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Differential analysis of fish meal substitution with two soybean meals on juvenile pearl gentian grouper

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Fermented soybean meal and soybean protein concentrate are products of soybean that have been processed physically or biologically, and their use as an alternative to fish meal results in a significant reduction in the effects of anti-nutritional factors (ANFs) in soybean on aquatic species. Replacing fish meal with soybean protein concentrate and fermented soybean meal can meet the high protein requirements of carnivorous fish while effectively reducing aquaculture costs; however, excessive substitution can also cause economic losses. In this study, we used transcriptome sequencing to investigate the impacts of fermented soybean meal and soybean protein concentrate on the growth and physiology of pearl gentian grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂) juveniles and to examine the mechanisms by which fermented soybean meal and soybean protein concentrate impair the intestinal condition of fish. Originally weighed 12.55 ± 0.06 g, the selected pearl gentian groupers were categorised into three treatment groups: one group was fed fish meal-based diets (FM, control group), one group was fed fish meal- and soybean protein concentrate-based diets (SPC40) and one group was fed fish meal- and fermented soybean meal-based diets (FSBM40), with the same crude protein and crude fat content in all three diets. The experiment was conducted for 10 weeks. The growth results showed that both the fermented soybean meal and soybean protein concentrate diets significantly inhibited the growth of the fish. Based on the results of enzyme activity, substance content and gene expression levels associated with intestinal damage and intestinal inflammation, it is highly likely that the fermented soybean meal and soybean protein concentrate diets affected the intestinal health of the fish and triggered intestinal inflammation. This study provides a theoretical basis to further explore the mechanism of soybean-initiated intestinal problems in fish.

KEYWORDS

Epinephelus fuscoguttatus **♀ × *Epinephelus lanceolatus* ♂, soybean protein concentrate, fermented soybean meal, intestinal inflammation, transcriptome sequencing**

1 Introduction

Many aquaculture species are carnivorous, and their traditional feeds require high levels of fish meal and fish oil. The booming aquaculture fuelled the market demand for fish meal, thus causing unbalanced growth between fishing for feed and food, decreasing the productive potential of fisheries and accelerating the depletion of traditional fisheries. Therefore, it is detrimental to the future growth of aquaculture (Zhang et al., 2020). Replacing fish meal with plant-based ingredients in aquaculture is feasible. Soybean meal is a common plant-based feed ingredient in aquatic feeds. However, it contains saponins, lectins, soybean antigenic proteins, trypsin inhibitors and other anti-nutritional factors (ANFs) that have been shown to adversely affect the digestion and absorption of some aquatic species (Sørensen et al., 2011). Soybean protein concentrate extraction mainly adopts the alcohol process, and its protein content is further increased and ANFs are substantially reduced (Yang et al., 2014). The level of ANFs in fermented soybean meal produced by microbial fermentation of soybean meal is also significantly reduced compared to soybean meal. During the fermentation process of soybean meal, soybean proteins can be broken down into smaller peptide proteins that are more easily digested and absorbed (Mugwanya et al., 2022). These products obtained by processing soybean meal through different processes have been used as alternative protein sources to fish meal in the culture of a variety of aquatic fish species.

Replacing fish meal with soybean meal and its processing products can essentially meet the nutritional requirements of most aquatic species' diets (Król et al., 2016; Uczay et al., 2019). However, fish farmed with high levels of soybean meal instead of fish meal often suffer from reduced growth performance, weakened immunity, disruption of body metabolism, and other adverse reactions during the breeding process. The intestinal mucosal barrier of fish is found to be damaged and intestinal health is affected, leading to intestinal inflammation (Rumsey et al., 1994; Urán et al., 2008). Studies have shown that replacing fish meal with soybean meal often causes soybean meal-induced enteritis (SBMIE) in carnivorous fish. SBMIE is a non-infectious subacute enteritis that triggers shortening of fish distal intestinal mucosal folds, widening of the lamina propria, reduction of absorptive cell vacuoles and inflammatory cell infiltration. This will greatly affect the growth and survival of fish and reduce production efficiency (van den Ingh et al., 1991). Numerous studies have shown that replacing fish meal with excessive amounts of soybean protein concentrate or fermented soybean meal can also affect the growth performance of fish by disrupting their intestinal structure and promoting an inflammatory response (Kissil et al., 2000; Deng et al., 2006; Shiu et al., 2015; He et al., 2020). The intestinal inflammation caused by soybean processing products following this substantial reduction in ANFs requires further study.

The intestine is the main digestive and absorption organ of fish, and the intestinal mucosa struggles in the front line against pathogens. An intact intestinal mucosal epithelial structure and sound intestinal function are particularly important for the intestinal health of fish (Brandl et al., 2017). Feeding excessive plant-based ingredients, such as soybean meal, can damage the fish's intestinal tract and also affect its intestinal permeability, allowing pathogens to invade (Hu et al., 2016). Moreover, the importance of the vast microbial communities in the intestines to maintain the integrity of the intestinal structure and function cannot be ignored, and harmful microbial infections resulting from an imbalance in the intestinal flora trigger the host to perform an inflammatory response (Nayak, 2010). The causes and specific mechanisms of intestinal inflammation in fish caused by the replacement of fish meal with plant-based raw materials, such as soybean meal, are still unclear, and past studies have tended to focus on the effects of ANFs in soybean meal on fish intestinal (Krogdahl et al., 2010). Fermented soybean meal and soybean protein concentrate contain fewer ANFs, and relatively little research has been done on their effects on fish intestinal health.

Pearl gentian grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂) is an excellent carnivorous marine hybrid fish that combines the excellent quality of both parents. With fast growth and strong disease resistance, the pearl gentian grouper has gradually become the largest grouper species cultured in China (Zhang et al., 2021). Research concerning the epistasis of pearl gentian grouper at the transcriptional level is relatively scarce and needs to be further improved. Since the advancement of the high-throughput sequencing technique, transcriptome sequencing has been commonly used in gene expression regulation. Comparative transcriptomics is now common in studying various aquatic species, which includes various aspects such as growth and development, toxicology and immune response (Chen et al., 2020). In this study, we fed pearl gentian grouper with soybean protein concentrate or fermented soybean meal in place of 40% fish meal, aiming to compare the differences in the distal intestine of fishes eating these two soybean processing products at the transcriptome level, analyse the mechanisms that induce enteritis and determine the impact of the highly replaced fish meal with these two soybean processing products on the growth and physiology of pearl gentian grouper.

2 Materials and methods

2.1 Experimental diets

There were three experimental diets, among which the control feed (FM) was mainly made of fish meal, while soybean protein concentrate (SPC40) or fermented soybean meal (FSBM40) was employed to substitute 40% fish meal protein in the remaining two groups, respectively. The contents of crude protein and crude fat in the three diets were the same. SPC40 and FSBM40 groups were supplemented with appropriate amounts of lysine and methionine to maintain equal lysine and methionine contents in the three

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diets. Table 1 shows the details of the diet formula and the approximate ingredients. Accurate weighing of raw materials that have been ground into fine powder according to the formulation and then mixed uniformly using a stepwise expansion method. Then, the weighed fine powder, fish oil, soybean oil, soy lecithin and an appropriate amount of water were mixed sequentially according to the formulation before being granulated by the twin-screw extruder. The granules with diameters of 2.0 mm and 3.0 mm were put at −20°C for later experiments after being air-dried to about 10% moisture.

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

Ingredients (%)	Diets		
	FM	SPC40	FSBM40
Red fish meal[a]	50.00	30.00	30.00
Soybean protein concentrate[b]	0.00	21.74	0.00
Fermented soybean meal[c]	0.00	0.00	23.89
Vital wheat gluten	5.00	5.00	5.00
Wheat flour	18.00	18.00	18.00
Casein	4.60	4.60	4.60
Gelatin	1.00	1.00	1.00
Fish oil	3.02	4.41	4.49
Soybean oil	2.00	2.00	2.00
Soybean lecithin	2.00	2.00	2.00
Microcrystalline cellulose	11.48	8.14	5.84
Calcium monophosphate	1.50	1.50	1.50
Ascorbic acid	0.05	0.05	0.05
Choline chloride	0.50	0.50	0.50
Vitamin premix[d]	0.30	0.30	0.30
Mineral premix[e]	0.50	0.50	0.50
Ethoxyquin	0.05	0.05	0.05
Lysine[f]	0.00	0.05	0.13
Methionine[f]	0.00	0.16	0.15
Proximate composition) (%, dry matter)			
Crude protein	50.97	50.63	50.45
Crude lipid	10.15	10.71	10.54

[a]The red fish meal obtained from Corporación Pesquera Inca S.A.C., Bayovar Plant, Peru. 72.53% crude protein and 8.82% crude fat on a dry matter basis.
[b]The soybean protein concentrate obtained from Zhanjiang Haibao Feed Co. Ltd (Zhanjiang, China). 70.72% crude protein on a dry matter basis.
[c]The fermented soybean meal obtained from Foshan CJ Biotechnology Co. Ltd (Foshan, China). 60.75% crude protein on a dry matter basis.
[d]Vitamin premix consisted of (g/kg premix): VB_1 17.00 g, VB_2 16.67 g, VB_6 33.33 g, VB_{12} 0.07 g, VK 3.33 g, VE 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 g, folic acid 4.17 g, inositol 102.04 g, cellulose 592.72 g;
[e]Mineral premix consisted of (g/kg premix): $FeSO_4{\cdot}H_2O$ 18.785 g. $ZnSO_4{\cdot}H_2O$ 32.0991 g, $MgSO_4{\cdot}H_2O$ 65.1927 g, $CuSO_5{\cdot}5H_2O$ 11.0721 g, $CoCl_2{\cdot}6H_2O$ (10%) 5.5555 g, KIO_3 0.0213 g, KCl 22.7411 g, Na_2SeO_3 (10%) 0.5555 g, zeolite powder 843.9777 g;
[f]Lysine and Methionine were added to balance amino acid with FM group.

2.2 Feeding management

The pearl gentian grouper juveniles used in the study were purchased from Zhanjiang, Guangdong, China, and the culture experiments were conducted in an indoor culture system at the Zhanjiang Marine Biological Research Base, China. Seven hundred and twenty fish weighing 12.55 ± 0.06 g were equally and randomly divided into 12 tanks (three treatment groups, four biological replicates per treatment group). The tank has a capacity of one cubic meter (stocking density of 60 fish per cubic meter of water) and is equipped with oxygenation equipment to prevent dissolved oxygen levels from falling below 7 mg L^{-1}. The fish were fed commercial feeds containing ≥520 g/kg of crude protein and ≥130 g/kg of crude fat content for one week before the culture experiment. During the culture process, satiety feeding was provided daily at 8:00 and 16:00 with a water temperature of 29 ± 1°C.

2.3 Sample collection

After the culture was carried out for 10 weeks and feeding was stopped for one day, the researchers counted and weighed the surviving fish in each of the 12 tanks to calculate the weight gain rate(WGR), specific growth rate (SGR), feed conversion ratio (FCR), and survival rate (SR). After weighing the fish, we anaesthetised it with eugenol (1:10,000), dissected and removed the distal intestine and immediately stored it in liquid nitrogen. The distal intestine was stored at −80°C for subsequent experiments.

The WGR, SGR, FCR and SR were calculated by the following formulas:

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

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

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

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

2.4 Biochemical analysis

To determine the activities of trypsin, total superoxide dismutase (T-SOD), glutathione peroxidase (GSH-Px) and the concentration of complement 3 (C3), complement 4 (C4) and immunoglobulin M (IgM) in the distal intestine of fish, the researchers used the fish ELISA kit manufactured by Shanghai

Jiang Lai Biotechnology Co., Ltd (Shanghai, China). The experiment was in strict accordance with the instructions. The protein content of the distal intestine was measured using the BCA method (Beyotime Biotechnology Co., Ltd., Shanghai, China) to calculate the relevant indicators.

2.5 Transcriptome sequencing and analysis

Oligo (dT) magnetic beads were employed to enrich the mRNA was enriched, and SMARTer PCR cDNA Synthesis Kit (Clontech, Japan) was used to synthesise the cDNA *via* reverse transcription amplification. cDNA fragments ≥4 kb were enriched by BluePippin screening. The full-length cDNA was ligated by SMRT dumbbell-shaped connector repair. The complete SMRT bell library was checked and sequenced using the Pacific Biosciences Sequel platform. Seven databases were used to annotate the full-length transcript: Gene Ontology, Kyoto Encyclopaedia of Genes and Genomes Orthology database, Swiss-Prot, Clusters of Orthologous Groups of proteins, Protein family, NCBI non-redundant nucleotide sequences and NCBI non-redundant protein sequences. In addition, four software were employed, including Diamond BLASTX, BLAST, Hmmscan and Blast2GO. Based on the thresholds of $|\mathrm{Log_2FC}| > 1$ and $P< 0.05$, the researchers carried out the screening of differentially expressed genes (DEGs).

2.6 RNA extraction and real-time quantitative PCR

Trizol (Invitrogen, Carlsbad, CA, USA) was employed to extract RNA from the distal intestinal tissues. The researchers then used NanoDrop 2000 (Thermo Fisher Scientific, USA) to measure RNA concentrations and RNA integrity on a 1% agarose gel. To prepare cDNA, RNA was processed using the Evo M-MLV Reverse Transcription Kit (Takara, Japan).

Real-time quantitative PCR was carried out on a PCR Mastercycler (Mastercycler® ep realplex, Eppendorf, Germany) using the SYBR® Premix ExTaq™ II Kit (Takara, Japan). The thermal profile included a 95°C cycle, 2 minutes for each and a compound cycle twice, which is 95°C for 15 seconds, 60°C for 10 seconds and 72°C for 20 seconds. Primer synthesis template sequences were referenced to the PacBio SMART pearl gentian grouper distal intestine tissue full-length transcriptome sequencing database obtained from past studies, as detailed in Table 2. Primers were synthesised by Bioengineering Co., Ltd. (Shanghai, China). The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was employed to carry out the target gene expressions.

2.7 Statistical analysis

In this study, the mean and standard deviation ($\bar{x} \pm$ SD) was used to present the data, and SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) was selected for a one-way analysis of variance (ANOVA). The Duncan multiple comparison test was performed to determine the significant differences between the groups. Statistically significant differences were described as $P< 0.05$.

3 Results

3.1 Growth performance

The WGR, SGR and FCR of pearl gentian grouper were significantly affected by high contents of soybean protein concentrate or fermented soybean meal in the diets ($P< 0.05$), as illustrated in Figure 1. The two comparative groups did not show a significant difference in WGR, SGR and FCR ($P > 0.05$), but the WGR and SGR were considerably lower, and the FCR was substantially higher in the FSBM40 group. Survival rates were not significantly different and were similar between the three groups ($P > 0.05$).

3.2 Biochemical indices

The outcomes suggested that compared to the FM group, both the SPC40 and FSBM40 groups increased fish intestinal trypsin, T-SOD and GSH-Px activity and decreased IgM, C3 and C4 protein content ($P< 0.05$) significantly. The SPC40 group had the highest trypsin, GSH-Px and T-SOD activity and the lowest IgM, C3 and C4 protein content (Table 3).

3.3 Immune-related gene expression

Table 4 shows the expression levels of anti-inflammatory and pro-inflammatory cytokines in the distal intestine of pearl gentian groupers. The mRNA in pro-inflammatory cytokines, including *TNFα*, *IL1β*, *IL8*, *IL12*, *IL17* and *IL32*, were higher in the FSBM40 and SPC40 groups ($P< 0.05$). Furthermore, compared with those in the FSBM40 group, the expressions of *IL32* and *TNFα* in the SPC40 group were considerably lower ($P< 0.05$). Among the anti-inflammatory cytokines, compared to the FM group, the mRNA in *TGFβ1*, *IL4*, *IL5* and *IL10* exhibited lower levels in the distal intestine of the SPC40 and FSBM40 groups ($P< 0.05$). In addition, compared to the FSBM40 group, the expression level of *IL5* was significantly higher in the SPC40 group ($P< 0.05$).

3.4 Analysis of differentially expressed transcripts

3.4.1 Statistics of DEGs

The statistical results of the DEGs screened based on differential analysis are shown in Table 5. It is indicated that by comparison with the FM group, there are 2,328 up-regulated genes and 1,748 down-regulated genes in the SPC40 group ($P< 0.05$), with a total of 4076 significantly differential genes. In addition, compared with the FM group, there are 1,457 genes significantly down-regulated and 2,005 genes significantly up-regulated in the FSBM40 group ($P<$

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TABLE 2 PCR primers for mRNA expression of intestinal immune-related genes in grouper.

Gene	Forward 5′-3′	Revise 3′-5′	Size (bp)
IL1β	AAGGTGGACGCCAACAGACA	GTTCACTGCAGGCTCAGGGA	153
IL8	TGTGGCACTCCTGGTTCTCC	GGGTTCACCTCCACCTGTCC	132
IL12	GACGGAGCATTTCCTGGTGG	TGCTCCAAGAGCTCGGGTAA	172
IL17	GAGAGGACGGTGTCTGTGTGG	CATGCACAGTTGAGGGTGTGG	101
IL32	CAGCAACAGTAGCAGCAGGC	CCATCCTCCTCAGCTCTGCC	176
TNFα	AACTGTGTGTCCCCACTGCC	CCACAGATGGCCCAGGTCAT	81
IL4	GCAGTGAGTGAAGCCATCGC	TGCAGTTCCTGATAGCGCGA	146
IL5	GGCCAACAGTCAAGATGTCTGCC	GAATGACCAGGAGCAGTTCAGTGT	160
IL10	ACACAGCGCTGCTAGACGAG	TAGACTTGTGCCACGACGGG	142
TGFβ1	CTTCTCCTCCTCCTCGCTGC	GATGTTGCTGAGGGCTTCGC	195
P65	TCAACCCAGTCCAAGCAGCA	GATGCTGCCAGCTGAACGTC	107
MyD88	GCATCTTGCGCTTCCTCACC	CCTGGTCCTTGGTTACGGCA	107
IκBα	ATGCAAAGGAGCAGCGTAACG	GAGGTTGGGGTCTGCTCCT	107
β-actin	GGCTACTCCTTCACCACCACA	TCTCCAAGGCAACGGGTCT	188
IgA	GATGGACCTGACAATAGC	AAAGATGTCCGCAACAC	151
pIgR	GTAACCACCGAGGGAGA	GCAAGTCGGTTAGGTCG	167

β-actin is the housekeeping gene in the experiment. IL, interleukin; TNFα, tumor necrosis factor α; TGFβ1, transforming growth factor β1; MyD88, myeloid differentiation factor 88; IgA, Immunoglobulin A; pIgR, Polyimmunoglobulin receptor.

0.05), for a total of 3,462 significantly different genes. As shown in Figure 2, there were 1,440 overlapping DEGs between the SPC40 and FSBM40 groups as compared to the FM group (Profile A), 2,636 DEGs unique to the SPC40 group (Profile B) and 2,022 DEGs unique to the FSBM40 group (Profile C).

3.4.2 Functional enrichment analysis

GO and KEGG were used to perform the functional enrichment and classification of the DEGs. The annotation outcomes for the three broad functional categories of GO (cellular component, molecular function and biological process) show that Profile A has 47 subclasses. In addition, in the biological process, the metabolic process (342) was the most enriched. Profile B comprises 53 subclasses, and the single-organism process (535) was the most enriched in the biological process. Profile C has 51 subclasses, of which the metabolic process (342) was the most enriched in the biological process category. Binding was the most enriched sub-categories in both Profile A (418), Profile B (670) and Profile C (554) among the molecular functional categories. Among the cellular component categories, the membrane was the most enriched sub-category in both Profile A (301), Profile B (430) and Profile C (276) (Figure 3).

According to the significant results of the KEGG enrichment analysis, in Profile A, 287 signalling pathways were enriched in Profile A, with 38 signalling pathways showing substantial change ($P< 0.05$). The largest proportion of these significantly changed pathways involved the digestive system-related pathways (5/38), lipid metabolism-related pathways (5/38) and those related to the metabolism of cofactors and vitamins (5/38), including the digestion and absorption of vitamin, fat and protein, mineral absorption, linoleic and alpha-linolenic acid metabolism, nicotinate and nicotinamide metabolism, arachidonic acid metabolism, vitamin B6 metabolism, thiamine metabolism, glycerophospholipid metabolism, ether lipid metabolism, retinol metabolism, folate biosynthesis and pancreatic secretion (Figure 4A). In Profile B, 326 signalling pathways were enriched, with 34 signalling pathways exhibiting a significant change ($P< 0.05$). Most of these significantly changed pathways were lipid metabolism-related (14/34) and digestive system-related (7/34), including the digestion and absorption of vitamin, protein, fat, steroid biosynthesis, synthesis and degradation of ketone bodies, alpha-Linolenic acid metabolism, sphingolipid metabolism, glycerophospholipid metabolism, mineral absorption, primary bile acid biosynthesis, bile secretion, arachidonic acid metabolism, linoleic acid metabolism, steroid hormone biosynthesis, fatty acid degradation, biosynthesis of unsaturated fatty acids, ether lipid metabolism, glycerolipid metabolism, carbohydrate digestion and absorption, pancreatic secretion and fatty acid biosynthesis (Figure 4B). In Profile C, 314 signalling pathways were enriched, with 56 signalling pathways showing significant change ($P< 0.05$), and most were related to infectious diseases (11/56) and the immune system (8/56), including Epstein-Barr virus infection, the intestinal immune network for IgA production, staphylococcus aureus infection, leishmaniasis, measles, B cell receptor signalling pathway, amoebiasis, natural killer cell-mediated cytotoxicity, Fc gamma R-mediated phagocytosis, tuberculosis, Th17 cell differentiation, legionellosis, hepatitis C, complement and coagulation cascades, hematopoietic cell lineage, African trypanosomiasis, Fc epsilon RI signalling pathway, toxoplasmosis and influenza A (Figure 4C).

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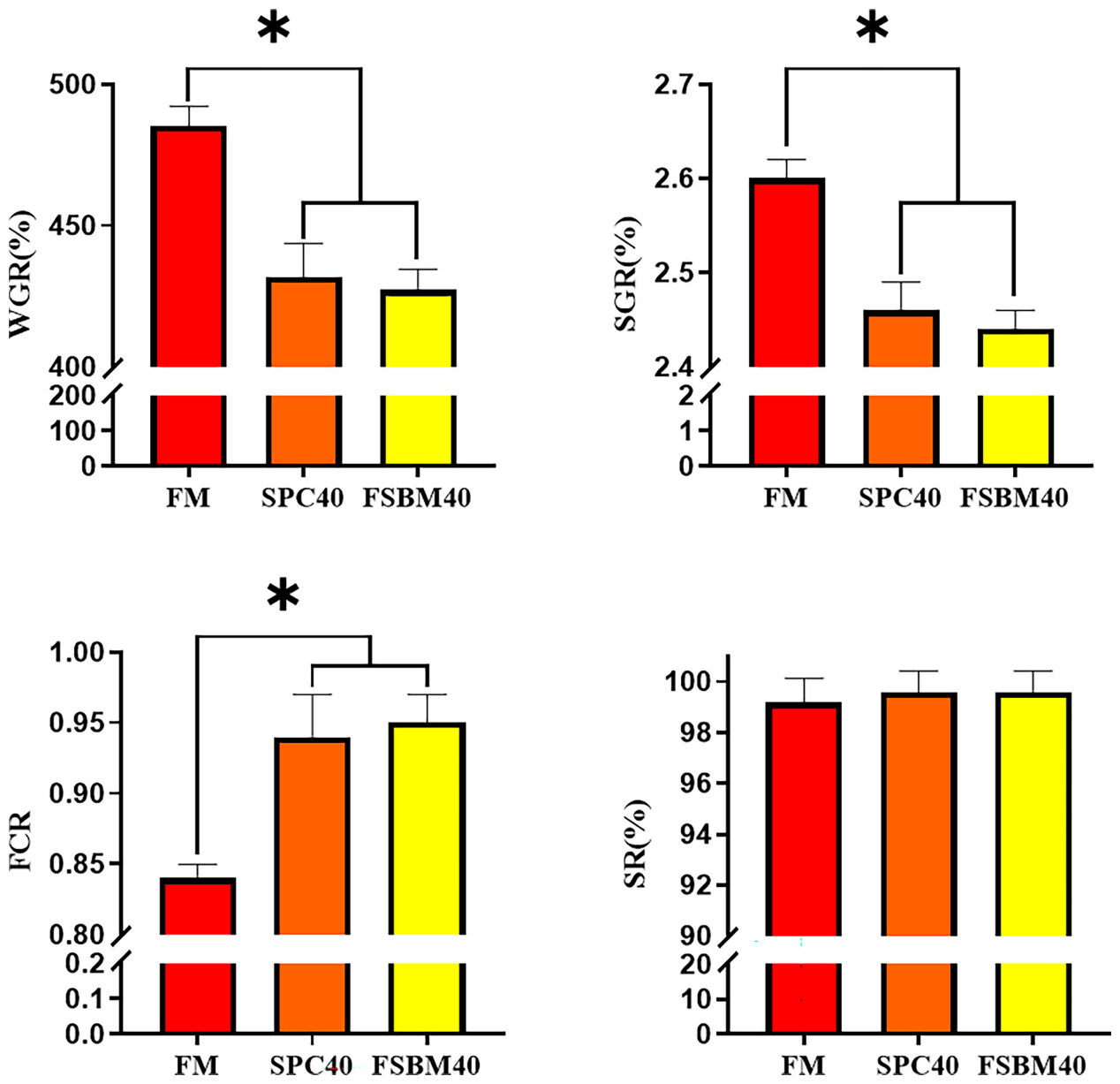

FIGURE 1
Effect of soybean protein concentrate or fermented soybean meal substitution of 40% fish meal protein on the growth of pearl gentian grouper (n=4). WGR, weight gain rate; SGR, specific growth rate; FCR, feed conversion ratio; SR, survival rates; *P< 0.05.

TABLE 3 Effect of different soybean processing products substitute for fish meal protein on the enzyme activities of pearl gentian grouper (n = 3).

Parameters	FM	SPC40	FSBM40
Trypsin (U/mg)	597.33 ± 75.53^{a}	1378.25 ± 97.99^{b}	1194.50 ± 90.84^{c}
IgM(μg/mg)	94.33 ± 4.22^{a}	37.77 ± 3.15^{b}	50.84 ± 5.60^{c}
C3 (μg/mg)	85.58 ± 5.31^{a}	39.97 ± 7.49^{b}	51.34 ± 7.73^{c}
C4 (μg/mg)	128.83 ± 10.17^{a}	60.32 ± 5.23^{b}	76.83 ± 8.28^{c}
T-SOD (U/mg)	78.23 ± 9.95^{a}	154.04 ± 13.35^{b}	135.55 ± 14.25^{b}
GSH-Px (U/mg)	167.20 ± 21.34^{a}	322.51 ± 22.74^{b}	286.12 ± 24.83^{b}

Data in the same row with different superscripts indicate significant differences ($P < 0.05$).

TABLE 4 Expression of immune-related genes in the distal intestine of pearl gentian grouper fed different soybean processing products diets (n=3).

Gene	FM	SPC40	FSBM40
IL1β	1.16 ± 0.16^{a}	1.58 ± 0.09^{b}	1.74 ± 0.23^{b}
IL8	1.00 ± 0.08^{a}	1.86 ± 0.16^{b}	1.62 ± 0.04^{b}
IL12	1.01 ± 0.14^{a}	1.86 ± 0.16^{b}	2.25 ± 0.36^{b}
IL17	1.00 ± 0.07^{a}	1.37 ± 0.05^{b}	1.35 ± 0.01^{b}
IL32	1.01 ± 0.14^{a}	1.12 ± 0.21^{a}	1.42 ± 0.16^{b}
TNFα	1.01 ± 0.15^{a}	1.33 ± 0.03^{b}	4.09 ± 0.61^{c}
IL4	1.01 ± 0.15^{a}	0.40 ± 0.02^{b}	0.48 ± 0.06^{b}
IL5	1.00 ± 0.05^{a}	0.55 ± 0.10^{b}	0.40 ± 0.05^{c}
IL10	1.03 ± 0.06^{a}	0.56 ± 0.04^{b}	0.56 ± 0.03^{b}
TGFβ1	1.00 ± 0.08^{a}	0.18 ± 0.05^{b}	0.13 ± 0.01^{b}

Data in the same row with different superscripts indicate significant differences ($P < 0.05$).

3.5 Validation of the transcriptome data by qRT-PCR

To confirm the reliability of the full-length transcriptome sequencing method, the real-time quantitative PCR findings of nine immune-related genes, *IL4*, *IL5*, *IL10*, *TGFβ1*, *P65*, *MyD88*, *IκBα*, *IgA* and *pIgR*, were compared with the results of the transcriptome sequencing method used in this experiment (Figure 5).

4 Discussion

Although the use of plant protein instead of fish meal can effectively reduce the production cost of aquaculture, excessive use is not conducive to the digestion and absorption of certain aquatic species, which is more intuitive in terms of its growth performance (Barros et al., 2002; Yun et al., 2017). Compared to the FM group, the growth performance of both the soybean protein concentrate and fermented soybean meal groups was inhibited, which was reflected by the significantly changed values of WGR, SGR and FCR. Wang et al. (2020) concluded that the optimal percentage of fish meal substitution with soybean protein concentrate for juvenile pearl gentian grouper was 37.32% and that excessive substitution was detrimental to the results of growth. In studies on both *Sparus aurata* juveniles and *Paralichthys olivaceus* juveniles, it was found that their growth capacity decreased continuously with increasing soybean protein concentrate content (Kissil et al., 2000; Deng et al., 2006). However, Zhao et al. (2010) showed that completely replacing fish meal with soybean protein concentrate did not significantly affect the SGR of Nile tilapia (*Oreochromis niloticus*) fry. In addition, replacing fish meal completely with soybean protein concentrate did not affect the growth of *Litopenaeus vannamei* in a 72-day culture (Sá et al., 2013). This may be related to differences in species and feeding habits. Studies on the impacts of fermented soybean meal on aquatic animals are less frequent than those on soybean protein concentrate, and the growth performance of different species is affected by fermented soybean meal, showing different results. *Acanthopagrus schlegeli* ingestion of diets with fermented soybean meal replacing 32% and below of pollack fish meal had no significant effect on its SGR (Azarm and Lee, 2014). He et al. (2020) observed that replacing 30% fish meal with fermented soybean meal had no significant effect on the growth of *Micropterus salmoides* juveniles, however, increasing the replacement amount to 60% resulted in an increase in FCR and a decrease in WGR. In this study, compared to the FM and SPC40 groups, where fish meal was replaced with 40% soybean protein concentrate, WGR and SGR were found to be lower when fish meal was replaced with 40% fermented soybean meal. The study of *Epinephelus coioides* by Shiu et al. (2015) also concluded that growth performance was significantly diminished when fish meal was replaced with 40% fermented soybean meal. Soybean protein concentrate and fermented soybean meal have substantially eliminated the ANFs they contain, but there are still residuals that can cause growth problems in some fish and seem more pronounced in carnivorous fish.

TABLE 5 Different soybean processing products substituted for fishmeal in the distal intestine of the pearl gentian grouper for comparison of significantly different genes (n = 4).

Number	SPC40 vs FM	FSBM40 vs FM
Up	2328	2005
Down	1748	1457
Total	4076	3462

The replacement of fish meal with soybean meal as a protein source ensures dietary protein content and its amino acid composition is appropriate. However, previous reports have often suggested that soybean meal dietary manipulation affects multiple physiological responses in a variety of fish that can affect the digestive tract. Among these, the most widely studied is SBMIE, a non-infectious subacute enteritis (Baeverfjord and Krogdahl, 1996; Urán et al., 2008; Hedrera et al., 2013). ANFs are widely considered one of the causes of this type of enteritis, and although two soybean processing products, soybean protein concentrate and fermented

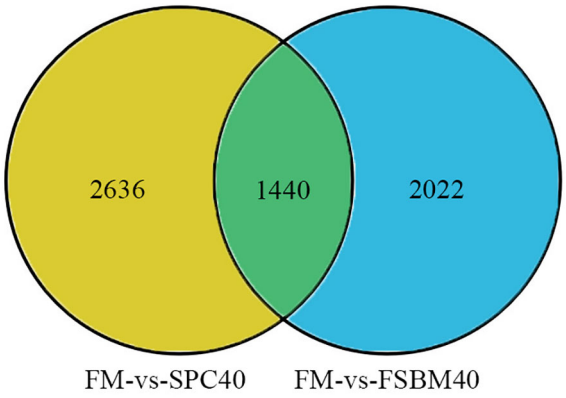

FIGURE 2
Venn map analysis of DEGs in the distal intestine of pearl gentian grouper (n = 4).

soybean meal, have been effective in eliminating most of the ANFs, the physiological status of pearl gentian grouper fed diets containing soybean processing products was distinctive from that of the FM group (Krogdahl et al., 2010). The soybean protein concentrate and fermented soybean meal in the diet resulted in a substantial increase in trypsin activity in the distal intestine of pearl gentian grouper, similar to the findings of Lilleeng et al. (2007) in SBMIE-affected Atlantic salmon (*Salmo salar*). It has also been found in humans and other animals with intestinal inflammation, which may be related to a decrease in the degradation and reabsorption capacity of trypsin due to intestinal damage (Motta et al., 2011). In the study, the changes in the levels of the immune-related components such as C3, C4 and IgM in the distal intestinal tissues indicate that excessive addition of the two soybean processing products mentioned above during culture impairs the immune function of pearl gentian grouper. Adaptive and innate immunity are two acknowledged components of the immune system of teleost fish (Uribe et al., 2011). The complement pathway belongs to innate immunity and is crucial in defending against pathogens. The functions of C3 and C4, important bioactive proteins in this pathway, have been described in various teleost fish, such as *Oreochromis niloticus* (Bai et al., 2022), *Ctenopharyngodon idella* (Meng et al., 2019), *Paralichthys olivaceus* (Wu et al., 2022) and *Oncorhynchus mykiss* (Løvoll et al., 2006). IgM is the main immunoglobulin in teleost fish and the immune response it is involved in belongs to the acquired immunity (Hikima et al., 2011). After adding soybean protein concentrate and fermented soybean meal to the feed, the levels of these three immune-related components in the intestinal tissue of pearl gentian grouper decreased significantly, which may be related to their impaired intestinal immune function. In terms of anti-oxidants, compared with the FM group, the GSH-Px and T-SOD activity in the experimental group that substituted fish meal with soybean processing products showed a significant difference, and the plant material in the diet may cause oxidative stress in the intestinal tissues of pearl gentian grouper. Similarly, Wang et al. (2020) showed that feeding pearl gentian grouper with soybean protein concentrate substitution for fish meal significantly affects its antioxidant enzyme activity. Many reports of protein source substitution in aquatic animal feeds have explored antioxidant capacity, and numerous studies of fish meal substitution with other protein sources fed to aquatic species have shown that excessive substitution is detrimental to the oxidative and antioxidant balance of the aquatic species organism (Uczay et al.,

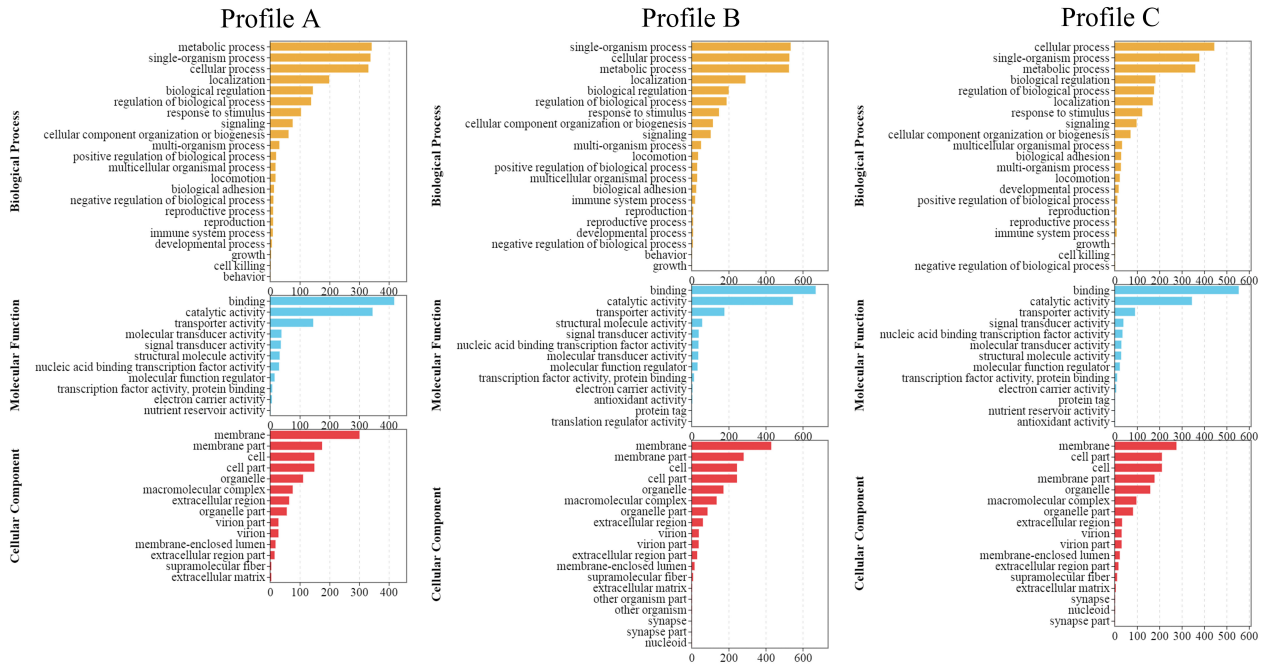

FIGURE 3
Results of GO enrichment analysis of DEGs enrichment under three broad functional categories (n = 4).

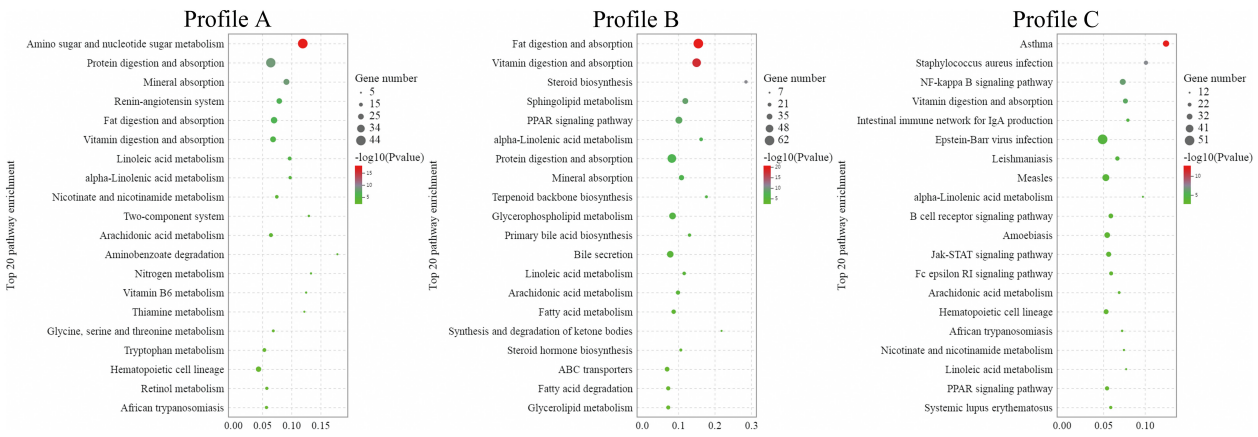

FIGURE 4
The top 20 differentially significant enrichment pathways in KEGG enrichment analysis of DEGs (n = 4).

2019; Pervin et al., 2020; Shen et al., 2020; Wang et al., 2021). Severe oxidative and antioxidant imbalances damage the gut and other tissues of aquatic species, endangering their health and survival.

The analysis of the molecular responses of fish to different diets can provide a reference value for the nutritional needs of aquaculture species (Król et al., 2016). We employed the RNA-seq technique to analyse the impact of the soybean protein concentrate diet and fermented soybean meal diet on pearl gentian grouper. The third-generation sequencing technology, single-molecule real-time (SMRT), developed by Pacific Biosciences, was employed in our research to address the shortcoming of second-generation sequencing, which can only analyse small fragment sequences. There were more DEGs between the SPC40 and FM groups than between the FSBM40 and FM groups in terms of number, and about 35% of the DEGs between the SPC40 and FM groups were also found in the DEGs between the FSBM40 and FM groups. This indicates that there are large differences in the effects of these two soybean meals in the diets

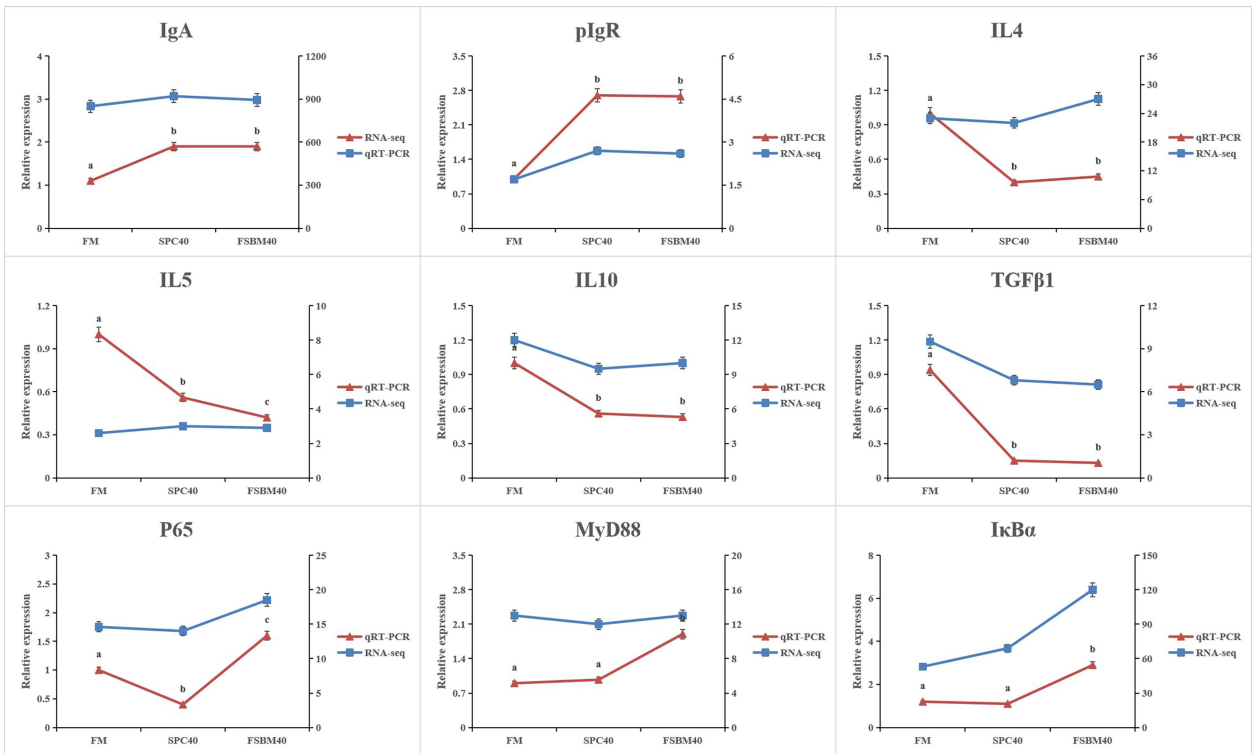

FIGURE 5
Comparison of RNA Seq and qRT-PCR results (n = 4). The mRNA expression level of qRT-PCR was normalized by *β-actin*. The relative expression level in RNA-seq analysis was calculated by FPKM value. Different letters assigned to the lines represented significant differences between the groups at $P < 0.05$.

on fish at the transcriptional level. Functional enrichment analysis allows for the classification of differential genes and the description of gene functions. Three profiles were investigated using GO enrichment analysis and KEGG enrichment analysis, from which the similarities and differences of the impact of soybean protein concentrate or fermented soybean meal substitutes on pearl gentian grouper were inferred. The results of KEGG enrichment in Profile A revealed significant differences in multiple digestion and absorption pathways in fish, including the digestion and absorption of protein, fat and vitamin and mineral absorption, which corroborated the findings that both soybean protein concentrate and fermented soybean meal diets significantly affected fish growth performance. The activity of digestive enzymes in the fish intestine is influenced by the nutrients in the diet. The significant effect of feeding soybean protein concentrate and fermented soybean meal diets on the digestion and absorption of pearl gentian grouper may be due to changes in digestive enzyme activity, and the residual ANFs in soybean protein concentrate and fermented soybean meal may be one of the main reasons for changing the intestinal environment of fish and thus affecting digestive enzyme activity (Li et al., 2014; Liu et al., 2017). The nutrition of the soy processed product diet is not as nutritionally balanced as that of the fish meal diet, and fish growth receives an impact and responds through compensatory behaviour. This includes a series of means to promote biosynthesis, in which the secretion of many other substances related to digestive enzymes, in addition to digestive enzymes, is also improved (Kemski et al., 2020). In this research, the transcriptomic outcomes revealed that the soybean protein concentrate diet significantly impacted the fat digestion and absorption pathway in fish and that the bile secretion pathway was also greatly affected. Among the membrane protein, the second largest family is the solute carrier (SLC) superfamily. The structure of the various proteins in the SLC superfamily varies considerably, but their functions are united, and SLC transporter proteins are crucial in the absorption of nutrients (César-Razquin et al., 2015). In addition, the disruption of intestinal homeostasis in human Crohn's disease patients is associated with *SLC22A4* and *SLC22A5* mutations affecting L-carnitine absorption (Fortin et al., 2009). Another study found that SBMIE decreases the expression level of the *SLC22A5* gene associated with fatty acid metabolism (Sahlmann et al., 2013). The differential genes under the soybean processed product diet in the enrichment analysis results also included various SLC transporter protein genes, such as *SLC22A5*, *SLC26A3*, *SLC23A1*, and *SLC4A4*.

Diets that have an excessively detrimental effect on the intestinal environment can promote intestinal tissue damage and inflammation. More research is needed to determine the cause of SBMIE in carnivorous fish caused by plant protein sources such as soybean. In the present study, according to the outcomes of inflammation-related gene expression levels, the soybean protein concentrate diet and fermented soybean meal diet significantly increased the expression levels of multiple pro-inflammatory genes while reducing the expression levels of multiple anti-inflammatory genes in fish intestinal tissues compared to the FM group. It has been shown that a similar phenomenon occurs in fish with SBMIE (Hedrera et al., 2013; Sahlmann et al., 2013; Wu et al., 2018). In this study, the amount of soybean protein concentrate and fermented soybean meal used to substitute fish meal may have exceeded the tolerance level of pearl gentian grouper, causing intestinal inflammation. Urán et al. (2008) found that the expression levels of inflammatory genes in the distal intestine of carp (*Cyprinus carpio*) were also affected by soybean meal in the diet. In the early stages of feeding the soybean meal diet, the expression of pro-inflammatory cytokines *TNF*α and *IL1*β was significantly up-regulated, but there was no downregulation of anti-inflammatory cytokine *IL10* expression in the intestinal tissues of carp. The other difference is that carp will gradually adapt to the soybean meal diet and normalise their intestinal inflammatory gene expression levels. The digestive system of fish differs depending on their dietary habits, and the protein requirements of carnivorous aquaculture fish are high. There is no effective treatment for intestinal inflammation in carnivorous fish induced by replacing fish meal with plant material to reduce costs. Undoubtedly, the reduction of ANFs in plants is effective, but the use of soybean processing products after a substantial reduction in ANFs did not produce better results in this experiment. Some studies have shown that the soybean meal component of the diet is detrimental to the stabilisation of the intestinal flora of teleost fish, which may be one of the causes of SBMIE (Nayak, 2010; Miao et al., 2018; Liu et al., 2019). In addition, whether adding dietary additives to improve nutrition and intestinal flora can effectively relieve intestinal inflammation needs further study.

To sum up, soybean protein concentrate or fermented soybean meal components in feed can lead to a significant reduction in the growth performance of juvenile pearl gentian grouper. Although the differences in the SR of the fish were not significant, the intestinal health of the SPC40 and FSBM40 groups had been severely challenged. The DEGs obtained from the transcriptomic analysis of the distal intestine in this study can help further explore the mechanisms of food-borne intestinal inflammation caused by soybean components of this species, providing a theoretical basis for solving SBMIE and thus promoting green and sustainable aquaculture.

Data availability statement

The data presented in the study are deposited in the NCBI Sequential Read Archive (SRA) repository, accession number PRJNA664623 and PRJNA66441.

Ethics statement

The animal study was reviewed and approved by the ethics review board of Guangdong Ocean University.

Author contributions

AP designed and took part in the whole process of the experiment, and wrote the draft of this manuscript. BT and ZW co-conceived the experiment, revised the draft critically for important intellectual content. YX and RX participated in the

experiments and WZ revised the first draft. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Authors ZW and RX are employed by Guangdong Evergreen Feed industry 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.

Publisher's note

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