Animal Nutrition 12 (2023) 171–185

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

KeAi
CHINESE ROOTS
GLOBAL IMPACT

Original Research Article

Soybean protein concentrate causes enteritis in juvenile pearl gentian groupers (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂)

Wei Zhang [a, b, c], Beiping Tan [a, b, c, *], Junming Deng [a, b, c], Qihui Yang [a, b, c], Shuyan Chi [a, b, c], Aobo Pang [a, b, c], Yu Xin [a, b, c], Yu Liu [a, b, c], Haitao Zhang [c]

[a] *Laboratory of Aquatic Animal Nutrition and Feed, College of Fisheries, Guangdong Ocean University, Zhanjiang, Guangdong 524088, China*
[b] *Aquatic Animals Precision Nutrition and High Efficiency Feed Engineering Research Center of Guangdong Province, Zhanjiang, Guangdong 524088, China*
[c] *Key Laboratory of Aquatic, Livestock and Poultry Feed Science and Technology in South China, Ministry of Agriculture, Zhanjiang, Guangdong 524088, China*

ARTICLE INFO

Article history:
Received 25 November 2021
Received in revised form
8 August 2022
Accepted 10 August 2022
Available online 17 August 2022

Keywords:
Epinephelus fuscoguttatus ♀ × *E. lanceolatus* ♂
Enteritis
Intestinal flora
Intestinal structure
Soybean protein concentrate

ABSTRACT

Due to diminishing fish meal (FM) supplies, superb protein (PRO) sources are needed for aquaculture, such as soy-based PRO. However, these can cause enteritis and even intestinal injury in fish when used at high proportions in feed. This research examines the effects of substituting soybean protein concentrate (SPC) for FM on the growth performance and intestinal balance of pearl gentian groupers and investigates the mechanism of SPC-induced enteritis. Experimental fish ($n = 720$) were fed 1 of 3 following diets: (1) a 50% FM diet (control), (2) a diet with 20% of the FM substituted with SPC (group SPC20), and (3) a 40% SPC-substituted diet (SPC40). Fish were fed for 10 wk iso-nitrogenous (50% PRO) and iso-lipidic (10% lipid) diets. Groups SPC20 and SPC40 showed significantly lower developmental performance and intestinal structures than control. Group SPC40 had significantly higher expressions of pro-inflammatory-related genes, such as interleukin 1β (*IL1β*), *IL12*, *IL17* and tumor necrosis factor α and significantly lower expressions of anti-inflammatory-related genes, such as *IL5*, *IL10* and transforming growth factor β1. Biochemical and 16S high-throughput sequencing showed that the abundance and functions of intestinal flora in group SPC40 were significantly affected ($P < 0.05$), and there were significant correlations between operational taxonomic unit abundance variations and inflammatory gene expressions at genus level ($P < 0.05$). The second- and third-generation full-length transcriptome sequence was used to analyze the mechanism of SPC-induced enteritis in pearl gentian groupers, which showed that enteritis induced by SPC may be caused by disturbances to intestinal immune function induced by an imbalance in intestinal nutrition and metabolism, such as the intestinal immunity network for IgA production pathway. However, it remains unclear as to which intestinal immune or nutritional imbalance is most important in enteritis development. This study provides a basis for further research into soy PRO-related enteritis in fish.

© 2023 The Authors. Publishing services by Elsevier B.V. on behalf of KeAi Communications Co. Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Fish meal (FM) is a superb protein (PRO) source for aquaculture feeds as it is palatable, easy to digest and absorb, and has a relatively comprehensive essential nutrient profile (Shekarabi et al., 2021). However, in recent years, due to increasingly limited marine sources, the supply of FM has struggled to meet the continuously increasing demands of aquaculture and consequently has become expensive. Therefore, new PRO sources with low cost and stable supply are urgently needed. Soy products have great potential in this regard (Kalhoro et al., 2018; Shui et al., 2021).

* Corresponding author.
E-mail address: bptan@126.com (B. Tan).

Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2022.08.006

2405-6545/© 2023 The Authors. Publishing services by Elsevier B.V. on behalf of KeAi Communications Co. Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Currently, soy products are widely used in aquacultural feeds, including soybean meal (SBM), soybean PRO concentrate (SPC), fermented soybean meal, and soy PRO isolates. Among these products, SBM is a by-product of soybean oil extraction that is widely used in aquaculture as a plant-based PRO source. However, SBM contains a variety of anti-nutritional factors (ANF), such as saponins, soybean lectin, antigen PRO, trypsin inhibitor, phytic acid, soybean oligosaccharides and isoflavones, which make it unsuitable for replacing FM at high proportions as can cause metabolic disorders and even organ damage in fish (NRC, 2011; Chen, 2009). The SPC is a product obtained from SBM by removing the soybean oil and low-molecular-weight soluble PRO. Due to its specific processing technology, SPC has a lower antigen degree, higher amino acid content, better granulation, stable quality, and longer storage time than SBM (Xiang, 2017). The SPC preparation process mainly involves alcohol extraction using defatted SBM as a raw material to remove soluble components such as oligosaccharides, while the PRO remains insoluble. The SPC extracted by alcohol loses little nutritional value during processing while the ANF are mostly removed; however, research has shown that the solubility of alcohol-extracted SPC is low and its nitrogen solubility index is decreased to about 10%, which limits its application (Zheng et al., 2020).

It is known that an excessive SBM content in feed causes enteritis in many aquaculture fish species. Soybean meal-induced enteritis (SBMIE) is currently widely exploited as a model for research on fish enteritis (Hu et al., 2016). It has been found that SBM causes enteritis and other negative effects on the intestinal health of fish, including Atlantic salmon (Baeverfjord and Krogdahl, 1996), rainbow trout (Gu et al., 2016), carp (Urán et al., 2008) and zebrafish (Fuentes-Appelgren et al., 2014). SBMIE mainly occurs in tissue of the hindgut, which is the main site for PRO absorption by endocytosis and so is more sensitive to intestinal illnesses caused by food infection (Bakke-Mckellep et al., 2000). Although SPC contains much fewer ANF than SBM, studies have still found that excessive substitution of SPC may cause intestinal tissue damage in fish (Hien et al., 2017; Feng et al., 2017). However, previous studies on the effects of SPC on fish are largely based on the activities and gene expression levels of single or several related key enzymes. At the transcriptome level, SBMIE studies have mainly used DNA microarray technology (Kortner et al., 2012), and the detailed mechanisms involved remain unclear. By using high-throughput sequencing and transcriptional sequencing technologies, such as "3 + 2" transcriptome sequencing that has high flux and accuracy, a better understanding of SPC-induced enteritis in groupers can be achieved at a molecular level on the whole level of transcription of genes.

The hybrid pearl gentian grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂) is well-known as the Cantang fish and is a promising aquaculture species, which has been cultivated at large scale in China and across the world (Giri et al., 2020). Related research using DNA microarray technology has preliminarily found that different plant proteins have different effects on the transcription of Atlantic salmon on the whole level (Tacchi et al., 2012; Król et al., 2016). Our previous study analysed the SBMIE mechanism in pearl gentian groupers using full-length transcriptome sequencing and found that the TLR-mediated NF-κB signalling pathway plays an important role. In addition, the intestinal flora abundance changed significantly in SBMIE groupers (Zhang et al., 2021b). Related studies also found that dietary SPC can cause intestinal flora variations and intestinal sensitization and structural damage in Atlantic salmon (Green et al., 2013). However, few systematic reports have investigated the mechanism of enteritis and intestinal flora changes induced by dietary SPC on full-length transcriptome sequence level.

The present study investigated the effects of SPC on the growth and physiology of pearl gentian groupers. We also investigated the mechanism of enteritis and intestinal flora profile variations induced by SPC through conducting "3 + 2" full-length transcriptome and 16SrDNA sequencing, an approach that has not been reported before. The results provide a reference for improving the intestinal wellbeing of fish fed soy-based PRO.

2. Materials and methods

2.1. Animal ethics

The study was approved by the Expert Committee of the Fisheries College of Guangdong Ocean University. All methods were carried out according to relevant guidelines and regulations.

2.2. Test diets

The chemical compositions of the test diets are presented in Table S1. Red FM feed containing 72.53% crude PRO and 8.82% crude lipid was supplied by Corporación Pesquera Inca S.A.C., (Bayovar Plant, Peru). The SPC was bought from Zhanjiang Haibao Feed Co. Ltd. (Zhanjiang, China) and contained 70.72% crude PRO. Three isonitrogenous (nearly 50% crude PRO) and iso-lipidic (10% total lipid) test diets were formulated by replacing 0%, 20%, and 40% of FM PRO with SPC PRO; these were named FM (control), SPC20 and SPC40, respectively. Lysine and methionine were used in the test diets for amino acid balance (Miao et al., 2018). The starting materials were made into a fine powder and passed through a 60-mesh sieve, then weighed precisely to create the test diets. The constituents were mixed homogeneously by a sequential expansion approach (Zhang et al., 2018). Subsequently, a homogeneous mixture was obtained by adding deionized water and lipids and stirring. Then, the compound was processed with a pelletizer into 2.0- and 3.0-mm diameter pellets. After air-drying, the moisture contents of the pellets decreased to about 10%. The pellets were then sealed in plastic bags and stored at −20 °C until use. The diets' essential amino acids were determined (Sichuan Will Testing Technology Co., Ltd., Sichuan, China) and are displayed in Table S2. The ANF in the SPC diets were determined by high-performance liquid chromatography (ZheBo Testing Technology Co., Ltd., Guangdong, China) and are presented in Table S3.

2.3. Feeding trial and breeding management

Healthy female juvenile groupers (initial weight about 9 g) were bought from Zhanjiang, China. They were acclimated to the experimental environment for 1 wk by commercial diet (Haida Aquatic Feed Co., Ltd., Zhanjiang, China). Before the experiment, all fish were fasted for 24 h and divided into groups after being anaesthetized with eugenol. Then, the 720 fish with similar size were randomly distributed to 12 cylindrical fiberglass tanks (1,000 L) and divided into 3 groups. There were 4 replicates in each group and the fish were fed twice every day at 08:00 and 16:00 until apparent satiation for 10 wk. Feed consumption was counted by the method described in our previous study (Zhang et al., 2019). The experiment was performed at the Zhanjiang Marine Biological Research Base, China. Every tank was constantly aerated with an air stone to maintain an oxygen concentration near saturation. The temperature and light cycle were that of a natural environment. The temperature was 29 ± 1 °C, the ammonia and nitrate concentrations were less than 0.03 mg/L, and the dissolved oxygen concentration was no less than 7 mg/L. For the first 14 d, 60% of the water in each tank was changed daily, then 100% of the water was changed daily.

2.4. Sample collection

When the feeding experiment ended, the fish were fasted for 24 h before sampling. Then, after counting and weighing all fish in each tank, their weight gain rate (WGR), specific growth rate (SGR), feed conversion ratio (FCR) and survival rate (SR) were determined. Subsequently, the final body weight (FBW) and hepatosomatic index (HSI) were determined.

To determine enzyme activity, 6 fish were taken from each tank at random. Blood was taken from the caudal vein after fish were anaesthetized with eugenol (1:10,000). Then, the serum was centrifuged at 3,500 × *g* at 4 °C for 10 min after the blood was stored at 4 °C overnight. The serum was stored at −80 °C until analysis of enzyme activity. Then, the hindgut and liver were excised and any mesenteric and adipose tissues were removed. Tubes were used to collect some of the hindgut and liver samples from each fish, frozen in liquid nitrogen and stored at −80 °C for enzyme activity analysis. Some hindgut samples were cut and put in tubes with RNAlater overnight at 4 °C, and stored at − 0 °C for gene expression detection.

For intestinal flora and transcriptome sequencing, 8 fish were taken from each tank at random and their hindgut tissues sampled and washed with deionized water to remove the residual. These samples were placed in cryopreservation tubes and immersed in liquid nitrogen, with half used for 16S high-throughput sequencing and half used for transcriptome sequencing.

2.5. Histological observation of enteritis

Three fish were taken from each tank at random. Samples of the hindgut about 0.5 cm in length were placed into 4% paraformaldehyde widespread tissue fixative (Servieobio Technology Co., Ltd., Wuhan, China) for 24 h until haematoxylin and eosin staining (H&E) and Tunnel staining. The plica height/width, lamina propria width, microvilli length and goblet cell number were determined (Zhang et al., 2021a; Liu et al., 2019). The sections were analysed and photographed by optical microscopy (Olympus CKX41, Tokyo, Japan). Furthermore, the apoptosis was also observed by ortho fluorescence microscope (Nikon Eclipse C1, Tokyo, Japan) and the numbers of apoptosis cells were recoded (Gu et al., 2018).

2.6. Analysis of biochemical indicators

The bicinchoninic acid (BCA) approach was used to analyse the intestine, liver and serum samples stored at −80 °C (Beyotime Biotechnology Co., Ltd., Shanghai, China). The enzyme activities of trypsin (Try), total superoxide dismutase (T-SOD), glutathione reductase (GR) and glutathione peroxidase (GPx) in hindgut were determined using the fish ELISA kits. The immunoglobulin M (IgM), complement 3 (C3), complement 4 (C4) and malondialdehyde (MDA) concentrations were determined using the fish ELISA kits. The serum lysozyme (LYS) and liver alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using the fish ELISA kits. All ELISA kits were supplied by Shanghai Jianglai Biotechnology Co., Ltd. (Shanghai, China). All processes were carried out according to the manufacturer's instructions.

2.7. Immune-related gene expressions

The aggregate RNA of hindgut was taken using a Trizol kit (Invitrogen, Carlsbad, USA) according to the methods in the supplied manual. Briefly, 1% agarose gel was used to detect RNA sample integrity. The qualified RNA samples were prepared to cDNA by an Evo M-MLV reverse transcription kit (Takara, Japan) and stored at −20 °C for further use. The primers were laid out by Primer Premier 5.0 software according to the conditions and were incorporated by Shenggong Bioengineering Co., Ltd. (Shanghai, China). The sequences of primers designed in this study were obtained from the PacBio SMRT transcriptome sequencing of pearl gentian grouper hindgut conducted in a laboratory. Primers were designed for pro-inflammatory genes, such as interleukin (*IL*)*1β*, *IL12*, *IL17*, and tumor necrosis factor (*TNF*) α and anti-inflammatory genes, such as *IL5*, *IL10*, and transforming growth factor (*TGF*) *β1* (Table S4). The interior control genes were β-actin. The degrees of gene expression were identified by qRT-PCR (Mastercycler Ep Realplex, Eppendorf, Germany). The PCR reaction conditions were the following: 95 °C for 2 min, 1 cycle; 95 °C for 15 s, 60 °C for 10 s, and 72 °C for 20 s, 40 cycles. The expressions of the target genes were determined by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.8. Intestinal structure-related gene expressions

The hindgut tissues were treated and their gene expressions detected as mentioned above. The primers of aquaporin genes included aquaporin (*aqp*) *1*, *aqp4*, *aqp8*, *aqp9*, *aqp10*, *aqp11*, and *aqp12*; the primers of tight junction PRO genes included junctional adhesion molecules (*jam*), occludin (*occ*), claudin3 (*cla3*), claudin12 (*cla12*), claudin15 (*cla15*), zonula occludens (*ZO*)*-1*, ZO-*2*, and ZO-*3*; the primers of ion transport carrier genes included Na^+, K^-, $2Cl^-$ co-transporter (*nkcc*), guanylin (*gua*), Na^+, K^--ATP (*nka*)*α-1*, *nkaβ-1*, *nkaγ-1*, and chloride channel (*clc*). The primers are displayed in Table S4.

2.9. High-throughput analysis of intestinal flora 16S sequencing

The E.Z.N.A. kit (Omega Bio-Tek, Norcross, U.S.) was used for the extraction of total microbial genome DNA from hindgut samples according to the manufacturer's instructions. The sequencing detection and data analysis were performed by Gene Denovo Co., Ltd. (Guangzhou, China). The raw data was stored in the NCBI Sequential Read Archive (SRA) database under accession number PRJNA666309. The detailed analysis steps are supplied in Supplementary File.

In order to analyse the relationship between operational taxonomic unit (OTU) abundance changes of intestinal flora and the inflammatory genes, the canonical correlation analysis (CCA) was performed between OTU abundance with significant differences in intestinal flora and inflammatory genes in intestinal tissue.

2.10. The "3 + 2" transcriptome sequencing

The "3 + 2" full-length transcriptome sequencing process was performed on the PacBio Sequel and Illumina HiSeq 4000 platforms. Sequencing detection and data analysis were performed by Gene Denovo Co., Ltd. (Guangzhou, China). The raw reads of PacBio SMRT and Illumina sequencing are reserved in the NCBI SRA under accession numbers PRJNA664623 and PRJNA66441, respectively. The detailed procedures of the "3 + 2" full-length PacBio SMRT and RNA-Seq sequencing processes are supplied in Supplementary File.

2.11. Analysis of differential genes (DEGs)

In this experiment, the fish in group SPC40 showed obvious symptoms of enteritis. Therefore, the DEG in the hindgut of fish from groups SPC40 were screened to investigate the DEG distributions in immune-related signalling paths. The screening threshold of the genes was $|\log2FC| > 1$ and $P < 0.05$, which were identified as DEGs. Subsequently, the Kyoto Encyclopedia of Genes

and Genomes (KEGG) annotation analysis was performed for analysis of the DEGs. Finally, the signal paths relevant to immune diseases/system, infectious diseases, and signal transduction were analysed according to KEGG annotation results ($P < 0.05$).

2.12. Validation of real-time quantitative PCR (RT-qPCR) and Western blotting

To certify the precision of the "3 + 2" transcriptome sequencing results, RNA was extracted from the same batch of hindgut samples to carry out RT-qPCR. Eighteen representative genes in toll-like receptor/myeloid differentiation factor 88/nuclear factor kappa-B (TLR-MyD88-NF-κB) and the intestinal immune network for immunoglobulin A (IgA) production pathway way were selected, including *TLR1*, *TLR2*, *TLR3*, *TLR5*, *TLR8*, *TLR9*, *TLR13*, *TLR21*, *TLR22*, *IgA*, *pIgR*, *IL4*, *IL5*, *IL10*, *TGFβ1*, *MyD88*, *iκBα*, and *p65*. The primer layouts, syntheses and template sources of all genes showed no differences from the above-mentioned ones (Table S4). The interior control genes were β-actin. The expressions of the genes were identified by RT-qPCR. The reaction conditions were the following: 95 °C for 2 min, 1 cycle, 40 cycles at 95 °C for 15 s, 60 °C annealing for 10 s, and 72 °C for 20 s. All tests were carried out in triplicate. The melting curve was analysed to test the target specificity. The RT-qPCR data were counted applying the $2^{-\Delta\Delta CT}$ approach (Livak and Schmittgen, 2001). Then, the key proteins, such as IκBα, p65 and IgA in NF-κB and intestinal immune network for IgA production signaling pathways were determined by Western blotting.

2.13. Statistical analysis

The growth parameters were counted as the following equations:

Weight gain rate (WGR, %) = 100 × (final body weight − initial body weight)/initial body weight.

Specific growth rate (SGR, %/d) = 100 × (ln final body weight − ln initial body weight)/days.

Feed conversion ratio (FCR) = feed intake/(final body weight − initial weight).

Hepatosomatic index (HSI, %) = 100 × (hepatic weight/body weight).

Survival rate (SR, %) = 100 × (final fish number/initial fish number).

The results were explored by unidirectional ANOVA after homogeneity difference testing in SPSS 22.0 (SPSS Inc., Chicago, USA) and are presented as means ± standard deviation, with $P < 0.05$ indicating a significant difference.

3. Results

3.1. Growth performance

There were no fish deaths during the feeding experiment. Table 1 shows that the FBW, WGR, and SGR dropped significantly in the experimental groups ($P < 0.05$), with the lowest values observed in group SPC40. The FCR was significantly higher in the experimental groups ($P < 0.05$), with group SPC40 presenting the highest value. The HSI and SR showed no significant differences among groups ($P > 0.05$).

Table 1
Effects of varied degrees of SPC substitute for fish meal protein on the development of pearl gentian groupers ($n = 3$).[1]

Parameters	FM	SPC20	SPC40
IBW, g	12.55 ± 0.00	12.55 ± 0.03	12.55 ± 0.02
FBW, g	73.82 ± 0.52^a	70.02 ± 1.32^b	66.73 ± 0.87^c
WGR, %	485.14 ± 7.08^a	457.96 ± 17.30^b	431.73 ± 12.00^c
SGR, %/d	2.60 ± 0.02^a	2.53 ± 0.05^b	2.46 ± 0.03^c
FCR	0.84 ± 0.01^c	0.89 ± 0.03^b	0.94 ± 0.03^a
HIS, %	2.43 ± 0.45	2.12 ± 0.35	2.36 ± 0.39
SR, %	99.17 ± 0.96	99.17 ± 0.96	99.58 ± 0.84

FM = fish meal; SPC = soybean protein concentrate; IBW = initial body weight; FBD = final body weight; WGR = weight gain rate; SGR = specific growth rate; FCR = feed conversion ratio; HSI = hepatosomatic index; SR = survival rate.
$^{a-c}$Values in the same row with different superscripts indicate significant differences, $P < 0.05$.
[1] SPC20 and SPC40 refer to 20% and 40% SPC protein alternative degree to FM protein, respectively.

3.2. Histological evaluation

The results showed that with increases in the degree of SPC substitution, the plica height/width, microvilli length and goblet cell number decreased significantly in group SPC40 ($P < 0.05$), while the lamina propria width increased significantly in group SPC40 ($P < 0.05$) (Fig. 1 and Table 2). Besides, the proportion of apoptotic cells increased significantly with dietary SPC addition ($P < 0.05$; Fig. 2 and Table 2). The results indicate that high levels of dietary SPC caused obvious enteritis characteristics.

3.3. Determination of biochemical indexes

Table 3 shows that the enzyme activities of Try, T-SOD, GR, and GPx in hindgut increased significantly with the degree of dietary SPC substitution, with all groups showing significant differences ($P < 0.05$). However, the concentrations of IgM, C3, and C4 showed significant decreases in all groups ($P < 0.05$). In liver tissues, the enzyme activities of ALT and AST showed significant increases with the degree of dietary SPC substitution. Similarly, the enzyme activity of LYS in serum increased significantly with the degree of dietary SPC substitution.

3.4. Immune-related gene expressions

Table 4 shows that with increases in dietary SPC, the expressions of pro-inflammatory related genes *IL1β*, *IL12*, *IL17* and *TNFα* were significantly higher in group SPC40 ($P < 0.05$). There were no significant variances between groups FM and SPC20 ($P > 0.05$). Table 5 shows that with increases in dietary SPC, the expressions of anti-inflammatory-related genes of *IL5*, *IL10* and *TGFβ1* were significantly lower in group SPC40 ($P < 0.05$). The expression of *IL5* was also significantly lower in group SPC20 than in group FM ($P < 0.05$). The expressions of *IL10* and *TGFβ1* of group SPC20 were not significantly different from those of group FM ($P > 0.05$).

3.5. Intestinal structure-related gene expressions

Table 6 shows that in comparison with the FM control group, the expressions of aquaporin genes in hindgut, such as a*qu1*, *aqu4*, *aqu8*, *aqu9*, *aqu11*, and a*qu12*, decreased with increases in dietary SPC ($P < 0.05$). The minimum value was in group SPC40, which had a higher expression of a*qu10* ($P < 0.05$).

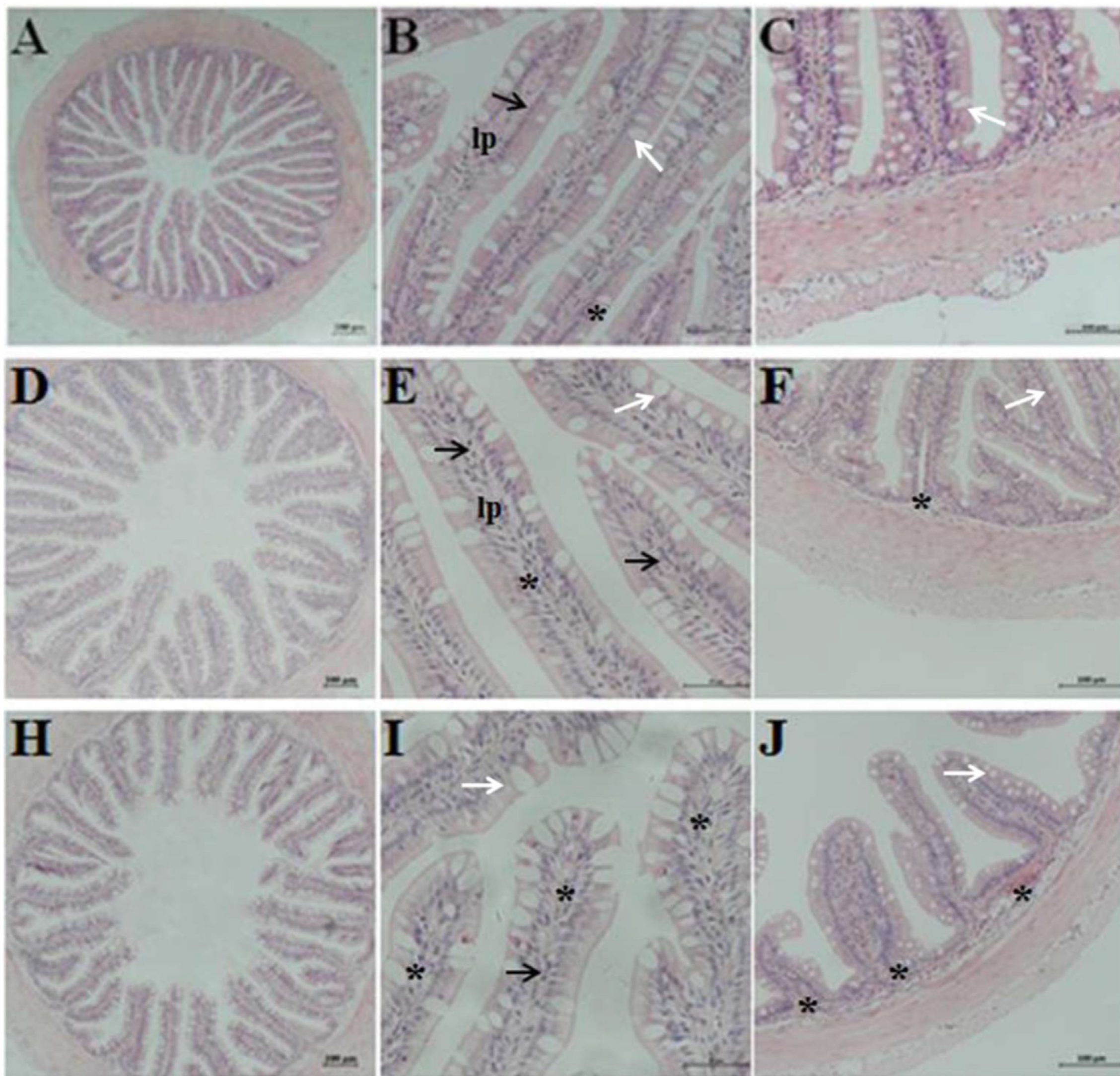

Fig. 1. Hematoxylin-eosin staining of the hindgut in pearl gentian groupers (scale bar = 100 μm). (A, B, and C) The group fed fish meal control diets. (D, E, and F) The group fed SPC20 diets. (H, I, and J) The group fed SPC40 diets. (A, D, and H) Pictures of dropping height and rising fusion of the mucosal folds. (B, E, and I) Pictures of rising width and cellular (leucocyte) infiltration (asterisk) of the lamina propria (black arrows). (C, F, and J) Pictures of increased width and cellular (leucocyte) infiltration of the submucosa. The white arrows represent goblet cells. SPC20 and SPC40 represent 20% and 40% SPC protein alternative degree to fish meal protein, respectively. lp = lamina propria.

Table 2
Effects of varied degrees of SPC substitute for fish meal protein on the intestinal morphology of pearl gentian groupers (n = 10).[1]

Parameters	FM	SPC20	SPC40
Plica height/width	7.80 ± 0.63^a	7.57 ± 0.48^a	4.82 ± 0.53^b
Lamina propria width, μm	16.65 ± 5.78^b	18.01 ± 3.27^b	35.09 ± 5.49^a
Microvilli length, μm	24.10 ± 2.06^a	22.72 ± 1.25^a	14.98 ± 3.42^b
Goblet cell, pcs	49.50 ± 2.38^a	42.50 ± 2.65^b	31.50 ± 1.29^c
Apoptotic cell, pcs	12.75 ± 2.99^c	30.00 ± 7.26^b	51.75 ± 8.18^a

FM = fish meal control group; SPC = soybean protein concentrate; pcs = pieces.
[a–c]Values in the same row with different superscripts indicate significant differences, P < 0.05.
[1] SPC20 and SPC40 refer to 20% and 40% SPC protein alternative degree to FM protein, respectively.

Table 7 shows that, in comparison with control, the expressions of tight-junction PRO genes, such as *jam*, *cla3* and *ZO-1*, were similar in group SPC20 (P > 0.05) and significantly higher in group SPC40 (P < 0.05). The expressions of *cla12* and *cla15* were significantly lower in group SPC40 than in groups FM and SPC20 (P < 0.05). Similarly, there were no significant differences between groups FM and SPC20 (P > 0.05). The expressions of *occ*, *ZO-2* and *ZO-3* did not differ among groups (P > 0.05).

Table 8 shows that, in group FM, the expressions of ion transport carrier genes, such as *gua*, *nkaα-1* and *clc*, were significantly lower than in groups SPC20 and SPC40 (P < 0.05) and similar to those in groups SPC20 and SPC40 (P > 0.05). There were no differences in *nkaβ-1* and *nkaγ-1* expression among groups (P > 0.05).

3.6. Analysis of intestinal flora 16S sequencing

3.6.1. Composition and abundance of intestinal flora

In the Venn diagram, 138 OTU are shared among the three groups, and PCoA analysis shows that the data for groups FM and SPC20 fell within the same confidence ellipse, while group SPC40's

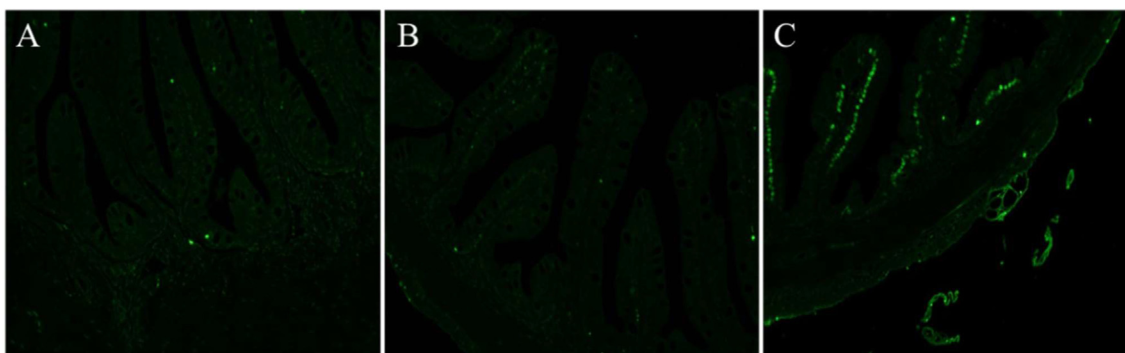

Fig. 2. The histomorphological images of hindgut tissue TUNEL staining of pearl gentian groupers. (A to C) The groups fed fish meal, SPC20 and SPC40 diets, respectively. The green fluorescence represented apoptosis cells. SPC20 and SPC40 represent 20% and 40% SPC protein alternative degree to fish meal protein, respectively. SPC = soybean protein concentrate.

Table 3
Effects of varied degrees of SPC substitute for fish meal protein on the enzyme activities of pearl gentian grouper ($n = 3$).[1]

Parameters	FM	SPC20	SPC40
Try, U/mg	597.33 ± 75.53^c	1,187.00 ± 103.28^b	1,378.25 ± 97.99^a
IgM, μg/mg	94.33 ± 4.22^a	50.74 ± 4.19^b	37.77 ± 3.15^c
C3, μg/mg	85.58 ± 5.31^a	51.22 ± 8.04^b	39.97 ± 7.49^b
C4, μg/mg	128.83 ± 10.17^a	76.83 ± 4.52^b	60.32 ± 5.23^c
T-SOD, U/mg	78.23 ± 9.95^c	135.65 ± 12.28^b	154.04 ± 13.35^a
GR, U/g	32.49 ± 7.07^c	61.93 ± 6.47^b	71.65 ± 6.31^a
GPx, U/mg	167.20 ± 21.34^c	257.18 ± 32.15^b	322.51 ± 22.74^a
ALT, U/g	25.53 ± 3.34^c	42.55 ± 4.51^b	48.55 ± 4.23^a
AST, U/g	26.88 ± 4.02^b	45.32 ± 3.99^a	50.36 ± 3.72^a
LYS, U/g	5.45 ± 0.47^b	5.65 ± 0.95^b	7.64 ± 0.64^a

FM = fish meal control group; SPC = soybean protein concentrate; Try = trypsin; IgM = immunoglobulin M; C3 = complement 3; C4 = complement 4; T-SOD = total superoxide dismutase; GR = glutathione reductase; GPx = glutathione peroxidase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; LYS = lysozyme.
$^{a-c}$Values in the same row with different superscripts indicate significant differences, $P < 0.05$.
[1] SPC20 and SPC40 refer to 20% and 40% SPC protein alternative degree to FM protein, respectively.

Table 4
Effects of SPC alternative for fish meal protein on the pro-inflammatory gene expression in hindgut of pearl gentian groupers ($n = 3$).[1]

Gene	FM	SPC20	SPC40
IL1β	1.16 ± 0.16^b	1.01 ± 0.11^b	1.58 ± 0.09^a
IL12	1.01 ± 0.14^b	1.12 ± 0.22^b	1.86 ± 0.16^a
IL17	1.00 ± 0.07^b	0.99 ± 0.12^b	1.37 ± 0.05^a
TNFα	1.01 ± 0.15^b	0.89 ± 0.21^b	1.33 ± 0.03^a

FM = fish meal control group; SPC = soybean protein concentrate; *IL* = interleukin; *TNF* = tumor necrosis factor.
^{a,b}Values in the same row with different superscripts indicate significant differences, $P < 0.05$.
[1] SPC20 and SPC40 refer to 20% and 40% SPC protein alternative degree to FM protein, respectively.

data can be distinguished from those of FM and SPC20 (Figs. S1 and S2). The detailed sequencing information and OTU diversity analysis were presented in Supplementary File. In terms of relative abundance, the top 5 phyla were Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Fusobacteria (Table S5). With increases in dietary SPC, the abundance of Proteobacteria significantly decreased in group SPC40 compared to FM control (MetaStat, $P < 0.01$). The abundances of Firmicutes, Bacteroidetes and Actinobacteria were significantly higher in group SPC40 (MetaStat, $P < 0.01$; Fig. 3A, Table S6).

Table 5
Effects of SPC alternative for fish meal protein on the anti-inflammatory gene expression in hindgut of pearl gentian groupers ($n = 3$).[1]

Gene	FM	SPC20	SPC40
IL5	1.00 ± 0.05^a	0.81 ± 0.05^b	0.55 ± 0.10^c
IL10	1.01 ± 0.08^a	1.03 ± 0.06^a	0.56 ± 0.04^b
TGFβ1	1.00 ± 0.08^a	1.01 ± 0.08^a	0.18 ± 0.05^b

FM = fish meal control group; SPC = soybean protein concentrate; *IL* = interleukin; *TGF* = transforming growth factor.
$^{a-c}$Values in the same row with different superscripts indicate significant differences, $P < 0.05$.
[1] SPC20 and SPC40 refer to 20% and 40% SPC protein alternative degree to FM protein, respectively.

Table 6
Effects of different degrees of SPC on gene expression of intestinal aquaporin in hybrid groupers ($n = 3$).[1]

Gene	FM	SPC20	SPC40
aqu1	1.00 ± 0.04^a	0.91 ± 0.09^a	0.52 ± 0.06^b
aqu4	1.02 ± 0.22^a	0.72 ± 0.04^b	0.14 ± 0.02^c
aqu8	1.00 ± 0.09^a	1.04 ± 0.21^a	0.72 ± 0.12^b
aqu9	1.00 ± 0.04^b	1.19 ± 0.13^a	0.15 ± 0.02^c
aqu10	1.01 ± 0.14^b	1.15 ± 0.11^b	1.54 ± 0.10^a
aqu11	1.01 ± 0.16^a	1.16 ± 0.11^a	0.58 ± 0.13^b
aqu12	1.00 ± 0.05^a	0.75 ± 0.06^b	0.32 ± 0.14^b

FM = fish meal control group; SPC = soybean protein concentrate; *aqu* = aquaporin.
$^{a-c}$Values in the same row with different superscripts indicate significant differences, $P < 0.05$.
[1] SPC20 and SPC40 refer to 20% and 40% SPC protein alternative degree to FM protein, respectively.

Table 7
Effects of different degrees of SPC on the gene expression of intestinal tight junction protein in hybrid groupers ($n = 3$).[1]

Gene	FM	SPC20	SPC40
jam	1.00 ± 0.07^b	0.99 ± 0.04^b	1.26 ± 0.02^a
occ	1.02 ± 0.23	1.03 ± 0.07	0.90 ± 0.13
cla3	1.00 ± 0.04^b	0.97 ± 0.10^b	1.21 ± 0.08^a
cla12	1.00 ± 0.09^a	1.10 ± 0.12^a	0.85 ± 0.07^b
cla15	1.00 ± 0.10^a	1.04 ± 0.16^a	0.83 ± 0.04^b
ZO-1	1.01 ± 0.15^a	1.26 ± 0.35^a	3.75 ± 0.06^b
ZO-2	1.01 ± 0.13	0.92 ± 0.20	1.08 ± 0.33
ZO-3	1.01 ± 0.20	0.79 ± 0.16	0.84 ± 0.16

FM = fish meal control group; SPC = soybean protein concentrate; *jam* = junctional adhesion molecules; *cla* = claudin; *ZO* = zonula occludens.
^{a,b}Values in the same row with different superscripts indicate significant differences, $P < 0.05$.
[1] SPC20 and SPC40 refer to 20% and 40% SPC protein alternative degree to FM protein, respectively.

Table 8
Effects of different degrees of soybean protein concentrate on the gene expression of intestinal ion transporter protein in hybrid groupers (n = 3).[1]

Gene	FM	SPC20	SPC40
nkcc	1.02 ± 0.29	1.02 ± 0.03	1.22 ± 0.07
gua	1.02 ± 0.22[a]	0.92 ± 0.12[a]	0.76 ± 0.11[b]
nkaα-1	1.01 ± 0.14[a]	0.89 ± 0.12[a]	0.66 ± 0.10[b]
nkaβ-1	1.01 ± 0.16	0.90 ± 0.11	0.94 ± 0.16
nkaγ-1	1.02 ± 0.23	1.04 ± 0.14	1.06 ± 0.03
clc	1.01 ± 0.18[a]	0.93 ± 0.03[a]	0.82 ± 0.06[b]

FM = fish meal control group; SPC = soybean protein concentrate; *nkcc* = Na^+, K^-, 2Cl$^-$ co-transporter; *gua* = guanlin; *nka* = Na^+, K^--ATP; *clc* = chloride channel.
[a,b]Values in the same row with different superscripts indicate significant differences, $P < 0.05$.
[1] SPC20 and SPC40 refer to 20% and 40% SPC protein alternative degree to FM protein, respectively.

In terms of relative abundance, the top 10 genera were *Photobacterium*, *Faecalibacterium*, *Vibrio*, *Weissella*, *Bacteroides*, *Bifidobacterium*, *Dialister*, *Staphylococcus*, *Stenotrophomonas*, and *Subdoligranulum* (Table S7). The abundance of *Photobacterium* was significantly lower in group SPC40 than in FM control (MetaStat, $P < 0.01$), while the abundances of *Faecalibacterium*, *Bacteroides*, *Bifidobacterium*, *Dialistern*, *Subdoligranulum*, *Roseburia*, *Lachnoclostridium*, *Blautia*, *Megamonas*, *Alistipes*, and *unidentified_Ruminococcaceae* were significantly higher in group SPC40 (MetaStat, $P < 0.01$; Fig. 3B).

3.6.2. Functional prediction

Functional prediction analysis between groups FM and SPC40 was carried out using FAPROTAX software. It provides a clustered heat map displaying the top 20 most abundant intestinal flora grouped according to their functions, the names of which include aerobic_chemoheterotrophy, chemoheterotrophy, fermentation, aromatic_compound_degradation, photoautotrophy, oxygenic_photoautotrophy, cyanobacteria, human_pathogens_all, nitrite_respiration and animal_parasites_or_symbionts (Fig. 4A). Among all the predicted functions, compared to control, the functional abundances of fermentation, aerobic_chemoheterotrophy and chemoheterotrophy were significantly lower in group SPC40 (t-test, $P < 0.05$; Fig. 4B), while xylanolysis, human_pathogens and animal_parasites_or_symbionts were significantly higher in group SPC40 (t-test, $P < 0.05$; Fig. 4B).

3.7. Correlation analysis

Fig. 5 showed the correlations between OTU abundances at genus level with significant variations of intestinal flora and inflammatory genes. An envfit test was carried out to analyse whether the explanatory variable was significantly correlated with the response variables. In general, the results showed that the OTU abundance variations were significantly correlated with the inflammatory genes. Among the 12 OTU abundances with significantly variations, *Photobacterium* was positively correlated with anti-inflammatory genes and the rest were positively correlated with pro-inflammatory genes (Table 9).

3.8. Transcriptome sequencing analysis

3.8.1. Statistics of DEG

There were 4,076 DEG identified in group SPC40 compared to control group FM, among which 2,328 were up-regulated and 1,478 were down-regulated (Table 10). The cluster heat map indicated that the DEG might be well clustered within each group, suggesting that the sample procedures were rational and the data quality was high (Fig. 6). The significantly up-regulated gene set was named Profile A (Table S8), and the significantly down-regulated gene set was named Profile B (Table S9).

3.8.2. KEGG enrichment analysis

KEGG enrichment analysis was performed on Profiles A and B. The Profile A results suggested that 312 paths were enriched, and 38 of them were enriched significantly (Fig. 7A). Among all the paths, 81 were associated with immune diseases/system, infectious diseases and signal transduction, of which 15 paths were significantly enriched. In other words, 39.47% (15/38) of all significantly enriched paths were connected with immune diseases/system, infectious diseases and signal transduction.

The Profile B enrichment results indicate that 300 paths were enriched, and 44 of them were enriched significantly (Fig. 7B). Among these paths, only one was associated with immune diseases/system, infectious diseases and signal transduction, with 28 paths being significantly enriched, which named two-component system. That is to say, 2.27% (1/44) of all the significantly enriched paths were related to immune diseases/system, infectious diseases and signal transduction. However, 81.82% (36/44) of the significantly enriched paths were connected with the digestive system, lipid metabolism, the metabolism of terpenoids and polyketides, carbohydrate metabolism and amino/vitamins metabolism. Most of these significantly enriched paths are closely related to the digestion and absorption of nutrients.

3.8.3. Validation of the transcriptome by RT-qPCR

To certify the precision of transcriptome sequencing, 18 genes connected with immune and inflammatory development were chosen for RT-qPCR: *TLR1*, *TLR2*, *TLR3*, *TLR5*, *TLR8*, *TLR9*, *TLR13*, *TLR21*, *TLR22*, *IgA*, *pIgR*, *IL4*, *IL5*, *IL10*, *TGFβ1*, *MyD88*, *iκBα*, and *p65*. Fig. 8 shows that the RT-qPCR test results are generally in line with the transcriptome sequencing data, which also further verifies the countability of the “3 + 2” full-length transcriptome sequencing strategy.

3.8.4. Western blotting

The key proteins in the NF-κB and intestinal immune network related to IgA-production signalling pathways were analysed (Fig. 9). The results show that the ratios of p-IκBα to IκBα and n-p65 to t-p5 were not significantly increased in group SPC40 ($P < 0.05$), while the ratio of p-IgA to IgA was significantly increased in group SPC40 ($P < 0.05$). The Western blotting analysis also supports the results of transcriptome sequencing.

4. Discussion

The present research shows that replacing FM with SPC has significant effects on the pearl gentian grouper growth, intestinal morphology, intestinal flora, and gene expressions in hindgut. Faudzi et al. (2017) found that 60% SPC replacement of FM (in a 50% basal FM diet) significantly decreased the growth of brown-marbled groupers with an initial body weight (IBW) about 6.1 g. Our study shows that the FBW, WGR and SGR of pearl gentian groupers (IBW about 12.55 g) decreased significantly with 20% and 40% SPC replacement in a 50% basal FM diet. We compared the potential biomarkers in the intestinal contents of pearl gentian groupers with SPC- and SBM-induced enteritis and found that although the SPC used in this study contained fewer ANF than the SBM, it did not provide any significant advantages to the fish (Zhang et al., 2021b). This may be because ANF are not the only major influence, or because the studied growth period was not long enough. SBMIE in fish is also known as non-contagious subacute enteritis. Its main histological characteristics are shortened mucosal folds,

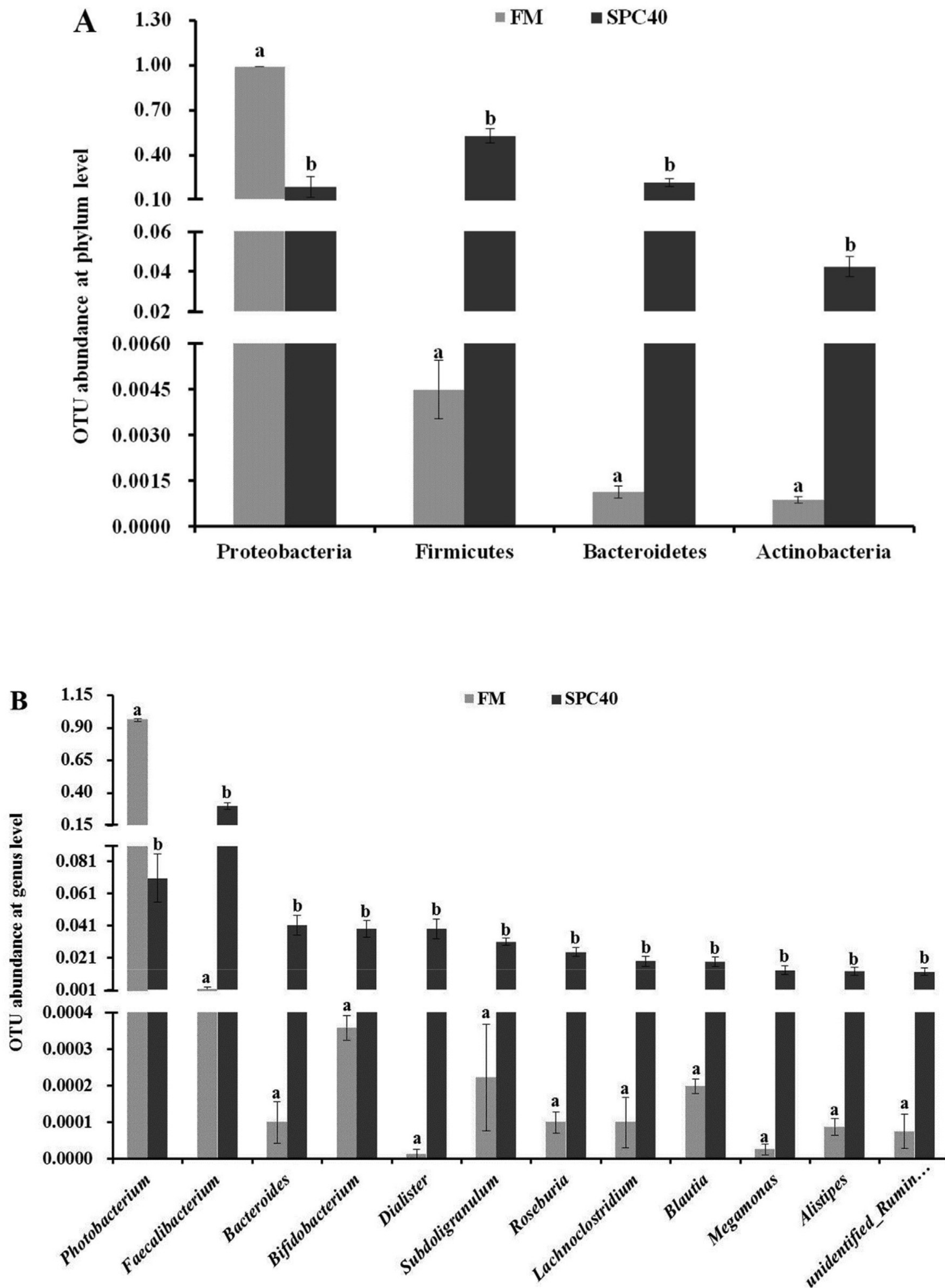

Fig. 3. The operational taxonomic unit (OTU) composition and abundance of intestinal flora in pearl gentian groupers fed by 40% soybean protein concentrate (SPC) substitution degree. (A) Phylum level and (B) genus level ($n = 4$). FM = fish meal; SPC40 = 40% SPC protein alternative degree to fish meal protein. Different letters on the bars represent significant differences among the groups at $P < 0.05$.

swelling of the lamina propria and submucosa, infiltration of different inflammatory cells and reduced absorption of vacuoles by intestinal epithelial cells (Sahlmann et al., 2013). In the present study, the enzyme activities related to intestinal immunity and structural integrity were negatively affected in pearl gentian groupers in a state of SPC-induced enteritis.

In addition, an increase of pro-inflammatory gene expressions may inhibit the expressions of tight junction PRO genes. Alsadi et al.

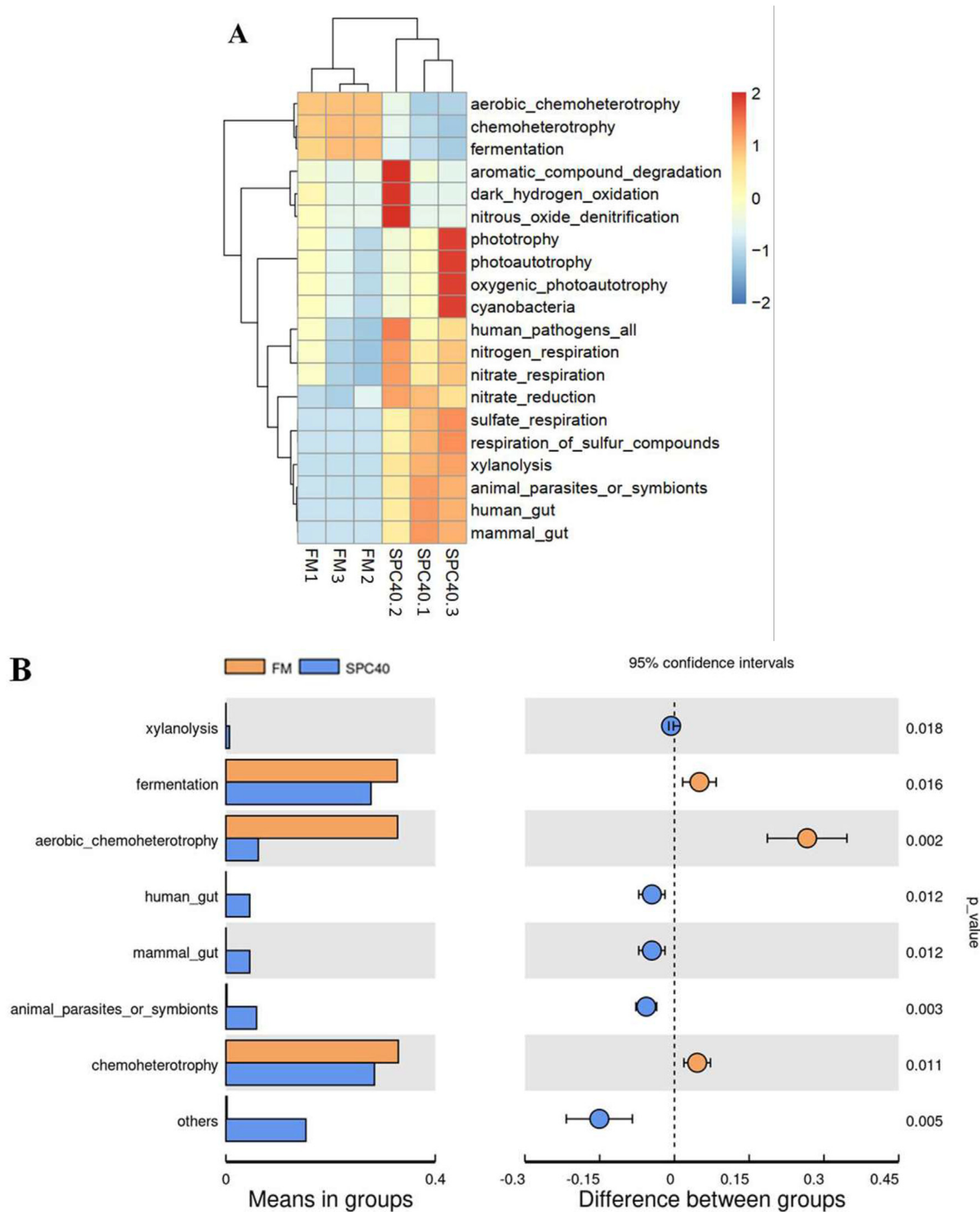

Fig. 4. The intestinal flora functional prediction of pearl gentian groupers fed by fish meal (FM) and SPC40 diets. (A) Top 20 functional clustering heatmap by FAPROTAX software; (B) significance tests of functional difference analysis (n = 3, one sample lost). SPC = soybean protein concentrate; SPC40 = 40% SPC protein alternative degree to FM protein.

(2009) showed that most of the pro-inflammatory elements, such as *IL1β*, *IFN-γ2* and *TNFα*, can lead to the destruction of the tight junction barrier of epithelial cells. Pan et al. (2017) also found that down-regulated expressions of the inflammatory genes *IL1β*, *IL6*, *IL8*, *IL15*, *IL17d*, *IFN-γ2* and *tnfα* are negatively correlated with the expressions of the tight junction PRO genes *cla3*, *-b*, *-c*, *occ* and *ZO-1*, which is consistent with the present study.

Healthy intestinal flora is essential to fish health. Intestinal flora is influenced by various factors, including breed, developmental phase and environmental factors. Of course, the dietary composition is also important (Kim and Kim, 2013). Previous studies found that Atlantic salmon fed SPC had greater intestinal flora diversity than those fed an FM-based diet, leading to the presence of bacteria that are usually absent from marine fish (Green et al., 2013). It has been pointed out that the dominant bacterial phyla in the intestinal flora of pearl gentian grouper are Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria (Li, 2019). The present study found similar results; however, the abundance of the dominant bacteria in each group varied according to the SPC amount. With SPC addition, the abundance of Proteobacteria

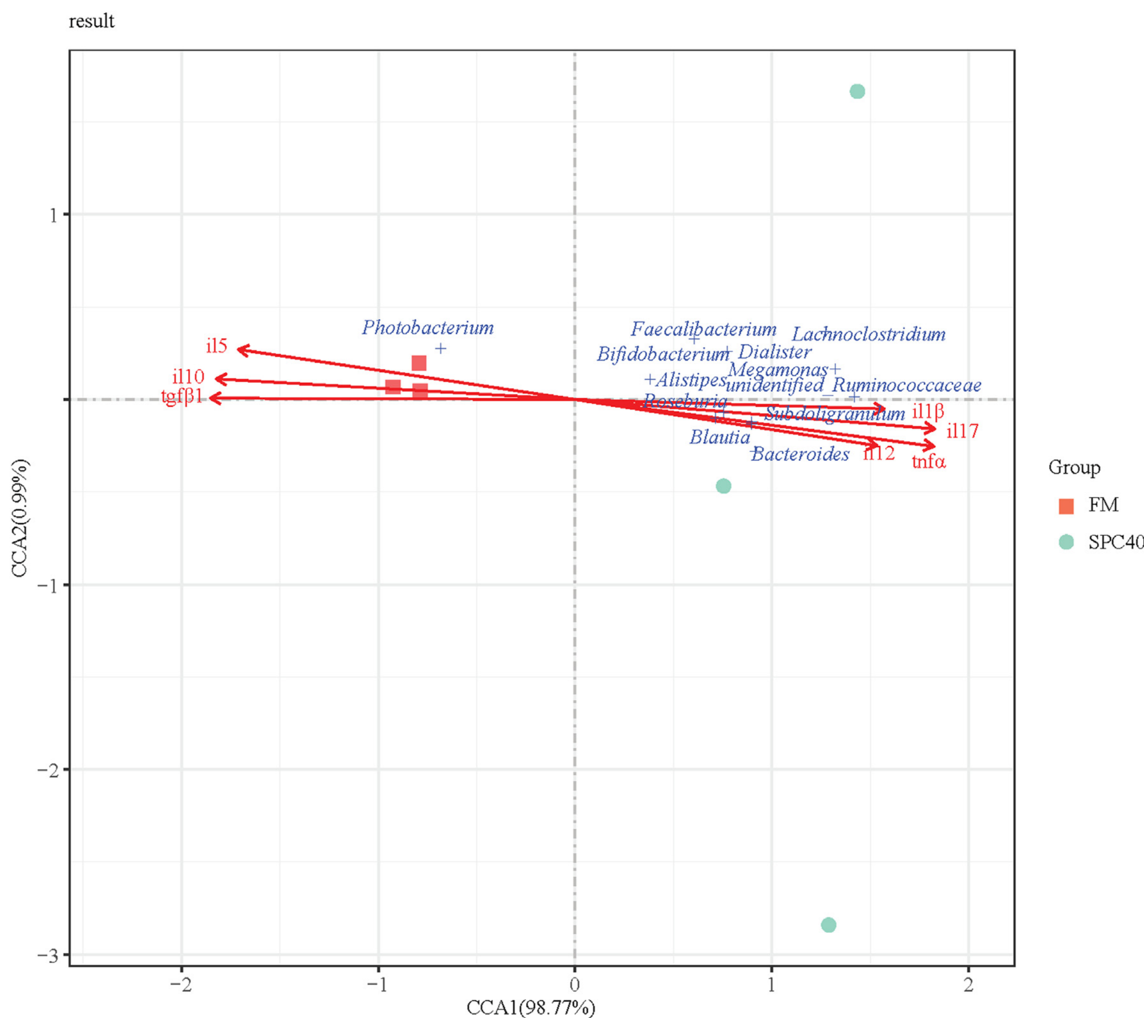

Fig. 5. The canonical correlation analysis (CCA) between the operational taxonomic unit (OTU) abundances of intestinal flora at genus level and the inflammatory genes ($n = 3$, one sample lost). In CCA plot, the arrows represent explanatory variables and the points represent response variables. The lengths of the arrows represent the strength of the influence of the explanatory variable on the response variable; the longer lengths indicate greater influences. The angles between the arrows and coordinate axis represent the correlation between the explanatory variable and coordinate axis; smaller angles indicate stronger correlation. A sample point located in the same direction as the arrow indicates that the changes in explanatory and response variables are positively correlated, while in the opposite direction which indicates a negative correlation. The value in the coordinate axis label in the plot represents the interpretation proportion of the explanatory variables' combination and the response variable variation. SPC = soybean protein concentrate; SPC40 = 40% SPC protein alternative degree to fish meal (FM) protein.

Table 9
The CCA association analysis between OTU abundance with significant variarions of intestinal flora and inflammatory gene expressions in hindgut ($n = 4$).

Gene	CCA1	CCA2	r^2	*P*-value
IL1β	0.9995	−0.0326	0.683	0.032
IL12	0.9905	−0.1379	0.9398	0.004
IL17	0.9962	−0.0866	0.9341	0.008
TNFα	0.987	−0.1607	0.668	0.051
IL5	−0.9877	0.1561	0.8318	0.023
IL10	−0.9981	0.0609	0.9254	0.013
TGFβ1	−1	0.0037	0.9527	0.014

CAA = canonical correlation analysis; OTU = operational taxonomic unit; IL = interleukin; TNF = tumor necrosis factor; TGF = transforming growth factor.

Table 10
Contrast of significant varied genes between soybean meal and soybean protein concentrate substitute for fish meal protein in hindgut of pearl gentian groupers ($n = 4$).

Number	SPC40 vs. FM
Up	2,328
Down	1,748
Total	4,076

FM = fish meal; SPC40 = 40% SPC protein alternative degree to FM protein.

dropped in group SPC40, while those of Firmicutes, Bacteroides and Actinobacteria increased. As SPC contains non-starch polysaccharides and phytoestrogens, the observed increases in Firmicutes and Bacteroides may be related to these bacteria using those substances as energy sources (Zhu et al., 2019; Zheng et al., 2010). Meanwhile, related studies have reported that the occurrence of enteritis may be accompanied by a decrease in Proteobacteria abundance and increases in Firmicutes and Bacteroidetes abundance (Li, 2019). The genus *Photobacterium* belongs to the Vibrionaceae family and comprises facultative anaerobic bacteria that are widely distributed in seawater and on the body surfaces and in the digestive tracts of some marine fish (Nayak, 2010). Although the *Photobacterium* abundance was significantly decreased in group SPC, it is not clear whether this was caused by a decrease in the FM content of the SPC-substituted diets. Research in humans has pointed out that the diversity of *Faecalibacterium* is related to

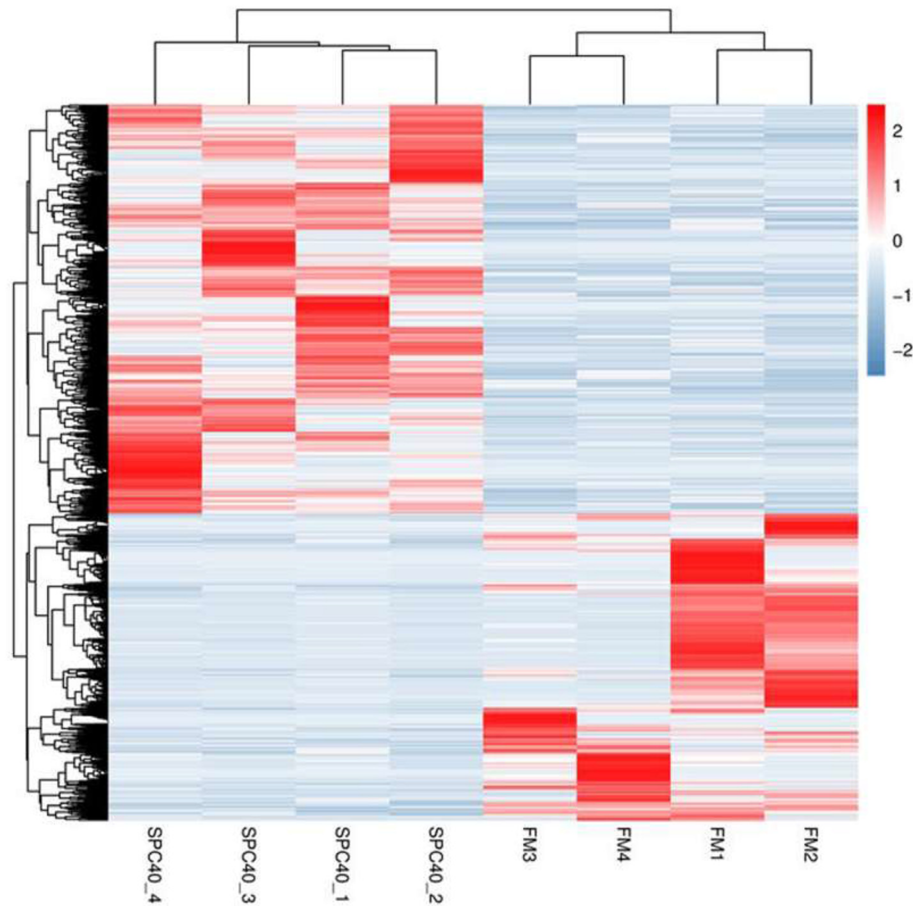

Fig. 6. Cluster heat map of significantly varied genes in the hindgut of pearl gentian groupers at varied alternative degrees of soybean protein concentrate ($n = 4$). SPC = soybean protein concentrate; SPC40 = 40% SPC protein alternative degree to fish meal (FM) protein.

illness status and is lower in the western population and in individuals with enteritis or obesity (Filippis et al., 2020). The present study found that the abundance of *Faecalibacterium* was lower in group SPC40 than in FM control, which may be due to intestinal illness caused by high levels of SPC in the diet. *Bacteroides* is a common bacterium in appendicitis and septicaemia. *Bifidobacterium* is commonly used as a probiotic and its abundance was higher in SPC-fed fish in this study, which may indicate a positive regulatory effect of SPC on intestinal flora. *Dialist* is also closely related to intestinal illness and is almost non-existent in patients with depression (Król et al., 2016; Mireia et al., 2019). The abundance of *Lachnoclostridium* is higher in the intestines of mice with ulcerative colitis than in healthy ones but decreases after probiotic treatment (Wang, 2017). *Blautia* is associated with obesity and intestinal inflammation in children (Benítez-Páez et al., 2020). The abundance of *Megamonas* was significantly increased in patients with colorectal cancer (Yachida et al., 2019). *Alistipes* is a late sub-branch genus of the Bacteroidetes phylum, which is commonly related to chronic intestinal inflammation (Parker et al., 2020). Ruminococaceae is a key floral family in Crohn's illness and ulcerative colitis and is tightly connected with the recurrence of Crohn's illness after surgical intervention (Schirmer et al., 2019). Little research has investigated the effects of SPC on fish intestinal flora; however, our experiment found that a high replacement level eventually affected intestinal function and increased pathogenic bacteria. For example, the abundance of intestinal flora with the animal_parasites_or_symbionts function was significantly increased in group SPC40. This is also reflected in the transcriptome analysis, such as in the Two-component system signalling pathway, which is significant in the response of bacteria to external stimuli and completion of the pathogenic process (Rajagopal et al., 2010).

To further investigated the enteritis characteristic caused by SPC, the present study analysed the intestinal tissues of pearl gentian groupers fed each diet using “3 + 2” full-length transcriptome sequencing. In our previous research, the SBMIE in pearl gentian groupers analysed through the same transcriptome technology was accompanied by significant changes in the NF-κB signalling pathway, antigen processing and presentation, the B-cell receptor signalling pathway and the AMPK signalling pathway (Zhang et al., 2021b). However, in present experiment, the transcriptome of groupers fed SPC was significantly different from those fed SBM. Only 17.2% of the total number of DEGs had similar expression patterns, which indicates that there were great differences in the metabolic strategies of groupers fed SBM or SPC (Zhang, 2020). The transcriptome analysis showed that group SBM40 found a relatively conservative signalling pathway, such as NF-κB, which is similar to previous studies on fish SBMIE (Zhang et al., 2021b). Although the transcriptional profile of group SPC40 was significantly different from that of group SBM40, there were also enriched signalling pathways tightly connected with intestinal immunity, such as the intestinal immunity network for IgA production. The intestine is the largest lymphoid tissue in the human body. A significant characteristic of intestinal immunity is its capability to produce a huge amount of non-inflammatory IgA antibodies as the first line of defence against microorganisms (Mestecky et al., 1999). Few studies have been carried out on the

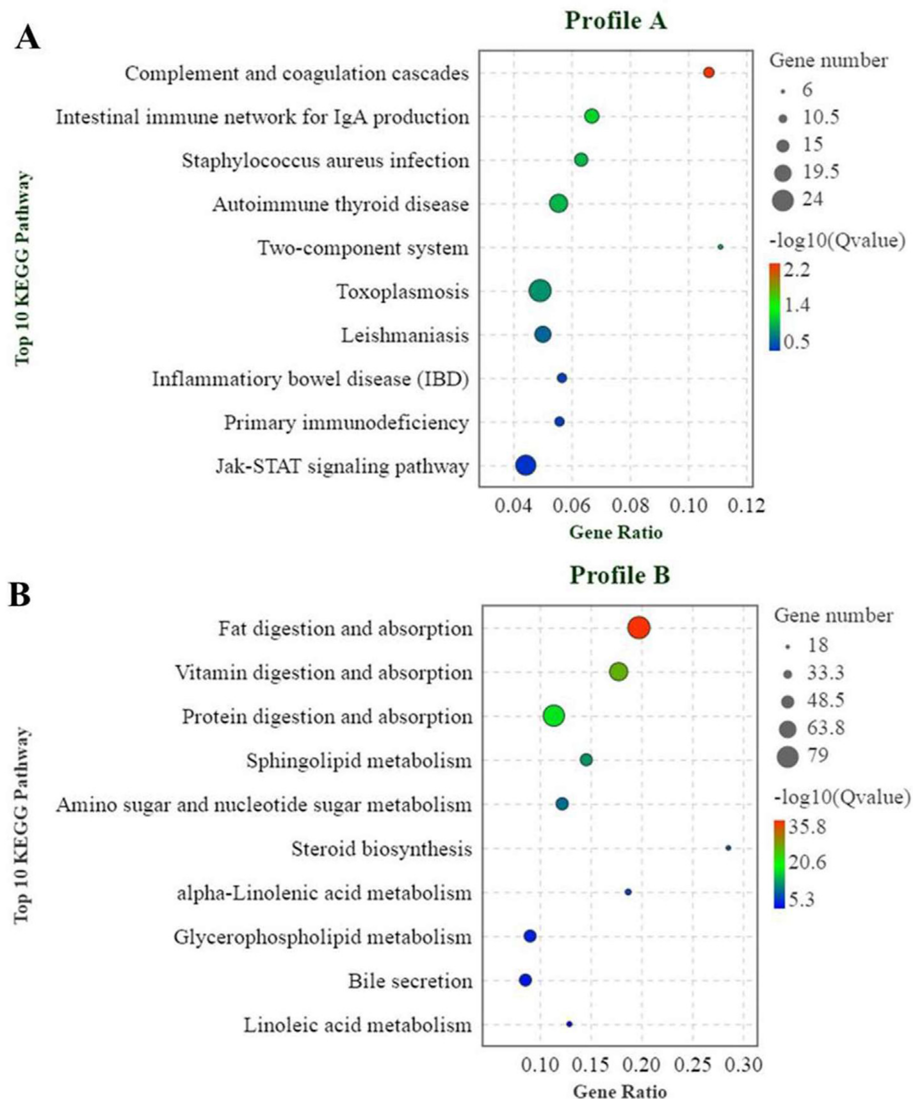

Fig. 7. Significant KEGG enrichment pathways of DEGs in SPC40 group in hindgut of pearl gentian groupers. (A) Up-regulation and (B) down-regulation KEGG pathways ($n = 4$). KEGG = Kyoto Encyclopedia of Genes and Genomes; DEGs = differential genes; SPC = soybean protein concentrate; SPC40 = 40% SPC protein alternative degree to fish meal protein.

signalling pathways of the intestinal immunity network related to IgA production in fish. Research on mammals has found that one significant immune response after intestinal flora colonization is the generation of IgA by immune cells in gut-connected lymphoid tissue (Suzuki and Fagarasan, 2008). In aseptic animals, IgA-secreting cells in the intestinal mucosa decreased sharply and were almost absent in newborns before colonization by symbiotic bacteria (Benveniste et al., 1971). Compared with other Ig isoforms, IgA is the most plentiful one secreted by mucosal cells. In human studies, at least 80% of plasma cells are positioned in the intestinal lamina propria, which produce more immunoglobulin IgA (40 to 60 mg/kg per day) than other Ig isoforms (van Egmond et al., 2001). Studies have shown that the intestinal epithelium is significant in maintaining intestinal homeostasis, and recognizes bacterial products and secretes cytokines through TLRs, thereby enhancing epithelial obstacles and regulating local immune responses (Rakoff-Nahoum et al., 2004). Research on mammals suggests that IgA generation is caused by the interaction of special antigens with innate immune receptors, such as tlr2, tlr4 and tlr9 (Suzuki and Fagarasan, 2008). Additionally, the TLR–NF–κB signalling pathway is significant in the inflammation process and immune response (Tan et al., 2016). The above discussion highlights the function of the TLR-MyD88-NF-κB and the intestinal immune network for immunoglobulin A (IgA) production pathway of intestine, as related to enteritis in pearl gentian groupers fed SBM and SPC.

Our previous research found nine TLR members in the intestinal tissues of pearl gentian groupers: TLR1, TLR2, TLR3, TLR5, TLR8, TLR9, TLR13, TLR21 and TLR22 (Zhang, 2020). Currently, at least 20 kinds of TLRs have been identified in fish. Among the TLRs in present study, *TLR1*, *TLR2*, *TLR3*, *TLR5*, *TLR9*, *TLR21* and *TLR22* are considered bacterial ligands in fish (Cerutti et al., 2008). Our previous study found that the expressions of *TLR5*, *TLR8*, *TLR9*, *TLR21* and *TLR22* increased significantly with SBM replacement of FM (Zhang et al., 2021b), while the expressions of *TLR5*, *TLR8*, *TLR9* and *TLR22* increased significantly after SPC substitution for FM in this study. After SBM or SPC replacement, intestinal pathogenic microorganisms may activate TLR signal transduction via a range of

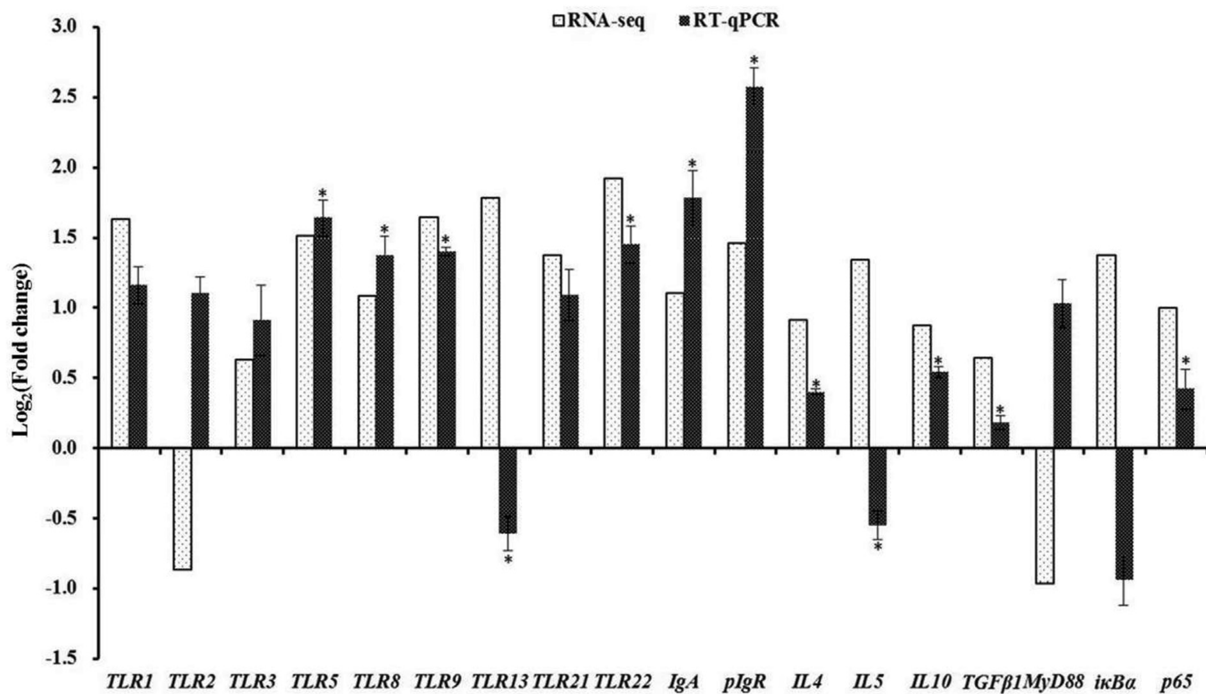

Fig. 8. The contrast of RNA-Seq and RT-qPCR results and the expressions of representative genes in Toll-like receptor/myeloid differentiation factor 88/nuclear factor kappa-B (TLR-MyD88-NF-κB) and intestinal immune network for IgA production in hindgut were chosen to confirm the precision of "3 + 2" transcriptome sequencing. The relative expression degree in RNA-seq analysis was counted by FPKM value. Asterisks distributed on the column showed significant variances between the groups at $P < 0.05$ ($n = 4$). FPKM = fragments per kilobase of exon model per million mapped fragments.

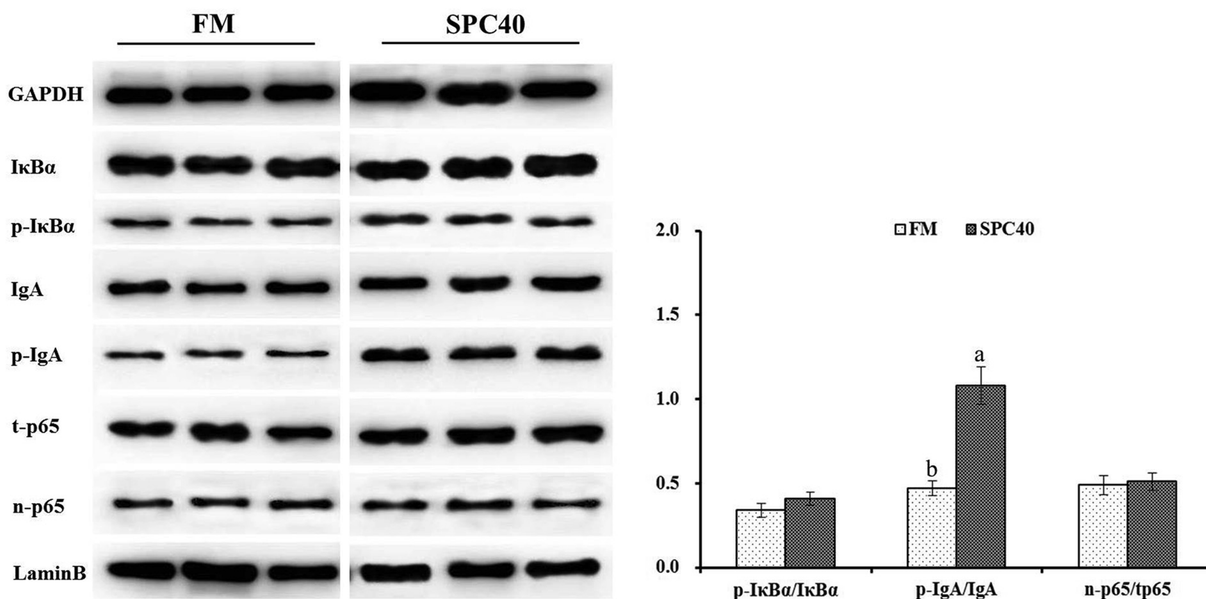

Fig. 9. Western blotting analysis of activations of NF-κB and intestinal immune network for IgA production signaling pathways in the hindgut of pearl gentian groupers fed the diets of 40% SPC PRO substitution for fish meal PRO ($n = 3$). Different letters on the bars represent significant differences among the groups at $P < 0.05$. GAPDH and LaminB were selected as the total protein and nuclear protein internal parameters, respectively. All Western blotting were obtained from a complete gel band. SPC = soybean protein concentrate; SPC40 = 40% SPC protein alternative degree to fish meal (FM) protein. PRO = superb protein; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; IκBα = inhibitor of NF-κBα; p-IκBα = phosphorylation inhibitor of NF-κBα; t-p65 = total p65; n-p65 = nucleus p65; IgA = immunoglobulin A; p-IgA = phosphorylation immunoglobulin A.

bacterial elements or products, respectively (Zhang et al., 2022). However, there are some differences in the types of TLRs used.

Transcriptome analysis showed that a high level of dietary SPC had a wide range of significant effects (81.82%) on the signalling pathways involved in intestinal digestion and nutrition absorption. However, in the SPC groups, only 39.47% of the DEGs had significant effects on immune and illness-related signalling pathways. Therefore, SPC-induced enteritis may be due to a disturbance in intestinal immune function caused by an imbalance in intestinal nutrition metabolism. Our synchronous transcriptome research has also

found that in the state of SBMIE, there is a general inhibition of pathways associated with nutritional digestion and absorption (67.44%; Zhang et al., 2021b). However, whether intestinal immunity or metabolic imbalance plays the leading role in enteritis development remains unclear and warrants further exploration. Studying soy protein-induced enteritis is of great significance, both in aquaculture and in most terrestrial animals including human beings, and further development of this field is required.

5. Conclusion

The present study found that 20% and 40% SPC substitution levels had negative effects on the growth and intestinal health of pearl gentian groupers. The 40% SPC diet caused obvious enteritis symptoms. The groupers' intestinal flora abundance and functions differed significantly after excessive SPC substitution for FM. The full-length transcriptome analysis showed that TLR-mediated intestinal immune network for IgA production signalling pathway played an important role in SPC-induced enteritis, but the types of TLRs involved were some different from that in SBM-induced enteritis. The immune-related signalling pathways that were significantly affected made small contributions to SPC-induced enteritis. However, signalling pathways related to nutrient digestion and absorption were generally inhibited in SPC-induced enteritis of pearl gentian grouper.

Author contributions

Wei Zhang was engaged in the whole experiment, and formulated the draft of this manuscript; **Beiping Tan** designed the experiment; **Junming Deng** composed and amended the draft critically; **Qihui Yang** and **Shuyan Chi** took part in the tests and amended the first draft; **Aobo Pang** and **Yu Xin** completed the data analysis; **Yu Liu** and **Haitao Zhang** approved the final version.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgements

This study received assistance from the National Key R&D Program of China (2019YFD0900200). We appreciate the support provided by the Key Laboratory of Aquatic, Livestock and Poultry Feed Science and Technology in South China, Ministry of Agriculture.

Appendix Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2022.08.006.

References

Alsadi R, Boivin M, Ma T. Mechanism of cytokine modulation of epithelial tight junction barrier. Front Biosci 2009;14:2765–78.

Baeverfjord G, Krogdahl Å. Development and regression of soybean meal induced enteritis in Atlantic salmon, *Salmo salar* L., distal intestine: a comparison with the intestines of fasted fish. J Fish Dis 1996;19:375–87.

Bakke-Mckellep AM, Press CM, Baeverfjord G, Krogdahl Å, Landsverk T. Changes in immune and enzyme histochemical phenotypes of cells in the intestinal mucosa of Atlantic salmon, *Salmo salar* L. with soybean meal-induced enteritis. J Fish Dis 2000;23:115–27.

Benítez-Páez A, Pugar EMGD, López-Almela I, Moya-Pérez N, Sanz Y. Depletion of *Blautia* species in the microbiota of obese children relates to intestinal inflammation and metabolic phenotype worsening. mSystems 2020;5:e00857.

Benveniste J, Lespinats G, Salomon JC. Serum and Secretory IgA in axenic and holoxenic mice. J Immunol 1971;107:1656–62.

Cerutti A, Rescigno M. The biology of intestinal immunoglobulin a responses. Immunity 2008;28:740–50.

Chen W. Effects of antinutritional factors on the utilization of soybean proteins by Japanese flounder, *Paralichthys olivaceus*. Qingdao: Ocean University of China; 2009.

Faudzi NM, Yong ASK, Shapawi R, Senoo S, Takii K. 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. Aquacult Res 2017;49:431–41.

Feng J, Wang P, He JJ, Lou JY, Dang H, Deng R. Effects of soybean protein concentrate instead of fish meal on serum biochemical indexes and liver histology of juvenile large yellow croaker (*Larimichthys crocea*). J Fish Sci China 2017;24: 268–77.

Filippis FD, Pasolli E, Ercolini D. Newly explored *Faecalibacterium* diversity is connected to age, lifestyle, geography, and disease. Curr Biol 2020;30:4932–43.

Fuentes-Appelgren P, Opazo R, Barros L, Feijoó CG, Romero J. Effect of the Dietary inclusion of soybean components on the innate immune system in Zebrafish. Zebrafish 2014;11:41–9.

Giri NA, Muzaki A, Marzuqi M, Sudewi. Optimizing protein, lipid and carbohydrate levels in diets for growth of juvenile hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus*♂). Earth Environ Sci 2020;584(7pp):012030.

Green TJ, Smullen R, Barnes AC. Dietary soybean protein concentrate-induced intestinal disorder in marine farmed Atlantic salmon, *Salmo salar* is associated with alterations in gut microbiota. Vet Microbiol 2013;166:286–92.

Gu M, Bai N, Zhang Y, Krogdahl Å. Soybean meal induces enteritis in turbot *Scophthalmus maximus* at high supplementation levels. Aquaculture 2016;464: 286–95.

Gu M, Jia Q, Zhang ZY, Bai N, Xu XJ, Xu BY. Soya-saponins induce intestinal inflammation and barrier dysfunction in juvenile turbot (*Scophthalmus maximus*). Fish Shellfish Immunol 2018;77:264–72.

Hien TTT, Phu TM, Tu TLC, Trung NB, Phu TM, David B. Effects of replacing fishmeal with soy protein concentrate on growth, feed efficiency, and digestibility in diets for snakehead, *Channa striata*. Aquacult Res 2017;48:3174–81.

Hu HB, Kortner TM, Gajardo K, Chikwati E, Tinsley J, Krogdahl Å. Intestinal fluid permeability in Atlantic Salmon (*Salmo salar* L.) is affected by dietary protein source. PLoS One 2016;11:e0167515.

Kalhoro H, Zhou J, Hua Y, Ng WK, Ye L, Zhang J, et al. Soy protein concentrate as a substitute for fish meal in diets for juvenile *Acanthopagrus schlegelii*: effects on growth, phosphorus discharge and digestive enzyme activity. Aquacult Res 2018;49:1896–906.

Kim DH, Kim DY. Microbial diversity in the intestine of olive founder (*Paralichthys olivaceus*). Aquaculture 2013;414:103–8.

Kortner TM, Skugor S, Penn MH, Mydland LT, Djordjevic B, Hillestad M, et al. Dietary soyasaponin supplementation to pea protein concentrate reveals nutrigenomic interactions underlying enteropathy in Atlantic salmon (*Salmo salar*). BMC Vet Res 2012;8:101.

Król E, Douglas A, Crampton VO, Speakman JR, Secombes CJ, et al. Differential responses of the gut transcriptome to plant protein diets in farmed Atlantic salmon. BMC Genomics 2016;17:156–72.

Li RM. The effects of inositol and zinc in low-fishmeal dietary on nutritional physiological function for juvenile *Litopenaeus vannamei* and *Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂, respectively. Zhanjian: Guangdong Ocean University; 2019.

Liu Y, Chen ZC, Dai JH, Yang P, Xu WQ, Ai QH, et al. 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 2019;88:65–75.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\triangle\triangle CT}$ method. Methods 2001;25:402–8.

Mestecky J, Moro I, Underdown BJ. In: Ogra P, et al., editors. Mucosal immunology. Academic Press San Diego); 1999. p. 133–52.

Miao S, Zhao C, Zhu J, Hu J, Dong X, Sun L. Dietary soybean meal affects intestinal homoeostasis by altering the microbiota, morphology and inflammatory cytokine gene expression in northern snakehead. Sci Rep 2018;8:113–23.

Mireia VC, Gwen F, Youssef D, Tigchelaar EF, Wang J, Tito RY. The neuroactive potential of the humangut microbiota in quality of life and depression. Nat Microbiol 2019;4:623–32.

Nayak SK. Role of gastrointestinal microbiota in fish. Aquacult Res 2010;41: 1553–73.

NRC. Nutrient requirements of fish and shrimp. Washington, D.C.: The National Academic Press; 2011.

Pan JH, Feng L, Jiang WD, Wu P, Kuang SY, Tang L, et al. Vitamin E deficiency depressed fish growth, disease resistance, and the immunity and structural integrity of immune organs in grass carp (*Ctenopharyngodon idella*): referring to NF-κB, TOR and Nrf2 signaling. Fish Shellfish Immunol 2017;60:219–36.

Parker BJ, Wearsch PA, Veloo ACM, Rodriguez-Palacios A. The genus alistipes: gut bacteria with emerging implications to inflammation, cancer, and mental health. Front Immunol 2020;11:906.

Rajagopal L, Vo A, Silvestroni A, Rubens CE. Regulation of cytotoxin expression by converging eukaryotic-type and two-component signaling mechanisms in Streptococcus agalactiae. Mol Microbiol 2010;62:941–57.

Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by Toll-Like receptors is required for intestinal homeostasis. Cell 2004;118:229–41.
Sahlmann C, Sutherland BJG, Kortner TM, Koop BF, Krogdahl A, Bakke AM. Early response of gene expression in the distal intestine of Atlantic salmon (*Salmo salar* L.) during the development of soybean meal induced enteritis. Fish Shellfish Immunol 2013;34:599–609.
Schirmer M, Garner A, Vlamakis H, Xavier RJ. Microbial genes and pathways ininflammatory bowel disease. Nat Rev Microbiol 2019;17:497–511.
Shekarabi SPH, Mehrgan MS, Banavreh A, Foroudi F. Partial replacement of fishmeal with corn protein concentrate in diets for rainbow trout (*Oncorhynchus mykiss*): effects on growth performance, physiometabolic responses, and fillet quality. Aquacult Res 2021;52:249–59.
Shui XM, Lin K, Zhao XT, Huang J, He JJ, Lou YD, et al. Effects of soy protein concentrate on growth and intestinal microbiota of large yellow croaker *larimichthys crocea*. J Fish Sci China 2021;28:37–47.
Suzuki K, Fagarasan S. How host-bacterial interactions lead to IgA synthesis in the gut. Trends Immunol 2008;29:523–31.
Tacchi L, Secombes CJ, Bickerdike R, Adler MA, Venegas C, Takle H, et al. Transcriptomic and physiological responses to fishmeal substitution with plant proteins in formulated feed in farmed Atlantic salmon (*Salmo salar*). BMC Genom 2012;13:363–84.
Tan P, Dong X, Mai KS, Xu W, Ai QH. 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 Immunol 2016;59:398–405.
Urán PA, Gonçalves AA, Taverne-Thiele JJ, Schrama JW, Rombout JHWM. Soybean meal induces intestinal inflammation in common carp (*Cyprinus carpio* L.). Fish Shellfish Immunol 2008;25:751–60.
van Egmond M, Damen CA, van Spriel AB, Vidarsson G, van Garderen E, van de Winkel JGJ. IgA and the IgA Fc receptor. Trends Immunol 2001;22:205–11.
Wang CSE. The mechanism of inhibition effect of probiotics on ulcerative colitis carcinogenesis and the analysis of differences in intestinal microbiota. Beijing: Beijing Union Medical University Hospital; 2017.
Xiang FQ. Effect of sea bass on growth, immune and intestinal flora by soy protein concentrate. Changsha: Hunan Agricultural University; 2017.
Yachida S, Mizutani S, Shiroma H, Shiba S, Nakajima T, Sakamoto T, et al. Metagenomic and metabolomic analyses reveal distinct stage-specific phenotypes of the gut microbiota in colorectal cancer. Nat Med 2019;25:968–76.
Zhang W. Study on the differential mechanism of intestinal mucosal barrier damage in *Epinephelus fuscoguttatus* ♀× *E. lanceolatus* ♂ caused by three kinds of soybean protein. Zhanjian: Guangdong Ocean University; 2020.
Zhang W, Tan BP, Deng JM, Dong XH, Yang QH, Chi SY, et al. Mechanisms by which fermented soybean meal and soybean meal induced enteritis in marine fish juvenile pearl gentian grouper. Front Physiol 2021a;12:646853.
Zhang W, Tan BP, Deng JM, Yang QH, Chi SY, Pang AB, et al. PRR-mediated immune response and intestinal flora profile in soybean meal induced enteritis of pearl gentian groupers, *Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus*. Front Immunol 2022;13:814479.
Zhang W, Tan BP, Deng JM, Zhang HT. Multiomics analysis of soybean meal induced marine fish enteritis in juvenile pearl gentian grouper, *Epinephelus fuscoguttatus* ♀× *Epinephelus lanceolatus* ♂. Sci Rep 2021b;11:23319.
Zhang W, Tan BP, Ye GL, Wang JX, Dong XH, Yang QH, et al. Identification of potential biomarkers for soybean meal-induced enteritis in juvenile pearl gentian grouper, *Epinephelus lanceolatus* ♂ × *Epinephelus fuscoguttatus*. Aquaculture 2019;512:734337.
Zhang W, Wang FM, Tan BP, Dong XH, Zhang HT, Chi SY, et al. Effect of the dietary phosphatidylcholine at different growth stages of Pacific white shrimps, *Litopenaeus vannamei*. Aquacult Nutr 2018;25:1–12.
Zheng HY, Yan GS, Marquez S, Andler S, Dersjant-Li Y, de Mejia EG. Molecular size and immunoreactivity of ethanol extracted soybean protein concentrate in comparison with other products. Process Biochem 2020;96:122–30.
Zhu R, Li L, Wu LF, Li M, Yu Z, Duan J, et al. Soy protein concentrate instead of fish meal processing status and impact on fish. Soybean Sci 2019;38:317–21.