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Partial fishmeal protein replacement with peptides from swine blood modulates the nutritional status, immune response, and intestinal microbiota of hybrid groupers (female *Epinephelus fuscoguttatus*× male *E. lanceolatus*)

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ABSTRACT

This study investigated the effects of replacing fish meal (FM) protein with peptides from swine blood (PSB) on the growth, digestion, and absorption capacity of hybrid groupers (female *Epinephelus fuscoguttatus* × male *Epinephelus lanceolatus*). It also analyzed their inflammatory-related gene expression, and intestinal microbiota. Four isonitrogenous and isolipidic experimental feeds were formulated to replace 0% (FM), 25% (PSB25), 50% (PSB50), and 75% (PSB75) of the FM protein with PSB. Each experimental diet was fed to triplicate groups of 30 hybrid grouper juveniles for 8 weeks. No significant differences in the weight gain and specific growth rates were found among the FM, PSB25, and PSB50 groups. The intestinal digestive enzyme (lipase, amylase, trypsin, and pepsin) and brush border enzyme (Na^{+}/K^{+}-ATPase, creatine kinase, and γ-glutamyl transpeptidase) activities showed no significant differences among the FM, PSB25, and PSB50 groups. Meanwhile, trypsin, pepsin. Creatine kinase, and γ-glutamyl transpeptidase activities in the PSB group were significantly higher than those of the FM group. No significant differences in the expression levels of the toll-like receptor 21 (TLR21), myeloid differentiation factor 88 (MyD88), and inflammatory cytokines (TNF-α, IL-2, IL-8, and IL-10) were observed among the four groups. The intestinal microbiota results showed no significant differences in alpha diversity among the four groups. At the genus level, at the genus level, *Lactobacillus* relative abundance was significantly reduced in the PSB10 group compared to that in the FM group. However, no significant differences were found among the FM, PSB50, and PSB75 groups. However, no significant differences were found among the FM, PSB50, and PSB75 groups. These results demonstrated that up to 50% of the FM protein in the feed can be replaced by PSB without adversely affecting the nutritional status and inflammatory response in hybrid groupers. Replacing FM with PSB significantly influences the microbial profile but not the diversity

1. Introduction

Global fishmeal (FM) supplies are insufficient to meet the increasing feed demands of aquaculture, and using FM in the diet must be decreased (Król et al., 2016). Therefore, it is important to find suitable alternative protein sources for FM replacements in the diet to ensure the sustainable development of aquaculture. These alternative protein sources contain plant and animal proteins. Plant protein sources used in aquatic feed are generally limited due to anti-nutritional factors and amino acids composition imbalances (Gorissen et al., 2018; Luo et al., 2011; Zhang et al., 2019). Therefore, underutilized animal protein sources may better reduce the use of FM in aquatic diets.

Feed-derived bioactive peptides are defined as protein fragments with specific sequences that have nutritional value and a positive impact

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on bodily functions. They, ultimately, influence health (Kitts and Weiler, 2003). Bioactive peptides may bring different beneficial effects, such as immunomodulating, antimicrobial, and antioxidant activities. They may also modulate nutrients digestion and absorption and enhance mineral absorption and bioavailability (Kitts and Weiler, 2003; Madureira et al., 2010; Tai et al., 2020). In mice, fish protein hydrolysates containing active biopeptides could enhance immunocompetence without inducing any inflammatory immune reaction in the intestine. Further, the phagocytic activity of peritoneal macrophages was enhanced, and interleukin 4, interleukin 6, and interleukin 10 were significantly increased in the lamina propria of the small intestine (Duarte et al., 2006). Bioactive colostral peptides have also been evidenced to differentially modulate the innate immune response of murine intestinal epithelial cells (Jørgensen et al., 2010). Single fermented poultry by-product meal (FPBM), tuna hydrolysate (TH), or a mixture of PBM and black soldier fly (*Hermetia illucens*) larvae enhanced intestine digestive enzyme activities and improved intestinal health as fishmeal replacements in the different fish species feed (Chaklader et al., 2019; Dawood et al., 2020; Siddik et al., 2018, 2019, 2020).

Peptides from swine blood (PSB) are a by-product produced by separating peptides from hygienically collected and screened healthy swine blood (Yu et al., 2005). There is little biological degradation of amino acids in high protein PSB (89% dry matter), given the low-temperature processing method (spray-drying) (Wang et al., 2014; Yang, 2004). PSB scavenging free radicals in the body indicate that it has a strong antioxidant capacity (Fang et al., 2006). Adding 5.0 g/kg PSB to the diet could improve the antioxidant capacity and immune function in Jian carp (*Cyprinus carpio* var. Jian) (Wang et al., 2014). In vitro, PSB could also promote the non-specific immunity of grass carp (*Ctenopharyngodon idella*) via a dose-dependent manner (Jia et al., 2014). Based on the advantages shown above, it can be speculated that PSB is a promising alternative protein source for FM protein in fish feed.

The intestine is an important functional organ directly interacts with food. It also absorbs nutrients and energy, while still acting as a barrier to prevent bacteria and endotoxin in the cavity from spreading to organs and tissues (Magnotti and Deitch, 2005). The fish intestine's microbiota contributes to regulating nutrient digestion, immune responses, intestinal differentiation, disease resistance, and pathogen colonization (Nayak, 2010). The dietary composition can cause biological changes in the host by shaping the intestinal microbial community of fish and altering the metabolism and population size of key symbiotic species (Ringø et al., 2006). In some aquaculture species, animal-based diets induced changes in intestinal microbiota composition. Then, they suppressed intestinal health concerning feed digestibility and intestinal immunity (Gajardo et al., 2017). The study demonstrated that animal by-product meals, as replacements to FM, gave good results regarding growth performances. Further, they did not induce significant changes in the gut microbial richness of rainbow trout aquafeed (Rimoldi et al., 2018). An increased number of *Lactobacillus and Streptococcus* and decreased number of *Aeromonas* at genus in the distal intestine of marron fed FPBM (Siddik et al., 2019). Enzymatic hydrolysates from poultry by-products (EHPB) composed of a mixture of free amino acids and di-, tri-, and oligo-peptides as a protein source. This influenced the microbial community structure and significantly decreased the abundance of Vibrio in turbot (*Scophthalmus maximus*) (Hao et al., 2020). In this study, the effects of PSB diets on fish gut microbiota composition were probed.

The hybrid grouper (female *Epinephelus fuscoguttatus* × male *Epinephelus lanceolatus*) is a carnivorous fish species used in aquaculture. It is farmed primarily in China, given its growth of market demand in recent years. Relevant research continues to be conducted on the replacement of FM for hybrid groupers with different animal proteins (Jin, 2018; Yao et al., 2018). This study aims to examine the effects of FM protein replacement with PSB on growth, intestinal digestion and absorption capacity, inflammation-related gene expression, and microbial flora in hybrid groupers. The development of novel animal proteins can offer tremendous benefits for the sustainable development of aquaculture.

2. Materials and methods

2.1. Ethical statement

We used hybrid grouper juveniles after approval by the Animal Research and Ethics Committee of Guangdong Ocean University, China. All experiments were conducted according to its guidelines.

2.2. Experimental diets

PSB was obtained from Beijing HongshunYangyuan Biotech Co., Ltd. (Beijing, China). Its processing mainly involved anti-freezing by adding anticoagulant, storing below 10 °C, filtering, centrifugation, and spray-drying. The molecular weight distribution (%) of PSB is shown in Fig. 1. Four isonitrogenous (51% crude protein) and isolipidic (11% crude lipid) test feeds were formulated to replace 0% (FM), 25% (PSB25), 50% (PSB50) and 75% (PSB75) of the fish meal (FM) protein with peptides from swine blood (PSB) (Table 1). These isonitrogenous were based on the chemical compositions and amino acid profiles of the protein sources (Table 2). Methionine was supplemented to meet the amino acid requirements of groupers (Chi et al., 2015). All ingredients were carefully weighed according to the formulation and mixed in a Hobart-type mixer (JS-14S, Zhejiang Zhengtai Electric Co., Ltd., China). Fish oil and soybean lecithin were supplemented and thoroughly mixed. Following this, purified water was added to produce a suitable dough. Strip feeds of 2.5-mm diameters were formed using a double-helix extrusion mechanism. Finally, the feeds were air-dried and stored at −20 °C until feeding.

2.3. Feeding trial

Hybrid grouper juveniles (n = 540) were obtained from a native species fish farm in Zhanjiang, China. All fish were acclimated to a commercial diet for 1 week. Healthy, uniformly sized fish (mean ± standard deviation, SD, initial weight = 12.39 ± 0.33 g) were randomly distributed into four groups in triplicate. Hence, there was a total of 12 groups of 30 fish, each kept in a 500-L fiberglass tank. All fish were fed twice daily (08:00 and 17:00) until apparent satiation for 8 weeks. The water temperature was kept at 28–30 °C with dissolved oxygen (DO) > 5 mg/L and NH_4^+-N < 0.03 mg/L.

2.4. Digestibility trial

The digestibility trial was conducted during the feeding trial period. Yttrium trioxide (0.1%) (99.9%, Sinopharm Chemical Reagent Co., Ltd., Shang, China) was used as the external indicator in the four

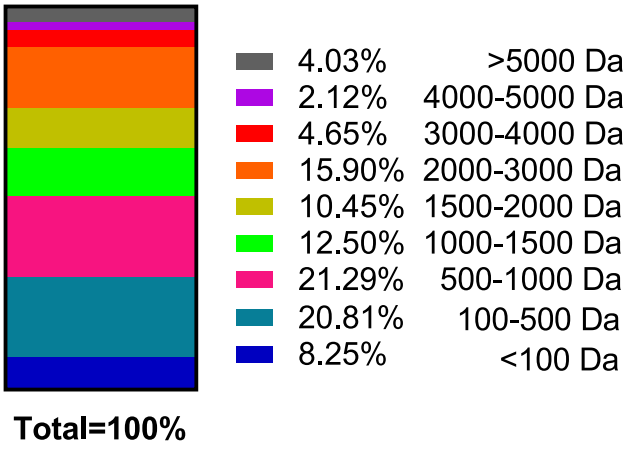

Fig. 1. Molecular weight distribution (%) of peptides from swine blood.

Table 1
Composition and nutrient level of the diets (% Dry matter).

Ingredients	Experimental diets			
	FM	PSB25	PSB50	PSB75
Brown fishmeal	55.00	41.25	27.50	13.75
Peptides from swine blood[a]	0.00	10.33	20.65	30.98
Casein	5.00	5.00	5.00	5.00
Wheat gluten meal	9.44	9.44	9.44	9.44
Wheat flour	16.00	16.00	16.00	16.00
Corn starch	5.27	7.59	9.52	9.90
Fish oil	3.62	4.95	6.25	7.50
Soybean lecithin	2.00	2.00	2.00	2.00
Methionine	0.12	0.31	0.51	0.71
Vitamin premix[b]	0.20	0.20	0.20	0.20
Mineral premix[b]	0.50	0.50	0.50	0.50
Others[c]	2.43	2.43	2.43	2.43
Microcrystalline cellulose	0.42	0.00	0.00	1.59
Total	100.00	100.00	100.00	100.00
Nutrient level (%)				
Crude protein	50.49	51.19	51.67	51.25
Crude lipid	11.79	11.01	11.72	11.03

[a] Obtained from Beijing Hongshun Yangyuan Biotech Co., Ltd. (Beijing, China).
[b] Obtained from Qingdao Master Biotech Co., Ltd. (Qingdao, China).
[c] Others: calcium monophosphate, 1.50%; attractant, 0.50%; choline chloride, 0.30%; yttrium trioxide, 0.10%; antioxidants, 0.03%.

Table 2
Proximate and essential amino acid compositions of BFM and PSB.

	BFM	PSB
Proximate analysis (% in dry matter)		
Crude protein	67.00	89.20
Crude lipid	10.38	11.60
Moisture	8.20	5.45
Essential amino acid (% in protein)		
Methionine	1.99	0.57
Lysine	5.07	8.80
Valine	3.18	7.47
Isoleucine	2.77	0.17
Phenylalanine	2.60	6.11
Leucine	4.93	12.15
Threonine	2.92	3.10
Histidine	1.35	7.20
Arginine	4.30	3.20

BFM: brown fishmeal; PSB: peptides from swine blood; Tryptophan was not analyzed.

experimental diets. After two weeks of acclimation, feces from each replicate were collected by siphoning 6–7 h after feeding. Briefly, once the feces were observed, they were immediately collected by gently siphoning, dried for 6 h at 65 °C, and stored at −20 °C until analysis. The fecal collection continued for ten weeks until 6 g dry weight of fecal material had been sufficiently collected for chemical analysis.

2.5. Sample collection

After eight weeks, all fish fasted for 24 h. Then, the fish from each tank were counted and weighed to calculate growth indexes. Three fish from each tank were randomly selected and stored at −20 °C for body composition analysis. The proximal intestines of two fish and the whole intestines of another two fish per tank were removed on ice, homogenized, and centrifuged at 3000 *g* and 4 °C for 10 min. The supernatant of each sample was collected and stored at −80 °C for enzyme activity analysis. The whole intestines of another two fish per tank were collected and immediately frozen in liquid nitrogen before being stored at −80 °C for relative gene expression analysis. The whole intestines from another three fish per tank were taken for microbiota analysis.

2.6. Proximate composition analysis

The body composition of hybrid groupers and the proximate nutrient level of the diets were measured according to standard methods (AOAC, 1995). Moisture was determined by drying the samples at 105 °C until a constant weight was obtained. Crude protein (N × 6.25) and crude lipids were determined via the Kjeldahl method, using an Auto Kjeldahl System (2300-Auto-analyzer, Foss, Sweden), and by Soxhlet extraction, using petroleum ether as a solvent, respectively. Crude ash was measured by calcination at 550 °C in a muffle furnace.

2.7. Enzyme activity analysis

The amylase, lipase, and trypsin activities from the proximal intestine were determined using commercially available kits. So were the Na^+/K^+-ATPase, creatine kinase, and γ-glutamyl transpeptidase activities from the whole intestine (Shanghai Enzyme-link Biotech Co., Ltd., Shanghai, China) following the manufacturer's instructions.

2.8. Gene expression analysis

The total RNA from the intestinal tract was prepared using Trizol Reagent (Transgen Biotech, Beijing, China). The integrity of total RNA samples was tested by electrophoresing on a 1.2% denaturing agarose gel. Meanwhile, the purity and concentration were determined by a spectrophotometer (NanoDrop ® ND-2000). Subsequently, total RNA was used to synthesize complementary DNA (cDNA) using the Prime Script™ RT reagent kit with a genomic DNA Eraser (Takara, China). All primer sequences are presented in Table 3. Real-time PCR assays were conducted on a CFX96 real-time PCR Detection System (Bio-Rad, Hercules, CA), with a 5 μL SYBR Green Master Mix (Takara, China). It was conducted according to the manufacturers' instructions. At the end of each reaction, a melting curve analysis was conducted to determine the specificity of production. Also, the amplification efficiency was analyzed according to the equation: E = 10(−1/slope) – 1. The expression levels of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.9. Analysis of intestinal microbiota

The DNA from the intestine tract was extracted using DNA isolation

Table 3
Primers pair sequences for real-time qPCR.

Genes	Nucleotide sequence (5′-3′)	Genbank accession no.
TLR21	F: GTCGTCCTATCACCGCATGA R: CGCCCTACGTAGCTGATTCC	JF738115.1
TLR22	F: CGAGCCAGGTAAACCCATCA R: CTCATCAAACAGGCGGAAGC	JQ965995.1
MyD88	F: AGCTGGAGCAGACGGAGTG R: GAGGCTGAGAGCAAACTTGGTC	JF271883.1
IL-2	F: GCCGACCTGGTTGTAATCCTCA R: ATCTCAAAGCCTGTCTCATTGGT	HM185491.1
IL-6	F: AGAGGCAAATTAATAAAAGGGAG R: CGGTTTCAAACGAGGAGCAGATC	JN806222.1
IL-8	F: GGCCGTCAGTGAAGGGAGTC R: TCAGAGTGGCAATGATCTCA	GU988706.1
IL-10	F: ACACAGCGCTGCTAGACGAG R: GGGCAGCACCGTGTTCAGAT	KJ741852.1
TNF-α	F: GTGGCCTACACGACTGCACC R: TACAAAGGGCCACAGTGAGA	FJ491411.1
TGF-β1	F: CGATGTCACTGACGCCCTGC R: AGCCGCGGTCATCACTTATC	GQ205390.1
β-actin	F: GATCTGGCATCACACCTTCT R: CATCTTCTCCCTGTTGGCTT	AY510710.2

TLR21: toll-like receptor 21; TLR22: toll-like receptor 22; MyD88: myeloid differentiation factor 88; IL-2: interleukin 2; IL-6: interleukin 6; IL-8: interleukin 8; IL-10: interleukin 10; 1 α; TGF-β1: transforming growth factor β1.

kits (Qiagen, Germany). PCR was performed to generate small fragments of the bacterial 16S-rRNA genes V3–V4 region using the common primers 341F/806R (F: CCTACGGRRBGCASCAGKVRVGAAT; R: GGACTACNVGGGTWTCTAATCC). Purified PCR amplicons were quantified using the PicoGreen dsDNA assay kit (Invitrogen, USA). The paired-end sequences (2 × 300 bp) were sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA, USA). The raw reads were deposited into the NCBI SRA database (SUB7760027).

The data were filtered, and chimeric sequences were removed. The resulting sequences were clustered via operational taxonomic units (OTUs) with a threshold of 97% sequence similarity. The silva database (https://www.arb-silva.de/) was used as the 16S-rRNA reference database. Venn analysis identified the unique and common OTUs using the R software (version 3.3.1). The representative sequences were analyzed by the RDP classifier (Wang et al., 2007).

The α-diversity indexes based on the OTUs were examined to obtain information on the community richness and diversity of each sample. A statistical analysis of the community structure based on taxonomic information was performed at each classification level. The significant differences in the community structure between the two groups were analyzed using the unweighted UniFrac method (Hamady et al., 2010).

2.10. Statistical analysis

Data were analyzed via a one-way analysis of variance (ANOVA) followed by Tukey's test with SPSS 22.0 software. *P*-values <0.05 were considered statistically significant. Comparisons of the intestinal microbiota of the two groups were calculated by Welch's *t*-test. All data are shown as means ± standard deviation (SD).

3. Results

3.1. Growth performance

The survival rate (SR) was not influenced by the dietary treatment level ($P > 0.05$, Table 4). The final body weight (FBW), weight gain rate (WGR), and specific growth rate (SGR) gradually decreased with increasing replacement levels. However, no significant differences in FBW, WGR, or SGR were found among the FM, PSB25, and PSB50 groups ($P > 0.05$). The feed conversion ratio (FCR) in the PSB75 group was significantly higher than that of the other groups ($P < 0.05$).

Table 4
Growth performance of the hybrid groupers fed the different diets.

Indexes	Experimental diets				*P*-value
	FM	PSB25	PSB50	PSB75	
FBW (g)	70.13 ± 2.90^{b}	65.46 ± 0.03^{b}	62.65 ± 6.74^{b}	46.94 ± 3.06^{a}	0.002
WGR[a] (%)	465.51 ± 23.39^{b}	427.89 ± 0.24^{b}	405.25 ± 54.36^{b}	278.55 ± 24.67^{a}	0.001
SGR[b] (%/d)	3.06 ± 0.06^{b}	2.97 ± 0.00^{b}	2.99 ± 0.10^{b}	2.44 ± 0.06^{a}	0.011
FCR[c]	0.91 ± 0.01^{b}	0.93 ± 0.01^{b}	0.89 ± 0.01^{b}	1.06 ± 0.04^{a}	0.000
SR[d] (%)	98.89 ± 1.92	98.89 ± 1.92	100 ± 0.00	100 ± 0.00	0.596

FBW: final body weight; WGR: weight gain rate; SGR: specific growth rate; FCR: feed conversion ratio; SR: survival rate. Values are means ± SD of three replicates. Different superscript letters in each row show significant differences among treatments by Tukey's test ($P < 0.05$). The same as below.
[a] WGR (%) =100 × (final body weight – initial body weight)/initial body weight.
[b] SGR (%/d) =100 × (ln (final body weight) – ln (initial body weight))/d.
[c] FCR = total diet intake/total wet weight gain.
[d] SR (%) = 100 × the final fish number/the initial fish number.

3.2. Apparent digestibility

The apparent digestibility coefficient (ADC) of dry matter in the PSB25 group was significantly lower than that of the other groups ($P < 0.05$, Table 5). Crude protein ADC increased with increasing replacement levels ($P < 0.05$). The FM group had the highest body crude lipid ADC, which was significantly higher than that of the other groups ($P < 0.05$).

3.3. Body composition

No significant differences in moisture were found among the treatments ($P > 0.05$, Table 6). Crude protein in the PSB75 group was significantly lower than that in the other groups ($P < 0.05$). Fish fed the PSB50 and PSB75 diets had significantly higher crude lipid levels than the fish fed the FM and PSB25 diets ($P < 0.05$). In contrast, the ash of the treatments followed the opposite trend to that of crude lipid.

3.4. Activities of intestinal digestive and brush border enzymes

The activities of the intestinal digestive enzymes (lipase, amylase, trypsin, and pepsin) and brush border enzymes (Na^+/K^+-ATPase, CK, and γ-GT) gradually increased with increasing replacement levels (Table 7). However, no significant differences in lipase, pepsin, and Na^+/K^+-ATPase activities were found among the four groups ($P > 0.05$). The activities of amylase, trypsin, CK, and γ-GT in the PSB75 group were significantly higher than those in the FM group ($P < 0.05$).

3.5. Immune-related gene expression

The expression levels of the TLR21, TLR22, MyD88, TNF-α, IL-2, IL-6, IL-8, and TGF-β1 genes were down-regulated in the PSB-fed fish relative to the FM-fed fish. Meanwhile, no significant changes in TLR21, MyD88, TNF-α, IL-2, IL-8, and IL-10 expression were found among the treatments ($P > 0.05$, Fig. 2). The expression level of the TLR22 gene in the FM group was significantly higher than that in the other groups ($P < 0.05$). The expression levels of the IL-6 gene in the FM and PSB25 groups were significantly higher than those in the PSB50 and PSB75 groups ($P < 0.05$). The expression level of the TGF-β1 gene in the FM group was significantly higher than that in the PSB50 group ($P < 0.05$).

3.6. Analysis of intestinal microbiota

An average of 453 OTUs and 53,311 effective sequencing lengths were obtained in each group (Fig. 3A). A total of 32, 8, 26, and 28 OTUs were uniquely identified in the FM, PSB25, PSB50, and PSB75 groups, respectively (Fig. 3B). A total of 191 OTUs were commonly identified among the four groups. No significant differences in the four parameters of ACE, Chao 1, Shannon, and Simpson were found among treatment groups ($P > 0.05$, Table 8).

At the phylum level, Firmicutes, Proteobacteria, and Bacteroidetes

Table 5
Apparent digestibility coefficient (ADC)[a] of the hybrid groupers fed the different diets.

Items	Experimental diets				*P*-value
	FM	PSB25	PSB50	PSB75	
Dry matter	0.80 ± 0.01^{b}	0.78 ± 0.00^{a}	0.80 ± 0.01^{b}	0.80 ± 0.02^{b}	0.003
Crude protein	0.94 ± 0.00^{a}	0.95 ± 0.00^{b}	0.96 ± 0.00^{c}	0.97 ± 0.00^{c}	<0.001
Crude lipid	0.94 ± 0.01^{c}	0.82 ± 0.02^{a}	0.84 ± 0.01^{ab}	0.85 ± 0.02^{b}	<0.001

[a] ADC of dry matter (%) = 100 × [1– (dietary Y_2O_3 level/feces Y_2O_3 level)].
[a] ADC of nutrients (%) = 100 × [1– (dietary Y_2O_3 level/feces Y_2O_3 level) × (feces nutrient level/dietary nutrient level)].

Table 6
Proximate composition of the bodies of hybrid groupers fed the different diets.

Items	Experimental diets				*P*-value
	FM	PSB25	PSB50	PSB75	
Protein	58.49 ± 0.64^{b}	60.00 ± 0.39^{b}	58.47 ± 0.43^{b}	55.67 ± 0.39^{a}	0.001
Lipid	24.97 ± 025^{b}	23.11 ± 0.27^{a}	26.26 ± 0.37^{c}	27.21 ± 0.18^{c}	0.001
Ash	15.03 ± 0.14^{c}	15.52 ± 0.30^{c}	14.10 ± 0.11^{b}	13.30 ± 0.08^{a}	0.001
Moisture	71.27 ± 0.24	71.12 ± 0.62	71.75 ± 0.04	71.36 ± 0.36	0.409

had higher relative abundances in each group (Fig. 4A). At the genus level, the top-four relative abundances per group are described below (Fig. 4B): *Halomonas* (14.53%), *Clostridium_sensu_stricto_1* (7.96%), *Bacteroides* (7.61%), and *Streptococcus* (7.25%) in the FM group. *Bacteroides* (55.08%), *Vibrio* (5.88%), *Clostridium_sensu_stricto_1* (4.47%), and f_Peptostreptococcaceae_Unclassified (2.98%) in the PSB25 group. *Halomonas* (8.69%), *Trichococcus* (6.78%), *Clostridium_sensu_stricto_1* (6.30%), and f_Peptostreptococcaceae_Unclassified (6.21%) in the PSB50 group. *Finally*, *Pseudomonas* (11.66%), *Bacteroides* (9.01%), *Arcobacter* (5.93%), and *Streptococcus* (5.43%) in the PSB75 group.

The relative abundances of the five bacterial genera between pairs of groups are shown in Fig. 5. PSB-containing groups had a lower relative abundance of *Lactobacillus* than the FM group. There were no significant

Table 7
The intestinal digestive and brush border enzyme activities (U/gprot) of the hybrid grouper fed the different diets.

Items	Experimental diets				*P*-value
	FM	PSB25	PSB50	PSB75	
Proximal intestine					
Lipase	3.87 ± 0.45	4.22 ± 0.91	4.76 ± 0.97	6.06 ± 0.68	0.068
Amylase	1.34 ± 0.20^{a}	1.50 ± 0.08^{a}	2.40 ± 0.56^{b}	2.77 ± 0.27^{b}	0.002
Trypsin	17,075.83 ± 275.81^{a}	21,562.49 ± 552.55^{ab}	21,587.49 ± 3155.68^{ab}	22,679.65 ± 841.42^{b}	0.049
Pepsin	0.95 ± 0.06	1.05 ± 0.10	1.11 ± 0.25	1.14 ± 0.25	0.612
Whole intestine					
Na^{+}/K^{+}-ATPase	115.24 ± 19.44	149.12 ± 11.31	151.22 ± 25.36	151.79 ± 24.04	0.210
CK	0.67 ± 6.14^{a}	1.00 ± 0.15^{ab}	1.02 ± 0.07^{ab}	1.17 ± 0.29^{b}	0.042
γ-GT	0.38 ± 0.03^{a}	0.46 ± 0.10^{ab}	0.59 ± 0.07^{ab}	0.62 ± 0.09^{b}	0.014

CK, creatine kinase; γ-GT, γ-glutamyl transpeptidase.

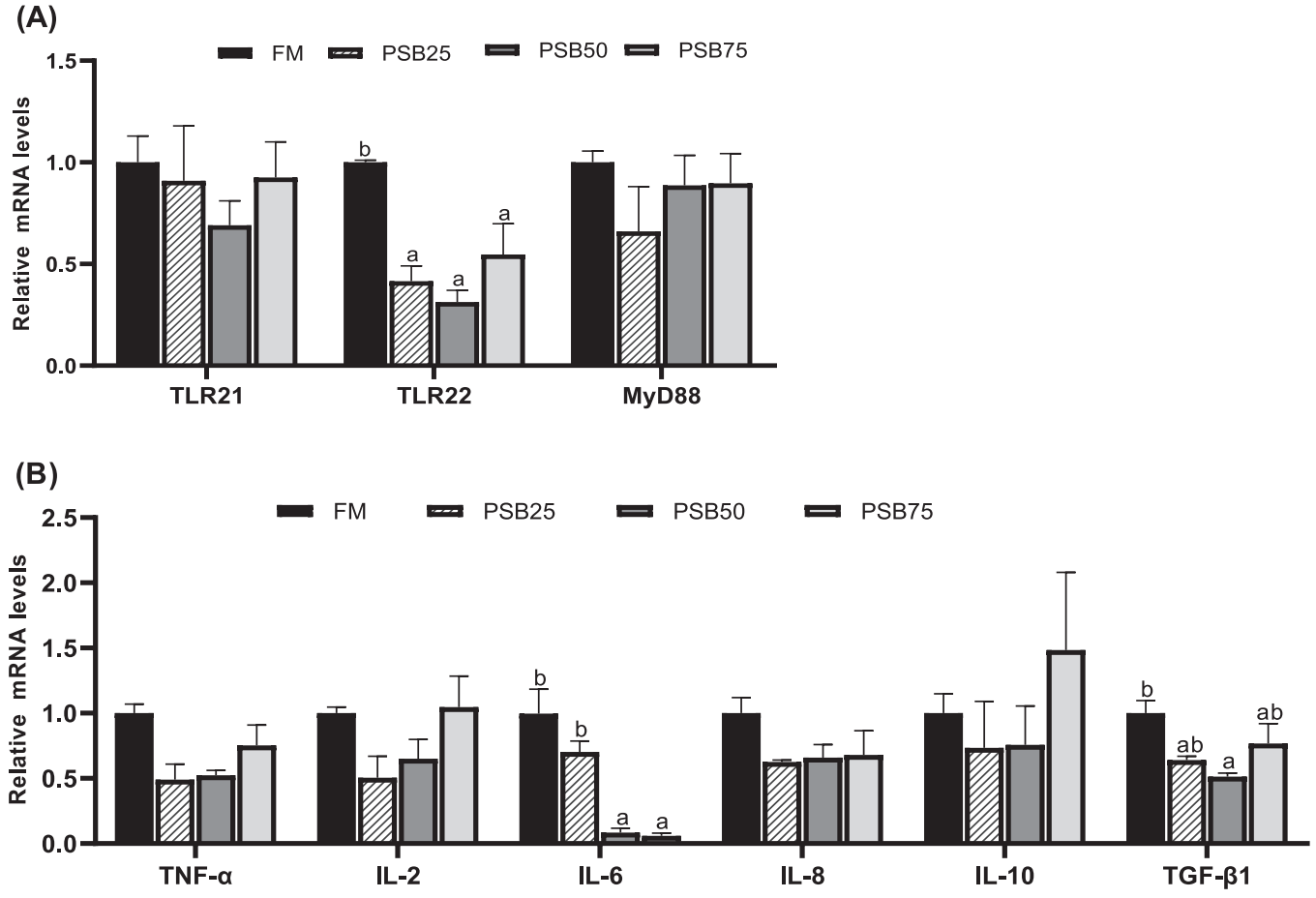

Fig. 2. Relative mRNA expression levels of the TLRs signaling (A)- and inflammatory (B) related genes in hybrid groupers fed the different diets. Values are means ± SD of the three replicates. Bars of the same gene bearing different letters show significant differences among treatments by Tukey's test ($P < 0.05$).

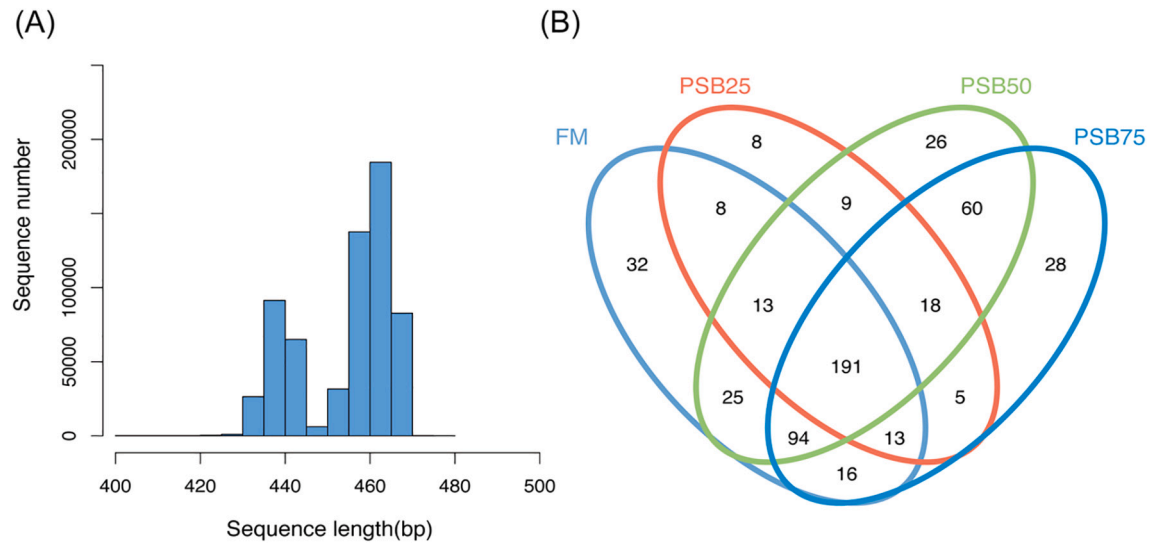

Fig. 3. Effective sequence length distribution (A) and Venn diagrams (B) demonstrating the distribution of the operational taxonomic units (OTUs) in the four groups of fish fed different diets.

Table 8
Community richness and diversity analysis of the intestinal tract of the hybrid grouper fed the different experimental diets based on alpha diversity indexes.

Items	Experimental diets				*P*-value
	FM	PSB25	PSB50	PSB75	
ACE	264.26 ± 26.27	194.78 ± 22.01	273.51 ± 63.72	288.38 ± 31.60	0.078
Chao1	266.40 ± 27.89	192.87 ± 25.82	274.27 ± 64.60	288.10 ± 30.42	0.077
Shannon	5.60 ± 0.99	3.20 ± 2.11	5.61 ± 0.26	5.81 ± 0.39	0.081
Simpson	0.95 ± 0.05	0.62 ± 0.30	0.95 ± 0.01	0.95 ± 0.01	0.068

differences in the relative abundances of *Halomonas*, *Exiguobacterium*, or *Parabacteroides* between the FM and PSB25 groups ($P > 0.05$). However, the relative abundance of *Nesterenkonia* was significantly higher in the PSB25 group than in the FM group ($P < 0.05$). No significant differences in the relative abundances of *Acinetobacter*, *Idiomarina*, or *Mesonia* were observed between the FM and PSB50 groups ($P > 0.05$). However, the relative abundance of *Bacteroides* was significantly higher in the PSB50 group than in the FM group ($P < 0.05$). There were no significant differences in the relative abundances of *Halomonas*, *Pseudomonas*, *Arcobacter*, or *Weissella* between the FM and PSB75 groups ($P > 0.05$).

4. Discussion

This study found that up to 50% of the FM protein can be replaced by PSB without adversely affecting growth performance and digestion and absorption capacity. The growth performance of fish was related to their digestion and absorption capacity, which could be reflected by the activities of digestive enzymes and brush border enzymes (Mitra et al., 2008; Zhou et al., 2020). Peptides with molecular weight less than 1000 Da account for more than 50% of PSB. That was the main reason for groupers obtaining better growth. Absorption between low-molecular-

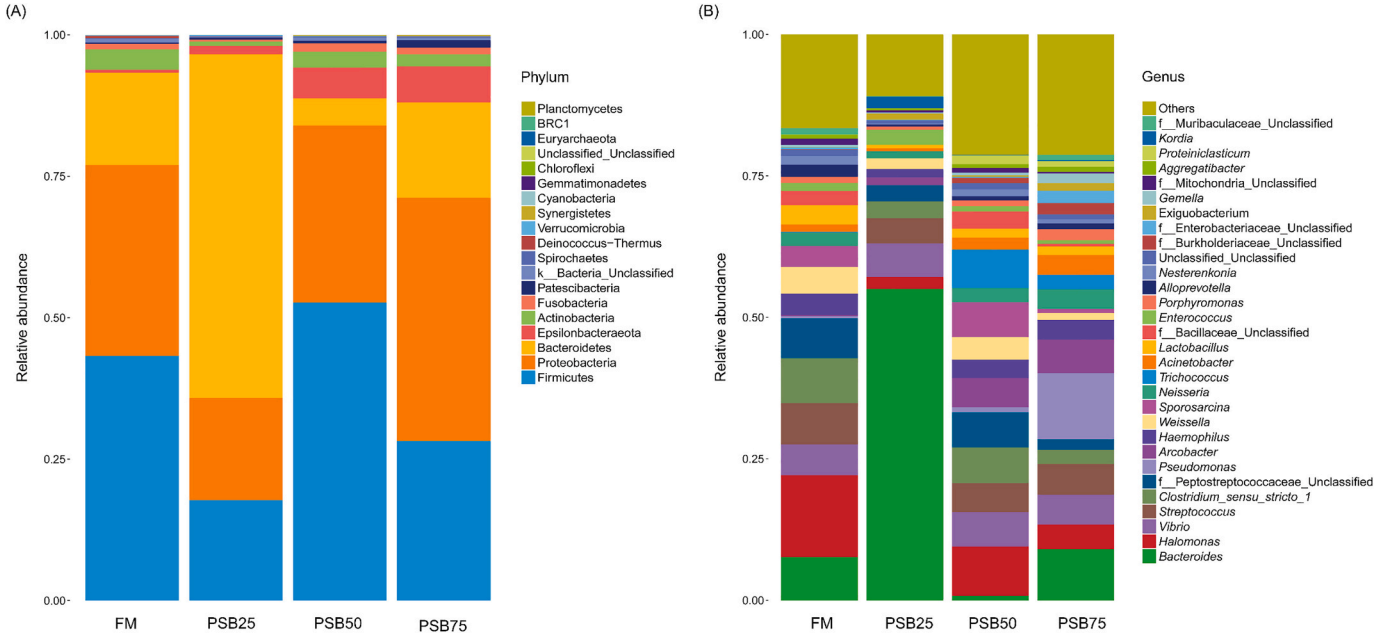

Fig. 4. Analysis of relative abundance of species at phylum (A) and genus (B) levels in the four groups of fish fed different diets.

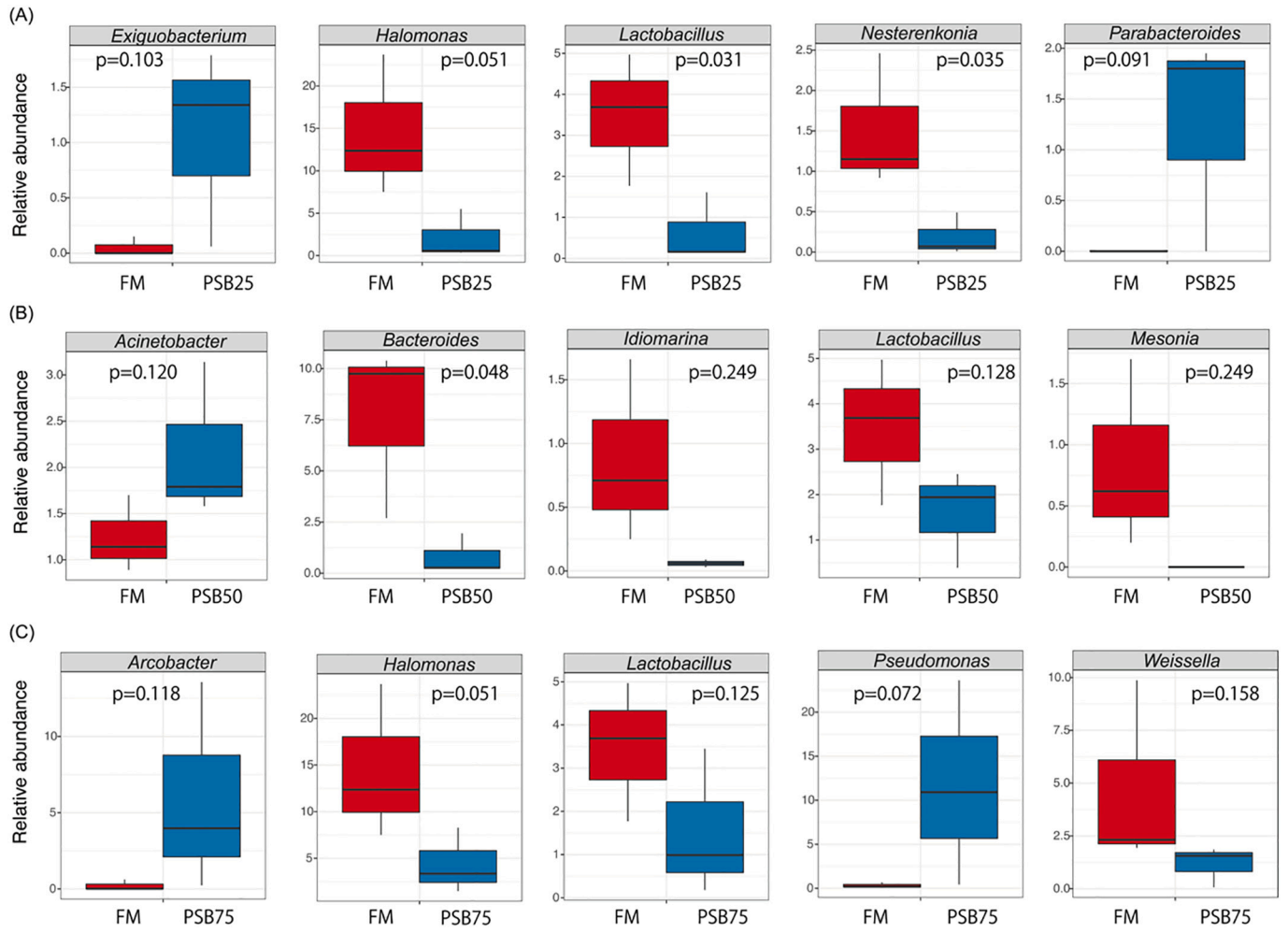

Fig. 5. Comparisons of microbe abundance in group FM with that in groups (A) PSB25, (B) PSB50, and (C) PSB75. Boxplots are presented for each of the five microbe genera with the greatest differences in abundance between the groups ($P < 0.05$ in all cases).

weight peptides and intact proteins is asynchronous. Intact proteins must be broken down into small molecules by proteases in the digestive tract to be absorbed and utilized by fish. Meanwhile, most of the low-molecular-weight peptides can be directly absorbed and utilized, increasing the protein absorption rate and promoting fish growth (Hao et al., 2020). Further, small peptides improve the digestive enzyme activity and antioxidant capacity of fish, which has been evidenced in a series of studies (Dong et al., 2015; Dossou et al., 2018; Singh et al., 2014). In this study, fish fed the PSB75 diet demonstrated significantly enhanced intestinal trypsin, pepsin, CK, and γ-GT activities compared with the FM group. However, reductions in growth and body protein contents were found in the PSB75 group. This is similar to Hao et al.'s (2020) previous report, suggesting that excessive EHPB impeded turbot growth. The malnutrition due to excessive digestion and absorption of nutrients may lead to metabolic burden and reduce the anabolism of proteins and increase blood glucose contents in hybrid groupers when 75% of the FM protein is replaced by PSB. Delcroix et al. (2015) reported that the nature of the marine protein hydrolysate is essential for the development and health of Sea bass (*Dicentrarchus labrax*) larvae. Still, a high proportion of small peptides is not a sufficient criterion to assess dietary value.

The immune status of fish was closely related to inflammation that was initiated and regulated by inflammatory cytokines (Sun et al., 2018). TLRs are critical for detecting pathogens by recruiting the interleukin-1 receptor (IL-1R)-associated kinase via MyD88 and subsequently inducing nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPK) (Akira and Hoshino, 2003). TLR22 can also activate the MyD88-dependent pathway to enhance the production of pro-inflammatory cytokines (Zhang et al., 2014). In the present study, no significant changes in the TLR21, MyD88, and inflammatory cytokine-related genes expression in the grouper intestine were found among the four groups. This suggests that FM protein in the feed could be replaced by PSB without adverse effects on the inflammation response at the transcription level. It was related to the fact that PSB was mainly rich in di-, tri-, and oligo-peptides. Dietary supplementation with the krill hydrolysate composed of free amino acids, di-, tri-, and oligo-peptides could improve the innate immunity of olive flounder (*Paralichthys olivaceus*) (Khosravi et al., 2015). For another animal protein, Marron fed FPBM enriched in antimicrobial peptides significantly increased the microvilli number and gene expression of IL-8, IL-10, and interleukin 17F (IL-17F) genes in the distal intestine (Siddik et al., 2020). As the important constituent of the mucosal immune system, the gut-associated lymphoid tissue (GALT) constructs a local immune environment that is both defensive and tolerant. The gastrointestinal tract is the main portal of pathogen entry in teleost fish (Wu et al., 2016). Interestingly, previous studies reported that fish mast cell granules contain histamine. Although this was true only for species belonging to the largest and most evolutionarily advanced order of teleosts, the Perciformes. This group includes Nile tilapia (*Oreochromis niloticus*), sea bass, and seabream (*Sparus aurata*) (Galindo-Villegas et al., 2016; Mulero et al., 2007). The inflammatory response in the gut was clearly demonstrated to be regulated by the direct action of histamine on

professional phagocytes (Galindo-Villegas et al., 2016). Therefore, the intestinal inflammatory response of the hybrid grouper may be exerted by histamine released by mast cells in the GALT. However, further confirmation of this inference is strongly recommended to study the role of histamine on groupers' intestinal immunity.

The dietary composition can cause biological changes in the host fish by changing the intestinal microbial community, thereby altering the metabolism and population size of key symbiotic species (Ringø et al., 2006). Fish gut microbiota is key for regulating nutrient digestion, immune response, intestinal differentiation, disease resistance, and potential colonization by pathogens (Gómez and Balcázar, 2008; Nayak, 2010). There were no significant differences among the treatments in the diversity and abundance of microbiota in this study. A similar result was observed in hybrid groupers fed a cottonseed protein concentrate diet (Ye et al., 2020a). Also, our results show that the intestinal flora of this species was mainly composed of Firmicutes, Proteobacteria, and Bacteroidetes taxa. These phyla usually constitute the core intestinal flora of hybrid groupers, regardless of diet type (Ye et al., 2020a, 2020b, 2020c). Proteobacteria, Bacteroidetes, and Firmicutes represent up to 90% of the gut microbiota in various marine and freshwater species (Apper et al., 2016; Liu et al., 2018; Rimoldi et al., 2018; Ringø et al., 2016). The similarity of the bacterial taxa in the gut microbiota of multiple fish species indicates that these bacteria are involved in important host gut functions, such as digestion, nutrient absorption, and immune response (Ghanbari et al., 2015). At the genus level, the gut microflora of the PSB75 group was dominated by *Pseudomonas*, *Bacteroides*, and *Arcobacter* and was accompanied by significantly low growth compared with the FM group. In terms of potentially pathogenic bacteria, some species of *Pseudomonas* could exert harmful effects on fish health: *Pseudomonas plecoglossicida* and *Pseudomonas anguilliseptica* infections in *Plecoglossus altivelis* and *Gadus morhua* L. have been respectively reported (Park et al., 2000; Ferguson et al., 2004). *Pseudomonas stutzeri* could cause bacteremia, generalized sepsis, and local infections such as meningitis, endocarditis, and conjunctivitis (Yao et al., 2015). Helicobacter pylori infection lead to gastric mucosa inflammation and damage to the gastric epithelium. Also, the members of genus *Arcobacter* have been regarded as emergent entero-pathogens and potential zoonotic agents (Collado and Figueras, 2011). The possible reason for poorer growth at a high level of PSB could be could be the presence of potentially pathogenic *Pseudomonas* and *Arcobacter*. *Bacteroides* abundance in fish and human gut have been described widely while a lower level of *Bacteroides* is reported to have a strong correlation in the progression of several diseases in humans (Zhou and Zhi, 2016; Egerton, et al., 2018). Notably, the fish fed the PSB25 diet had a higher relative abundance of *Bacteroides* compared with the other groups. This reflects the complexity of the intestinal flora responses to different dietary intakes (Flint et al., 2012). The specific reasons for this finding require further study.

In conclusion, the study's findings showed that up to 50% of the FM protein in the feed can be replaced by PSB without adversely affecting the nutritional status and inflammatory response in hybrid groupers. Although the digestion and absorption capacities of fish increased at a higher replacement level (75%) of PSB to FM in the feed, fish growth declined. The possible reason for poorer growth at a high level of PSB could be the presence of potentially pathogenic *Pseudomonas* and *Arcobacter*.

Declaration of Competing Interest

There are no conflicts to declare.

Acknowledgement

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