled in the water twice every 12 h within 1 day. The final bacterial concentration of addition was 1.0×10^7 CFU mL^{-1}. All the experimental groups were conducted in triplicates.

The relative production rate of PHB was calculated according to **Eq. 1**.

$$\text{Relative production rate (RPR, \%)} = \frac{\text{PR in different treated groups}}{\text{PR in untreated SS group}} \times 100. \quad (1)$$

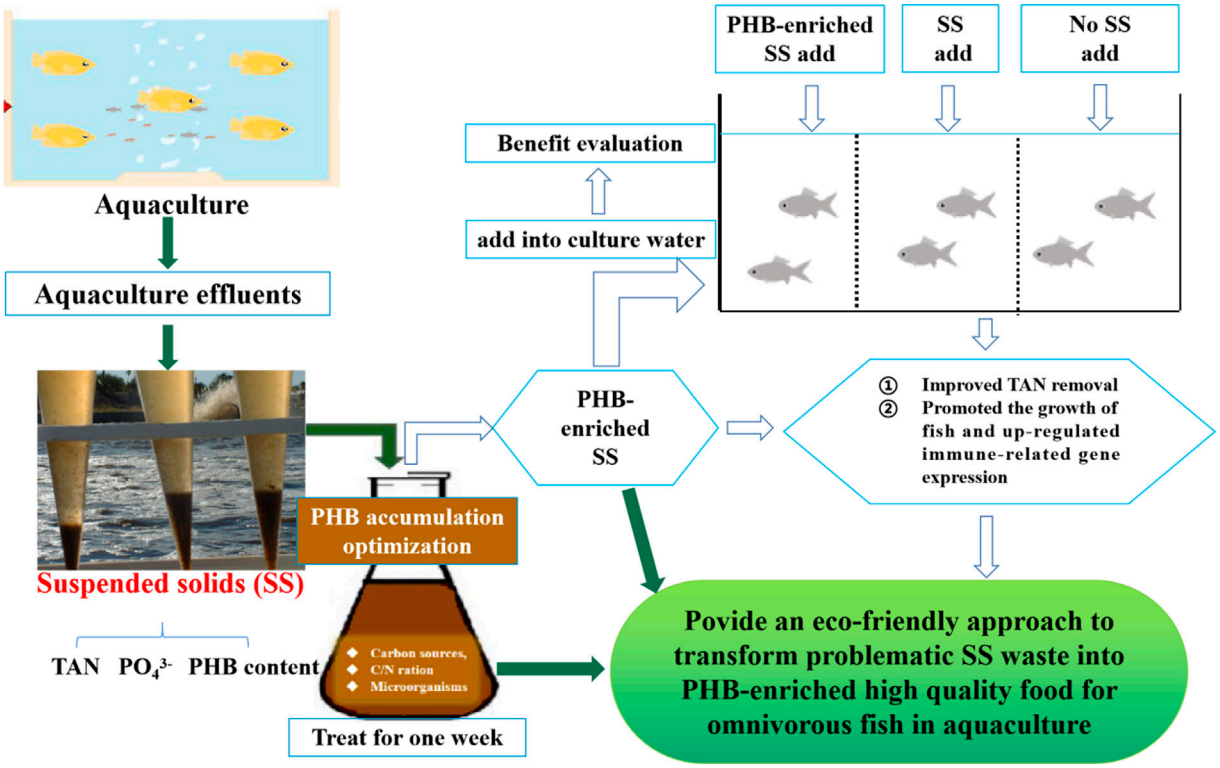


FIGURE 1 | Graphical representation of the aquaculture suspended solid (SS) treatment process by adding carbons considering PHB accumulation.

2.5 Benefit Evaluation of Poly-β-Hydroxybutyrate-Enriched Suspended Solid Treatment in the Aquaculture System

2.5.1 Poly-β-Hydroxybutyrate-Enriched Suspended Solid Treatment

Based on the results from part 2.4 (**Figure 6**), PHB-enriched SS were obtained by adjusting the C/N ratio, carbon source, and probiotics addition. In brief, SS were randomly collected from three indoor concrete tanks and three outdoor ponds, which cultured fish and shrimp (**Table 2**). Then, the collected SS were diluted in sterilized freshwater at 1:10 (dry weight of SS:volume of freshwater). These SS were treated and kept at a C/N ratio of 12 by the addition of wheat bran twice every 12 h within 1 day under 24 h of continuous aeration for 7 days at pH 8.0 and temperature 25°C. Meanwhile, the cocktail of *Pseudomonas* and *Lactobacillus* was added at day 1 and day 3.

2.5.2 Effects of Suspended Solid Treatment on the Removal Rate of Total Ammonia Nitrogen

At day 0 and day 7, the TAN concentration in the supernatant was measured according to the indophenol method of Koroleff (1976). The removal percentage of TAN from SS solutions was calculated according to **Eq. 2**.

$$Removal\ (\%) = \frac{(Ic - Fc)}{Ic \times 100} \quad (2)$$

where *Ic* is the initial concentration (mg L^{-1}) of TAN (day 0), and *Fc* is the final concentration (mg L^{-1}) (day 7).

2.5.3 The Nutritional Composition of Suspended Solids and Their Effects on the Growth Performance of Gibel Carp

2.5.3.1 Nutritional Composition Analysis of Suspended Solids

In order to test whether SS could be up-taken by fish, and determine the composition of SS that affects fish production, we first detected the nutritional composition of SS. The nutritional composition was analyzed as our previous description (Zhang et al., 2018). In brief, basal SS were collected using the Imhoff cone and dried in an oven at 105°C to constant weight or freeze-dried to analyze the proximate composition, including the crude protein, lipid, ash, and total amino acid content (AOAC, 1995). The PHB content in the PHB-enriched SS and untreated SS was analyzed as described in **Section 2.2.2**.

2.5.3.2 Effects of Suspended Solids on the Growth Performance of Gibel Carp

Gibel carp is a main ornamental freshwater culture species, and it can uptake SS in water as food sources (Zhang et al., 2018). Thus, in this study, gibel carp was chosen as a representative ornamental species to evaluate the potential usage of treated SS in aquaculture. Gibel carp (mean body weight of 12.03 g) was bought from a fish farm at Dafeng, Jiangsu province. Prior to the

TABLE 1 | Characterization of basal suspended solids (SS) collected from aquaculture system.

Parameters	Initial SS from indoor concrete tank (gibel carp)	Unit
pH	8.12±0.05	-
Temperature	27.20±0.26	°C
Phosphate ($PO_4^{3-}P$)	9.45±1.04	mg L^{-1}
Nitrate ($NO_3^{-}N$)	14.57±0.91	mg L^{-1}
Nitrite ($NO_2^{-}N$)	1.88±0.03	mg L^{-1}
Ammonium ($NH_4^{+}N$)	3.15±0.48	mg L^{-1}
Total ammonia nitrogen (TAN)	19.60±1.37	mg L^{-1}
Total phosphorus (TP)	11.10±0.40	mg L^{-1}
PHB content	7.27±0.91	% (w/w dry weight)

PHB, poly-β-hydroxybutyrate.

experiments, fish were acclimated for 2 weeks in a tank with continuous aeration at water pH of 7.4–8.2, and temperature of 25–27°C under a 12D/12L cycle. Fish were fed with a commercial diet (Tongwei, China) three times daily at 3% of their body weight, and 30% of water was exchanged. After acclimation, 30 individuals were sampled and separately weighed. Other fish were then randomly distributed into the experimental tanks.

Three groups were set, including the PHB-enriched SS addition (SSA), untreated SS addition (USSA), and no SS addition (NSSA) groups (**Figure 1**). The PHB content in PHB-enriched SS and untreated SS was 17.34 ± 4.76% and 7.06 ± 1.76%, respectively (**Supplementary Table S2**). Each group was housed in quadruplicate plastic tanks (size: 60 cm × 50 cm × 40 cm) for 30 days, and each tank included 20 fish. None of the fish in these three groups were fed with a commercial diet in order to test whether SS could be up-taken by fish, and further ensure positive effects of PHB in SS, that is, treated SS could potentially be used as feed. Total suspended solid (TSS) concentration is a good indicator for evaluating suspended solids. Based on the previous study (Zhang et al., 2018), the optimal TSS concentration for gibel carp was 600–800 mg L^{-1}. Thus, TSS concentration in PHB-enriched SSA and USSA groups of the present study was kept at 600 mg L^{-1} through mixing aerated freshwater and stock SS water as previously described by Zhang et al. (2018), and TSS concentration was set at 10 mg L^{-1} (close to TSS concentration in natural freshwater) in the NSSA group by daily water-exchange. The TSS concentration was determined according to the Standard Methods for Examination of Water and Wastewater (APHA, 2012). During 30 days of feeding experiment, all fish were cultured at pH of 7.4–8.2, and a temperature of 25 ± 2°C under a 12D/12L cycle. The dead individuals would be cleaned up immediately.

At 30 days, all the fish from each group were weighed to calculate the growth parameters, including weight gain (WGR), specific growth rate (SGR), and thermal growth coefficient (TGC) according to **Eqs 3–5**.

$$\mathrm{WGR}\ (\%) = \frac{100 \times [\mathrm{FW}(\mathrm{g}) - \mathrm{IW}(\mathrm{g})]}{FW}, \quad (3)$$

$$\mathrm{SGR}(\%\ \mathrm{day}-1) = \frac{100 \times (\mathrm{LnFW} - \mathrm{LnIW})}{\mathrm{time}\ (\mathrm{days})}, \quad (4)$$

$$\mathrm{TGC} = \left[\left(\sqrt[3]{\mathrm{FW}} - \sqrt[3]{\mathrm{IW}} \right) / \mathrm{Txt} \right] \times 1000, \quad (5)$$

where FW and IW are the final and initial body weight, respectively, T is duration of the experiment in days, and t is the mean daily water temperature.

2.5.3.3 Effects of Suspended Solid Treatment on the Immune-Related Gene Expression of Gibel Carp

At day 30, five fish were sampled from each group, and anesthetized with tricaine methane sulfonate (MS-222) at 200 mg L^{-1} for immune-related gene transcription analysis. The spleen and gill (50–100 mg each tissue) were sampled, immediately immersed in 500 μL RNAiso Plus (Sigma), and stored at −80°C until total RNA extraction. RNeasy mini kit (Qiagen, Valencia, CA, United States) was used to extract total RNA (Zhang et al., 2017; Qiao et al., 2019a), and the quantity and purity of RNA were analyzed using a Nanodrop ND-1000 spectrophotometer. First-strand cDNA synthesis was conducted using the PrimeScript™ first-strand cDNA Synthesis kit (Takara Bio, Dalian, China) and the $\mathrm{Oligo}^{\mathrm{dT}}$Primer. Six immune-related genes, including heat shock protein 70 (*hsp70*), tyrosine-protein kinase (*JAK*), interleukin-11 (*IL-11*), serine/threonine-protein kinase mTOR (*mTOR*), phosphatidylinositol 3-kinase regulatory subunit alpha (*PIK3R1*), and intelectin (*ITLN*), were selected to evaluate the different treatment of SS on the immune-related gene expression of gibel carp. The gene-specific primer list is shown in **Supplementary Table S1**, and quantitative real time PCR (qRT-PCR) performance was conducted as described in our previous publications (Zhang et al., 2018; Qiao et al., 2020). The relative transcriptional levels of different genes were determined using the following formula: ΔCt = Ct (target) - Ct (internal). The relative fold changes of a specific gene in fish from the PHB-enriched SSA and USSA groups were compared to those from the NSSA group using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

2.6 Data Processing and Statistical Analysis

All data are represented as mean ± standard deviation from triplicated or quadruple samples. Statistical analysis was conducted by Duncan's multiple range test using one-way ANOVA with SPSS software (version 24.0). The significant difference was set at p value less than 0.05.

3 RESULTS

3.1 Characterization of Suspended Solids

The pH of the collected SS was 8.12 ± 0.05, and the temperature was 27.20 ± 0.26°C. The concentrations of NO_3^-–N, NO_2^-–N, NH_4^+–N, TAN, PO_4^{3-}–P, and TP in SS were 14.57 ± 0.91 mg L^{-1}, 1.88 ± 0.03 mg L^{-1}, 3.15 ± 0.48 mg L^{-1}, 19.6 ± 1.37 mg L^{-1}, 9.45 ± 1.04 mg L^{-1}, and 11.10 ± 0.40 mg L^{-1}, respectively. The content of PHB in SS was 7.27 ± 0.91% (w/w dry weight) (**Table 1**). Apparently, nitrogen and phosphorus are the dominant elements in SS.

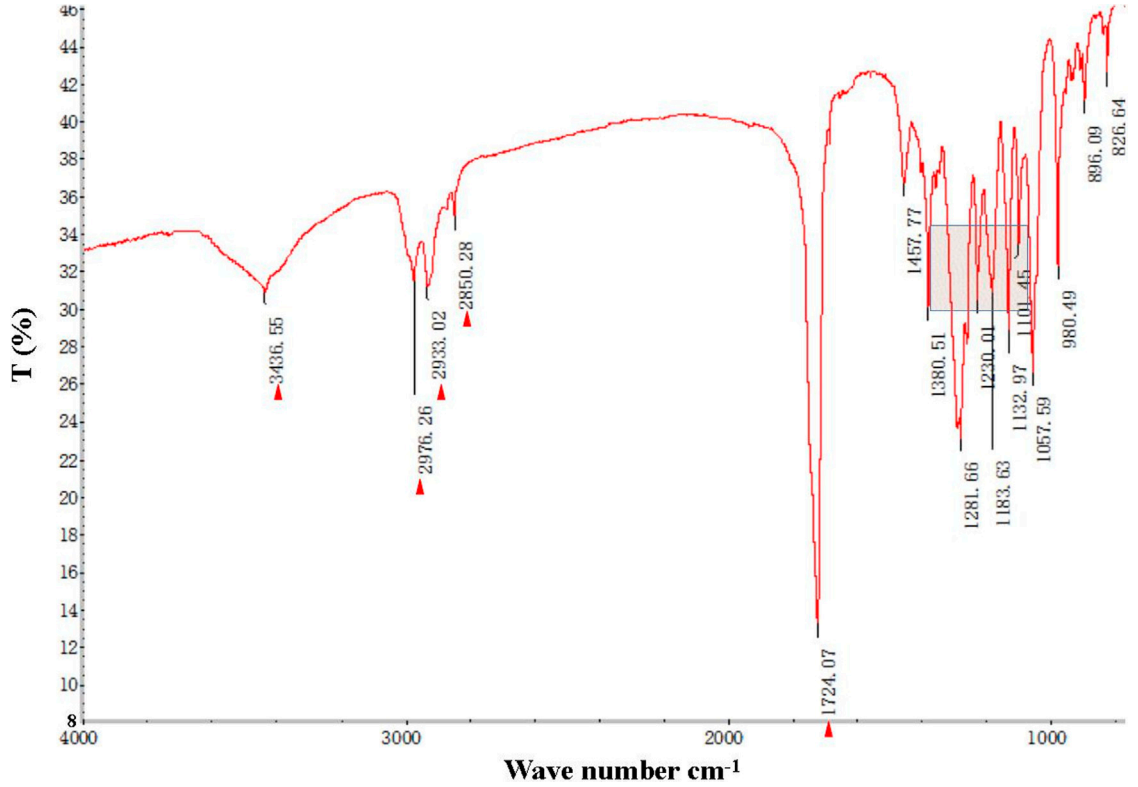


FIGURE 2 | Fourier transform infrared (FT-IR) spectra of PHB extract from SS. Red triangle and box with blue line mean bands of PHB.

3.2 Polymer Structure and Material Properties of Poly-β-Hydroxybutyrate in Suspended Solids

The FT-IR spectra of the extracted PHB were measured to confirm the practical structure (**Figure 2**). The band at 1724 cm^{-1} corresponded to the stretching of the C=O bond, whereas a series of intense bands located at 980–1,457 cm^{-1} corresponded to the stretching of the C–O bond of the ester group. The methylene C–H stretching vibration near 2,933 cm^{-1} was also observed. The presence of absorption bands at 1724 cm^{-1} and 1,281 cm^{-1} in the extracted PHB sample were characteristics of C=O and C–O stretching groups, and identical to standard PHB. GC–MS analysis showed that the retention time of the extracted PHB sample was 2.214 min (**Figure 3**). SEM–EDS analysis showed that the content of elements C and O in the PHB extract from SS was 63.56% of carbon and 35.93% of oxygen, respectively (**Figure 4**). These data confirmed that our extract PHB is the same as the standard PHB.

3.3 Bacterial Diversity Related to Poly-β-Hydroxybutyrate Accumulation in Suspended Solids

We analyzed the bacterial diversity in SS (**Supplementary Figure S1**). The plateau OTU rarefaction curve after the similarity cutoff of 97% indicated that the sequencing data were reasonable. Furthermore, the Shannon index and relative abundance suggested that the sequences obtained can represent most bacteria in each sample.

The results of OTU analysis using 16S rRNA gene copy numbers from known bacterial genomes showed that the relative abundance of ɤ-Proteobacteria, β-Proteobacteria, α-Proteobacteria, Saprospirae, Flavobacteria, Deltaproteobacteria, Cytophagia, and Fusobacteria was 24%, 21%, 19%, 7%, 7%, 2%, 2%, and 1%, respectively, at the class level (**Figure 5A**). At the family and genus levels, the relative abundance of *Comamonas*, Xanthomonadaceae_unclassified, *Acinetobacter*, Saprospiraceae_unclassified, Xanthobacteraceae_unclassified, *Wautersiella*, *Pseudomonas*, and Myxococcales_unclassified were 9%, 6%, 5%, 5%, 5%, 4%, 3%, and 3%, respectively (**Figure 5B**). Thus, *Comamonas*, *Acinetobacter*, and *Pseudomonas* are the most dominant bacteria related to PHB production in SS.

3.4 Poly-β-Hydroxybutyrate Accumulation Optimization

To improve PHB production, the effects of cultivation conditions (C/N ratio, carbon sources, and probiotic addition) during the treatment process were studied. The results showed that more PHB could be produced in SS at a C/N ratio of 12 with wheat bran as the carbon source, and the bacterial addition of a cocktail of *Pseudomonas* and *Lactobacillus*, through which the relative production rate could be improved by ~200% (**Figure 6**).

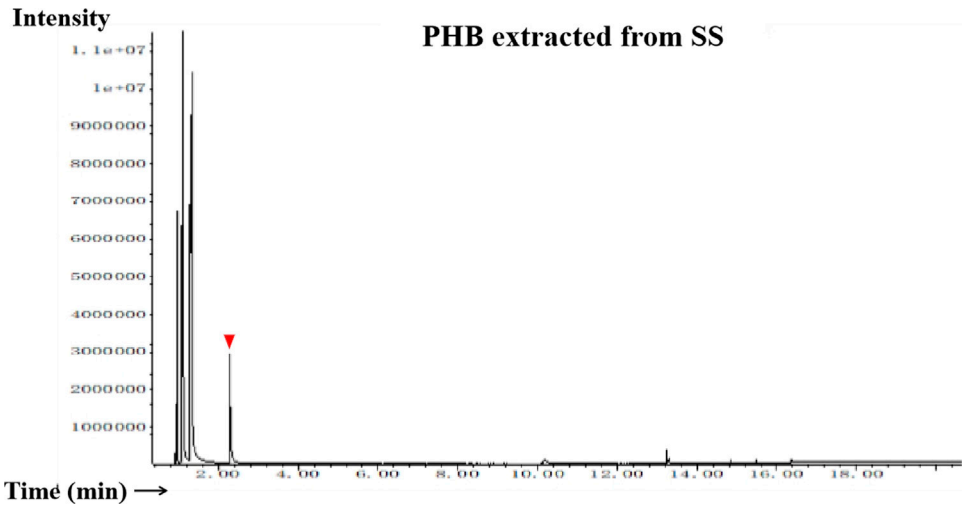


FIGURE 3 | Gas chromatogram of PHB extract from SS. Red triangle means the retention time.

3.5 Benefit Evaluation of Poly-β-Hydroxybutyrate-Enriched Suspended Solids in Aquaculture System

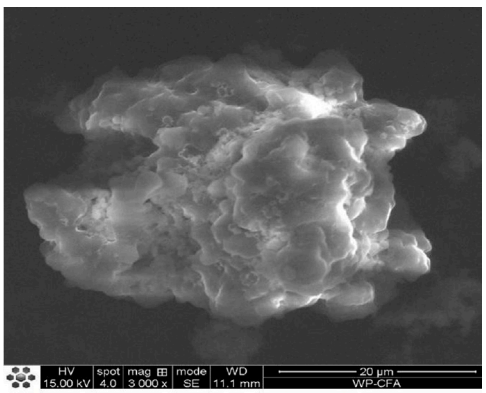
3.5.1 Poly-β-Hydroxybutyrate-Enriched Suspended Solids Improved TAN Removal From Suspended Solids

The remediation rate in the aquaculture effluents for TAN was more than 87%. The remediation rates in SS collected from the indoor concrete tank 1 (cultured gibel carp), indoor concrete tank 2 (cultured gibel carp), indoor concrete tank 3 (cultured shrimp), outdoor pond 1 (cultured gibel carp), outdoor pond 2 (polyculture of freshwater fish), and outdoor pond 3 (cultured shrimp), were 87.19 ± 5.73%, 91.11 ± 8.94%, 87.76 ± 6.27%, 88.55 ± 3.86%, 90.17 ± 7.49%, and 80.06 ± 6.31%, respectively (**Table 2**). The final TAN concentration after treatment was below the tolerance range of fish or shrimp.

3.5.2 Poly-β-Hydroxybutyrate-Enriched Suspended Soilds Promoted Fish Growth

The content of crude protein, crude lipid, ash, and total amino acids in basal SS was 29.84% (percentage of dry matter), 3.16%, 19.09%, and 14.96%, respectively. The PHB contents in the untreated SS and PHB-enriched SS were 7.06 ± 1.76% and 17.34 ± 4.76%, respectively (**Supplementary Table S2**).

The WG and SGR of gibel carp in the PHB-enriched SSA and USSA groups were significantly higher than those of the NSSA group. SS could be up-taken by gibel carp as a food source from water, and PHB-enriched SS had a greater effect on the growth performance. The WG, SGR, and survival rate in the PHB-enriched SSA group were 39.15%, 1.66% d^{-1}, and 100%, while those in the NSSA group were -50.38%,-1.36% d^{-1} and 50%, respectively (**Table 3**). In the USSA group, the survival rate (23.3 ± 0.03%) was lower, possibly owing to the higher concentration of NH_4^+–N and NO_2^-–N, and non-feeding in the untreated SS.



Norm. mass percent (%)

Spectrum	C	O	Al
p112230-by 1	64.36	35.15	0.49
p112230-by 2	63.90	35.45	0.64
p112230-by 3	62.41	37.19	0.40
Mean value:	63.56	35.93	0.51
Sigma:	1.02	1.10	0.12
Sigma mean:	0.59	0.63	0.07

FIGURE 4 | Scanning electron microscopy observation of PHB extract from SS.

3.5.3 Poly-β-Hydroxybutyrate-Enriched SS Up-Regulated the Immune-Related Gene Expression in Fish

We fed gibel carp with the PHB-enriched SS and examined the immune-related gene expression. The untreated SS were used as the control. The data showed that SS supplementation could up-regulate mRNA expression of the six immune-related genes. In particular, the expression levels of genes *hsp70*, *JAK*, and *mTOR*

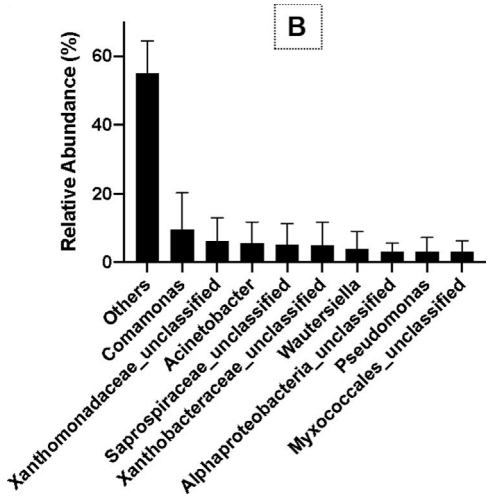


FIGURE 5 | Bacterial diversity at the class level **(A)** and the genus level **(B)** in SS by MiSeq sequencing.

in both the gills and spleen were up-regulated in the PHB-enriched SSA group. Compared to the negative control NSSA group, the expressions of *hsp70* and *JAK* in the spleen from the PHB-enriched SSA group were significantly up-regulated by 105.36-fold and 53.79-fold, respectively. The mRNA expressions in the gills of the experimental group were 69.45-fold and 81.74-fold of that in the control group. The transcriptional levels of the genes in the survival fish of the USSA group were higher than those in the NSSA group, but lower than those in the PHB-enriched SSA group (**Figure 7**).

4 DISCUSSION

PHB exists in SS from both the traditional aquaculture system and zero-water exchange system modulated by the carbon source addition. In the traditional aquaculture system, the content of pure PHB in the untreated basal SS was lower than the content of crude PHB from SS with carbon source addition during the aquaculture process (Ruan et al., 2011). GC–MS analysis showed that the retention time of our purified PHB was 2.214 min, while the PHB standard (Sigma) was 2.189 min and PHB from *Achromobacter marplatensis* was 2.21 min (Pol et al., 2017). In the infrared spectrum, the purified PHB from SS had an obvious band at 1724 cm^{-1} which was the absorption peak of hydroxyl in carboxylic acid. A series of bands at 2,850 cm^{-1}, 3,436 cm^{-1}, 2,976 cm^{-1}, and 2,933 cm^{-1}, were observed, corresponding to the stretching vibration of the -CH, -CO, $-CH_3$, and $-CH_2$, which were the typical characteristics of PHB. We also observed a large amount of C–O vibration between 1,057 cm^{-1} and 1,380 cm^{-1}, which is consistent with the results reported by Rodríguez-Contreras et al. (2017). These data confirmed that SS from the traditional aquaculture system contained PHB and SS has the potential for PHB production.

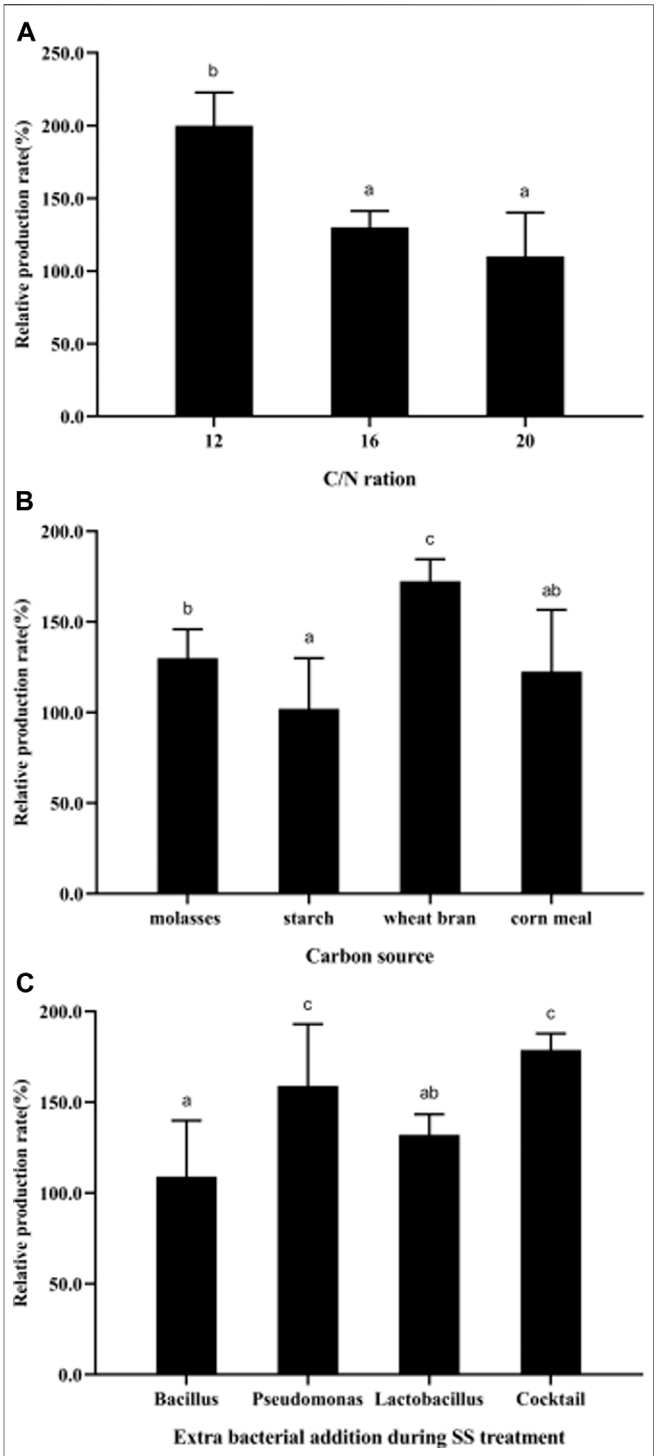


FIGURE 6 | Parametric optimization for high-yield PHB production during SS treatment. **(A)**, C/N ration; **(B)**, carbon sources; **(C)**, probiotics widely used in practical aquaculture. All the data are presented as mean ± SE. Values marked with different small letters indicates significant differences among groups ($p < 0.05$). The strain information used in this study is: *Bacillus*, *Bacillus subtilis*; *Pseudomonas*, *Pseudomonas putida*; Cocktail, cocktail of *Pseudomonas* and *Lactobacillus*.

TABLE 2 | Effect of the PHB-enriched treatment on total ammonia nitrogen (TAN) removal rate from suspended solids (SS).

SS samplings	Initial concentration of TAN (mg L^{-1})	Final concentration of TAN (mg L^{-1})	Removal (Mean, %)
SS from indoor concrete tank 1 (gibel carp)	16.7	2.14	87.19
SS from indoor concrete tank 2 (gibel carp)	19.9	1.77	91.11
SS from indoor concrete tank 3 (shrimp)	21.4	2.62	87.76
SS from outdoor pond 1 (gibel carp)	11.7	1.34	88.55
SS from outdoor pond 2 (polyculture of freshwater fish species)	18.3	1.7	90.17
SS from outdoor pond 3 (shrimp)	13.8	1.51	89.06

TABLE 3 | Growth performance of gibel carp (*Carassius auratus gibelio*) at day 30 after fed with PHB-enriched suspended solids.

Items	Groups		
	PHB-enriched suspended solid addition	Untreated suspended solid addition	No suspended solid addition
Initial weight (g)	12.37±1.45	11.77±1.48	11.94±1.03
Final weight (g)	$20.33±3.18^{c}$	$13.39±2.72^{b}$	$7.94±1.16^{a}$
WG (%)	$39.15±12.93^{c}$	$12.10±2.36^{b}$	$-50.38±3.46^{a}$
SGR (% day^{-1})	$1.66±0.37^{c}$	$0.43±0.12^{b}$	$-1.36±0.07^{a}$
TGC	$0.56±0.14^{c}$	$0.13±0.08^{b}$	$-0.39±0.05^{a}$
Survival rate (%)	$100.00±7.37^{c}$	$31.67±3.33^{a}$	$50.00±3.85^{b}$

The data (mean ± standard deviation) were calculated from quadruplicate tanks, and analyzed through Duncan's multiple range test using one-way ANOVA with SPSS software (version 24.0).

WG, weight gain; SGR, special growth rate; TGC, thermal growth coefficient.

Values marked with a different superscript for the same row are significantly different among groups ($p < 0.05$).

PHB, as an intracellular energy store, is synthesized by microbes, and PHB yield is closely related to the C/N ratio, carbon sources, and bacterial diversity (Crab et al., 2012; Qiao et al., 2018; Amadu et al., 2021). Thus, the primary bacterial diversity in SS associated with PHB accumulation was analyzed, and then the accumulation condition optimized. The microbial community in SS, activated sludge, and aquaculture wastes is more complex than a single bacterial medium. The predominant phyla in SS from this study were Proteobacteria, Saprospirae, and Flavobacteriia. The predominant genera were *Comamonas*, Xanthomonadaceae_unclassified, *Acinetobacter*, Saprospiraceae_unclassified, Xanthobacteraceae_unclassified, *Wautersiella*, *Pseudomonas* and *Myxococcales*_unclassified. Similarly, in the activated sludge, the main functional microorganisms are phosphorus accumulating organisms (0.43%–5.34%) and glycogen accumulating organisms (0.16%–10.08%). Eight microorganisms were predicted as major PHB accumulators (Ju et al., 2008; Wang et al., 2013). Proteobacteria, Acidobacteria, and Burkholderiales were the dominant bacterial population associated with PHB production in the activated sludge (Wang et al., 2013; Miao et al., 2016), and the genera related to denitrification were identified as *Paracoccus*, *Ottowia*, *Theresa*, and Comamonadaceae_unclassified, accounting for 46.21% of total bacteria. Thus, *Proteobacteria*, *Comamonas*, *Acinetobacter*, and *Pseudomonas* in SS are potentially related to PHB production. Similar results were also reported in wastewater and activated sludge (Ju et al., 2008; Wang et al., 2013; Miao et al., 2016). Based on the bacteria isolated from SS using the Sudan Black B staining method (data not shown), and probiotics used in the aquaculture practice, the effects of bacterial strains including *Bacillus* sp., *Pseudomonas* sp., and *Lactobacillus* sp. on PHB accumulation in SS were studied. The results showed that *Pseudomonas* sp. and *Lactobacillus* sp. had more effective improvement in PHB production.

The C/N ratio and carbon sources are the two key factors in stimulating SS formation and PHB accumulation (Avnimelech, 1999; Fontenot et al., 2007; Albuquerque et al., 2010; Amadu et al., 2021). In the present study, we recommended the optimal C/N ratio is 12:1, which is slightly higher than that in shrimp cultured wastewater (C/N ratio of 10:1) (Fontenot et al., 2007), but much lower than that of previous studies which did not add additional bacteria supplementation in water (Wu et al., 2016; Alsafadi & Al-Mashaqbeh, 2017; Pol et al., 2017; Rodríguez-Contreras et al., 2017). Also, the cost of organic carbon can directly affect the economic profitability of culture industry (Kiani et al., 2020; Iber et al., 2021). Wheat bran as a carbon source promoted more PHB production than molasses, starch, and corn meal. These four kinds of carbon resources can significantly reduce the cultural costs (Solaiman et al., 2006). Previous studies showed that olive mill wastewater could be used as the sole carbon source to produce PHAs by *Haloferax mediterranei* (Alsafadi & Al-Mashaqbeh, 2017). In solid-state fermentation, *Cupriavidus necator* cultured in the medium with soy cake or soy cake with sugarcane molasses could produce PHB (Oliveira et al., 2007; Sharma et al., 2016). In pure bacterial culture, the carbon source significantly affects PHB production by *Pseudomonas* sp. (Matsusaki et al., 1998), *Bacillus aryabhattai* (Balakrishna Pillai et al., 2017), and *C. necator* (Sharma et al., 2016). The maximum PHB production by *B. aryabhattai* was

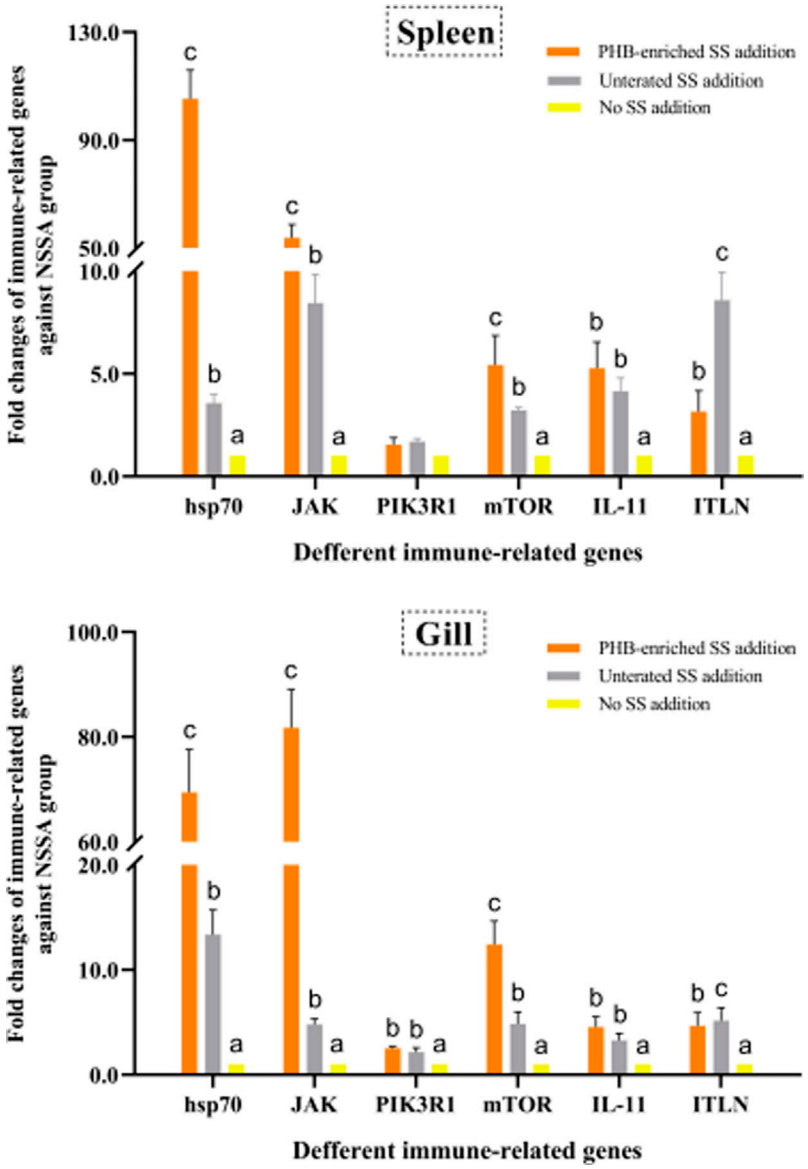


FIGURE 7 | Effects of PHB-IRenriched and untreated SS addition in culture water on the immune-related gene expression in the spleen and gills of gibel carp (*Carassius auratus gibelio*).

obtained when glucose was used as the sole carbon source, rather than fructose, maltose, starch, or glycerol (Balakrishna Pillai et al., 2017). Taken together, we recommended the C/N ratio of 12, wheat bran as carbon source, and mixed bacterial addition of *Pseudomonas* sp. and *Lactobacillus* sp., which could improve the PHB accumulation during SS treatment.

PHB enrichment in SS could eliminate TAN efficiently. The nitrogen in SS is one of detrimental elements in aquaculture systems and the main components of fish fecal droppings (Lazzari & Baldisserotto, 2008; Lananan et al., 2014; Dauda et al., 2019; Rommozzi et al., 2020). In this study, the TAN removal rate of PHB enrichment was more than 87%, which is higher than bioremediation treatment in shrimp culture (68.53%) (Tsukuda et al., 2015), but lower than microalga treatment (100%) (Viegas et al., 2021). As reported previously, for hydroponic bio-filtration, TAN removal was 0.18–0.22 g m^{-2} d, TN removal was 2.64–4.32 g m^{-2} d; for sand biofiltration, TN in a rainbow trout (*Oncorhynchus mykiss*) culture system was reduced from 17.52 mg L^{-1}–14.11 mg L^{-1}. For biofloc technology, the removal of TAN in shrimp and mullet culture systems was 0.12–0.17 mg L^{-1}, which was safe for the aquaculture species. TAN can be determined by measuring the ammonia and ammonium ions (Dauda et al., 2019; Kır et al., 2019).

Although PHB is an eco-friendly material and can be degraded into CO_2 and H_2O, whether the treated SS (PHB-enriched SS) has the potential to be recycled and reused in the culture system still

remains unclear. Previous reports demonstrated that PHB supplementation in diet and culture water can improve the growth, immunity, and disease resistance of aquatic animals, such as tilapia (*Oreochromis mossambicus*), soiny mullet (*Liza haematocheila*), Pacific white shrimp (*Litopenaeus vannamei*), and giant river prawn (*Macrobrachium rosenbergii*) (Nhan et al., 2010; Schryver et al., 2010; Deng et al., 2014; Xu et al., 2014; Meirong et al., 2018; Qiao et al., 2019a, 2019b, 2020). Dietary PHB supplementation can up-regulate the mRNA expression of some immune-related genes such as *Hsp70*, *MHCs*, *TLRs*, *IL-8*, *hepcidin*, *pbpA*, and *AOX* in fish (Qiao et al., 2019a, 2020). Additionally, PHB can enhance the disease resistance of aquatic animals against both bacterial and viral infections (Deng et al., 2014; Defoirdt et al., 2018; Van Hung et al., 2019; Qiao et al., 2020). In the present study, PHB-enriched SS can be up-taken by gibel carp, which is in accordance with the previous studies which confirmed that SS can be up-taken by omnivorous fish and shrimp (Nhan et al., 2010; Meirong et al., 2018; Wu et al., 2018; Zhang et al., 2018). The nutritional composition of SS was similar to that composition of SS obtained from the tilapia and shrimp BFT system (Luo et al., 2014; Rajkumar et al., 2016), which can meet the demands of fish and shrimp (Luo et al., 2014; Rajkumar et al., 2016; Zhang et al., 2018). Compared with the untreated SS, the PHB-enriched SS (more than 2-fold) showed positive effects on fish growth and immunity. The WG, SGR, and survival rate of fish cultured in water that contained PHB-enriched SS were higher than those of untreated SS and no SS supplementation groups without feeding, which might be more related to higher PHB accumulation, rather than the probiotics stimulation. Accordingly, it was reported that PHB-accumulating bacteria can improve the survival and growth of shrimp, artemia, and fish more effectively than single probiotics (Gao et al., 2018; Laranja et al., 2018; Krasaesueb et al., 2019). The WG (39.15%) and SGR (1.66% day^{-1}) in the PHB-enriched SS group were higher than those in the bacterial single treatment group, which were 20.46% and 0.40% day^{-1}, respectively (Xu, 2019). It suggested that 'PHB + probiotics' treatment is much better than single probiotic usage in fish culture. The lowest survival rate of gibel carp in the untreated SS group without feeding was observed, which might be caused by higher NO_2^- concentration and non-feeding. NO_2^- is an intermediate toxic compound, which affects the hemoglobin in carrying oxygen and threatens to destabilize electrolytes (Jiang et al., 2014). As a result, the higher concentration of NO_2^- in water causes stress, weakens growth, damages the internal organs, and reduces disease tolerance of aquatic animals, and thus harms aquaculture farming (Hurtado et al., 2016). A lower survival rate (50.00 ± 3.85%) in the no SS addition group was also observed, which is considered to relate to the 1 month non-feeding as reported by Zhang et al. (2018). The 1 month survival rate of gibel carp (41.02 ± 3.2 g) in the non-feeding freshwater culture group was 50.00 ± 7.37% (Zhang et al., 2018). PHB-enriched SS could up-regulate the expressions of the immune-related genes, such as *hsp70*, *ITLN*, and *JAK* in the spleen and gills of gibel carp. The spleen is an immune organ of fish (Li et al., 2014), and the gills are one of the first organs directly exposed to the water environment, and are considered as a major organ related to the mucosal immunity (Murray et al., 2007; Andrews et al., 2010). The *hsp70*, *ITLN*, and *JAK* play important roles in the immune response of gibel carp against both viral and bacterial infections (Podok et al., 2014a, 2014b; Xu et al., 2014). Hsp70, as a molecular chaperone, plays very important roles in anti-oxidation, anti-apoptosis, cellular immunity, and innate immune response (Wachstein et al., 2012). The expression of ITLN in gibel carp fed only with bioflocs at a TSS concentration of 600 mg L^{-1} for 30 days was up-regulated by 78.1-fold (Zhang et al., 2018). The present study would provide a better food source to aquatic animals, and greatly simplify the addition process of PHB in diets due to its water insolubility and reduction of the costs of commercial products.

Thus, from an ecological point of view, PHB can be enriched in SS to reduce nitrogen emission and be non-toxic to the environment, since polymer PHB is naturally degraded into CO_2 and H_2O. Notedly, this study provides a novel method for using SS in aquaculture systems to reduce waste emission. From an economic point of view, PHB acclimation is derived from low-cost raw materials, and PHB-enriched SS can be reused as a food source to promote fish growth and immunity. It provides cheaper food, and a simple and feasible approach to apply PHB in aquaculture.

5 CONCLUSION

PHB exists in SS from aquaculture system. PHB accumulation in SS is related to Proteobacteria, *Comamonas*, *Acinetobacter*, and *Pseudomonas*. Optimizing the carbon source and microbial addition increased PHB production in SS. The PHB-enrichment SS reduced TAN, improved fish growth, and boosted fish immunity. We studied SS waste in the aquaculture system and developed an approach to transforming SS waste into high-quality fish food.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This study was conducted in accordance with the regulations for the administration of laboratory animals in Jiangsu province, China.

AUTHOR CONTRIBUTIONS

GQ: supervision, conceptualization, review and editing, and funding acquisition. XL: methodology, software, analysis, and writing—original draft. MZ: project administration, reviewing and editing, and funding acquisition. YS, YZ, PC, YS, and PC: resources, methodology, software, analysis. ZZ and PL: sampling from outdoor ponds and indoor tanks and funding acquisition.

JL, ZQ, and ZW: supervision, resources, writing—review and editing, and validation. EW: English editing.

FUNDING

This research was financially supported by the National Nature Science Foundation of China (Grant no. 32071265, 31602179), and partially by the National Key R&D Program of China (2019YFD0900302), and by the Projects on Social Science of Philosophy in Colleges and Universities of Jiangsu Province (2020SJA 1923), and by the Projects on High-Quality Development of Fisheries of Yancheng (YCSCY2021020, YCSCY2021014).

ACKNOWLEDGMENTS

We thank JL, Julian Ramelow, and EW for English editing. We also thank the eight reviewers for very constructive feedback and suggestions for article structure organization, data analysis, and literature.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.797625/full#supplementary-material

REFERENCES

Ahmed, N., Thompson, S., and Glaser, M. (2019). Global Aquaculture Productivity, Environmental Sustainability, and Climate Change Adaptability. *Environ. Manage.* 63, 159–172. doi:10.1007/s00267-018-1117-3

Albuquerque, M. G. E., Torres, C. A. V., and Reis, M. A. M. (2010). Polyhydroxyalkanoate (PHA) Production by a Mixed Microbial Culture Using Sugar Molasses: Effect of the Influent Substrate Concentration on Culture Selection. *Water Res.* 44 (11), 3419–3433. doi:10.1016/j.watres.2010.03.021

Alsafadi, D., and Al-Mashaqbeh, O. (2017). A One-Stage Cultivation Process for the Production of Poly-3-(hydroxybutyrate-Co-Hydroxyvalerate) from Olive Mill Wastewater by *Haloferax Mediterranei*. *New Biotechnol.* 34, 47–53. doi:10.1016/j.nbt.2016.05.003

Amadu, A. A., Qiu, S., Ge, S., Addico, G. N. D., Ameka, G. K., Yu, Z., et al. (2021). A Review of Biopolymer (Poly-β-Hydroxybutyrate) Synthesis in Microbes Cultivated on Wastewater. *Sci. Total Environ.* 756, 143729. doi:10.1016/j.scitotenv.2020.143729

Andrews, M., Battaglene, S., Cobcroft, J., Adams, M., Noga, E., and Nowak, B. (2010). Host Response to the Chondracanthid copepod *Chondracanthus goldsmidi*, a Gill Parasite of the Striped trumpeter, *Latris lineata* (Forster), in Tasmania. *J. Fish. Dis.* 33, 211–220. doi:10.1111/j.1365-2761.2009.01107.x

AOAC, Association of Official Analytical Chemists (1995). *Official Methods of Analysis*. 16th ed. Arlington, VA: AOAC International.

APHA (2012). *Standard Methods for the Examination of Water and Wastewater*. 22nd. Washington DC: American Public Health Association.

Arun, A., Murugappan, R. M., David Ravindran, A. D., Veeramanikandan, V., and Balaji, S. (2006). Utilization of Various Industrial Wastes for the Production of Poly-β-hydroxybutyrate (PHB) by *Alcaligenes Eutrophus*. *Afr. J. Biotechnol.* 5 (17), 1524–1527. doi:10.4314/ajb.v5i17.43150

Avnimelech, Y. (1999). Carbon/nitrogen Ratio as a Control Element in Aquaculture Systems. *Aquaculture* 176 (3–4), 0–235. doi:10.1016/S0044-8486(99)00085-X

Badiola, M., Mendiola, D., and Bostock, J. (2012). Recirculating Aquaculture Systems (RAS) Analysis: Main Issues on Management and Future Challenges. *Aquacultural Eng.* 51, 26–35. doi:10.1016/j.aquaeng.2012.07.004

Balakrishna Pillai, A., Jaya Kumar, A., Thulasi, K., and Kumarapillai, H. (2017). Evaluation of Short-Chain-Length Polyhydroxyalkanoate Accumulation in *Bacillus Aryabhattai*. *Braz. J. Microbiol.* 48 (3), 451–460. doi:10.1016/j.bjm.2017.01.005

Bangar, C., Mhaske, P., Parasur, V., and Pawar, S. (2017). "Comparative Study of Removal of Dairy Waste Characteristics by Using Various Natural and Chemical Coagulants," in Special Issue: National Conference "MOMENTUM-17.*Int. J. Res. Adv. Technol.*Vol. 14

Becke, C., Schumann, M., Geist, J., and Brinker, A. (2020). Shape Characteristics of Suspended Solids and Implications in Different Salmonid Aquaculture Production Systems. *Aquaculture* 516, 734631. doi:10.1016/j.aquaculture.2019.734631

Bengtsson, S., Werker, A., Christensson, M., and Welander, T. (2008). Production of Polyhydroxyalkanoates by Activated Sludge Treating a Paper Mill Wastewater. *Bioresour. Technol.* 99 (3), 509–516. doi:10.1016/j.biortech.2007.01.020

Benrebah, F., Prevost, D., Yezza, A., and Tyagi, R. (2007). Agro-industrial Waste Materials and Wastewater Sludge for Rhizobial Inoculant Production: a Review. *Bioresour. Technol.* 98, 3535–3546. doi:10.1016/j.biortech.2006.11.066

Carpine, R., Raganati, F., Olivieri, G., Hellingwerf, K. J., Pollio, A., Salatino, P., et al. (2018). Poly-β-hydroxybutyrate (PHB) Production by *Synechocystis* PCC6803 from CO_2: Model Development. *Algal Res.* 29, 49–60. doi:10.1016/j.algal.2017.11.011

Chua, A. S. M., Takabatake, H., Satoh, H., and Mino, T. (2003). Production of Polyhydroxyalkanoates (PHA) by Activated Sludge Treating Municipal Wastewater: Effect of pH, Sludge Retention Time (SRT), and Acetate Concentration in Influent. *Water Res.* 37 (15), 3602–3611. doi:10.1016/S0043-1354(03)00252-5

Crab, R., Defoirdt, T., Bossier, P., and Verstraete, W. (2012). Biofloc Technology in Aquaculture: Beneficial Effects and Future Challenges. *Aquaculture* 356-357, 351–356. doi:10.1016/j.aquaculture.2012.04.046

Das, S. K., Sathish, A., and Stanley, J. (2018). Production of Biofuel and Bioplastic from *Chlorella Pyrenoidosa*. *Mater. Today Proc.* 5 (83), 16774–16781. doi:10.1016/j.matpr.2018.06.020

Dauda, A. B., Ajadi, A., Tola-Fabunmi, A. S., and Akinwole, A. O. (2019). Waste Production in Aquaculture: Sources, Components and Managements in Different Culture Systems. *Aquacult. Fish.* 4 (3), 81–88. doi:10.1016/j.aaf.2018.10.002

De Schryver, P., Sinha, A. K., Kunwar, P. S., Baruah, K., Verstraete, W., Boon, N., et al. (2010). Poly-β-hydroxybutyrate (PHB) Increases Growth Performance and Intestinal Bacterial Range-Weighted Richness in Juvenile European Sea Bass (*Dicentrarchus labrax*). *Appl. Microbiol. Biotechnol.* 86 (5), 1535–1541. doi:10.1007/s00253-009-2414-9

De Schryver, P., and Verstraete, W. (2009). Nitrogen Removal from Aquaculture Pond Water by Heterotrophic Nitrogen Assimilation in Lab-Scale Sequencing Batch Reactors. *Bioresour. Technol.* 100 (3), 1162–1167. doi:10.1016/j.biortech.2008.08.043

Defoirdt, T., Mai Anh, N. T., and De Schryver, P. (2018). Virulence-inhibitory Activity of the Degradation Product 3-hydroxybutyrate Explains the Protective Effect of Poly-β-Hydroxybutyrate against the Major Aquaculture Pathogen *Vibrio campbellii*. *Sci. Repsci. Rep.* 8 (1), 7245. doi:10.1038/s41598-018-25385-w

Deng, K. Y., Kong, J., Meng, X. H., Luo, K., Cao, B. Y., Liu, N., et al. (2014). Effects of Poly-β-Hydroxybutyrate on WSSV Resistance in *Fenneropenaeus chinensis*. *J. Fish. Sci. China* 8, 1167–1174. doi:10.1074/jbc.M113.510743

Fontenot, Q., Bonvillain, C., Kilgen, M., and Boopathy, R. (2007). Effects of Temperature, Salinity, and Carbon: Nitrogen Ratio on Sequencing Batch Reactor Treating Shrimp Aquaculture Wastewater. *Bioresour. Technol.* 98 (9), 1700–1703. doi:10.1016/j.biortech.2006.07.031

Gao, M., Du, D., Bo, Z., and Sui, L. (2019). Poly-β-hydroxybutyrate (PHB)-accumulating Halomonas Improves the Survival, Growth, Robustness and Modifies the Gut Microbial Composition of Litopenaeus *Vannamei postlarvae*. *Aquaculture* 500, 607–612. doi:10.1016/j.aquaculture.2018.10.032

Gao, Y., Guo, L., Shao, M., Hu, F., Wang, G., Zhao, Y., et al. (2020). Denitrification Performance Evaluation and Kinetics Analysis with Mariculture Solid Wastes (MSW) Derived Carbon Source in marine Recirculating Aquaculture Systems (RAS). *Bioresour. Technol.* 313, 123649. doi:10.1016/j.biortech.2020.123649

Ge, S., and Champagne, P. (2017). Cultivation of the marine Macroalgae *Chaetomorpha Linum* in Municipal Wastewater for Nutrient Recovery and Biomass Production. *Environ. Sci. Technol.* 51 (6), 3558–3566. doi:10.1021/acs.est.6b06039

Ge, S., Peng, Y., Qiu, S., Zhu, A., and Ren, N. (2014). Complete Nitrogen Removal from Municipal Wastewater via Partial Nitrification by Appropriately Alternating Anoxic/aerobic Conditions in a Continuous Plug-Flow Step Feed Process. *Water Res.* 55, 95–105. doi:10.1016/j.watres.2014.01.058

Ge, S., Qiu, S., Tremblay, D., Viner, K., Champagne, P., and Jessop, P. G. (2018). Centrate Wastewater Treatment with *Chlorella vulgaris*: Simultaneous Enhancement of Nutrient Removal, Biomass and Lipid Production. *Chem. Eng. J.* 342, 310–320. doi:10.1016/j.cej.2018.02.058

Gibson, T. F., Watanabe, W. O., Losordo, T. M., Whitehead, R. F., and Carroll, P. M. (2020). Evaluation of Chemical Polymers as Coagulation Aids to Remove Suspended Solids from marine Finfish Recirculating Aquaculture System Discharge Using a Geotextile Bag. *Aquacultural Eng.* 90, 102065. doi:10.1016/j.aquaeng.2020.102065

Han, P., Lu, Q., Fan, L., and Zhou, W. (2019). A Review on the Use of Microalgae for Sustainable Aquaculture. *Appl. Sci.* 9 (11), 2377. doi:10.3390/app9112377

Heiderscheidt, E., Tesfamariam, A., Pulkkinen, J., Vielma, J., and Ronkanen, A.-k. (2020). Solids Management in Freshwater-Recirculating Aquaculture Systems: Effectivity of Inorganic and Organic Coagulants and the Impact of Operating Parameters. *Sci. Total Environ.* 742, 140398. doi:10.1016/j.scitotenv.2020.140398

Henriksson, P. J. G., Belton, B., Jahan, K. M.-e., and Rico, A. (2018). Measuring the Potential for Sustainable Intensification of Aquaculture in Bangladesh Using Life Cycle Assessment. *Proc. Natl. Acad. Sci. U.S.A.* 115 (12), 2958–2963. doi:10.1073/pnas.1716530115

Hesni, M. A., Hedayati, A., Qadermarzi, A., Pouladi, M., Zangiabadi, S., and Naqshbandi, N. (2020). Using Chlorella Vulgaris and Iron Oxide Nanoparticles in a Designed Bioreactor for Aquaculture Effluents Purification. *Aquacultural Eng.* 90, 102069. doi:10.1016/j.aquaeng.2020.102069

Holm-Nielsen, J. B., Al Seadi, T., and Oleskowicz-Popiel, P. (2009). The Future of Anaerobic Digestion and Biogas Utilization. *Bioresour. Technol.* 100, 5478–5484. doi:10.1016/j.biortech.2008.12.046

Hrabak, O. (1992). Industrial Production of Poly-β-Hydroxybutyrate. *FEMS Microbiol. Lett.* 103, 251–255. doi:10.1016/0378-1097(92)90317-h

Hurtado, C. F., Cancino-Madariaga, B., Torrejón, C., and Villegas, P. P. (2016). Separation of Nitrite and Nitrate from Water in Aquaculture by Nanofiltration Membrane. *Desalination Water Treat.* 57, 26050–26062. doi:10.1080/19443994.2016.1160440

Iber, B. T., Okomoda, V. T., Rozaimah, S. A., and Kasan, N. A. (2021). Eco-friendly Approaches to Aquaculture Wastewater Treatment: Assessment of Natural Coagulants Vis-A-Vis Chitosan. *J. Bioresour. Technol.* 15, 100702. doi:10.1016/j.biteb.2021.100702

Jiang, Q., Dilixiati, A., Zhang, W., Li, W., Wang, Q., Zhao, Y., et al. (2014). Effect of Nitrite Exposure on Metabolic Response in the Freshwater Prawn *Macrobrachium nipponense*. *Cent. Eur. J. Biol.* 9, 86–91. doi:10.2478/s11535-013-0167-4

Jochum, M., Moncayo, L. P., and Jo, Y.-K. (2018). Microalgal Cultivation for Biofertilization in rice Plants Using a Vertical Semi-closed Airlift Photobioreactor. *PLoS One* 13 (9), e0203456. doi:10.1371/journal.pone.0203456

Ju, Z. Y., Forster, I., Conquest, L., Dominy, W., Kuo, W. C., and David Horgen, F. (2008). Determination of Microbial Community Structures of Shrimp Floc Cultures by Biomarkers and Analysis of Floc Amino Acid Profiles. *Aquac. Res.* 39 (2), 118–133. doi:10.1111/j.1365-2109.2007.01856.x

Karri, R. R., Sahu, J. N., and Chimmiri, V. (2018). Critical Review of Abatement of Ammonia from Wastewater. *J. Mol. Liquids* 261, 21–31. doi:10.1016/j.molliq.2018.03.120

Kiani, S., Kujala, K., T. Pulkkinen, J., Aalto, S. L., Suurnäkki, S., Kiuru, T., et al. (2020). Enhanced Nitrogen Removal of Low Carbon Wastewater in Denitrification Bioreactors by Utilizing Industrial Waste toward Circular Economy. *J. Clean. Prod.* 254, 119973. doi:10.1016/j.jclepro.2020.119973

Kır, M., Sunar, M. C., and Gök, M. G. (2019). Acute Ammonia Toxicity and the Interactive Effects of Ammonia and Salinity on the Standard Metabolism of European Sea Bass (*Dicentrarchus labrax*). *Aquaculture* 511, 734273. doi:10.1016/j.aquaculture.2019.734273

Kokou, F., and Fountoulaki, E. (2018). Aquaculture Waste Production Associated with Antinutrient Presence in Common Fish Feed Plant Ingredients. *Aquaculture* 495, 295–310. doi:10.1016/j.aquaculture.2018.06.003

Koroleff, F. (1976). Determination of Nutrients: 8. Total and Organic Nitrogen," in *Methods of seawater analysis*. Editor K. A. Grasshoff, 161–181.

Krasaesueb, N., Incharoensakdi, A., and Khetkorn, W. (2019). Utilization of Shrimp Wastewater for Poly-β-Hydroxybutyrate Production by *Synechocystis* Sp. PCC 6803 Strain ΔSphU Cultivated in Photobioreactor. *Biotechnol. Rep.* 23, e00345. doi:10.1016/j.btre.2019.e00345

Lananan, F., Abdul Hamid, S. H., Din, W. N. S., Ali, N. a., Khatoon, H., Jusoh, A., et al. (2014). Symbiotic Bioremediation of Aquaculture Wastewater in Reducing Ammonia and Phosphorus Utilizing Effective Microorganism (EM-1) and Microalgae (*Chlorella* sp.). *Int. Biodeterioration Biodegradation* 95, 127–134. doi:10.1016/j.ibiod.2014.06.013

Laranja, J. L. Q., De Schryver, P., Ludevese-Pascual, G. L., Amar, E. C., Aerts, M., Vandamme, P., et al. (2018). High Amorphous Poly-Beta-Hydroxybutyrate (PHB) Content in a *Probiotic bacillus* Strain Displays Better Protective Effects *In Vibrio* -challenged Gnotobiotic Artemia. *Aquaculture* 487, 15–21. doi:10.1016/j.aquaculture.2018.01.005

Law, J. H., and Slepecky, R. A. (1961). Assay of Poly-B-Hydroxybutyric Acid. *J. Bacteriol.* 82 (1), 33–36. doi:10.1128/jb.82.1.33-36.1961

Lazzari, R., and Baldisserotto, B. (2008). Nitrogen and Phosphorus Waste in Fish Farming. *Bol. Inst. Pesca* 34 (4), 591–600.

Li, G., Zhao, Y., Wen, L., Liu, Z., Yan, F., and Gao, C. (2014). Identification and Characterization of microRNAs in the Spleen of Common Carp Immune Organ. *J. Cel. Biochem.* 115 (10), 1768–1778. doi:10.1002/jcb.24843

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

Luo, G., Gao, Q., Wang, C., Liu, W., Sun, D., Li, L., et al. (2014). Growth, Digestive Activity, Welfare, and Partial Cost-Effectiveness of Genetically Improved Farmed tilapia (*Oreochromis niloticus*) Cultured in a Recirculating Aquaculture System and an Indoor Biofloc System. *Aquaculture* 422-423 (423), 1–7. doi:10.1016/j.aquaculture.2013.11.023

Matsusaki, H., Manji, S., Taguchi, K., Kato, M., Fukui, T., and Doi, Y. (1998). Cloning and Molecular Analysis of the Poly(3-Hydroxybutyrate) and Poly(3-Hydroxybutyrate- Co -3-hydroxyalkanoate) Biosynthesis Genes in *Pseudomonas* Sp. Strain 61-3. *J. Bacteriol.* 180 (24), 6459–6467. doi:10.1128/JB.180.24.6459-6467.1998

Meixner, K., Troschl, C., Fritz, I., and Drosg, B. (2017). Cyanobacteria for Bioplastic Production. Univ. Nat. Resour. Life Sci. Vienna Dep. Agrobiotechnology. IFA-Tulln Inst. *Environ. Biotechnol.*, 27049. doi:10.13140/RG.2.2.32919.27049

Miao, L., Wang, S., Li, B., Cao, T., Zhang, F., Wang, Z., et al. (2016). Effect of Carbon Source Type on Intracellular Stored Polymers during Endogenous Denitritation (ED) Treating Landfill Leachate. *Water Res.* 100, 405–412. doi:10.1016/j.watres.2016.05.010

Murray, H. M., Leggiadro, C. T., and Douglas, S. E. (2007). Immunocytochemical Localization of Pleurocidin to the Cytoplasmic Granules of Eosinophilic Granular Cells from the winter Flounder Gill. *J. Fish. Biol.* 70, 336–345. doi:10.1111/j.1095-8649.2007.01452.x

Naughton, S., Kavanagh, S., Lynch, M., and Rowan, N. J. (2020). Synchronizing Use of Sophisticated Wet-Laboratory and In-Field Handheld Technologies for Real-Time Monitoring of Key Microalgae, Bacteria and Physicochemical Parameters Influencing Efficacy of Water Quality in a Freshwater Aquaculture Recirculation System: A Case Study from the Republic of Ireland. *Aquaculture* 526, 735377. doi:10.1016/j.aquaculture.2020.735377

Naumann, D., Helm, D., and Labischinski, H. (1991). Microbiological Characterizations by FT-IR Spectroscopy. *Nature* 351 (6321), 81–82. doi:10.1038/351081a0

Nhan, D. T., Wille, M., De Schryver, P., Defoirdt, T., Bossier, P., and Sorgeloos, P. (2010). The Effect of Poly β-hydroxybutyrate on Larviculture of the Giant Freshwater Prawn (*Macrobrachium rosenbergii*). *Aquaculture* 302 (1–2), 76–81. doi:10.1016/j.aquaculture.2010.02.011

Oliveira, F. C., Dias, M. L., Castilho, L. R., and Freire, D. M. G. (2007). Characterization of Poly(3-Hydroxybutyrate) Produced by *Cupriavidus Necator* in Solid-State Fermentation. *Bioresour. Technol.* 98 (3), 633–638. doi:10.1016/j.biortech.2006.02.022

Olusegun, A., Babatunde, D., and Abiodun, O. (2016). Haematological Response of *Clarias gariepinus* Juveniles Reared in Treated Wastewater after Waste Solids Removal Using Alum or *Moringa Oleifera* Seed Powder. *Int. J. Aquacult.* 6. doi:10.5376/ija.2016.06.0011

Podok, P., Wang, H., Xu, L., Xu, D., and Lu, L. (2014b). Characterization of Myeloid-specific Peroxidase, Keratin 8, and Dual Specificity Phosphatase 1 as Innate Immune Genes Involved in the Resistance of Crucian Carp (*Carassius auratus Gibelio*) to Cyprinid Herpesvirus 2 Infection. *Fish Shellfish Immunol.* 41 (2), 531–540. doi:10.1016/j.fsi.2014.10.001

Podok, P., Xu, L., Xu, D., and Lu, L. (2014a). Different Expression Profiles of Interleukin 11 (IL-11), Intelectin (ITLN) and Purine Nucleoside Phosphorylase

5a (PNP 5a) in Crucian Carp (*Carassius auratus* Gibelio) in Response to Cyprinid Herpesvirus 2 and *Aeromonas Hydrophila*. *Fish Shellfish Immunol.* 38 (1), 65–73. doi:10.1016/j.fsi.2014.03.001

Pol, A. P., Erika, A. C., Alexey, N., Christina, E., Rybak, Jürgen., Martin, K., et al. (2017). Bacterial Community and PHB-Accumulating Bacteria Associated with the wall and Specialized Niches of the Hindgut of the forest Cockchafer *(Melolontha Hippocastani*). *Front. Microbiol.* 8 (e51557), 291. doi:10.3389/fmicb.2017.00291

Qiao, G., Chen, P., Sun, Q., Zhang, M., Zhang, J., Li, Z., et al. (2020). Poly-β-hydroxybutyrate (PHB) in Bioflocs Alters Intestinal Microbial Community Structure, Immune-Related Gene Expression and Early Cyprinid Herpesvirus 2 Replication in Gibel Carp (*Carassius auratus* Gibelio). *Fish Shellfish Immunol.* 97, 72–82. doi:10.1016/j.fsi.2019.12.045

Qiao, G., Sun, Q., Zhang, M., Xu, C., Lv, T., Qi, Z., et al. (2019a). Antioxidant System of Soiny Mullet (*Liza haematocheila*) Is Responsive to Dietary Poly-β-Hydroxybutyrate (PHB) Supplementation Based on Immune-Related Enzyme Activity and De Novo Transcriptome Analysis. *Fish Shellfish Immunol.* 95, 314–327. doi:10.1016/j.fsi.2019.10.042

Qiao, G., Xu, C., Sun, Q., Xu, D.-H., Zhang, M., Chen, P., et al. (2019b). Effects of Dietary Poly-β-Hydroxybutyrate Supplementation on the Growth, Immune Response and Intestinal Microbiota of Soiny Mullet (*Liza haematocheila*). *Fish Shellfish Immunol.* 91, 251–263. doi:10.1016/j.fsi.2019.05.038

Qiao, G., Zhang, M., Li, Y., Xu, C., Xu, D.-H., Zhao, Z., et al. (2018). Biofloc Technology (BFT): An Alternative Aquaculture System for Prevention of Cyprinid Herpesvirus 2 Infection in Gibel Carp (*Carassius auratus Gibelio*). *Fish Shellfish Immunol.* 83, 140–147. doi:10.1016/j.fsi.2018.09.015

Qiu, S., Shen, Y., Zhang, L., Ma, B., Amadu, A. A., and Ge, S. (2020a). Antioxidant Assessment of Wastewater-Cultivated *Chlorella Sorokiniana* in *Drosophila melanogaster*. *Algal Res.* 46, 101795. doi:10.1016/j.algal.2020.101795

Qiu, S., Wang, L., Chen, Z., Yang, M., Yu, Z., and Ge, S. (2021b). An Integrated Mainstream and Sidestream Strategy for Overcoming Nitrite Oxidizing Bacteria Adaptation in a Continuous Plug-Flow Nutrient Removal Process. *Bioresour. Technol.* 319, 124133. doi:10.1016/j.biortech.2020.124133

Rajkumar, M., Pandey, P. K., Aravind, R., Vennila, A., Bharti, V., and Purushothaman, C. S. (2016). Effect of Different Biofloc System on Water Quality, Biofloc Composition and Growth Performance in *Litopenaeus vannamei* (Boone, 1931). *Aquac. Res.* 47 (11), 3432–3444. doi:10.1111/are.12792

Rodríguez-Contreras, A., García, Y., Manero, J. M., and Rupérez, E. (2017). Antibacterial PHAs Coating for Titanium Implants. *Eur. Polym. J.* 90, 66–78. doi:10.1016/j.eurpolymj.2017.03.004

Rommozzi, E., Giannakis, S., Giovannetti, R., Vione, D., and Pulgarin, C. (2020). Detrimental vs. Beneficial Influence of Ions during Solar (SODIS) and Photo-Fenton Disinfection of *E. coli* in Water: (Bi)carbonate, Chloride, Nitrate and Nitrite Effects. *Appl. Catal. B: Environ.* 270, 118877. doi:10.1016/j.apcatb.2020.118877

Rosa, J., Lemos, M. F. L., Crespo, D., Nunes, M., Freitas, A., Ramos, F., et al. (2020). Integrated Multitrophic Aquaculture Systems - Potential Risks for Food Safety. *Trends Food Sci. Technol.* 96, 79–90. doi:10.1016/j.tifs.2019.12.008

Ruan, Y.-J., Zhu, L., and Xu, X.-Y. (2011). Study on the Flocs Poly-β-Hydroxybutyrate Production and Process Optimization in the Bio-Flocs Technology System. *Bioresour. Technol.* 102 (16), 7599–7602. doi:10.1016/j.biortech.2011.05.028

Sharma, P. K., Fu, J., Spicer, V., Krokhin, O. V., Cicek, N., Sparling, R., et al. (2016). Global Changes in the Proteome of Cupriavidus Necator H16 during Poly-(3-Hydroxybutyrate) Synthesis from Various Biodiesel By-Product Substrates. *AMB Expr.* 6 (1). doi:10.1186/s13568-016-0206-z

Solaiman, D. K. Y., Ashby, R. D., Foglia, T. A., and Marmer, W. N. (2006). Conversion of Agricultural Feedstock and Coproducts into Poly(hydroxyalkanoates). *Appl. Microbiol. Biotechnol.* 71 (6), 783–789. doi:10.1007/s00253-006-0451-1

Sun, H., He, Z., Zhang, M., Yen, L., Cao, Y., Hu, Z., et al. (2020). Spatial Variation of Soil Properties Impacted by Aquaculture Effluent in a Small-Scale Mangrove. *Mar. Pollut. Bull.* 160, 111511. doi:10.1016/j.marpolbul.2020.111511

Tsukuda, S., Christianson, L., Kolb, A., Saito, K., and Summerfelt, S. (2015). Heterotrophic Denitrification of Aquaculture Effluent Using Fluidized Sand Biofilters. *Aquacultural Eng.* 64, 49–59. doi:10.1016/j.aquaeng.2014.10.010

Van Hung, N., Bossier, P., Hong, N. T. X., Ludeseve, C., Garcia-Gonzalez, L., Nevejan, N., et al. (2019). Does Ralstonia Eutropha , Rich in Poly-β Hydroxybutyrate (PHB), Protect Blue Mussel Larvae against Pathogenic Vibrios ? *J. Fish Dis.* 42 (6), 777–787. doi:10.1111/jfd.12981

Venkateswar Reddy, M., Mawatari, Y., Yajima, Y., Seki, C., Hoshino, T., and Chang, Y.-C. (2015). Poly-3-hydroxybutyrate (PHB) Production from Alkylphenols, Mono and Poly-Aromatic Hydrocarbons Using *Bacillus* Sp. CYR1: A New Strategy for Wealth from Waste. *Bioresour. Technol.* 192, 711–717. doi:10.1016/j.biortech.2015.06.043

Viegas, C., Gouveia, L., and Gonçalves, M. (2021). Aquaculture Wastewater Treatment through Microalgal. Biomass Potential Applications on Animal Feed, Agriculture, and Energy. *J. Environ. Manage.* 286, 112187. doi:10.1016/j.jenvman.2021.112187

Wachstein, J., Tischer, S., Figueiredo, C., Limbourg, A., Falk, C., Immenschuh, S., et al. (2012). HSP70 Enhances Immunosuppressive Function of CD4+CD25+FoxP3+ T Regulatory Cells and Cytotoxicity in CD4+CD25− T Cells. *PLoS ONE* 7 (12), e51747. doi:10.1371/journal.pone.0051747

Wang, Y., Cai, J., Lan, J., Liu, Z., He, N., Shen, L., et al. (2013). Biosynthesis of Poly(hydroxybutyrate-Hydroxyvalerate) from the Acclimated Activated Sludge and Microbial Characterization in This Process. *Bioresour. Technol.* 148, 61–69. doi:10.1016/j.biortech.2013.08.102

Wu, H., Fan, Z., Jiang, X., Chen, J., and Chen, G.-Q. (2016). Enhanced Production of Polyhydroxybutyrate by Multiple Dividing *E. coli*. *Microb. Cel Fact.* 15 (1), 128–140. doi:10.1186/s12934-016-0531-6

Wu, Z. B., Gatesoupe, F.-J., Li, T. T., Wang, X. H., Zhang, Q. Q., Feng, D. Y., et al. (2018). Significant Improvement of Intestinal Microbiota of Gibel Carp (*Carassius auratus* Gibelio) after Traditional Chinese Medicine Feeding. *J. Appl. Microbiol.* 124 (3), 829–841. doi:10.1111/jam.13674

Xu, L., Podok, P., Xie, J., and Lu, L. (2014). Comparative Analysis of Differential Gene Expression in Kidney Tissues of Moribund and Surviving Crucian Carp (*Carassius auratus Gibelio*) in Response to Cyprinid Herpesvirus 2 Infection. *Arch. Virol.* 159 (8), 1961–1974. doi:10.1007/s00705-014-2011-9

Xu, M., Tremblay, P. L., Ding, R., Xiao, J., Wang, J., Kang, Y., et al. (2020). Photo-augmented PHB Production from CO_2 or Fructose by Cupriavidus Necator and Shape-Optimized CdS Nanorods. *Sci. Total Environ.* 753, 142050. doi:10.1016/j.scitotenv.2020.142050

Xu, X., Ma, B., Lu, W., Feng, D., Wei, Y., Ge, C., et al. (2020). Effective Nitrogen Removal in a Granule-Based Partial-Denitrification/anammox Reactor Treating Low C/N Sewage. *Bioresour. Technol.* 297, 122467. doi:10.1016/j.biortech.2019.122467

Xu, Y. (2019). *Isolation, Identification and Efficacy Analysis of Probiotics from the Intestinal Tract of Gibel Carp (Carassius auratus* Gibelio*)*. Master thesis (Dalian: Dalian Ocean University), 23–41. (in Chinese with English Abstract).

Yun, L., Yu, Z., Li, Y., Luo, P., Jiang, X., Tian, Y., et al. (2019). Ammonia Nitrogen and Nitrite Removal by a Heterotrophic *Sphingomonas* Sp. Strain LPN080 and its Potential Application in Aquaculture. *Aquaculture* 500, 477–484. doi:10.1016/j.aquaculture.2018.10.054

Zhang, M., Li, Y., Xu, D.-H., Qiao, G., Zhang, J., Qi, Z., et al. (2018). Effect of Different Water Biofloc Contents on the Growth and Immune Response of Gibel Carp Cultured in Zero Water Exchange and No Feed Addition System. *Aquac. Res.* 49 (4), 1647–1656. doi:10.1111/are.13620

Zhang, M., Xu, C., Zhao, Z., Wang, Z., Qiu, M., Li, Q., et al. (2017). Effects of Biofloc Technology on Growth Performance and Disease Resistance of Gibel Carp (*Carassius auratus* Gibelio). *J. Fish. Sci. China* 24 (3), 533–542. doi:10.3724/sp.j.1118.2017.16225

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

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

frontiers | Frontiers in Marine Science

TYPE Original Research
PUBLISHED 05 September 2022
DOI 10.3389/fmars.2022.862020

OPEN ACCESS

EDITED BY
Ikram Belghit,
Norwegian Institute of Marine Research (IMR), Norway

REVIEWED BY
Houguo Xu,
Yellow Sea Fisheries Research Institute (CAFS), China
Marta Silva,
Norwegian Institute of Marine Research (IMR), Norway

*CORRESPONDENCE
Alicia Estevez
alicia.estevez@irta.es

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

RECEIVED 25 February 2022
ACCEPTED 11 August 2022
PUBLISHED 05 September 2022

CITATION
Estévez A, Padrell L, Iñarra B, Orive M and San Martin D (2022) Brewery by-products (yeast and spent grain) as protein sources in rainbow trout (*Oncorhynchus mykiss*) feeds. *Front. Mar. Sci.* 9:862020. doi: 10.3389/fmars.2022.862020

Brewery by-products (yeast and spent grain) as protein sources in rainbow trout (*Oncorhynchus mykiss*) feeds

Alicia Estévez[1]*, Lluis Padrell[2], Bruno Iñarra[3], Mikel Orive[3] and David San Martin[3]

[1]Institute of Agrifood Research and Technology (IRTA) Sant Carles de la Rápita, Tarragona, Spain, [2]Institute of Agrifood Research and Technology (IRTA) Mas Bové, Tarragona, Spain, [3]AZTI, Food Research, Basque Research and Technology Alliance (BRTA), Bizkaia, Spain

A trial was conducted to analyze the effect of the inclusion of yeast and spent grain obtained from breweries in feeds for rainbow trout (*Oncorhynchus mykiss*), taking into account the availability of these by-products, produced in large quantities in Europe. The ingredients were assayed in both dried and hydrolyzed format and compared with a commercial dried or hydrolyzed yeast. According to the results, the inclusion of 20% yeast and 15% spent grain in the feed, formulated with only 15% inclusion of fish meal, produced similar results in growth among all the groups, a food conversion significantly lower for the control and spent grain formulated feeds, and rainbow trout muscle composition similar to the fish fed with a control commercial feed and showed a protein digestibility of 87%–89% without differences with the commercial yeast. Hydrolysis of the ingredients had no effects on the protein digestibility of the feeds. Protein digestibility of the ingredients was lower for spent grain. An inclusion rate not higher than 15% for spent grain is recommended. These industrial by-products can be a good source to reduce the use of plant-based ingredients and increase the sustainability of both sectors, brewery industry, and aquaculture.

KEYWORDS

Brewery by-products, valorisation, circular economy, protein digestibility, protein substitution, rainbow trout, fillet quality

Introduction

According to Food and Agriculture Organization (FAO) statistics for 2020 total aquaculture production in 2018 accounted for 82.1 million tonnes (51.3 million inland and 30.8 million of marine aquaculture). The contribution of aquaculture to fish production reached 46% in 2018 with inland aquaculture, producing the highest

quantities of farmed fish (57.2% of world total) mainly produced in freshwater (FAO, 2020). Whole fish and fish by-products are used in high quantities to produce fish meal and fish oil for animal feeding, and according to FAO, 2020, the sustainability of aquaculture depends on the use of other new ingredients to replace these fish-derived products. New ingredients need to be highly digestible to boost fish performance and reduce waste production, and they must be available in regular quantities and at competitive prices (Gatlin et al., 2007). Several protein sources have been used in recent years in experimental aquafeeds for freshwater and marine finfish, mostly derived from microalgae (Sarker et al., 2020) or insects (Cardinaletti et al., 2019; Randazzo et al., 2021a; Randazzo et al., 2021b). Terrestrial animal by-products have also been used although they are not considered acceptable by consumers (Naylor et al., 2009). As feed covers the largest cost of production in aquaculture that can reach 50%–70% of fish farmers production costs like in salmon (Ashe and Oglend, 2016), reducing the feed cost without compromising the quality or health of cultured fish may lead to a significant reduction in the total cost. That is the reason why, in the last decade, the interest in industrial by-products recovery, especially in those locally available and low-cost, to be used as alternative ingredients in feed, has increased significantly (Barrows et al., 2008).

More than 1.95 billion hectoliters of beer are produced worldwide (Conway, 2019), generating large amounts of organic waste, mostly spent yeast (BSY), and grain (BSG) that are considered high-quality ingredients for feeds due to their high content of protein, approximately 20% dry weight (DW) in the case of BSG (Robertson. et al., 2010; Aliyu and Bala, 2011; McCarthy et al., 2013) and 40%–50% DW for BSY (Mussatto. et al., 2006; Thomas and Rahman, 2006; Mussatto, 2009; Levic et al., 2010; Zhou et al., 2018).

In Europe (Eurostat, 2020) over 34 billion liters of beer containing alcohol were produced in 2020, equivalent to approximately 77 liters per inhabitant. BSG accounts for approximately 85% of all residues produced by the brewing industry (Aliyu and Bala, 2011), whereas BSY accounts for maximum 15% of total by-products generated (Kerby and Vriesekoop, 2017). These wastes from breweries are reused as terrestrial animal feed or for the production of bioethanol (Djuragic et al., 2010; Buffington, 2014). Several publications in recent years have identified these by-products as alternative ingredients in feeds for farmed fish in Europe (Oliva-Teles and Gonçalves, 2001; Cheng et al., 2004; Kaur and Saxena, 2004; Ozório et al., 2012; Castro et al., 2013; Sealey et al., 2014; Campos et al., 2018; Zhou et al., 2018; Zhang et al., 2018).

Brewers' spent grain is the major (approximately 85% in weight) by-product of beer industry (Mussatto et al., 2006). It has been used not only as human and livestock food (Murdock et al., 1981; Faccenda et al., 2017; Mussato et al., 2014) but also as feed ingredient for the ongrowing of crustaceans (Muzinic et al., 2004) and fish (Yamamoto et al., 1994; Cheng et al., 2004; Kaur and Saxena, 2004; Campos et al., 2018; Jayant et al., 2018) due to its hight content in protein and fiber as well as lipids, minerals, and vitamins (Mussatto et al., 2006; Mussato et al., 2014).

Brewers' spent yeast is the second largest by-product from brewing, and its disposal is considered an environmental problem. Several authors (Oliva-Teles and Gonçalves., 2001; Ebrahim and Abou-Seif, 2008; Ozório et al., 2012; Sealey et al., 2014) cited this by-product as a potential alternative to fish meal in the feed for cultured fish, porcine, and ruminants (Huige, 2006). Brewer's yeast is a source of protein, vitamins, and minerals (Ovie and Eze, 2014), and other bioactive compounds such as β-glucans, mannan oligosaccharides, vitamins, minerals, and nucleic acids (Ferreira et al., 2010) are also present in high quantities in this by-product.

The digestibility of these two ingredients has already been published by Nazzaro et al. (2021). The main objectives of the present study were as follows: 1) to establish the inclusion rate of hydrolyzed and non-hydrolyzed BSG and BSY and 2) to validate their use as aquafeed ingredients for rainbow trout (*Oncorhynchus mykiss*), as a model for carnivorous freshwater fish.

Materials and methods

Ingredients and experimental diet preparation

Mahou San Miguel, a Spanish brewery located in Lérida (Spain), provided the by-products that were stabilized before its inclusion in aquafeeds as in Nazzaro et al. (2021) and hydrolyzed as in San Martin et al. (2020). Four ingredients were obtained: (1) dried spent yeast (DSY; *Saccharomyces cerevisiae*), (2) hydrolyzed spent yeast (HSY), (3) dried spent grain (DSG), and (4) hydrolyzed spent grain (HSG) with a moisture lower than 10%. A commercial dried and hydrolyzed yeast was obtained from Aplicaciones Biológicas a la Nutrición S.L. (ABN, Madrid, Spain), being included in the feeds at the same rate as BSY, to compare the digestibility of this commercial yeast with that obtained from BSY and to evaluate its effects on fish growth.

The diets were formulated and extruded (4-mm die diameter, 5- to 6-mm pellet diameter) at Institute of Agrifood Research and Technology (IRTA, Mas Bové, Tarragona, Spain). The extrusion (Rosal extruder, model RS50, Spain) was carried out at 700 rpm with a final temperature of 110°C at 140 kg/h using 20% water vapor to reach 85°C–90°C at precondition. The diets were formulated using a commercial fish meal (Super Prime 70 LT, Corpesca, Spain) to meet the nutritional requirements of rainbow trout (FAO, 2020) (Table 1). Yttrium oxide (Y_2O_3, Sigma Aldrich, Spain) was used as an inert marker (0.2 g kg^{-1}) for the evaluation of digestibility. All the feeds were iso-proteic and iso-lipidic and were formulated including 10% and 20% of DSY, HSY, dried yeast from ABN company

10.3389/fmars.2022.862020

TABLE 1 Formulation of feeds used in the trial.

Ingredient (g)	CTRL	DSY 10	HSY 10	DSY 20	HSY 20	DSG 7.5	HSG 7.5	DSG 15	HSG 15
Soy bean meal	6.00	4.06	4.06	1.00	1.00	6.00	6.00	2.52	2.52
Wheat gluten	21.78	19.52	19.52	16.78	16.78	20.40	20.40	18.40	18.40
Soycomeal[f]	17.00	17.00	17.00	17.00	17.00	17.00	17.00	17.00	17.00
Fish meal[a]	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00
Fish oil[c]	7.75	7.50	7.50	7.38	7.38	7.74	7.74	7.72	7.72
Soya oil	5.85	5.78	5.78	5.77	5.77	5.91	5.91	6.12	6.12
Lutavit C Aquastab 35 %	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Dicalcium Phosphate[g]	0.83	0.70	0.70	0.65	0.65	0.75	0.75	0.65	0.65
Choline chloride[f]	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.27
Lysine HCl	0.36	0.09	0.09			0.20	0.20		
Mineral mix[d]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin premix[d]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Wheat starch[b]	15.95	16.77	16.77	13.60	13.60	17.66	17.66	14.65	14.65
Soy lecithin	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Ytrium oxide[e]	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Brewer's spent grain						7.50	7.50	15.00	15.00
Brewer's spent yeast		10.00	10.00	20.00	20.00				
Total Fish meal	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00
Total Vegetable meal	44.78	40.58	40.58	34.78	34.78	50.90	50.90	52.92	52.92
FM/FO	15/7.7	15/7.5	15/7.5	15/7.4	15/7.4	15/7.7	15/7.7	15/7.7	15/7.7
Crude protein (% DW)	45.46	43.57	44.52	44.86	43.23	44.07	44.19	43.48	43.70
Crude fat (%DW))	15.50	15.65	16.24	16.41	16.00	16.25	16.92	16.38	17.43
Water (%)	91.50	91.55	91.60	91.70	91.90	91.60	92.10	92.00	92.20
Ash (%DW)	5.30	5.35	5.70	5.80	5.60	5.65	5.70	5.30	5.50

The same formulation was used for dried and hydrolyzed yeast and spent grain. Changes in corn gluten, wheat gluten, and soybean meal, to account for total plant meal inclusion, were made to ensure diets were isonitrogenous. CTRL, control diet; DSY, dried spent yeast; HSY: hydrolyzed spent yeast; DSG, dried spent grain; HSG, hydrolyzed spent grain; DYABN, dried commercial yeast from ABN; HYABN, hydrolyzed commercial yeast from ABN. Total vegetable meal includes the amounts of spent grain added to DSG and HSG feeds.
A. Super Prime LT fishmeal Corpesca, Chile.
B. Cargill, Brenntag, Spain.
C. Eurocoyal, Barcelona, Spain.
D. Tecnovit, Tarragona, Spain.
E. Sigma, Spain.
F. Andres Pintaluba S.A., Spain.

(DYABN), and hydrolyzed yeast from ABN (HYABN) and 7.5% and 15% of DSG and HSG to the basal mixture (Table 1). Feeds including the commercial yeast from ABN have the same formulation and inclusion rate (10% and 20%) than the feeds with brewery SY included. This new ingredients were included in the feeds replacing gluten and plant-derived meals.

Fish rearing and fecal collection

Rainbow trout specimens were obtained from Aiguanatura dels Ports (Tarragona, Spain) transported to IRTA by road and kept in quarantine for 14 days and fed using the *Premium* commercial feed for rainbow trout from Skretting. Trouts were distributed in twenty-two 200-L fiber glass tronco-conical tanks with a purge system to collect uneaten feed pellets, in groups of 15 fish (body weight 77.90 ± 8.97 g) per tank. The tanks were supplied with filtered freshwater in a recirculation system (IRTAMar™) and maintained at 20°C with natural light and photoperiod and salinity of 1.5 ppt.

At the end of the trial, all the fish were individually weighted and the growth calculated using relative growth rate (RGR, %) and specific growth rate (SGR, %) as in the formula:

$$RGR = (Wf - Wi)/Wi \times 100$$

$$SGR = (LnWf - LnWi)/t \times 100$$

where Wi and Wf are the fish weight at the beginning (Wi) and at the end (Wf) of the feeding period, and t is the time (days) between Wf and Wi.

Five fish per tank were dissected, and liver was weighted to calculate hepatosomatic index (HSI) as in the following formula:

$$HSI = Weight\ of\ liver\ of\ fish/Body\ weight\ of\ fish \times 100$$

The experimental diets were randomly assigned to the tanks and fed in triplicates, once per day, during 60 days using Arvotec (Finland) automatic feeders. The amount of feed was calculated according to the theoretical weight of the fish and the feeding tables provided by Skretting. Every day, the feed provided was registered, as well as the uneaten feed, collected every day from the bottom center drain of the tanks, dried in an oven for 24 h, and weighted to calculate feed conversion ratio (FCR) and protein efficiency ratio (PER).

$$FCR = Feed\ consumed\ /\ (Final - Initial\ weight)$$

$$PER = (Final - Initial\ weight)\ /\ Protein\ consumed$$

In the case of the fish fed with ABN commercial yeast only, one replicate per treatment was used due to the high number of tanks used in the trial and because the main purpose of including this treatment was to compare the digestibility of both types of yeast (commercial vs. obtained from brewery).

Feces were collected by abdominal stripping (as described by Nazzaro et al., 2021) in anesthetized fish (MS 222, 47 ppm, 5-min exposure, Pharmaq, Spain), in alternate days during 2 weeks, to collect enough quantities for chemical and Ytrium analyses, before the final sampling and carried out in all the tanks fed with the control and brewery by-products ingredients. Fecal samples were freeze-dried and stored at −20°C until chemical analyses.

In the final sampling (day 60), five fish per tank were eviscerated, and the weight of viscera and liver was recorded. Samples of whole liver and muscle taken using a standardized cut (2 cm wide cut behind the dorsal fin on the left lateral side of the fish) were collected and kept at −20°C for biochemical analyses.

The ADCs of the experimental diets were calculated according to Maynard et al. (1979) using only the feeds with the highest inclusion rate (20% for DY and 15% for SG)

$$ADC\,(\%) = 100 \times (1 - (dietary\ Y_2O_3 level/feces\ Y_2O_3 level) \times (feces\ nutrient\ or\ energy\ level\ /\ dietary\ nutrient\ or\ energy\ level)\,.$$

The ADCs of the test ingredients were estimated according to NRC (2011):

$$ADC_{SG}(\%) = ADC_{test} + [(ADC_{test} - ADC_{ref}) \times ((0.85 \times D_{ref})/(0.15 \times D_{ing}))]$$

$$ADC_{SY}(\%) = ADC_{test} + [(ADC_{test} - ADC_{ref}) \times ((0.8 \times D_{ref})/(0.2 \times D_{ing}))]$$

Where:

ADC_{test} = *ADC (%) of the experimental diet;*

ADC_{ref} = *ADC (%) of the reference diet;*

D_{ref} = *g/kg nutrient (or MJ/kg gross energy) of the reference diet (DM basis);*

D_{ing} = *g/kg nutrient (or MJ/kg gross energy) of the test ingredient (DM basis).*

Chemical analyses

The feces samples were dehydrated by freeze-drying (LyoAlfa 6, Telstar, USA) before chemical analyses to avoid nutritional losses or alterations. The biochemical analyses of the diets, ingredients, feces, and muscle and liver of the fish were performed in duplicates according to the standard methods of the Association of Official Analytical Chemists (AOAC, 2006). Dry matter (105°C for 14 h, AOAC 925.09), ash incineration in a muffle furnace (Nabertherm, Germany 500°C for 5 h, AOAC, 942.05), crude protein (Dumas's procedure using Nitrogen analyser FP-528 Leco, USA, AOAC 968.06, with N correction factor of 6.25), and crude fat (Büchi Extraction System B-811, Switzerland, AOAC 920.39) were analyzed in all the samples obtained. Acid catalyzed transmethylation (Christie, 1982) and purification of methyl esters (TLC plates) were carried out before the fatty acid analysis carried out by gas–liquid chromatography (Thermo TraceGC, Villalta et al., 2005). Ytrium oxide content in diets and feces was determined according to Garantun-Tjeldsto et al. (2006) by inductively coupled plasma–mass spectrometry (Agilent Technologies 7700x).

Data analysis

All the biochemical analyses were carried out in duplicates using pooled samples of each tank. In the case of commercial yeast with only one replicate, the results were not considered in the comparisons among treatments.

Initial and final weight (n = 60), HSI (n = 3), growth rates (SGR and RGR, n = 3), feed conversion (FCR, n = 3), protein efficiency (PER, n = 3), biochemical composition of muscle and liver (n = 3), and apparent digestibility coefficient (ADC) data (n = 2) were tested for normality of variances using Levene's test before being submitted to a one-way analyses of variance (ANOVA) using Sigma Plot 12.0 program (Systat Software, Inc., USA). The differences were considered statistically significant if $P < 0.05$ after using a Holm-Sidak *post hoc* test to perform pair wise comparisons of means.

Results

Table 1 shows the formulation and proximate composition of the feeds used in the study including brewery by-products, and the formulation used for the inclusion of spent yeast (SY) was also used for the formulation using yeast from a commercial company (ABN, Spain). Table 2 shows the composition of the brewery by-products used in the trial.

TABLE 2 Proximate composition and amino acid profile of the ingredients derived from the brewery industry [spent yeast (SY) and spent grain (SG)] dried (D) and hydrolyzed (H), used in the formulation of the feed.

	DSY	HSY	DSG	HSG
Dry matter (DM, g/kg)	941.90	890.50	920.00	981.10
Ash (g/kg DM)	42.36	43.46	39.13	61.05
Crude protein (g/kg DM)	478.50	463.11	247.07	217.92
Crude fat (g/kg DM)	3.74	5.01	84.23	116.81
Gross energy (MJ/kg)	19.90	19.92	21.69	20.65
Phosphorus (g/kg DM)	9.66	9.77	5.33	3.47
Crude fiber (g/kg DM)	6.79	6.96	187.83	165.94
Starch (g/kg DM)	218.60	225.15	39.02	34.96
Vitamin B2 (ppm)	2.76	5.61	0.43	1.43
β-glucan (g/kg DM)	80.9	90.4	0.88	0.00
Essential amino acids (g/kg DM)				
Arginine	25.16	23.81	12.83	10.70
Histidine	12.10	11.45	6.52	5.61
Lysine	31.74	28.86	9.57	7.34
Threonine	24.31	23.47	9.13	8.05
Isoleucine	23.46	22.68	10.22	8.05
Leucine	35.14	33.69	24.78	18.45
Valine	26.54	25.27	12.72	10.50
Methionine	8.17	7.75	5.11	3.87
Phenylalanine	23.04	22.46	14.46	11.31
Non-essencial amino acids (g/kg DM)				
Tyrosine	17.20	15.72	9.57	8.05
Aspartic acid	47.88	45.82	17.17	14.47
Glutamic acid	60.30	58.28	52.39	36.90
Alanine	29.41	28.30	15.11	11.72
Glycine	18.90	18.30	8.80	7.95
Proline	22.30	21.22	25.11	18.24
Hydroxyproline	<0.3	<0.3	<0.3	<0.3
Serine	25.37	24.26	11.20	8.77

Final weight was significantly higher for DSY and HSY groups (Table 3), although SGR and RGR were not significantly different among the groups of fish fed with brewery by-products (DSY, HSY, DSG, and HSG) included at two different levels and those fed with the control diet (CTRL). FCR and PER results were significantly lower for the CTRL and HSG-fed groups, whereas those fed with spent yeast (DSY and HSY) show the highest values.

ADCs of the feeds and ingredients are shown in Table 4. Digestibility of protein was 86.6%–89.9% in the experimental diets, being higher in the case of CTRL. The ADC of lipids varied from 80.9% to 88.7%, and the lowest digestibility was found for CTRL and the highest for the spent yeast (both dried and hydrolyzed), although no significant differences were detected (ANOVA, $P = 0.053$, $n = 2$).

Protein digestibility coefficients were higher than 75% for SY and the commercial yeast (YABN), whereas SG had statistically significant lower values between 50% and 65%. No improvement in digestibility of the ingredients was obtained after hydrolysis of yeast and spent grain.

Muscle and liver protein and lipid content results are shown in Table 5. A significantly higher protein content was recorded in the muscle of fish fed with HSY included at 20% and HYABN commercial yeast included at 10%, showing values approximately 71%–72%, and the lowest values were obtained in the fish from DSG, HSG, and CTRL groups. Lipids were higher in the muscle (10% lipids) of fish from DSY 20 group, whereas the lowest values were found in CTRL, HSG 7.5, and HYABN 10 (5.6% lipids). In the liver, the highest protein content (52%–54% DW) was recorded in DSG 7.5 and HSG 15 groups, whereas it was lower in those fed with DSY and HSY included at 10% and 20%, as well as in the fish fed with HYABN at 10%. Lipid content was higher in in the liver of fish fed with DYABN included at 10% (15.5% lipids) and lower in those fed with HSY at 10% (9% lipids) with the rest of the groups showing levels between 11% and 14%.

TABLE 3 Initial and final weight of rainbow trout used in the inclusion trial (n = 60 per treatment) and results in hepatosomatic index (HSI; five fish per tank, n = 3), specific growth rate (SGR; n = 3), relative growth rate (RGR; n = 3), feed conversion ratio (FCR; n = 3), and protein efficiency ratio (PER; n = 3).

	Initial weight (g)		Final weight (g)		HSI		SGR		RGR		FCR		PER	
	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD
CTRL	79.33	8.65	174.28	34.56 b	2.03	0.61 b	1.29	0.05	119.44	6.67	1.29	0.05 b	1.19	0.07 b
DSY10%	77.10	9.33	217.88	48.84 a	4.71	2.05 a	1.70	0.19	200.95	8.53	1.81	0.05 a	2.01	0.09 a
DSY20%	77.42	10.07	219.68	28.75 a	3.76	0.88 ab	1.71	0.09	184.13	15.25	1.71	0.09 a	1.84	0.15 a
HSY10%	78.82	9.60	217.37	45.87 a	4.26	1.28 ab	1.66	0.05	175.33	8.80	1.66	0.05 a	1.75	0.09 a
HSY20%	77.56	9.67	222.80	31.48 a	4.46	1.44 a	1.73	0.04	187.30	7.42	1.73	0.04 a	1.87	0.07 a
DSG7.5%	77.95	8.80	193.38	37.55 ab	2.94	1.51 ab	1.49	0.11	148.11	16.11	1.49	0.11 ab	1.48	0.16 ab
DSG15%	77.63	7.52	192.90	29.14 ab	2.88	0.88 ab	1.49	0.03	148.48	5.30	1.49	0.03 ab	1.48	0.05 ab
HSG7.5%	77.39	8.79	178.40	38.85 b	2.14	0.53 b	1.37	0.04	130.66	5.53	1.37	0.03 b	1.31	0.06 b
HSG15%	77.92	8.37	175.73	39.02 b	2.32	0.53 b	1.33	0.15	125.08	20.14	1.33	0.15 b	1.25	0.20 b
DYABN10%	77.41	9.55	206.19	62.02 ab	3.30	0.73 ab	1.61		166.37		1.61		1.66	
DYABN20%	78.13	10.06	228.63	43.21a	2.50	0.07 ab	1.76		192.62		1.76		1.93	
HYABN10%	77.19	8.10	216.36	35.36 ab	4.07	0.85 ab	1.69		180.31		1.69		1.80	
HYABN20%	78.15	10.45	202.94	38.58 ab	3.47	0.79 ab	1.56		159.67		1.56		1.60	
ANOVA			P < 0.001		P < 0.001		P=0.007		P=0.07		P < 0.001		P < 0.001	

Different letters indicate statistically significant differences (ANOVA, P < 0.05, n = 3). CTRL, control diet; DSY, dried spent yeast; HSY, hydrolyzed spent yeast; DSG, dried spent grain; HSG, hydrolyzed spent grain; DYABN, dried commercial yeast from ABN; HYABN, hydrolyzed commercial yeast from ABN. Fish fed with commercial yeast from ABN were not included in the statistics; only one tank was used per treatment.

Tables 6, 7, 8 show the fatty acid profile of feeds, muscle, and liver, respectively. Muscle and liver fatty acid content reflected the fatty acid composition of the feeds used. Figure 1 summarizes the fatty acid profile of the muscle with all the groups showing similar profiles although the muscle of fish fed with spent yeast (dried or hydrolyzed) included at 20% shows a lower N-3 PUFA content and higher MUFA, the fish fed with spent grain show higher levels of N-6, and those fed with DSY included at 10% show a profile closer to the CTRL.

Discussion

In the last years, considerable efforts have been made to look for alternative ingredients to fish meal and oil, and most of the research focused in looking for sustainable ingredients to reduce wastes. Several products such as fish trimmings (Stevens et al., 2018), insects (IPIFF, 2018), algae (Loveday, 2019), and by-products derived from the processing industry and microbial biomass (Hua et al., 2019) have centered most of this research. Brewery by-products have also been considered as good ingredients for marine and freshwater fish culture, because of their content of protein (40%–50% for spent yeast), lipids, vitamin B2, β-glucans, and mannan-oligosaccharides. Recently, a review by Agboola et al. (2021) about the use of yeast in feed production and fish aquaculture suggested the need for a large-scale production of yeast at affordable cost. In the case of breweries, spent yeast and spent grain are produced in very high quantities, but they need to be dewatered and dried before being used in aquafeeds. San Martin et al. (2020) designed these

TABLE 4 Apparent digestibility coefficients of feeds and ingredients (Av±SD) used in the inclusion trial.

Apparent Digestibility of the Feed

	CTRL	DSY 20%	HSY 20%	DSG 15%	HSG 15%	DYABN 20%	HYABN 20%
Protein	89.86±1.70	86.63±2.35	86.80±2.40	87.62±2.66	86.63±2.44	87.71±1.23	87.15±1.17
Lipids	80.87±1.67	87.44±1.76	88.65±1.65	84.20±1.81	82.07±1.53	85.86±2.05	86.14±2.06
Apparent Digestibility Coefficients of Ingredients							
		DSY 20%	HSY 20%	DSG 15%	HSG 15%	DYABN 20%	HYABN 20%
Protein		74.52±1.62a	75.37±1.47a	65.10±2.90b	49.93±3.23c	78.62±1.27a	76.09±1.35a

DSY: Dried spent yeast. HSY: Hydrolysed spent yeast. DSG: Dried spent grain. HSG: Hydrolysed spent grain. DYABN: Dried commercial yeast from ABN. HYABN: Hydrolysed commercial yeast from ABN. Different letters indicate significant differences (ANOVA, P<0.001, n=2).

TABLE 5 Protein and lipid content (% dry weight. DW) of the muscle and liver of rainbow trout fed the experimental diets.

	MUSCLE				LIVER			
	Total Lipids (% DW)		Protein (% DW)		Total Lipids (% DW)		Protein (% DW)	
	Av	SD	Av	SD	Av	SD	Av	SD
CTRL	5.62	0.22 d	62.13	0.57 c	14.84	0.21 ab	45.09	1.56 b
DSY 10%	6.67	0.22 c	69.83	3.27 ab	11.02	0.21 c	38.97	1.00 c
DSY 20%	10.74	0.02 a	64.97	0.98 abc	11.34	0.57 c	38.67	0.46 c
HSY 10%	7.74	0.03 c	64.66	1.82 abc	9.59	0.14 d	39.11	0.83 c
HSY 20%	9.33	0.03 b	72.3	3.00 a	11.63	0.11 c	39.75	0.18 c
DSG 7.5%	8.88	0.39 b	67.3	3.81 abc	13.32	0.34 b	52.14	0.33 a
DSG 15%	7.24	0.05 c	61.43	2.46 c	13.87	0.14 b	44.36	0.81 b
HSG 7.5%	5.61	0.19 d	60.47	3.64 c	13.4	0.17 b	48.66	1.67 b
HSY 15%	8.13	0.35 bc	68.43	0.80 abc	14.42	0.47 b	54.24	0.33 a
DYABN 10%	9.35	0.54 b	65.41	4.06 abc	15.47	0.32 a	47.17	0.36 b
DYABN 20%	6.71	0.21 c	62.08	0.64 b	11.6	0.03 c	41.58	1.25bc
HYABN 10%	5.63	0.23 d	71.05	1.08 a	10.97	0.01 c	38.65	1.08 c
HYABN 20%	9.16	0.32 b	62.75	2.89 b	11.97	0.28 c	45.93	0.01 b
ANOVA	P<0.001		P<0.001		P<0.001		P<0.001	

Different letters indicate significant differences (ANOVA, P<0.05, n=3). DSY: Dried spent yeast, HSY: Hydrolysed spent yeast, DSG: Dried spent grain, HSG: Hydrolysed spent grain, DYABN: Dried commercial yeast from ABN, HYABN: Hydrolysed commercial yeast from ABN.

processes of dewatering and drying, reducing energy consumption, being more economical and environmentally sustainable, and the quantities of DSY and grain can now provide a higher contribution in satisfying the protein demand of the global aquafeed industry. This was one of the objectives of the project LIFE-Brewery together with the trials carried out with gilthead sea bream (Estévez et al., 2021) and the present trial with rainbow trout, to show that, once these brewery by-products are processed, they can be used as ingredients in aquafeeds for high-value fish such as salmon, rainbow trout, or European sea bass and gilthead seabream. The main objective of the present study was to evaluate the inclusion of these products in rainbow trout feeds and re-evaluate the digestibility of both ingredients and feeds. Thus, the ADC values obtained in the present study were higher than those published by Nazzaro et al. (2021) probably as a consequence of the improvements introduced in the mechanical dewatering and drying processes, reducing the manipulation of spent yeast.

Brewer's yeast can be considered a good replacer of fish meal and plat-based proteins (Rumsey et al., 1990; Rumsey et al., 1991; Oliva-Teles and Gonçalves, 2001) in aquafeeds, and it has been included in commercial diets for several fish species, including salmonids (Ferreira et al., 2010). Similar to the results of the present study in rainbow trout, Rumsey et al., (1991) found that, when brewer's yeast was included at up to 25% of the diet, fish growth and feed conversion were better than with the control feed. Higher levels of brewer's yeast were less palatable, but fish did not appear to be adversely affected. More recent studies using grain distiller's dried yeast from the ethanol industry in feeds for rainbow trout showed that 37.5% replacement of fish meal protein and 18% replacement of total dietary protein did not reduce fish performance (Hauptman et al., 2014; Sealey et al., 2015).

Spent grain is the main solid waste produced by the beer industry (Farcas et al., 2017) reaching up to 85% of breweries by-products. It is daily produced in large breweries, and, due to its high moisture content and transport cost, it is generally used in the neighborhood of the breweries as animal feed or as a compost. There are not many publications related to the use of this raw material in aquafeeds, but, in a publication by Hertrampf and Piedad-Pascual (2012), they recommended not to include it in high amounts in the feeds due to its high fiber content and the low pelletizing ability and poor durability of the pellet. It has been mostly used in freshwater fish feeds (carp and Nile tilapia; Kaur and Saxena, 2004) with good results in growth and conversion. Cheng et al. (2004) found similar results as in the present trial using spent grain in the diet for rainbow trout, whereas other authors (Overland et al., 2013; Welker et al., 2014) found also that using dried grains (mostly maize) from the fuel ethanol industry replacing plant ingredients in diets for rainbow trout has good results in terms of growth and conversion, improved the protein digestibility of the diets, and did not have any effect on intestine structure and function.

The results obtained in the present study show that SY inclusion levels up to 20% produced very good results in rainbow trout growth, with final weights significantly higher than those of CTRL group, although no differences in SGR and RGR were observed. FCR and PER had significantly higher values for the

TABLE 6 Fatty acid composition (% of total FAs, only main fatty acids and totals are included) of the feeds used in the trial.

	CTRL		DSY 10%		DSY 20%		HSY 10 %		HSY 20%		DSG 7.5%		DSG 15%		HSG 7.5%		HSG 15%		DYABN 10%		DYABN 20%		HYABN 10%		HYABN 20%	
	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD
Total FAs (mg/g Lipids)	614.69	27.91	614.24	2.31	604.38	1.19	616.51	6.75	632.68	0.62	619.25	0.72	607.02	13.15	606.12	6.07	640.44	1.47	541.24	18.19	597.55	36.97	552.15	11.87	561.70	30.71
14:0	1.67	0.15	1.07	0.14	1.16	0.34	1.50	0.00	1.36	0.00	1.45	0.34	1.31	0.01	1.33	0.29	1.25	0.06	1.30	0.04	1.18	0.06	1.05	0.26	1.06	0.23
16:0	18.80	0.10	17.54	0.63	17.53	0.68	18.68	0.16	17.70	0.10	18.08	0.10	18.03	0.01	18.26	0.69	17.87	0.23	17.58	0.46	17.69	0.14	17.10	0.17	17.65	0.08
18:0	4.68	0.37	4.14	0.25	4.10	0.04	4.14	0.13	3.86	0.06	4.04	0.16	3.95	0.07	4.14	0.04	4.11	0.04	3.99	0.07	4.59	0.16	4.17	0.26	4.51	0.14
22:0	0.26	0.02	0.24	0.02	0.22	0.04	0.24	0.01	0.29	0.09	0.26	0.01	0.28	0.00	0.24	0.07	0.28	0.02	0.22	0.00	0.21	0.04	0.25	0.03	0.24	0.01
Total SAT	25.96	0.32 a	23.59	0.87 ab	23.67	1.02 ab	25.33	0.27 ab	23.33	0.12 ab	24.25	0.37ab	24.11	0.07 ab	24.32	0.80 ab	24.15	0.26 ab	23.73	0.33 ab	24.32	0.02 ab	23.12	0.29 b	23.80	0.53 ab
16:1	2.67	0.16	2.58	0.11	2.73	0.04	3.40	0.37	3.09	0.38	2.50	0.18	2.45	0.23	2.47	0.17	2.30	0.00	2.84	0.27	2.83	0.16	2.51	0.03	2.87	0.01
18:1n-9	24.31	1.81	24.27	0.63	24.67	0.38	24.46	0.45	24.70	0.17	23.91	0.07	23.74	0.66	23.54	0.12	23.78	0.09	24.43	0.45	24.49	0.01	24.79	0.07	24.53	0.33
Total MUFA	28.39	1.85	28.14	0.58	28.54	0.46	29.41	0.93	29.01	0.25	27.63	0.27	27.43	1.02	27.26	0.06	27.36	0.21	28.46	0.14	28.46	0.20	28.52	0.08	28.61	0.24
18:2n-6	33.27	0.92	33.66	0.20	33.67	0.51	29.37	0.05	33.40	0.78	34.99	0.19	35.36	0.57	35.38	0.74	36.19	0.29	33.66	0.04	32.97	0.09	34.15	0.06	33.45	0.62
18:3n-6	0.61	0.07	0.43	0.02	0.36	0.07	0.61	0.28	0.43	0.09	0.29	0.04	0.40	0.01	0.33	0.09	0.35	0.02	0.40	0.17	0.40	0.08	0.71	0.26	0.37	0.04
20:4n-6			0.32	0.05	0.33	0.01	0.34	0.04	0.29	0.02	0.27	0.03	0.28	0.03	0.28	0.01	0.24	0.03	0.34	0.00	0.27	0.00	0.34	0.03	0.33	0.01
Total n-6 PUFA	33.88	0.98 b	34.41	0.27 b	34.35	0.43 b	30.32	0.37 c	34.12	0.67 b	35.56	0.12 a	36.04	0.59 a	35.99	0.64 a	36.78	0.30 a	34.40	0.14 b	33.64	0.17 b	35.20	0.30 a	34.16	0.67 b
18:3n-3	3.01	0.16	3.25	0.05	3.33	0.04	3.12	0.21	3.25	0.02	3.38	0.12	3.15	0.06	3.36	0.12	3.31	0.02	3.10	0.19	3.34	0.05	3.25	0.11	3.28	0.05
20:5n-3	3.45	0.64 b	4.20	0.05 ab	3.91	0.12 ab	4.73	0.10 a	4.16	0.44 ab	3.64	0.16 b	3.39	0.16 b	3.65	0.06 b	3.22	0.28 b	3.95	0.02 ab	3.95	0.17 ab	3.76	0.29 ab	3.99	0.23 ab
22:6n-3	5.27	0.38 b	6.31	0.11 a	6.16	0.02 a	7.05	0.03 a	6.09	0.10 a	5.44	0.39 b	5.73	0.54 ab	5.37	0.15 b	5.14	0.05 b	6.21	0.18 a	6.08	0.22 a	6.04	0.18 a	6.00	0.08 a
Total n-3 PUFA	11.73	1.19 b	13.83	0.02 ab	13.40	0.15 ab	14.90	0.29 a	13.49	0.53 ab	12.52	0.75 b	12.37	0.35 b	12.38	0.09 b	11.68	0.35 b	13.37	0.33 ab	13.54	0.04 ab	13.11	0.66 ab	13.39	0.10 ab
Total PUFA	45.61	2.17	48.23	0.29	47.76	0.57	45.22	0.66	47.61	0.15	48.08	0.63	48.41	0.94	48.37	0.73	48.45	0.05	47.77	0.19	47.18	0.21	48.31	0.37	47.55	0.77

Different letters indicate significant differences (ANOVA, $P<0.05$, n=3). DSY: Dried spent yeast, HSY: Hydrolysed spent yeast, DSG: Dried spent grain, HSG: Hydrolysed spent grain, DYABN: Dried commercial yeast from ABN, HYABN: Hydrolysed commercial yeast from ABN.

10.3389/fmars.2022.862020

TABLE 7 Fatty acid composition (% of total FAs, only main fatty acids and totals are included) of the muscle of the fish collected at the end of the trial.

	CTRL		DSY 10%		DSY 20%		HSY 10%		HSY 20%		DSG 7.5%		DSG 15%		HSG 7.5%		HSG 15%		DYABN 10%		DYABN 20%		HYABN 10%		HYABN 20%	
	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD
Total FAs (mg/g Lipids)	634.92	5.71	612.17	7.80	650.01	21.16	654.08	17.07	643.00	7.31	648.14	8.07	630.52	27.46	657.32	12.91	659.71	12.91	622.60	6.81	661.74	3.10	648.85	12.06	608.02	7.72
16:0	20.09	0.57	20.95	0.06	20.03	0.08	21.71	0.25	20.27	0.47	21.15	0.27	19.65	0.07	20.70	0.15	20.23	0.25	20.16	0.00	20.28	0.67	21.42	0.34	20.35	0.16
18:0	4.92	0.12	4.75	0.05	5.07	0.07	4.93	0.12	5.26	0.13	4.80	0.09	4.90	0.02	4.72	0.03	4.89	0.06	5.00	0.24	4.90	0.13	5.02	0.12	5.21	0.07
Total SAT	27.03	0.40c	28.18	0.13bc	27.45	0.06c	29.30	0.19a	28.03	0.66b	28.58	0.14bc	26.76	0.04c	27.64	0.30c	27.31	0.22c	27.61	0.10c	27.36	1.07bc	28.84	0.19b	27.86	0.28bc
16:1	2.70	0.15	3.94	0.02	4.12	0.05	4.60	0.12	4.05	0.16	3.81	0.04	2.91	0.04	3.03	0.17	2.89	0.02	4.43	0.08	3.42	0.12	3.50	0.07	4.06	0.05
18:1n-9	21.94	0.83	21.66	0.14	26.02	0.22	23.08	0.30	24.81	0.23	23.42	0.05	22.10	0.38	21.43	0.12	22.48	0.16	24.51	0.19	23.21	0.11	20.27	0.07	25.93	0.10
18:1n-7	2.73	0.12	3.16	0.13	3.51	0.07	3.68	0.16	3.52	0.28	3.16	0.36	3.15	0.27	2.68	0.66	3.13	0.30	3.38	0.06	2.99	0.35	2.93	0.04	3.26	0.21
Total MUFA	28.64	0.89c	30.07	0.06c	35.22	0.23a	32.81	0.25b	33.93	0.62ab	31.73	0.38bc	29.52	0.05c	28.42	0.39c	29.78	0.17c	33.75	0.16ab	31.07	0.31b	28.07	0.18c	34.81	0.31a
18:2n-6	19.21	0.35	18.67	0.01	19.62	0.29	16.06	0.04	19.68	0.43	20.49	0.22	21.06	0.10	21.11	0.00	21.53	0.19	18.93	0.30	17.91	1.10	16.78	0.37	18.78	0.14
20:4n-6	1.02	0.02	0.76	0.00	0.70	0.04	0.79	0.05	0.67	0.03	0.76	0.06	0.82	0.00	0.94	0.04	0.72	0.09	0.68	0.02	0.87	0.21	0.96	0.01	0.72	0.02
Total n-6 PUFA	20.98	0.27b	19.80	0.03c	20.74	0.22b	17.26	0.08c	20.74	0.46ab	21.81	0.29a	22.38	0.10a	22.61	0.01a	22.80	0.25a	19.95	0.30c	19.14	1.30c	18.12	0.41c	19.94	0.05c
18:3n-3	1.87	0.07	2.03	0.02	2.09	0.12	1.78	0.04	1.99	0.18	2.06	0.06	2.14	0.05	1.89	0.02	2.12	0.11	2.07	0.03	2.23	0.11	1.80	0.10	2.03	0.01
20:4n-3	0.39	0.03	0.31	0.02	0.33	0.00	0.28	0.01	0.29	0.01	0.26	0.01	0.33	0.04	0.28	0.05	0.28	0.01	0.29	0.01	0.29	0.01	0.32	0.00	0.26	0.01
20:5n-3	2.87	0.23 a	2.23	0.09c	1.79	0.06d	2.20	0.10c	1.93	0.02cd	2.30	0.00c	2.37	0.00c	2.56	0.05b	2.18	0.10c	1.85	0.03d	2.19	0.08c	2.45	0.03b	1.76	0.03d
22:5n-3	0.92	0.01	0.75	0.06	0.63	0.00	0.71	0.07	0.67	0.02	0.76	0.04	0.78	0.02	0.84	0.01	0.73	0.00	0.61	0.03	0.76	0.03	0.72	0.02	0.62	0.01
22:6n-3	16.81	1.25b	16.11	0.42b	11.27	0.23c	15.15	0.44b	11.88	0.66c	11.91	0.07c	15.18	0.11b	15.17	0.15b	14.25	0.05b	13.46	0.26bc	16.39	0.45b	19.25	0.89a	12.25	0.02c
Total n-3 PUFA	23.35	1.56a	21.95	0.22b	16.59	0.04c	20.63	0.36b	17.31	0.82c	17.89	0.22a	21.35	0.09b	21.33	0.08b	20.11	0.30b	18.72	0.32	22.39	0.55	24.97	0.76	17.39	0.01
Total PUFA	44.33	1.29	41.75	0.19	37.33	0.18	37.88	0.44	38.04	1.28	39.70	0.51	43.73	0.01	43.94	0.09	42.91	0.05	38.67	0.02	41.53	0.75	43.09	0.35	37.33	0.04
DHA/EPA	5.85	0.03a	7.24	0.49b	6.31	0.34ab	6.90	0.52a	6.16	0.42ab	5.17	0.03a	6.42	0.04ab	5.93	0.18ab	6.54	0.27ab	7.27	0.001b	7.48	0.46b	7.86	0.45b	6.94	0.14b
EPA/ARA	2.81	0.16	2.95	0.12	2.56	0.22	2.78	0.31	2.89	0.17	3.05	0.24	2.90	0.01	2.72	0.05	3.04	0.50	2.74	0.03	2.58	0.53	2.54	0.04	2.44	0.11
n-3/n-6	1.11	0.09b	1.11	0.01b	0.80	0.01a	1.20	0.01b	0.83	0.02a	0.82	0.00a	0.95	0.01a	0.94	0.00a	0.88	0.02a	0.94	0.03a	1.17	0.11b	1.38	0.07c	0.87	0.00a

Different letters indicate significant differences (ANOVA, $P<0.05$, n=3). DSY: Dried spent yeast, HSY: Hydrolysed spent yeast, DSG: Dried spent grain, HSG: Hydrolysed spent grain, DYABN: Dried commercial yeast from ABN, HYABN: Hydrolysed commercial yeast from ABN

10.3389/fmars.2022.862020

TABLE 8 Fatty acid composition (% of total FAs, only main fatty acids and totals are included) of the liver of the fish collected at the end of the trial.

	CTRL		DSY 10%		DSY 20%		HSY 10%		HSY 20%		DSG 7.5%		DSG 15%		HSG 7.5%		HSG 15%		DYABN 10%		DYABN 20%		HYABN 10%		HYABN 20%	
	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD	Av	SD
Total FAs (mg/g Lipids)	538.51	4.70	512.01	2.37	542.67	4.44	525.88	6.76	524.95	5.35	522.46	6.73	523.34	10.17	538.31	1.86	524.15	3.97	531.38	2.20	539.89	21.46	569.52	7.86	578.82	13.70
16:0	16.80	0.21	15.28	0.23	15.38	0.10	16.76	0.19	15.73	1.06	18.33	0.19	19.51	0.26	18.69	0.54	20.23	0.59	20.90	0.91	18.12	0.46	15.84	0.09	15.61	0.41
18:0	7.48	0.36	7.14	0.19	7.48	0.07	7.24	0.00	7.08	0.08	8.94	0.14	8.85	0.14	8.23	0.05	9.53	0.05	9.94	0.14	8.06	0.04	6.91	0.14	8.01	0.01
Total SAT	25.30	0.53b	23.87	0.07b	23.88	0.13b	25.29	0.28b	24.11	1.50b	28.29	0.37ab	29.37	0.25ab	27.83	0.68ab	30.74	0.57a	32.04	1.17a	27.01	0.64ab	24.03	0.21b	24.45	0.43b
16:1	1.35	0.08	3.26	0.10	2.71	0.04	2.46	0.03	2.65	0.26	1.78	0.07	1.99	0.14	1.66	0.09	1.46	0.06	3.85	0.69	1.91	0.46	2.97	0.12	2.31	0.28
18:1n-9	13.44	0.62	17.44	0.10	16.67	0.21	15.81	0.14	17.67	0.23	14.11	0.19	18.61	0.36	14.40	0.15	15.22	0.42	24.75	0.45	15.53	0.27	15.83	0.10	14.38	0.07
18:1n-7	2.05	0.00	3.08	0.04	2.97	0.19	3.30	0.00	2.82	0.16	2.46	0.12	2.69	0.05	2.40	0.23	2.44	0.21	3.85	0.03	2.79	0.22	3.13	0.04	2.45	0.11
Total MUFA	19.49	0.99c	26.49	0.01b	25.43	0.02b	25.59	0.21b	26.36	0.21b	21.09	0.11bc	25.96	0.45b	20.59	0.37c	21.48	0.69bc	36.24	1.17a	23.26	0.65b	24.82	0.04b	21.98	0.05bc
18:2n-6	9.26	0.24	7.78	0.00	7.62	0.10	6.12	0.03	7.94	0.62	8.71	0.06	13.25	0.49	10.91	0.09	10.89	0.66	9.04	0.20	8.23	0.06	6.31	0.00	7.88	0.12
20:4n-6	4.20	0.07	4.18	0.09	4.84	0.11	4.19	0.06	4.26	0.35	4.78	0.07	3.27	0.14	4.14	0.08	4.21	0.10	2.61	0.22	4.63	0.17	4.63	0.09	5.50	0.05
Total n-6 PUFA	15.58	0.01bc	15.01	0.55bc	14.88	0.13bc	12.45	0.04c	14.50	0.85bc	15.75	0.01bc	18.97	0.33a	16.93	0.01ab	17.56	0.93a	13.88	0.55bc	15.24	0.53bc	13.07	0.10bc	16.13	0.17b
18:3n-3	0.55	0.00	0.36	0.01	0.36	0.02	0.32	0.00	0.46	0.02	0.45	0.01	0.83	0.03	0.63	0.01	0.61	0.08	0.46	0.00	0.35	0.02	0.29	0.03	0.33	0.03
20:5n-3	2.75	0.01a	1.95	0.06b	1.98	0.03b	1.92	0.01b	1.97	0.13b	2.68	0.08a	1.95	0.02b	2.64	0.10a	2.36	0.04a	1.10	0.38d	1.76	0.09c	1.71	0.10c	1.93	0.01b
22:5n-3	0.78	0.01	0.59	0.01	0.56	0.02	0.56	0.02	0.64	0.01	0.70	0.04	0.52	0.01	0.67	0.06	0.55	0.05	0.31	0.09	0.49	0.01	0.54	0.05	0.56	0.01
22:6n-3	33.46	1.39a	30.27	0.41a	30.65	0.11a	32.52	0.01a	30.35	0.68a	29.02	0.41a	20.64	1.03b	28.85	0.91ab	24.92	0.11b	14.73	1.38c	30.27	0.63ab	33.99	0.18a	32.75	0.62a
Total n-3 PUFA	37.74	1.40a	33.26	0.47a	33.69	0.10a	35.43	0.02a	33.55	0.83a	33.02	0.43a	24.11	1.03b	32.98	1.06a	28.59	0.20ab	16.69	1.86c	32.98	0.71ab	36.63	0.08a	35.70	0.64a
Total PUFA	53.32	1.41	48.27	0.07	48.56	0.23	47.89	0.02	48.05	1.67	48.77	0.43	43.08	0.70	49.91	1.04	46.15	1.12	30.57	2.41	48.22	1.24	49.69	0.18	51.83	0.46
DHA/EPA	12.15	0.48a	15.55	0.30b	15.48	0.20b	16.90	0.11bc	15.42	0.65b	10.83	0.18a	10.56	0.40a	10.93	0.05a	10.57	0.15a	14.01	3.55b	17.21	0.51bc	19.86	1.21bc	16.95	0.26bc
EPA/ARA	0.66	0.01ab	0.47	0.01ab	0.41	0.00ab	0.46	0.01ab	0.24	0.30a	0.56	0.01b	0.60	0.02b	0.64	0.01ab	0.56	0.00b	0.42	0.11ab	0.38	0.01ab	0.37	0.03ab	0.35	0.00ab
n-3/n-6	2.42	0.09b	2.22	0.11b	2.26	0.01b	2.85	0.01b	2.32	0.01b	2.10	0.03b	1.27	0.08a	1.95	0.06ab	1.63	0.07a	1.20	0.09a	2.16	0.03b	2.80	0.01b	2.21	0.06b

Different letters indicate significant differences (ANOVA, P<0.05, n=3). DSY: Dried spent yeast, HSY: Hydrolysed spent yeast, DSG: Dried spent grain, HSG: Hydrolysed spent grain, DYABN: Dried commercial yeast from ABN, HYABN: Hydrolysed commercial yeast from ABN

10.3389/fmars.2022.862020

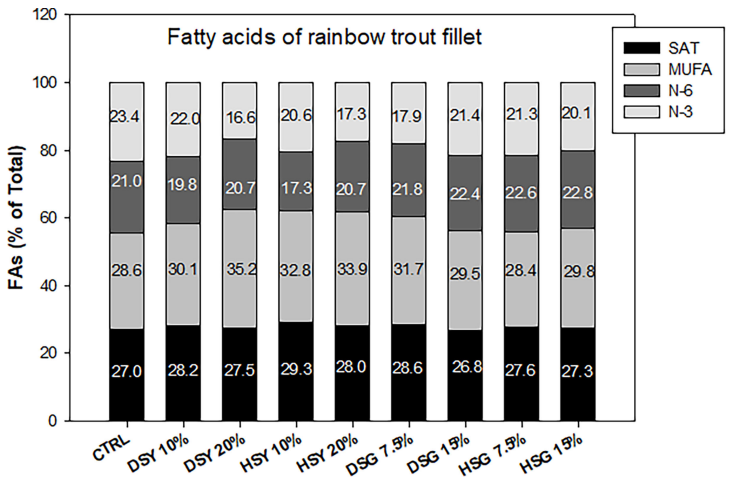


FIGURE 1
Fatty acid composition of the muscle of rainbow trout fed with the experimental diets. SAT, saturated; MUFA, monounsaturated; N-3, omega-3; N-6, omega-6 fatty acids.

fish fed with SY probably due to its lower digestibility compared with CTRL. In the case of SG, the results in final weight were similar to CTRL and SY-fed fish especially in the case of DSG, without differences in SGR and RGR, and, in this case, FCR was similar to that found for the CTRL group. In all the cases, the inclusion of brewery derived by-products did not affect fish final composition. Muscle composition at the end of the study shows a higher protein content in the fish fed with HSY included at 20% and a higher lipid levels in the fish fed with DSY included at 20%, as well as omega-3 fatty acid profile similar to those found in the fish fed with the control/commercial feed. In a previous study carried out with gilthead seabream (Estévez et al., 2021) using feeds with SY included up to 30%, very good results in growth and conversion were obtained even when the fish meal content was reduced to 10% in the formulation. However, in the same study using SG included up to 20%–30%, the growth of seabream and feed digestibility were reduced due to the high fiber content of SG. In that case, the final recommendation for seabream was the inclusion of 30% brewers' SY with a reduction in the use of fish meal, whereas for SG, the inclusion should not be higher than 15%.

As a conclusion, the inclusion of 20% of brewers' SY or 15% SG in the feed for carnivorous freshwater fish (rainbow trout) resulted in a higher final weight without differences in growth rates (SGR and RGR) than that obtained using a commercial feed, showing also good protein digestibility and food conversion. Final muscle nutritional composition was very similar to the results obtained for the control group, including the fatty acid composition. These by-products are produced in large quantities in Europe, and considering the results obtained, they can be considered a potential new source of protein and increase the sustainability of both brewery and aquaculture sectors. The valorization of Brewers' by-products as ingredients for the formulation of aquafeeds has an important favorable effect both on brewers and aquaculture environmental impact. In this regard, the comparison between the aquafeeds obtained using these by-products as ingredients and commercial aquafeed showed significant benefits, including a 6% reduction in climate change (Iñarra et al., 2022).

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Generalitat de Catalunya Ethics Committee.

Author contributions

AE carried out the trials with the fish, took samples and analysed the fillet and liver and wrote the manuscript; DS and BI work with the dewatering and drying of the spent yeast and spent grain and helped in writing the manuscript; LP formulated and prepared the feeds used in the trial and collaborate in writing the paper. All authors contributed to the article and approved the submitted version.

Funding

The authors acknowledge funding from Life BREWERY project (LIFE16ENV/ES/000160) that is co-funded by LIFE European Environment Programme.

Acknowledgments

All the brewer's by-products samples that have been used in this study have been provided by Mahou San Miguel company. Thanks are also due to Pau Sanchez, Sandra Molas, Marta Sastre, and Maria Curto for their help in fish maintenance and biochemical analyses and to Ramon Fontanillas for his help in the formulation of the feeds.

Conflict of interest

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

Publisher's note

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

References

Agboola, J. O., Overland, M., Skrede, A., and Hansen, J. O. (2021). Yeast as a major protein-rich ingredient in aquafeeds: a review of the implications for aquaculture production. *Rev. Aquaculture* 13, 949–970. doi: 10.1111/raq.12507

Aliyu, S., and Bala, M. (2011). Brewer's spent grain: A review of its potentials and applications. *Afr. J. Biotechnol.* 10, 324–331. doi: 10.5897/AJBx10.006

AOAC (2006). *Official methods of analysis of AOAC international (18th edition)* (Gaithersburg: MD: AOAC).

Ashe, F., and Oglend, A. (2016). The relationship between input-factor and output prices in commodity industries: The case of Norwegian salmon aquaculture. *J. commodity markets* 1, 35–47. doi: 10.1016/j.jcomm.2015.11.001

Barrows, F. T., Bellis, D., Krogdahl, A., Silverstein, J. T., Herman, E. M., Sealey, W. M., et al. (2008). Report of the plant products in aquafeed strategic planning workshop: An integrated, interdisciplinary research roadmap for increasing utilization of plant feedstuffs in diets for carnivorous fish. *Rev. Fisheries Sci.* 16, 449–455. doi: 10.1080/10641260802046734

Buffington, J. (2014). The economic potential of brewer's spent grain (BSG) as a biomass feedstock. *Adv. Chem. Eng. Sci.* 4, 308–318. doi: 10.4236/aces.2014.43034

Campos, I., Matos, E., Aragão, C., Pintado, M., and Valente, L. M. P. (2018). Apparent digestibility coefficients of processed agro-food by-products in European seabass (*Dicentrarchus labrax*) juveniles. *Aquaculture Nutr.* 24, 1274–1286. doi: 10.1111/anu.12665

Cardinaletti, G., Randazzo, B., Messina, M., Zarantoniello, M., Giorgini, E., Zimbelli, A., et al. (2019). Effects of graded dietary inclusion level of full-fat *Hermetia illucens* prepupae meal in practical diets for rainbow trous (*Oncorhynchus mykiss*). *Animals* 9, 251. doi: 10.3390/ani9050251

Castro, C., Pérez-Jiménez, A., Coutinho, F., Pousão-Ferreira, P., Brandão, T. M., Oliva-Teles, A., et al. (2013). Digestive enzymes of meagre (*Argyrosomus regius*) and white seabream (*Diplodus sargus*). *Effects dietary brewer's spent yeast supplementation. Aquaculture* 416–417, 322–327.

Cheng, Z. J., Hardy, R. W., and Huige, N. J. (2004). Apparent digestibility coefficients of nutrients in brewer's and rendered animal by-products for rainbow trout (*Oncorhynchus mykiss* (Walbaum)). *Aquaculture Res.* 35, 1–9. doi: 10.1111/j.1365-2109.2004.00941.x

Christie, W. W. (1982). *Lipid analysis* (Oxford, UK: Pergamon), 207.

Conway, J. (2019) *Beer production worldwide from 1998 to 2018*. Available at: https://www.statista.com/statistics/270275/worldwide-beer-production/.

Djuragic, O., Levic, J., and Serdanovic, S. (2010). Use of new feed from brewer by-products for breeding layers. *Rom. Biotech. Lett.* 15, 5559–5565.

Ebrahim, M. S. M., and Abou-Seif, R. A. (2008). Fish meal replacement by yeast protein (*Saccharomyces cerevisiae*) supplemented with biogenic l-carintine as a source of methionine plus lysine mixture in feed for Nile tilapia (*Oreochromis niloticus*) fingerlinges. *8th Int. Symposium Tilapia Aquaculture* 2008, 999–1099.

Estévez, A., Padrell, L., Iñarra, B., Orive, M., and San Martin, D. (2021). Brewery by-products (yeast and spent grain) as protein sources in gitlhead seabream (*Sparus aurata*) feeds. *Aquaculture* 543, 736921. doi: 10.1016/j.aquaculture.2021.736921

EUROSTAT (2020) *Happy international beer day*. Available at: https://ec.europa.eu/eurostat/web/products-eurostat-news/-/EDN-20200807.

Faccenda, A., Zambom, M. A., Castagnara, D. D., Avila, A. S., Fernandes, T., Eckstein, E. I., et al. (2017). Use of dried brewers' grains instead of soybean meal to feed lactating cows. *Rev. Bras. Zootecnia* 46, 39–46. doi: 10.1590/s1806-92902017000100007

FAO. 2020 *Nutritional requirements of rainbow trout*. Available at: http://www.fao.org/fishery/affris/species-profiles/rainbow-trout/nutritional-requirements/en/.

Farcas, A. C., Socaci, S. A., Mudura, E., Dulf, F. V., Vodnar, D. C., Tofana, M., et al. (2017). "Exploitation of brewing industry wastes to produce functional ingredients," in *Brewing technology*. Ed. M. Kanauchi (London: InTech). doi: 10.5775/intechopen.69231

Ferreira, I.M.P.L.V.O., Pinho, O., Vieira, E., and Tavarela, J. G. (2010). Brewer's *Saccharomyces* yeast biomass: Characteristics and potential applications. *Trends Food Sci. Technol.* 21, 77–84. doi: 10.1016/j.tifs.2009.10.008

Garantun-Tjeldsto, O., Otera, H., and Julshamn, K. (2006). Food ingestion in juvenile cod estimated by inert lanthanide markers - effects of food particle size. *ICES J. Mar. Sci.* 63, 311–319. doi: 10.1016/j.icesjms.2005.11.008

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. aquaculture research. *Aquac Res.* 38, 551–579. doi: 10.1111/j.1365-2109.2007.01704.x

Hauptman, B. S., Barrows, F. T., Block, S. S., Gaylord, T. G., Paterson, J. A., Rawles, S. D., et al. (2014). Evaluation of grain distillers dried yeast as a fish meal substitute in practical-type diets of juvenile rainbow trout. *Oncorhynchus mykiss. Aquaculture* 432, 7–14. doi: 10.1016/j.aquaculture.2014.03.026

Hertrampf, J. W., and Piedad-Pascual, F. (2012). *Handbook of ingredients for aquaculture feeds* (Switzerland: Springer Science & Business Media), 572.

Hua, K., Cobcroft, J. M., Cole, A., Condon, K., Jerry, D. R., Mangott, A., et al. (2019). The future of aquatic protein: Implications for protein sources in aquaculture diets. *One Earth* 1, 316–329. doi: 10.1016/j.oneear.2019.10.018

Huige., N. J. (2006). "Brewery by-products and effluents," in *Handbook of brewing*. Eds. F. G. Priest. and G. G. Stewart (Boca Raton: CRC Press), 656e713.

Iñarra, B., San Martin, D., Ramos, S., Cidad, M., Estévez, A., Fenollosa, R., et al. (2022). Ecodesign of new circular economy scheme for brewer's side streams. *Sustain. Chem. Pharm.* 18, 100727.

IPIFF (2018). *The European sector today: Challenges, opportunities and regulatory landscape* (Brussels: IPIFF Publication), 16.

Jayant, M., Hassan, M. A., Srivastava, P. P., Meena, D. K., Kumar, P., Kumar, A., et al. (2018). Brewer's spent grains (BSGs) as feedstuff for striped catfish, *Pangasianodon hypophthalmus* fingerlings: An approach to transform waste into wealth. *J. Cleaner Production* 199, 716–722. doi: 10.1016/j.jclepro.2018.07.213

Kaur, V. I., and Saxena, P. K. (2004). Incorporation of brewery waste in supplementary feed and its impact on growth in some carps. *Bioresource Technol.* 91, 101–104. doi: 10.1016/S0960-8524(03)00073-7

Kerby, C., and Vriesekoop, F. (2017). An overview of the utilisation of brewery by-products as generated by british craft breweries. *Beverages* 3, 24. doi: 10.3390/beverages3020024

Levic. J. Djuragic., O., and Sredanovic., S. (2010). Use of new feed from brewery by-products for breeding layers. *Romanian Biotechnol. Lett.* 15, 5559–5565.

Loveday, S. M. (2019). Plant protein ingredients with full functionality potential. *Nutri. Bull.* 45, 321–327. doi: 10.1111/nbu.12450

Maynard, L. A., Loosli, J. K., Hintz, H. F., and Warner, R. G. (1979). *Animal nutrition* (New York, NY: McGraw-Hill).

McCarthy, A. L., O'Callaghan, Y. C., Piggott, C. O., Fitzgerald, R. J., and O'Brien, N. M. (2013). Brewers' spent grain; bioactivity of phenolic component,its role in animalnutrition and potential incorporation in functional foods: areview. *Proc.Nutrition Soc* 72, 117–125. doi: 10.1017/S0029665112002820

Murdock, F. R., Hodgson, A. S., Robert, E., and Riley, (1981). Nutritive value of wet brewers grains for lactating dairy cows. *J. Dairy Sci.* 64, 1826–1832. doi: 10.3168/jds.S0022-0302(81)82771-3

Mussatto, S. I. (2009). "Biotechnological potential of brewing industry by-products," in *Biotechnology for agro-industrial residues utilisation: utilisation of agro-residues*. Eds. P. S. N. Nigam and A. Pandey (Dordrecht: Springer), 313–326. doi: 10.1007/978-1-4020-9942-7_16

Mussatto, S. I. (2014). Brewer's spent grain: Valuable feedstock for industrial applications. *J. Sci. Food Agric.* 94, 1264–1275. doi: 10.1002/jsfa.6486

Mussatto., S. I., Dragone., G., and Roberto., I. C. (2006). Brewers' spent grain: Generation. characteristics and potential applications. *J. Cereal Sci.* 43, 1–14. doi: 10.1016/j.jcs.2005.06.001

Muzinic, L. A., Thompson, K. R., Morris, A., Webster, C. D., Rouse, D. B., and Manomaitis, L. (2004). Partial and total replacement of fish meal with soybean meal and brewers' grains with yeast in practical diets for Australian red claw crayfish *Cherax quadricarinatus*. *Aquaculture* 230, 359–376. doi: 10.1016/S0044-8486(03)00420-4

Naylor, R. L., Hardy, R. W., Bureau, D. P., Chiu, A., Elliott, M., Forster, I., et al. (2009). Feeding aquaculture in an era of finite resources. *Proc. Natl. Acad. Sci.* 106, 15103–15110. doi: 10.1073/pnas.0905235106

Nazzaro, J., San Martin, D., Perez-Vendrell, A. M., Padrell, L., Iñarra, B., Orive, M., et al. (2021). Apparent digestibility coefficients of brewer's by-products used in feeds for rainbow trout (*Oncorhyncus mykiss*) and gilthead seabream (*Sparus aurata*). *Aquaculture* 530, 735796. doi: 10.1016/j.aquaculture.2020.735796

Oliva-Teles., A., and Gonçalves., P. (2001). Partial replacement of fishmeal by brewers yeast (*Saccharomyces cerevisae*) in diets for sea bass (*Dicentrarchus labrax*) juveniles. *Aquaculture.* 202 (3–4), 269–278.

Øverland, M., Karlsson, A., Mydland, L. T., Romarheim, O. H., and Skrede, A. (2013). Evaluation of *Candida utilis, kluyveromyces marxianus* and *Saccharomyces cerevisiae* yeasts as protein sources in diets for Atlantic salmon (*Salmo salar*). *Aquaculture 402-403* pp, 1–7.

Ovie, S. O., and Eze, S. S. (2014). Utilization of *Saccharomyces cerevisiae* in the partial replacement of fishmeal in *Clarias gariepinus* diets. *Int. J. Adv. Agric. Res.* 2, 83–88.

Ozório., R. O. A., Portz., L., Borghesi., R., and Cyrino., J. E. P. (2012). Effects of dietary yeast (saccharomyces cerevisia) supplementation in practical diets of tilapia (oreochromis niloticus). *Animals* 2 (1), 16–24.

Randazzo, B., Cardinaletti, G., Cerri, R., Giorgini, E., Belloni, A., Contó, M., et al. (2021b). *Hermetia illucens* and poultry by-product meals as alternatives to plant protein sources in gilthead seabream (*Sparus aurata*) diet: A multidisciplinary study on gish gut status. *Animals* 11, 677. doi: 10.3390/ani11030677

Randazzo, B., Zarantoniello, M., Gioacchini, G., Cardinaletti, G., Belloni, A., Giorgini, E., et al. (2021a). Physiological response of rainbow trout (*Oncorhynchus mykiss*) to graded levels of *Hermetia illucens* or poultry by-product as single or combined substitute ingredients to dietary plant proteins. *Aquaculture* 538, 736550. doi: 10.1016/j.aquaculture.2021.736550

Robertson., J. A., I'Anson., K. J. A., Treimo., J., Faulds., C. B., Brocklehurst., T. F., Eijsink., V. G. H., et al. (2010). Profiling brewers' spent grain for composition and microbial ecology at the site of production. *LWT - Food Sci. Technol.* 43 (6), 890–896. doi: 10.1016/j.lwt.2010.01.019

Rumsey, G. L., Hughes., S.G., Smith., R.R., and Kinsella., J.E. (1990). Use of dietary yeast Saccharomyces cerevisiae nitrogen by lake trout. *J. World Aquacult. Soc.* 21(3), 205–209.

Rumsey, G. L., Hughes., S.G., Smith., R.R., Kinsella., J.E., and Shetty., K.J. (1991). Digestibility and energy values of intact, disrupted and extracts from brewer's dried yeast fed to rainbow trout *Oncorhynchus mykiss*. *Anim. Feed Sci. Technol.* 33, 185–193.

San Martin, D., Orive, M., Iñarra, B., Castelo, J., Estévez, A., Nazzaro, J., et al. (2020). Brewers' spent yeast and grain protein hydrolysates as second-generation feedstuff for aquaculture feed. *Waste Biomass Valorization.* 11, 5307–5320. doi: 10.1007/s12649-020-01145-8

Sarker, P. K., Kapuscinski, A. R., McKuin, B., Fitzgerald, D. S., Nash, H. M., and Greenwood, C. (2020). Microalgae-blend tilapia feed eliminates fishmeal and fish oil, improves growth, and is cost viable. *Sci. Rep.* 10, 19328. doi: 10.1038/s41598-020-75289-x

Sealey, W. M., O'Neill, T. J., Peach, J. T., Gaylord, T. G., Barrows, F. T., and Block, S. S. (2015). Refining inclusion levels of grain distiller's dried yeast in commercial-type and plant-based diets for juvenile rainbow trout, *Oncorhynchus mykiss*. *J. World Aquaculture Soc.* 46 (4), 434–444. doi: 10.1111/jwas.12199

Sealey., W. M., Rawles., S. D., Block., S. S., Paterson., J. A., Hauptman., B. S., Barrows., F. T., et al. (2014). Evaluation of grain distillers dried yeast as a fish meal substitute in practical-type diets of juvenile rainbow trout *Oncorhynchus mykiss*. *Aquaculture* 432, 7–14. doi: 10.1016/j.aquaculture.2014.03.026

Stevens, J. R., Newton, R. W., Tlusty, M., and Little, D. C. (2018). The rise of aquaculture by-products: increasing food production, value, and sustainability through strategic utilisation. *Mar. Policy* 90, 115–124. doi: 10.1016/j.marpol.2017.12.027

Thomas., C. R., and Rahman., K. R. (2006). Brewery wastes. strategies for sustainability. *A review. Aspects Appl. Biol.* 80, 147–153.

Villalta, M., Estévez, A., Bransden, M. P., and Bell, J. G. (2005). The effect of graded concentrations of idetary DHA on growth, survival and tissue fatty acid profile of Senegal sole (*Solea senegalensis*) larvae during the *Artemia* feeding period. *Aquculture* 249, 353–365. doi: 10.1016/j.aquaculture.2005.03.037

Welker, T. L., Lim, C., Barrows, F. T., and Liu, K. (2014). Use of distiller´s dried grains with solubles (DDGS) in rainbow trout feeds. *Anim. Feed Sc. Tech.* 195, 47–57. doi: 10.1016/j.anifeedsci.2014.05.011

Yamamoto, T., Marcouli, P. A., Unuma, T., and Akiyama, T. (1994). Utilization of malt protein flour in fingerling rainbow trout diets. *Fish. Sci.* 60, 455–460. doi: 10.2331/fishsci.60.455

Zhang, P., Cao, S., Zou, T., Han, D., Liu, H., Jin, J., et al. (2018). Effects of dietary yeast culture on growth performance. *Immune response Dis. resistance gibel carp (Carassius auratus gibelio CAS III). Fish Shellfish Immunol.* 82, 400–407. doi: 10.1016/j.fsi.2018.08.044

Zhou, M., Liang, R., Mo, J., Yang, S., Gu, N., Wu, Z., et al. (2018). Effects of brewer's yeast hydrolysate on the growth performance and the intestinal bacterial diversity of largemouth bass (*Micropterus salmoides*). *Aquaculture* 484, 139–144. doi: 10.1016/j.aquaculture.2017.11.006

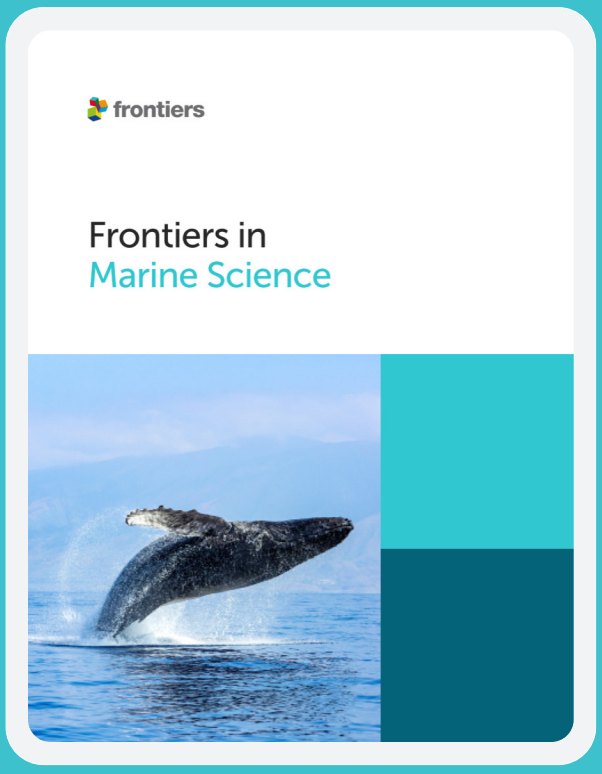
Frontiers in Marine Science

Explores ocean-based solutions for emerging global challenges

The third most-cited marine and freshwater biology journal, advancing our understanding of marine systems and addressing global challenges including overfishing, pollution, and climate change.

Discover the latest Research Topics

See more →



Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact