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Replacing fish meal with fermented rice protein in diets for hybrid groupers (*Epinephelus fuscoguttatus*♀× *Epinephelus lanceolatus*♂): Effects on growth, digestive and absorption capacities, inflammatory-related gene expression, and intestinal microbiota

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A R T I C L E I N F O

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A B S T R A C T

A feeding trial was carried out to investigate the effects of dietary fermented rice protein (FRP) on growth, digestive and absorption capacities, inflammatory-related gene expression, and intestinal microbiota in juvenile hybrid groupers (*Epinephelus fuscoguttatus*♀ × *Epinephelus lanceolatus*♂). A fish meal (FM)-based diet was used as a control for comparison with diets in which FM was replaced by FRP at percentages of 10 % (FRP10), 30 % (FRP30), and 50 % (FRP50). Each diet was fed to triplicate groups of fish for 56 days. We observed no significant differences in weight gain rate, specific growth rate, and protein productive value between the FM and FRP10 groups, but significantly lower rates in the FRP30 and FRP50 groups. The activities of the intestinal digestive enzymes (amylase, trypsin, and pepsin) and brush border enzymes (Na^+/K^+-ATPase, creatine kinase, and γ-glutamyl transpeptidase) were higher in the FRP-containing groups than in the FM group. The expression levels of immune-related genes (TLR22, MyD88, TNF-α, IL-2, IL-6, IL-8, and TGF-β1) were all down-regulated in FRP-fed fish. Intestinal microbiota showed no significant differences in alpha diversity among dietary groups. At the genus level, FRP-containing groups had lower relative abundances of *Bacteroides* and *Enterococcus* than the FM group. In conclusion, 10 % of the FM protein in the feed can be replaced by FRP without adverse effects on nutritional status. Replacing FM with FRP in the feed suppresses inflammatory function in hybrid groupers. It also has a critical influence on microbial profiles but not on diversity.

1. Introduction

Global fishmeal (FM) supplies are insufficient to meet the increasing feed demands of aquaculture. The use of FM in fish diets will need to be decreased (Król et al., 2016), so finding alternative protein sources is critical to the sustainable development of aquaculture. Over recent decades, studies have focused on vegetable protein sources such as soybean meal, cotton meal, rapeseed meal, corn protein meal, and wheat protein meal (Jiang et al., 2018). These plant proteins are less expensive than animal proteins but contain anti-nutritional factors and high protein molecular weights (Gorissen et al., 2018; Luo et al., 2011; Zhang et al., 2019), which limit their application in aquatic feed.

Rice is an important food crop worldwide. China is the largest producer of rice and produced 29.8 % of the world's rice in 2019 (National Bureau of Statistics, 2019). Rice protein, a by-product of rice processing, is reported to have excellent nutritional qualities (Yu, 2013), such as a more balanced amino acid composition and higher protein content than vegetable meal plant proteins. However, the solubility of rice protein is low due to the high temperatures used in the production process, which restricts the wide application of this protein. Fermented rice protein (FRP) produced by microbial fermentation methods not only has all the advantages of rice protein but also has good solubility, high acid and

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heat stability, and low viscosity (Yu, 2013). Rice and/or rice bran fermented with different strains of bacteria have been reported for use as food for humans and mammals.

A mixture of brown rice and rice bran fermented with *Aspergillus oryzae* (FBRA) can induce apoptosis in human acute lymphoblastic leukemia cells, mainly through the death receptor-mediated pathway and, supplementarily, through the tBid-mediated mitochondrial pathway (Horie et al., 2016). In rodents, FBRA has chemopreventive effects against chemical carcinogenesis (Katayama et al., 2003; Katyama et al., 2002; Kuno et al., 2004, 2006; Tomita et al., 2008) and inflammation-related carcinogenesis (Onuma et al., 2015; Phutthaphadoong et al., 2010). Black rice fermented with *Lactobacillus casei* attenuated osteoclastogenesis and ovariectomy-induced osteoporosis in rats (Lee et al., 2019). And that, obese mice fed rice fermented with *Bifidobacteria* had significant reductions in body and organ weights and re-established gut *Lactobacillus, Bifidobacterium,* and *Bacteroides* species (Ray et al., 2018). However, there are few studies on the application of FRP in aquatic animals. One study on crucian carp (*Carassius auratus*) showed that up to 30 % of the FM in the diet could be replaced by FRP fermented by *Bacillus subtilis* and *Saccharomyces cerevisiae* (2:1), with no effect on growth performance (Yu, 2013).

The hybrid grouper (*Epinephelus fuscoguttatus*♀ × *Epinephelus lanceolatus*♂) is a carnivorous fish species used in aquaculture. It is mainly farmed in China and has had increasing market demand in recent years. In recent years, research on replacing FM with different plant protein sources in hybrid grouper feed has been reported (Wang et al., 2020; Ye et al., 2020a, 2020b; Yin et al., 2018). This study aims to examine the effects of replacing dietary FM with FRP on growth performance, intestinal digestive and absorption capacities, inflammation-related gene expression, and microbial flora in the hybrid grouper. The development of novel plant proteins can have great 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, and all experiments were conducted according to its guidelines.

2.2. Experimental diets

The FRP in this study was supplied by Hubei Shuangding Agricultural Science and Technology Co., Ltd. (Jingzhou, China; 61.63 % crude protein and 4.61 % crude lipid on a dry matter basis). The FRP was fermented by the highly-active microbe *Aspergillus oryzae*, as described previously (Ochiai et al., 2013). High-quality FM-based feed was supplied by Guangdong Yuehai Feeds Group Co., Ltd. (Zhanjiang, China; 67.00 % crude protein and 10.38 % crude lipid on a dry matter basis). Four isonitrogenous (51 % crude protein) and isoenergetic (20 % gross energy) experimental diets were formulated by taking the original FM-based feed and replacing 0 %, 10 %, 30 %, or 50 % of the FM protein with FRP (Table 1). These diets are denoted as FM, FRP10, FRP30, and FRP50, respectively. Methionine and lysine were supplemented to meet the amino acid requirements of groupers (Chi et al., 2015; Li et al., 2019). All ingredients were carefully weighed 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, then purified water was added to produce a suitable dough. Strip feeds of 2.5-mm diameter were formed using a double-helix extrusion mechanism (F-75, South China University of Technology, China). Finally, the feeds were air-dried and stored at −20 °C until feeding.

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

Ingredients	Experimental diets			
	FM	FRP10	FRP30	FRP50
Brown fishmeal	55.00	49.50	38.50	27.50
Fermented rice protein[a]	0.00	5.98	17.94	29.90
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	5.69	4.64	2.54	0.27
Fish oil	3.62	4.20	5.35	6.47
Soybean lecithin	2.00	2.00	2.00	2.00
Lysine	0.00	0.00	0.00	0.20
Methionine	0.12	0.11	0.10	0.09
Vitamin premix[b]	0.20	0.20	0.20	0.20
Mineral premix[b]	0.50	0.50	0.50	0.50
Yttrium trioxide	0.10	0.10	0.10	0.10
Others[c]	2.33	2.33	2.33	2.33
Total	100.00	100.00	100.00	100.00
Nutrient level (%)				
Crude protein	50.49	51.08	50.53	50.87
Crude lipid	11.79	11.20	12.59	13.39
Gross energy	19.88	20.28	20.61	20.66
Nitrogen-free extract	18.23	20.55	22.48	22.86

[a] Essential amino acid contents: methionine, 1.80 %; lysine, 2.21 %; valine, 4.05 %; isoleucine, 2.81 %; phenylalanine, 3.80 %; leucine, 5.94 %; threonine, 2.52 %; histidine, 1.58 %; arginine, 5.53 %.
[b] Obtained from Qingdao Master Biotech (Qingdao, China).
[c] Others: calcium monophosphate, 1.50 %; attractant, 0.50 %; choline chloride, 0.30 %; antioxidants, 0.03 %.

2.3. Fish husbandry

Hybrid grouper juveniles (n = 540) were obtained from a native species fish farm (Zhanjiang, China). All fish were acclimated to a commercial diet for 1 week. Healthy and uniformly-sized fish (mean ± standard error of mean, S.E.M, initial weight = 12.39 ± 0.21 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 (99.9 %, Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) was used as the external indicator in the four 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

At the end of the 8th week, all fish were fasted for 24 h, then the fish from each tank were counted and weighed to calculate growth indices. Three fish from each tank were randomly selected and stored at −20 °C for body composition analysis. The stomach and proximal intestines of two fish and the whole intestines including proximal, mid, and distal 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 intestines of another two fish per tank were collected and immediately frozen in liquid nitrogen, then stored at −80 °C for relative

gene expression analysis. The intestines from another three fish per tank were taken for microbiota analysis.

2.6. Chemical 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℃ until a constant weight was obtained. Crude protein (N × 6.25) and crude lipids were determined by the Kjeldahl method using an Auto Kjeldahl System (2300-Auto-analyzer, Foss, Sweden) and by Soxhlet extraction using petroleum ether as solvent, respectively. Crude ash was measured by calcination at 550 °C in a muffle furnace.

2.7. Enzyme activity analysis

The amylase, lipase, trypsin, pepsin, Na^+/K^+-ATPase, creatine kinase, and γ-glutamyl transpeptidase activities were determined using commercial enzyme-link immunosorbent assay (ELISA) kits (Shanghai Enzyme-link Biotech Co. Ltd., Shanghai, China) following the manufacturer's instructions. The seven enzyme activities were determined by a double antibody sandwich method using ELISA kits (No. ml036449, No. ml036371, No. ml064285, No. ml676616, No. ml036470, No. ml036438, No. ml036466, respectively). All enzyme activities were recorded as specific activity (U g^{-1} tissue) intestine content.

2.8. Real-time quantitative RT-PCR

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, while 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 genomic DNA Eraser (Takara, China). We discovered previously that the use of some plant protein sources, such as cottonseed protein concentrate, soy protein concentrate, and peanut meal, to replace fish meal significantly affected the expression of immune-related genes (TLR22, MyD88, TNF-α, IL-1β, IL-6, TGF-β, and IL-10) (Wang et al., 2020; Ye et al., 2020a, b; Yin et al., 2018). All primer sequences in this study are listed in Table 2. According to the results of our preliminary experiment concerning the evaluation of internal control genes, β-actin was used as a reference gene to normalize cDNA loading. Real-time PCR assays were conducted on a CFX96 real-time PCR Detection System (Bio-Rad, Hercules, CA) with 5 μL SYBR Green Master Mix (Takara, China) according to the manufacturers' instructions. At the end of each reaction, a melting curve analysis was carried out to check the specificity of production. Also, the amplification efficiency was analyzed according to the equation: $E = 10(-1/\text{slope}) - 1$. The expression levels of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak, Schmittgen, 2001). Normalized gene expression for the control group (FM) was set at 1.

2.9. Analysis of intestinal microbiota

DNA from the intestinal tract was extracted using DNA isolation 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 (SUB7611221).

The data were filtered and chimeric sequences were removed. The resulting sequences were clustered by 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 was used to identify the unique and common OTUs using 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 community structure based on taxonomic information was performed at each classification level. The significant differences in community structure between two groups were analyzed using the unweighted UniFrac method (Hamady et al., 2010).

Table 2
Primers pair sequences for real-time qPCR.

Genes	Nucleotide sequence (5′-3′)	Genbank accession no.
b(0,+)AT1	F: CCCTCGTAGCCTACCTTTATACCT R: GCTGCTGCGGACAGACACTT	KR559237.1
B(0)AT1	F: CAATGCTAACGGGACAGG R: GGCAGAGTGGAGGTGATG	KR149369.1
PePT1	F: ATGGACATCAAGCCCAACAC R: CCGGTGACAGAGAAGACCAC	JX122768.1
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

b(0,+) AT1: b(0,+)-type amino acid transporter 1; B(0)AT1: system B(0) amino acid transporter; PePT1: peptide transporter 1; 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.

2.10. Statistical analysis

Data were analyzed by 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 two groups were calculated by Welch's t-test. All data are shown as means ± standard error of mean (S.E.M).

3. Results

3.1. Growth performance

The survival rate (SR) and feed intake (FI) were not influenced by dietary treatment levels ($P > 0.05$, Table 3). The final body weight (FBW), weight gain rate (WGR), specific growth rate (SGR), daily feed intake (DFI), and protein productive value (PPV) showed no significant differences between the FM and FRP10 groups ($P > 0.05$), but were significantly lower in the FRP30 and FRP50 groups ($P < 0.05$). The feed conversion ratio (FCR) trend among treatments was opposite to that of FBW, WGR, and SGR ($P < 0.05$).

Table 3
Effects of replacement fish meal by fermented rice protein on growth performance of juvenile hybrid groupers.

Indexes	Experimental diets			
	FM	FRP10	FRP30	FRP50
FBW (g)	70.13 ± 2.90[c]	70.64 ± 1.65[c]	57.20 ± 5.05[b]	46.87 ± 3.09[a]
WGR[a] (%)	465.51 ± 23.39[c]	469.72 ± 13.31[c]	361.28 ± 40.77[b]	277.98 ± 24.94[a]
SGR[b] (%/d)	3.13 ± 0.04[c]	3.11 ± 0.05[c]	2.81 ± 0.10[b]	2.37 ± 0.12[a]
FCR[c]	0.91 ± 0.01[a]	0.92 ± 0.03[a]	1.04 ± 0.08[b]	1.04 ± 0.07[b]
PPV[d] (%)	37.81 ± 0.24[b]	37.01 ± 0.64[b]	32.49 ± 1.75[a]	32.90 ± 1.04[a]
FI[e] (% BW/d)	2.26 ± 0.01	2.25 ± 0.01	2.27 ± 0.09	2.09 ± 0.03
DFI[f] (g/d/fish)	1.25 ± 0.03[c]	1.26 ± 0.02[c]	1.02 ± 0.05[b]	0.84 ± 0.03[a]
SR[g] (%)	98.89 ± 1.92	95.56 ± 7.70	96.67 ± 3.34	94.44 ± 5.09

FBW: final body weight; WGR: weight gain rate; SGR: specific growth rate; FCR: feed conversion ratio; PPV: protein productive value; FI: feed intake; DFI: average daily feed intake; SR: survival rate. Values are means ± S.E.M of three replications. Different superscript letters in each row show significant differences among treatments by Tukey's test ($P < 0.05$).
[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] PPV (%) = 100 × (final body weight × final body protein− initial body weight × initial body protein)/ total protein intake (Sandre et al., 2017).
[e] FI = 100 × total diet intake/ [(initial body weight + final body weight)/2 × days] (Yamamoto et al., 2002).
[f] DFI (g/day/fish) = feed consumption/fish number/days (Wu et al., 2018).
[g] SR (%) = 100 × the final fish number/the initial fish number.

3.2. Apparent digestibility

The apparent digestibility of dry matter and crude protein showed no significant differences between the FRP30 and FRP50 groups ($P > 0.05$, Table 4), but had significantly lower values than the FM and FRP10 groups ($P < 0.05$). The FRP30 group had a significantly higher apparent digestibility of crude lipid than other groups ($P < 0.05$).

3.3. Body composition

No significant differences in body composition, in terms of crude protein, crude ash, and moisture, were observed among treatments ($P > 0.05$, Table 5). There was no significant difference in crude lipid among the FM, FRP10, and FRP30 groups ($P > 0.05$), while the crude lipid of the FRP30 group was significantly lower than that of the FRP50 group ($P < 0.05$).

Table 4
Effect of replacement fish meal by fermented rice protein on dietary apparent digestibility of juvenile hybrid groupers.

Items	Experimental diets			
	FM	FRP10	FRP30	FRP50
Dry matter	80.56 ± 0.68[b]	80.97 ± 0.49[b]	73.90 ± 2.43[a]	74.88 ± 1.18[a]
Crude protein	93.68 ± 0.20[c]	92.48 ± 0.20[b]	87.68 ± 1.06[a]	88.17 ± 0.93[a]
Crude lipid	94.71 ± 0.53[b]	94.04 ± 1.36[b]	96.48 ± 1.27[c]	81.47 ± 1.60[a]

Values are means ± S.E.M of three replications. Different superscript letters in each row show significant differences among treatments by Tukey's test ($P < 0.05$).
ADC of dry matter (%) = 100 × [1− (dietary Y_2O_3 level/feces Y_2O_3 level)].
ADC of nutrients (%) = 100 × [1− (dietary Y_2O_3 level/feces Y_2O_3 level) × (feces nutrient level/dietary nutrient level)].

Table 5
Effects of replacement fish meal by fermented rice protein on body composition of juvenile hybrid groupers.

Items	Experimental diets			
	FM	FRP10	FRP30	FRP50
Crude protein	58.21 ± 0.59	57.41 ± 0.27	56.91 ± 1.25	56.96 ± 0.44
Crude lipid	25.97 ± 025[ab]	26.58 ± 0.28 [ab]	25.85 ± 1.17[a]	27.54 ± 0.27[b]
Crude ash	15.23 ± 0.35	15.59 ± 0.12	15.19 ± 0.22	15.29 ± 0.20
Moisture	71.27 ± 0.24	71.01 ± 0.74	71.30 ± 0.01	71.16 ± 0.21

Values are means ± S.E.M of three replications. Different superscript letters in each row show significant differences among treatments by Tukey's test ($P < 0.05$).

3.4. Activities of intestinal digestive and brush border enzymes

Intestinal lipase and pepsin activities showed no significant differences between the FM and FRP10 groups ($P > 0.05$, Table 6) but had significantly higher activities in the FRP30 and FRP50 groups ($P < 0.05$). Amylase, trypsin, and pepsin activities gradually increased with increasing dietary FRP levels ($P < 0.05$).

Na^+/K^+-ATPase, Creatine kinase (CK), and γ-Glutamyl transpeptidase (γ-GT) activities gradually increased with increasing dietary FRP levels ($P < 0.05$). Na^+/K^+-ATPase, CK, and γ-GT activities in the FRP50 group were significantly higher than those in the FM group ($P < 0.05$).

3.5. Expression of amino acid and peptide transporters and inflammatory-related genes in the intestinal tract

There were no significant differences in the expression levels of b (0,+)AT1 and B(0)AT1 genes among treatment groups ($P > 0.05$, Fig. 1). However, the expression levels of the PepT1 gene in the FM group were significantly higher than those in other groups ($P < 0.05$). The expression levels of TLR22, TNF-α, and IL-6 were significantly down-regulated in FRP-fed fish ($P < 0.05$). No significant changes in MyD88, IL-2, IL-8, or IL-10 expressions were observed among treatment groups ($P > 0.05$). The FRP30 group showed the highest TLR21 expression levels, which were significantly higher than those of the FM and FRP10 groups ($P < 0.05$). The trends in TGF-β1 among treatments were opposite to those in TLR21 ($P < 0.05$).

Table 6
Effect of replacement of fish meal by fermented riced protein on intestinal digestive enzyme and brush border enzymes activities (U/g tissue) of juvenile hybrid groupers.

Items	Experimental diets			
	FM	FRP10	FRP30	FRP50
Stomach				
Pepsin	0.92 ± 0.04[a]	0.93 ± 0.01[a]	1.16 ± 0.01[b]	1.22 ± 0.09[b]
Proximal intestine				
Lipase	3.86 ± 0.44[a]	3.77 ± 0.08[a]	5.12 ± 0.16[b]	5.98 ± 0.02[c]
Amylase	1.23 ± 0.02[a]	2.03 ± 0.26[b]	2.19 ± 0.28[b]	2.42 ± 0.03[b]
Trypsin	17075.83 ± 195.03[a]	19611.20 ± 546.08[b]	19650.21 ± 117.02[b]	26281.18 ± 507.08[c]
Whole intestine				
Na^+/K^+-ATPase	104.30 ± 4.29[a]	125.52 ± 7.75[ab]	129.33 ± 11.42[ab]	167.21 ± 17.27[b]
CK	0.68 ± 0.08[a]	0.87 ± 0.11[ab]	1.03 ± 0.05[b]	1.34 ± 0.03[c]
γ-GT	0.38 ± 0.03[a]	0.41 ± 0.03[ab]	0.51 ± 0.03[ab]	0.55 ± 0.09[b]

CK, creatine kinase; γ−GT, γ−glutamyl transpeptidase. Values are means ± S.E.M of three replications. Different superscript letters in each row show significant differences among treatments by Tukey's test ($P < 0.05$).

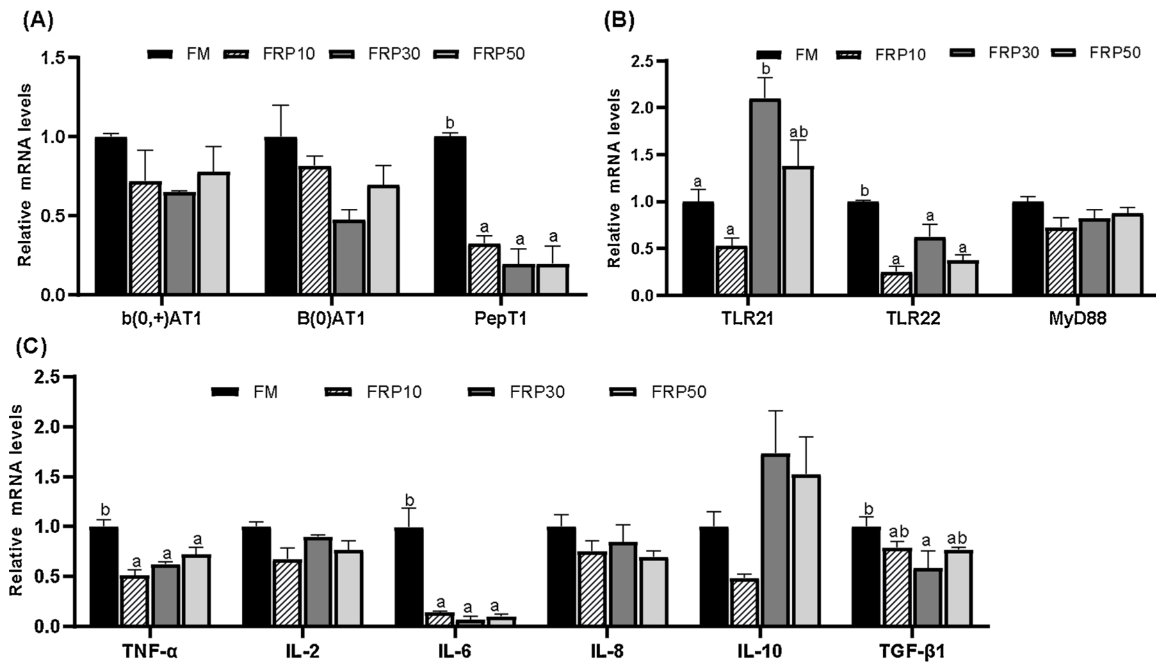

Fig. 1. Relative expression levels of transporters- and immune-related genes in hybrid groupers fed different diets. Values are means ± S.E.M of three replications. Bars of the same gene bearing the different letters show significant differences among treatments by Tukey's test ($P < 0.05$).

3.6. Analysis of intestinal microbiota

An average of 451 OTUs and 52,926 effective sequencing lengths were obtained in each group (Fig. 2A). Totals of 47, 35, 22, and 24 OTUs were uniquely identified in the FM, FRP10, FRP30, and FRP50 groups, respectively (Fig. 2B). A total of 295 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 7).

At the phylum level, Firmicutes, Proteobacteria, and Bacteroidetes had higher relative abundances in each group (Fig. 3A1). At the genus level, the top-four relative abundances per group are described below (Fig. 3A2): *Halomonas* (14.38 %), *Clostridium_sensu_stricto_1* (7.83 %), *Bacteroides* (7.43 %), and *Streptococcus* (7.19 %) in the FM group; *Vibrio* (12.03 %), *Halomonas* (9.50 %), *Clostridium_sensu_stricto_1* (9.25 %), and f_Peptostreptococcaceae_Unclassified (8.08 %) in the FRP10 group; *Streptococcus* (8.01 %), *Halomonas* (7.67 %), *Lactobacillus* (6.42 %), and *Haemophilus* (6.20 %) in the FRP30 group; and *Streptococcus* (8.04 %),

Table 7
Community richness and diversity analysis based on alpha diversity indexes in the intestinal tract of hybrid groupers fed different experimental diets.

Items	FM	FRP10	FRP30	FRP50	P-Values
ACE	299.44 ± 25.74	305.88 ± 36.67	320.69 ± 27.92	254.23 ± 30.56	0.490
Chao1	300.63 ± 28.61	308.84 ± 36.90	324.00 ± 24.19	257.94 ± 30.41	0.496
Shannon	5.70 ± 0.62	5.56 ± 0.20	6.34 ± 0.25	6.10 ± 0.17	0.442
Simpson	0.95 ± 0.03	0.93 ± 0.01	0.97 ± 0.01	0.97±±0.00	0.310

Values are means ± S.E.M of three replications. Different superscript letters in each row show significant differences among treatments by Tukey's test ($P < 0.05$).

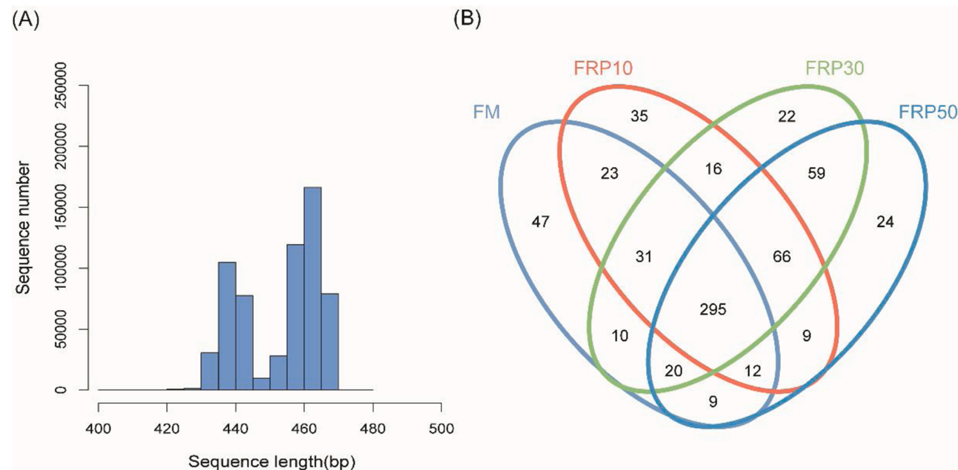

Fig. 2. Statistics of effective sequence length distribution (A) and Venn diagrams (B) demonstrating the distribution of the operational taxonomic units (OTU) in hybrid groupers fed different diets.

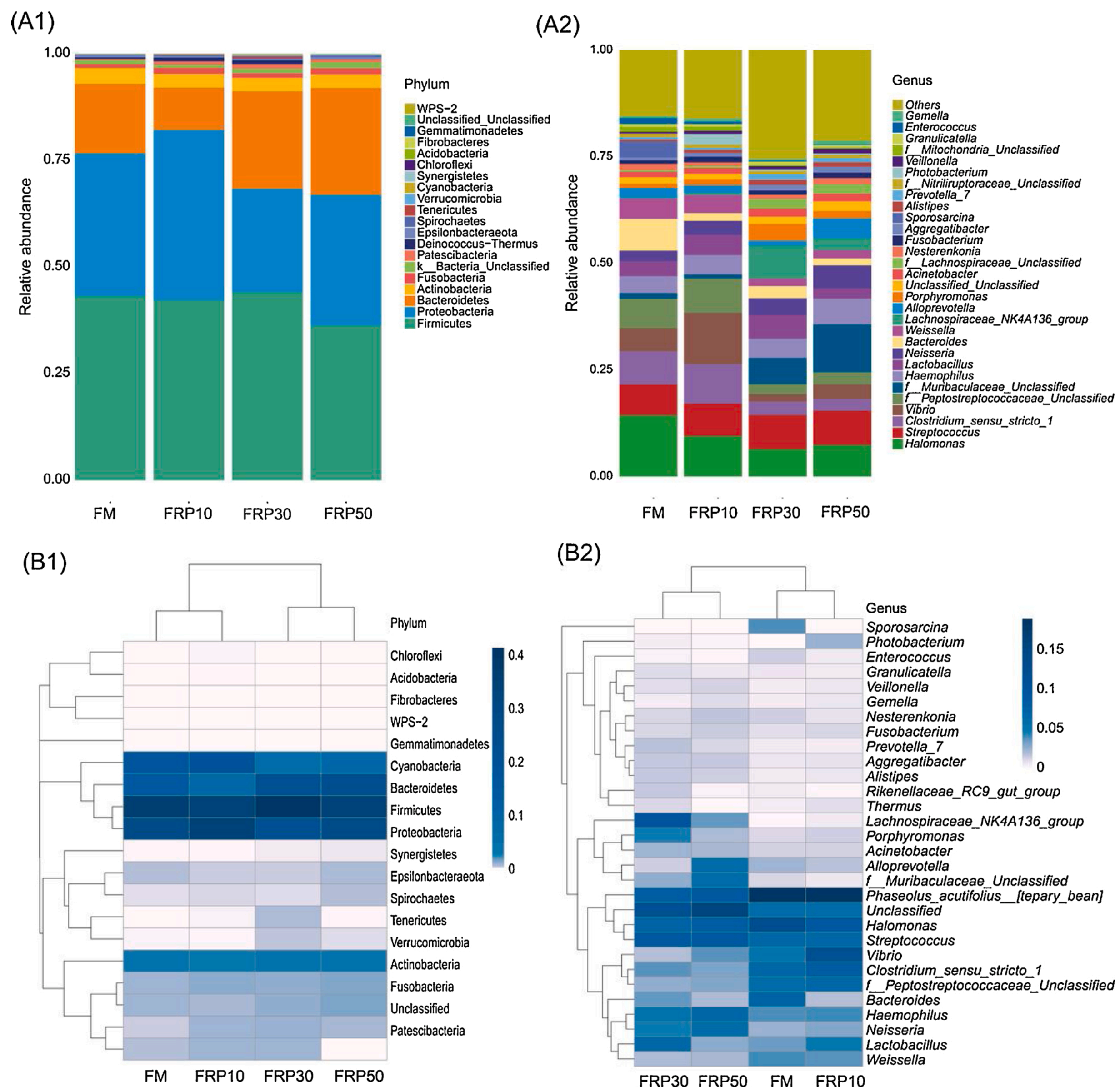

Fig. 3. Analysis of top-30 relative abundances of species at phylum (A1, B1) and genus (A2, B2) levels in the four groups of fish fed different diets.

Halomonas (7.40 %), *Haemophilus* (6.06 %), and *Neisseria* (5.40 %) in the FRP50 group. At the phylum and genus levels, the FM and FRP10 groups were clustered together, as were the FRP30 and FRP50 groups (Fig. 3B1, B2).

The relative abundances of the five bacterial genera between pairs of groups are shown in Fig. 4. FRP-containing groups had lower relative abundances of *Bacteroides* and *Enterococcus* than the FM group. There were no significant differences in the relative abundances of *Idiomarina*, *Nesterenkonia*, or *Photobacterium* between the FM and FRP10 groups ($P > 0.05$). No significant differences in the relative abundances of *Roseburia* and *Halomonas* were observed between the FM and FRP30 groups ($P > 0.05$). However, the relative abundance of *Acinetobacter* was significantly higher in the FRP30 group than in the FM group ($P < 0.05$). There were no significant differences in the relative abundances of *Enterovibro*, *Exiguobacterium*, or *Idiomarina* between the FM and FRP50 groups ($P > 0.05$).

4. Discussion

In this study, the fish fed the FRP10 diet had similar growth performance to those fed the FM diet. Both these groups had significantly higher performance than the FRP30 and FRP50 groups, suggesting that 10 % of the FM protein in the feed can be replaced by FRP without adverse effects on growth performance. Yu (2013) reported that crucian carp fed a diet where 30 % of the FM was replaced by FRP had similar growth performance to those fed an FM diet. Such a difference may be attributed to differences in feeding habits and feed formulations. A fermented soybean meal replacement level of 30 % caused no significant difference in growth performance compared to an FM diet in orange-spotted grouper (Epinephelus coioides, Hamilton) (Shiu et al., 2015). These results suggest that fermented plant protein products have great potential to partially replace FM in grouper diets. In terms of body composition, crude protein, crude ash, and moisture contents did not significantly differ among treatments, suggesting that the addition of FRP to grouper feed does not affect the nutritional composition of the fish. This is consistent with research on rainbow trout fish fed FM or fermented soybean meal diets without significant different on body compositions (Yamamoto et al., 2010). However, PPV showed no significant differences between the FM and FRP10 groups, but were significantly lower in the FRP30 and FRP50 groups. PPV not only

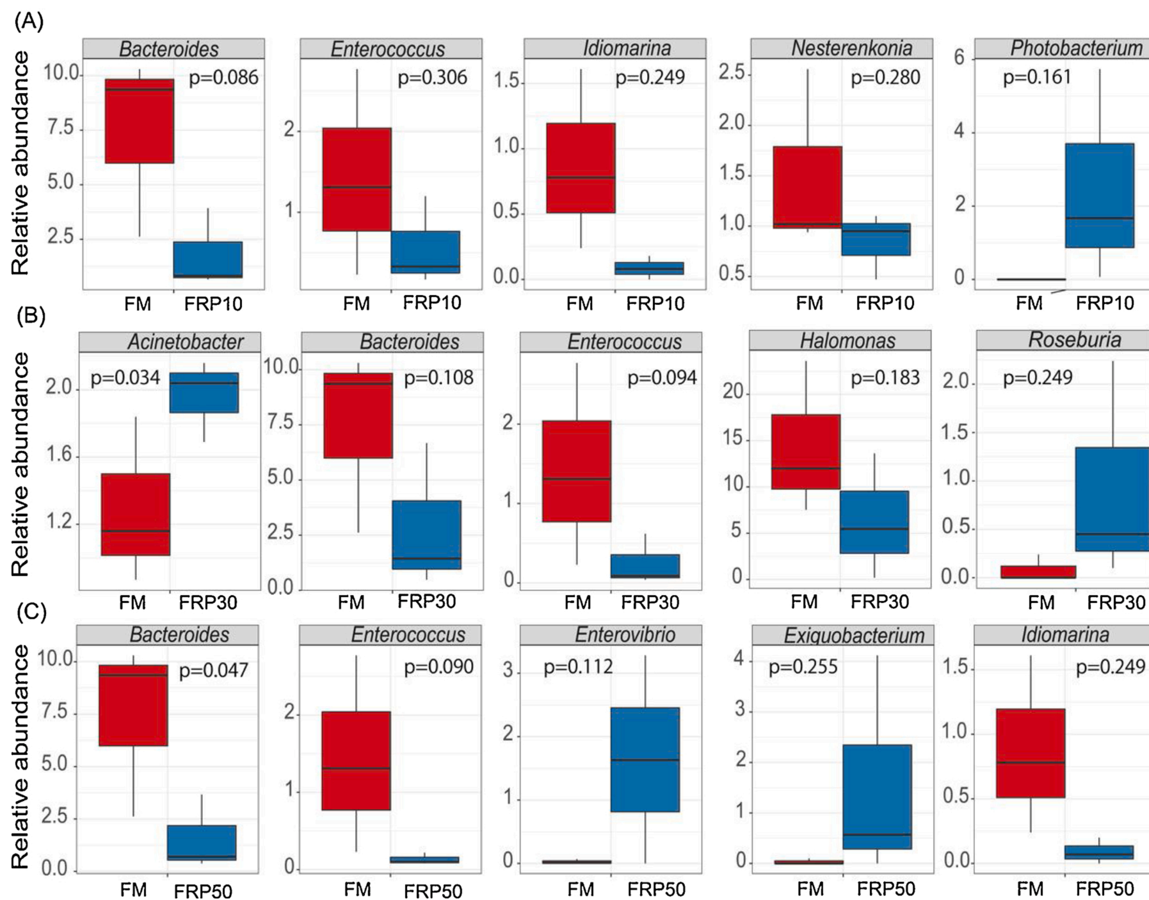

Fig. 4. Comparisons of microbe abundance in group FM with that in groups (A) FRP10, (B) FRP30, and (C) FRP50. Boxplots are presented for each of the five microbe genera with the greatest differences in abundance between the groups ($P < 0.05$ in all cases).

reflects the ability of fish protein synthesis and utilization, but also indirectly reflects nitrogen emission (Jia et al., 2013).

In the present study, the FRP30 and FRP50 fish had significantly lower dry matter and crude protein digestibility levels, lower growth performance, and DFI than other fish. In general, impaired growth performance of carnivorous fish fed plant protein-based diets is caused by reduced feed palatability and reluctant feed intake as a result of unfavorable diets taste (Nagel et al., 2012). When replacing a high percentage of dietary FM by FRP, anti-nutritional factors (ANFs) such as trypsin inhibitor or phytate may be potentially diminish diet taste, diet intake, nutrient absorption, and consequently growth. A study was reported that although fermentation of de-oiled rice bran (FDORB) with Rhizopus oryzae brought a significant reduction in the phytate and trypsin inhibitor contents than DORB (Ranjan et al., 2019). Besides reduced diet palatability and feed intake, even low levels of ANFs could have negative effects on dietary protein digestibility in carnivorous fish (Francis et al., 2001).

The presence of ANF especially residual protease inhibitors in plant protein can decrease the digestibility of dietary protein and harm digestive enzyme activity (Gatlin et al., 2007). In the present study, the specific activities of trypsin pepsin, amylase, and lipase increased with enhancing replacement of the FM with FRP. As we know, fish exocrine pancreas is the main site for digestive enzyme synthesis and secretion, and secretes numerous digestive enzymes into the intestinal lumen (Infante and Cahu, 2001). It has been demonstrated when protease inhibitors bind to proteases, it provokes the pancreas to secrete higher amounts of digestive enzymes to compensate the presence of the protease inhibitors and digest the dietary protein (Haard et al., 1996; Santigosa et al., 2008). This possible explanation also responds to the fact that high inclusion levels of the FRP resulted in a significantly reduced grouper growth.

The absorption of digested nutrition in fish intestine primarily depends on the activities of gut brush border enzymes, such as Na^+, K^+-ATPase, AKP, CK, and γ-GT (Tengjaroenkul et al., 2000; Chan et al., 2004). However, there is no information about the relationship between FRP and gut brush border-related enzymes in fish. Na^+/K^+-ATPase, a membrane-bound enzyme that actively transports Na^+ out of and K^+ into cells (Gal-Garber et al., 2003), played an important role in absorption of most of the amino acids and glucose (Geering, 1990). CK is involved in the couple of ATP and kinase (Liu et al., 2009). γ-GT is a membrane protein that may transport amino acids by the glutamyl cycle (Griffith and Meister, 1980). In this study, the replacement of FM with FRP in diets significantly increased the activities of the intestinal Na^+, K^+-ATPase, CK, and γ-GT and these were more evident in the FRP50 group. This may explain the increase in intestinal brush border enzyme activities in response to the increase in digesta. Also, protein is digested into amino acids and peptides, then absorbed and utilized by the body. Amino acids can be absorbed in the animal intestine and their transportation is regulated by amino acid transporters located in the intestine (Wang et al., 2009; Wu, 2013). In this study, the expression levels of b(0,+)AT1 and B(0)AT1 genes and the body protein contents were not significantly affected by the dietary FRP level. The results suggest that the replacement level of FRP does not significantly change the expression of amino acid transporters at the transcriptional level. PepT1 is one of the key intestinal transporters and plays a vital role in regulating oligopeptide transportation (Bakke et al., 2010; Song et al., 2017). In the present study, PePT1 gene expression was significantly down-regulated in FRP-fed fish, suggesting that the protein in FRP-containing feeds was easily digested into free amino acids instead of small peptides in the intestinal tract. However, PePT1 gene expression in the proximal

intestine of crucian carp was reported to be significantly up-regulated with increasing FRP replacement levels (Yu, 2013). This inconsistency may be attributed to differences in fermentation strains and fish species. So far, research on PepT1 shows that there are species-specific differences in its expression levels (Margheritis et al., 2013; Song et al., 2017).

The immune status of fish is closely associated with inflammation initiated and regulated by inflammatory cytokines (Sun et al., 2018). TLRs play a critical role in detecting pathogens by recruiting interleukin-1receptor (IL-1R)-associated kinase via MyD88 and, subsequently, inducing nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPK) (Akira, Hoshino, 2003). TLR22 can activate the MyD88-dependent pathway to enhance the production of pro-inflammatory cytokines (Zhang et al., 2014a). In this study, the expression levels of TLR22, MyD88, TNF-α, IL2, IL-6, IL-8, and TGF-β genes were all down-regulated in FRP-fed fish, suggesting that dietary FRP can suppress the occurrence of inflammation via the TLR22/MyD88 pathway. Similar results in mice showed that fermented brown rice and rice bran acted as effective chemopreventive agents against inflammation-related carcinogenesis by inhibiting inflammatory cell infiltration into inflammatory lesions (Onuma et al., 2015). Rice fermented with *Lactobacillus casei* treatment attenuated the receptor activator of NF-κB ligand-induced osteoclastic differentiation in RAW cells (Lee et al., 2019). In addition, our previous study reported that high inclusion level of cottonseed protein concentrate significantly down-regulated the mRNA levels of inflammation-related genes (IL-6, hepcidin, TGF-β, TLR-1, and TLR-3) and growth in the intestine of hybrid groupers (Yin et al., 2018), indicating that the intestinal inflammation was aggravated in fish fed with high replacement diet. In this study, FRP30 and FRP50 groups still showed a significantly lower DFI. Therefore, the downregulation of genes involved in inflammation response may be linked to the significantly reduced food consumption in groups FRP30 and FRP50.

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). The fish gut microbiota plays a key role in regulating nutrient digestion, immune responses, intestinal differentiation, disease resistance, and pathogens of potential colonization (Gómez, Balcázar, 2008; Nayak, 2010). In this study, there were no significant differences among treatments in the diversity and abundance of microbiota, and a similar result was observed in hybrid groupers fed a cottonseed protein concentrate diet (Ye et al., 2020b). Also, our results show that the intestinal flora of this species was mainly composed of Firmicutes, Proteobacteria, and Bacteroidetes. These phyla usually constitute the core intestinal flora of hybrid groupers regardless of diet type (Ye et al., 2020a, b, c). Proteobacteria, Firmicutes, and Bacteroidetes represent up to 90 % of the gut microbiota in various marine and freshwater fish 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, *Bacteroides* and *Enterococcus* have been reported as widely abundant in the fish gut (Egerton et al., 2018; Torrecillas et al., 2017; Wang et al., 2018) and have a strong correlation with the enteritis response in humans (Zhang et al., 2014b; Zhou and Zhi, 2016). In this study, the relative abundances of *Bacteroides* and *Enterococcus* declined with increasing dietary FRP content. It is worth noting that fish fed FRP-containing diets could suppress the expression of pro-inflammatory genes, which indicates that the decreased relative abundances of *Bacteroides* and *Enterococcus* in FRP-fed groups may be associated with suppression of inflammation. This relationship warrants further study. As well as, *Halomonas* sp., a potential probiotic, can modulate intestinal microflora and stimulate immunological levels in shrimp and, consequently, help prevent white spot syndrome virus infections (Zhang et al., 2009). In this study, the FM and FRP10 groups had higher relative abundance of *Halomonas* than that in other two groups, which were accompanied by significantly higher growth and digestibility of crude protein, implying that *Halomonas* might play an important role in regulating fish growth.

In conclusion, the study's findings showed that 10 % of the FM protein in the feed can be replaced by FRP without any adverse effect on hybrid grouper nutritional status. Replacing FM with FRP in the feed suppressed the inflammatory function in hybrid groupers, and had a critical influence on microbial profiles but not diversity.

CRediT authorship contribution statement

Yuanfa He: Methodology, Writing - original draft, Writing - review & editing. **Xinwei Guo:** Investigation, Software. **Beiping Tan:** Conceptualization, Funding acquisition. **Xiaohui Dong:** Formal analysis. **Qihui Yang:** Formal analysis. **Hongyu Liu:** Formal analysis. **Shuang Zhang:** Formal analysis. **Shuyan Chi:** Conceptualization, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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