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Effects of Lower Fishmeal With Hydrolyzed Fish Protein Powder on the Growth Performance and Intestinal Development of Juvenile Pearl Gentian Grouper (*Epinephelus fuscoguttatus* ♀ and *Epinephelus lanceolatus* ♂)

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The effect of hydrolyzed fish protein powder (HFP) on the growth, intestinal development, gene mRNA expression, and enzyme activity in the intestine and liver of juvenile hybrid grouper (*Epinephelus fuscoguttatus* ♀ and *Epinephelus lanceolatus* ♂) was assessed after an 8-week feeding trial. Seven isonitrogenous (50%) and isolipidic (9%) diets were fed to hybrid grouper with 0% (CT), 1% (H1), 1.5% (H2), 2% (H3), 2.5% (H4), 3% (H5), and 4% (H6) HFP. No significant difference ($p > 0.05$) in weight gain rate (WGR), specific growth rate (SGR), feed conversion ratio (FCR), and survival rate (SR) was observed in all the groups. The crude protein content in the H6 group was significantly higher than in the other groups ($p < 0.05$). Intestinal lipase and trypsin activity were significantly higher in H3 and H5 groups ($p < 0.05$). In the serum, superoxide dismutase (SOD) activity was significantly higher in H5 and H6 groups, while malondialdehyde (MDA) activity was lower ($p < 0.05$) compared to other treatments. Insulin-like growth factor (*IGF-I*) and target of rapamycin (*TOR*) mRNA expression levels in the intestine and muscle were significantly higher in the H2 group and H1 group ($p < 0.05$), respectively. The most abundant intestinal bacteria found at the genus level are *Acinetobacter*, *Vibrio*, and *Flavobacteriaceae.* The villus was significantly longer in hybrid grouper fed with different levels of HFP compared to the control, and fish in the H2 group had thicker intestinal muscle compared to the other groups ($p < 0.05$). In conclusion, the addition of HFP to the low fishmeal (FM) diets of juvenile grouper improved the intestinal development and increased the levels of intestinal digestive enzymes.

Keywords: hydrolyzed fish protein, pearl gentian hybrid grouper, small peptide, intestinal development, fishmeal

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INTRODUCTION

Aquaculture is anticipated to fulfill the global request for aquatic animals due to the reduction in capture fisheries since the 1990s (FAO, 2020). In aquaculture practice, feed accounts for over 50% of production cost, which is mainly composed of protein (Tacon and Metian, 2008). About 22 million tonnes of the world's fish production in 2018 was used for non-food purposes, of which 18 million tonnes were used to make fish oil and fishmeal (FM) (FAO, 2020). Traditionally, the most preferred dietary source of protein, which is the most expensive and important nutrient influencing fish growth and feed cost, especially in carnivorous fish, is obtained from FM due to its high digestibility, well-balanced amino acid, and rich source of essential n-3 fatty acids (Tacon and Metian, 2008; Olsen and Hasan, 2012). Environmental and ecological distress on the use of marine pelagic fish, limited supply, and increasing demand for FM has resulted in the intensive study of identifying viable alternative protein sources in aquafeed (FAO, 2020).

Processing fish produces a substantial amount of waste, which includes skin/scales, bones, swim bladders, roes, intestines, blood, and liver, representing about 57% of total weight. Large portions of these by-products, which contain a large amount of bioactive-rich materials, are wasted, discarded, or underutilized (Meeker, 2009; Kumar et al., 2018). Karayannakidis and Zotos (2016) stated that recycling these by-products into profitable goods for agriculture can be a waste management scheme. Quality protein in animal by-products can be hydrolyzed to obtain small molecular peptides which can act as a flavoring and good source of amino acids (Choi et al., 2012; Kumar et al., 2012).

Groupers are among the most common fish and extremely merchandized seafood in the Asia-Pacific region (FAO, 2020). Due to its rapid growth rate, hardiness to environmental conditions, high disease resistance, and high nutritional value, it is an ideal species for intensive aquaculture (Ch'ng and Senoo, 2008; Jiang et al., 2015; Arrokhman et al., 2017; Bunlipatanon and U-taynapun, 2017). With regard to marine fish culture output in China, groupers are ranked third (Yang et al., 2021). Hybrid grouper with a higher disease resistance, faster growth rate, and better feed conversion ratio (FCR) was produced at the University of Malaysia Sabah from brown-marbled grouper (*Epinephelus fuscoguttatus* ♀) and giant grouper (*Epinephelus lanceolatus* ♂) (Rahimnejad et al., 2015; Firdaus et al., 2016). The hybrid produced grows to 1 kg in a period of 6–7 months, while the parents require 8 months to 1 year to attain a similar weight (Arrokhman et al., 2017).

Nutrient utilization and growth performance are the conditions mostly used to assess substitute protein sources in aquafeeds, whereas intestinal health and immunity are often overlooked (Ye et al., 2019). Using hydrolyzed fish protein powder (HFP) to replace FM has been studied by Nguyen et al. (2012); Egerton et al. (2020), and Rimoldi et al. (2020) in gilthead sea bream (*Sparus aurata*), Pacific white shrimp (*Litopenaeus vannamei*), and Atlantic salmon (*Salmo salar*), respectively, but they focused mainly on growth and intestinal microbiota. This study aims to evaluate the effect of HFP on the growth, survival, whole-body composition, serum and liver physiological and biochemical indexes, intestinal morphology, digestive enzymes, gene mRNA expression, and intestinal microbiota in juvenile hybrid grouper.

MATERIALS AND METHODS

Experimental Diets

The HFP with 90% crude protein and 2% crude lipid (obtained from Maoming Xipu Biotechnology Co., Ltd.) was used as a substitute for FM in this experiment (**Table 1**). Seven fish diets containing crude protein (50%) and crude lipid (9%) were formulated, as shown in **Table 2**. The protein level of the formulated feed was balanced using cottonseed protein, which is in abundance and a good ingredient for fish feed, as observed by Yin et al. (2018). The group CT was fed with a diet that had no HFP replacing FM. Due to the high small peptides level, the experimental groups, namely, H1, H2, H3, H4, H5, and H6 were fed with diets containing 1, 1.5, 2, 2.5, 3, and 4% of HFP replacing the corresponding amount of FM, respectively. The raw materials were crushed after visual inspection and sieved using the 60 mm mesh screen. The raw ingredients were then weighed and mixed thoroughly using the V-mixer-type machine (JS-14S, Zhejiang Zhengtai Electrical Appliance Co., Ltd., China). Using a Hobart-type mixer (Food Mixer B60, Guangdong Henglian Food Machinery Co., Ltd., China), the ingredients were mixed with water and choline chloride to form a moist dough. The feed was pelletized into 2 and 2.5 mm granules, air-dried for 48 h, and the

TABLE 1 | Proximate composition of fishmeal (FM) and hydrolyzed fish protein powder (HFP).

	Ingredients	FM/%	HFP/%
Nutrient	Moisture	8.15	4.04
	Crude protein	64.32	90.02
	Crude lipid	7.57	2.00
Amino acid	Aspartate	5.76	7.14
	Threonine	2.79	3.14
	Serine	2.55	2.82
	Glutamine	8.53	12.14
	Glycine	3.89	11.36
	Alanine	4.21	6.71
	Cystine	0.63	0.04
	Valine	3.20	3.37
	Methionine	1.83	1.55
	Isoleucine	2.68	2.62
	Leucine	4.73	4.57
	Tyrosine	2.23	1.55
	Phenylalanine	2.76	2.44
	Lysine	4.97	5.04
	Histidine	1.98	1.58
	Arginine	3.70	4.92
	Proline	2.49	7.00
	*Tryptophan	0.00	0.44

**Tryptophan in FM was not determined (manufacturer's data).*

TABLE 2 | Formulation and nutrient composition of the experimental diets (% dry matter).

Diets and groups	CT	H1	H2	H3	H4	H5	H6
Fishmeal replacement ratio (%)	0	5	10	15	20	25	35
Poultry by-product meal	6.00	6.00	6.00	6.00	6.00	6.00	6.00
Fishmeal[b]	40.00	38.00	36.00	34.00	32.00	30.00	26.00
Hydrolyzed fish protein powder[a]	0.00	1.00	1.50	2.00	2.50	3.00	4.00
Cottonseed protein[b]	4.10	4.90	6.27	7.66	9.05	10.45	13.25
Soybean meal[b]	13.50	13.50	13.50	13.50	13.50	13.50	13.50
Wheat gluten[b]	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Wheat flour[b]	18.20	18.20	18.20	18.20	18.20	18.20	18.20
Soybean oil	1.80	1.80	1.80	1.80	1.80	1.80	1.80
Fish oil	1.35	1.55	1.68	1.79	1.90	2.00	2.20
Soy lecithin	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Choline Chloride	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Calcium monophosphate	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Vitamin C	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Premix (Vitamin + Mineral)[c]	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Attractant	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Total (%)	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Proximate composition[d]							
Crude protein	49.56	49.97	50.19	50.03	50.06	50.63	50.39
Crude lipid	9.11	8.47	9.36	9.80	8.84	8.61	8.88
Moisture	8.36	8.05	8.10	8.34	9.13	8.65	8.89
Ash	8.16	8.72	5.26	5.69	6.15	5.88	6.24

[a]Hydrolyzed fish protein powder was provided by Maoming Xipu Biotechnology Co., Ltd. [b]Ingredients purchased from Zhanjiang HaiBao Feed Factory, Zhanjiang, Guangdong, China. [c]Premix (vitamin + mineral) supplied the following per kilogram of the diet: vitamin A, 500,000 IU; vitamin D3, 100,000 IU; vitamin E, 4,000 mg; vitamin K3, 1,000 mg; vitamin B1, 500 mg; vitamin B2, 1,000 mg; vitamin B6, 1,000 mg; vitamin B12, 2; niacin, 4,000 mg; calcium pantothenate, 2,000 mg; folic acid, 100; biotin, 10; vitamin C, 15,000 mg; iron, 10,000 mg; copper, 300 mg; zinc, 5,000 mg; manganese, 1,200 mg; iodine, 80 mg; selenium, 30 mg; cobalt 20 mg (obtained from Zhanjiang Yuehai Feed Co., Ltd., Guangdong, China). [d]Proximate composition was measured values.

dried feed was stored in sealed plastic bags at −20°C until the experiment started.

Experimental Fish and Feeding Trial

Healthy pearl gentian groupers purchased from Hongyun seedling farm were used for the study. They were kept in aerated cement tanks for an acclimatization period of 3 weeks and hand-fed commercial diets (Dongwan No. 5 feed, China). This study was conducted using indoor fiberglass tanks (0.3 m^3) at the Marine Biological Research Base of Guangdong Ocean University with a natural photoperiod (12 h light/12 h dark) regime. A total of 630 hybrid groupers with no signs of disease were starved for 24 h, batch-weighed to determine an initial average weight (31.56 ± 0.04 g), and randomly distributed at a stocking density of 30 fish per tank. Each treatment was assigned to three replicate tanks. The experimental feed was fed manually twice a day (08:00 and 16:00) until a visually apparent satisfied state was reached, thus as much as they consume during feeding for 56 days. The amount of feed consumed for the period was recorded to check feed intake. Using single-air stones, aeration was provided, and the water temperature was 27.2 ± 1.32°C. Culture water was maintained by changing about 70% in the tanks daily. Daily, mortalities were checked, weighed, and recorded.

Sample Collection

Before the feeding trial, about 20 fish were randomly sampled and stored at −20°C for the initial chemical proximate composition analysis. To obtain the optimum levels of body metabolism, fish were starved for 24 h before the cessation of the experiment. The final number of fish and body weight were checked and recorded. Survival rate (SR), weight gain rate (WGR), FCR, and specific growth rate (SGR) were calculated based on the recordings. The weight and length of five fish per replicate were checked after which their viscera were harvested and weighed to establish their viscerosomatic index (VSI) and condition factor (CF).

$$\textit{Weight gain}\ (\%) = 100 \times \frac{\text{average final individual weight} - \text{average initial individual weight}}{\text{average initial individual weight}}$$

$$\textit{Specific growth rate}\ (\%/\textit{day}) = 100 \times \frac{\log_e \text{average final weight} - \log_e \text{average initial weight}}{\text{days of feeding}}$$

$$\textit{Feed conversion ratio} = \frac{\text{Feed consumed}}{\text{weight gain}}$$

$$\textit{Survival rate}\ (\%) = 100 \times \frac{\text{Final fish number}}{\text{Initial fish number}}$$

$$\text{Protein production value (PPV) (\%)} = 100 \times \frac{(\textit{Final weight} \times \textit{Crude protein}) - (\textit{Initial weight} \times \textit{Crude protein})}{\textit{Feed given} \times \textit{Crude protein}}$$

$$CF\ (\%) = 100 \times \left(\frac{\textit{final weight}}{(\textit{fish length})^3}\right)$$

$$VSI\ (\%) = 100 \times \frac{\textit{viscera weight}}{\textit{body weight}}$$

Nutrient Composition

Nutrient composition (e.g., moisture, crude protein, crude lipid, and ash) in feed and fish was determined using the standard methodology of the Association of Official Analytical Chemists (Baur and Ensminger, 1977).

Intestinal Enzyme Evaluation

Intestinal samples of 3 fish per replicate were weighed and homogenized in 0.9% aseptic saline as described by the specific operation method provided by the commercial kits obtained from the Nanjing Jiancheng Institute of Biological Engineering, China. Using the specific operation method and calculation method provided by the kit, amylase and lipase in the intestine were determined, and absorbance was measured using the microplate reader. Chymotrypsin, trypsin, and total protease activity in the intestine were checked by Shanghai Enzyme-Linked Biotechnology Co., Ltd., China.

Determination of Enzyme Activities

Blood was pooled from 3 fish per replicate using a 1-ml sterile syringe. Blood obtained was transferred into 1.5 ml Eppendorf tubes and stored at 4°C overnight. The blood samples were centrifuged at 4°C at 4,000 rpm for 15 min. The supernatant (serum) was collected and stored at −80°C to check the levels of total protein (TP), albumin (ALB), superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione peroxidase (GHS-PX) using the test kits (Nanjing Jiancheng Institute of Biological Engineering, China). The liver was harvested from the fish and stored at −80°C to check SOD and MDA activities using the test kits (Nanjing Jiancheng Institute of Biological Engineering, China). The absorbance was read with a microplate reader (Thermo Fisher Scientific, United States). The specific operation methods and calculation formulas were used referring to the test kit instructions. Using the isotope method, growth hormone (GH) and insulin (INS) in the serum were checked by Beijing North Biotechnology Research Co., Ltd.

RNA Extraction and Real-Time Quantitative Reverse Transcriptase PCR

The samples of the muscle, liver, and intestine were taken from 3 fish per replicate, and total RNA was extracted using the specific operation method of TRIzol (Invitrogen, United States) reagent. Using 1% agarose gel electrophoresis, the integrity of the extracted RNA was verified, and a spectrophotometer [NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific, United States)] was used to measure the concentration and purity of RNA. Reverse transcription was performed using the PrimeScript^{TM} kit (TaKaRa, China) and its method. The β-*actin* gene was used as the housekeeping gene, and real-time fluorescent quantitative PCR assays were conducted to detect gene expression levels for genes shown in **Table 3** using a quantitative thermal cycler (Bio-Rad CFX96; Bio-Rad Labs, Hercules, CA, United States). Relative expression levels of genes were calculated by $2^{-\Delta\Delta CT}$.

Intestinal Microbiota Analysis

The intestinal samples were sent to Beijing Biomarker Technologies Co., Ltd., for DNA extraction and PCR amplification using Illumina MiSep sequencing analysis. Using NucleoSpin Soil kit, the total genome DNA in the stool of the intestinal sample was extracted. Universal primers (338F: 5′-ACTCCTACGGGAGGCAGCA-3′ and 806R: 5′-GGACTACHVGGGTW TCTAAT-3′) were used to amplify the V3-V4 region of the 16S rRNA gene for Illumina deep sequencing. The microbiota alpha diversity, community composition, and community abundance at phylum and genus levels were performed by using the mothur (Schloss et al., 2009), a free online platform.

Intestinal Morphology

A grouper per replicate was randomly selected, and its mid intestine was harvested and stored in formaldehyde solution for hematoxylin and eosin (H&E) staining using the sectioning method. Results obtained were used for histological examination of the villus length (VL), villus width (VW), and muscle thickness (MT) using ImageJ software.

Statistical Analysis

All original data were subjected to statistical verification using one-way analysis of variance (ANOVA) after consistency and

TABLE 3 | Primer sequence used for real-time quantitative PCR analysis.

Gene	Primer sequence	Source
β-*Actin*	F:GGCTACTCCTTCACCACCACAG R: TCTGGGCAACGGAACCTCT	Liu et al., 2020
TOR	F: CCACTCTTTCTTTGCGGCTT R: GGGTTCTCGTCCCTCACTTG	Ren et al., 2020
IGF-1	F:TATTTCAGTAAACCAACAGGCTATG R:TGAATGACTATGTCCAGGTAAAGG	Wu et al., 2017
IFN-γ	F: TCCGTCAGGATTGAAACAGT R: CCTCCATCTTGGTGGTCAGTG	Liu et al., 2020
IL-β	F:ATGGCAACTGTTCCTGAACTCAACT R:TTTCCTTTCTTAGATATGGACAGGAC	Liu et al., 2020
FAS	F: CGGGTGTCTACATTGGGGTG R: GAATAGCGTGGAAGGCGTTT	Zou et al., 2019
LPL	F: TTCAACAGCACCTCCAAAACC R: GTGAGCCAGTCCACCACGAT	Zou et al., 2019

β-Actin-Beta Actin, TOR-Target of rapamycin, IGF-1-Insulin-like growth factor-I, IFN-γ-Interferon-gamma, IL-β-Interleukin beta, FAS-Fatty acid synthase, LPL-Lipoprotein lipase.

TABLE 4 | Effect of hydrolyzed fish protein powder on the growth and survival of juvenile pearl gentian hybrid grouper.

Group	WGR %	SGR %/d	FCR	SR %	CF (%)	VSI (%)
CT	265.28 ± 0.31	2.34 ± 0.01	0.89 ± 0.04	100.00 ± 0.00	3.25 ± 0.17^{b}	8.76 ± 0.17
H1	264.09 ± 13.13	2.35 ± 0.07	0.87 ± 0.01	100.00 ± 0.00	3.12 ± 0.12^{ab}	8.27 ± 0.12
H2	231.92 ± 4.20	2.17 ± 0.07	0.95 ± 0.03	97.78 ± 2.22	3.03 ± 0.16^{ab}	8.59 ± 0.16
H3	245.15 ± 4.26	2.24 ± 0.02	0.93 ± 0.01	100.00 ± 0.00	2.82 ± 0.02^{ab}	8.31 ± 0.02
H4	238.01 ± 1.51	2.24 ± 0.03	0.93 ± 0.01	100.00 ± 0.00	2.78 ± 0.46^{a}	8.58 ± 0.05
H5	251.31 ± 8.54	2.17 ± 0.03	0.85 ± 0.05	97.78 ± 2.22	2.81 ± 0.04^{ab}	8.29 ± 0.04
H6	236.36 ± 0.53	2.17 ± 0.03	0.95 ± 0.04	100.00 ± 0.00	2.74 ± 0.03^{a}	8.15 ± 0.03

Values are mean values of each group of hybrid grouper (three replicates) ± SE. Means in each row without superscript do not differ significantly ($p > 0.05$), while those with superscript differ significantly ($p < 0.05$). WGR, weight gain rate; SGR, specific growth rate; FCR, feed conversion ratio; SR, survival rate; CF, condition factor; VSI, viscerosomatic index.

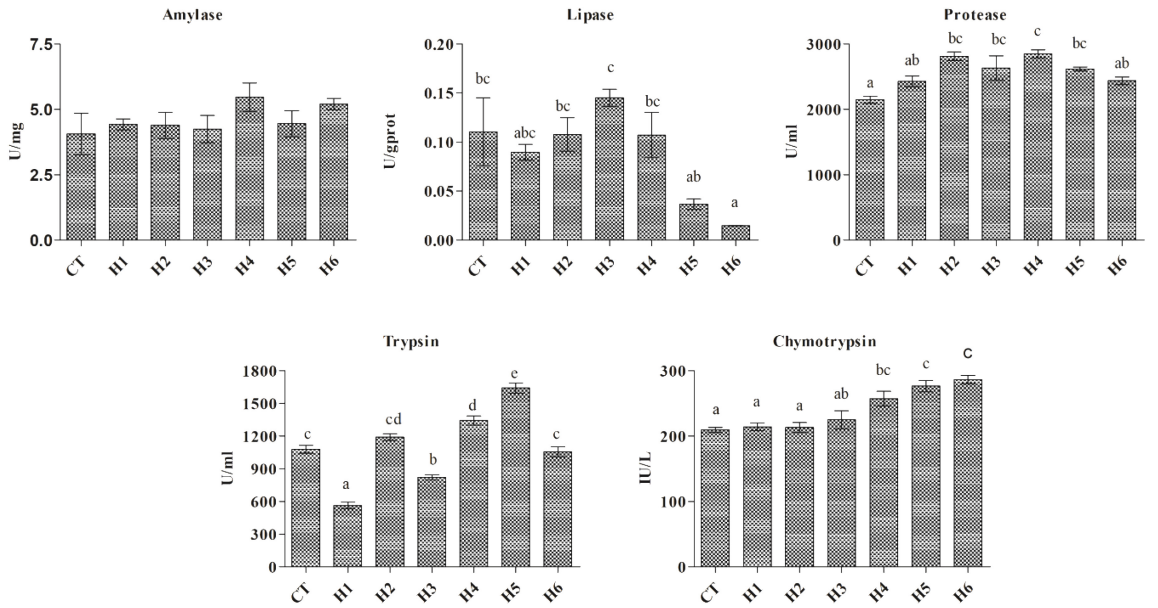

FIGURE 1 | Effect of HFP on digestive enzymes activity in the intestine of juvenile pearl gentian hybrid grouper. Data are presented as Means ± SE. Means without superscript do not differ significantly (P < 0.05), whiles those with superscript differ significantly (P < 0.05).

normality of data variance was checked. All statistical analyses were performed using the SPSS 22.0 for Windows and general differences were found to be significant at $p < 0.05$. Tukey's honest significant difference (HSD) test was used to compare the mean values between individual treatments. Data are represented as mean values of each group of hybrid grouper (three replicates) ± standard error (SE).

RESULTS

Growth Performance, Survival, and Body Composition

The hybrid grouper did not have a significant difference in WGR, SGR, FCR, SR, and VSI ($p > 0.05$) in all groups. CF of hybrid grouper in the CT group was not significantly different with H1, H2, H3, and H5 groups ($p > 0.05$) as summarized in **Table 3**.

In **Table 4**, it was observed that body moisture, crude lipid, and PPV did not show significant differences ($p > 0.05$) in all groups. The crude protein content in the H6 group was significantly higher than CT and H1 groups ($p < 0.05$). Ash content in H1, H2, H4, and H5 groups was significantly higher than the CT group but significantly lower than in the H3 group ($p < 0.05$).

Intestinal Digestive Enzyme Activity

No significant difference in intestinal amylase activity was observed among all the groups ($p > 0.05$), while lipase activity was significantly higher in the H3 group compared to H5 and H6 groups ($p < 0.05$). Intestinal trypsin activity was significantly lower in the H1 group ($p < 0.05$). Intestinal chymotrypsin activity was significantly lower in CT, H1, and H2 groups ($p < 0.05$). The highest intestinal protease activity was observed in the H4 group which did not differ significantly from H2, H3, and H5 groups (**Figure 1**).

Enzyme Activities in the Serum and Liver

In **Table 5**, the serum TP level in the H3 group was not significantly different from that of the H2, H4, H5, and H6

TABLE 5 | Effect of hydrolyzed fish protein powder on the enzyme activities in the serum and liver of juvenile pearl gentian hybrid grouper.

	CT	H1	H2	H3	H4	H5	H6
Serum							
TP	103.46 ± 2.78^{a}	104.15 ± 3.51^{a}	111.22 ± 6.59^{ab}	135.36 ± 3.87^{b}	116.75 ± 5.92^{ab}	108.29 ± 4.41^{ab}	127.59 ± 10.5^{ab}
ALB	7.52 ± 0.71	6.57 ± 0.40	7.58 ± 0.56	7.40 ± 0.38	7.17 ± 0.17	5.30 ± 0.66	7.88 ± 0.63
SOD	90.64 ± 4.01^{ab}	97.58 ± 2.31^{ab}	86.78 ± 1.39^{a}	81.70 ± 1.23^{a}	100.66 ± 6.63^{ab}	110.68 ± 5.24^{b}	109.44 ± 3.96^{b}
GSH-PX	372.86 ± 23.9	404.69 ± 4.28	325.71 ± 22.04	339.18 ± 44.44	351.83 ± 28.71	398.57 ± 22.65	354.69 ± 29.70
MDA	8.60 ± 0.27^{c}	5.17 ± 0.61^{ab}	7.36 ± 0.52^{bc}	8.77 ± 0.52^{c}	6.93 ± 0.26^{bc}	5.35 ± 0.61^{ab}	4.03 ± 0.17^{a}
GH	3.47 ± 0.46^{a}	5.09 ± 0.19^{abc}	6.02 ± 0.76^{bc}	5.82 ± 0.48^{bc}	4.95 ± 0.08^{abc}	6.44 ± 0.054^{c}	3.94 ± 0.36^{ab}
INS	16.39 ± 1.14^{a}	24.70 ± 0.79^{ab}	29.02 ± 1.95^{b}	25.91 ± 2.02^{ab}	25.44 ± 3.53^{ab}	28.22 ± 2.07^{b}	30.33 ± 1.75^{b}
Liver							
MDA	1.45 ± 0.09	1.80 ± 0.37	1.95 ± 0.36	2.04 ± 0.43	2.11 ± 0.32	2.52 ± 0.50	2.66 ± 0.10
SOD	3.76 ± 0.34	3.91 ± 0.43	4.60 ± 0.43	3.96 ± 0.33	3.92 ± 0.34	4.35 ± 0.18	4.00 ± 0.13

Values are mean values of each group of hybrid grouper (three replicates) ± SE. Means in each row without superscript do not differ significantly ($p > 0.05$), while those with superscript differ significantly ($p < 0.05$). GH, growth hormone; INS, insulin; TP, total protein; ALB, albumin; SOD, superoxide dismutase; MDA, malondialdehyde; GHS-PX, glutathione peroxidase.

TABLE 6 | Effect of hydrolyzed fish protein powder on mRNA *IGF-I* and *TOR* expression in the intestine, muscle, and liver of juvenile pearl gentian hybrid grouper.

	CT	H1	H2	H3	H4	H5	H6
Intestine							
IGF-I	0.37 ± 0.08^{a}	1.08 ± 0.08^{bc}	2.12 ± 0.18^{c}	1.60 ± 0.16^{bc}	1.08 ± 0.17^{bc}	0.96 ± 0.07^{ab}	1.42 ± 0.13^{bc}
TOR	1.02 ± 0.11^{a}	1.62 ± 0.20^{ab}	2.66 ± 0.18^{c}	2.23 ± 0.25^{bc}	1.48 ± 0.04^{a}	1.05 ± 0.07^{a}	1.67 ± 0.18^{ab}
Muscle							
IGF-I	0.79 ± 0.09^{ab}	1.37 ± 0.55^{b}	1.00 ± 0.04^{ab}	0.64 ± 0.04^{ab}	0.36 ± 0.05^{a}	0.59 ± 0.09^{ab}	1.09 ± 0.11^{ab}
TOR	0.99 ± 0.03^{abc}	1.47 ± 0.18^{c}	0.99 ± 0.18^{abc}	0.85 ± 0.06^{ab}	0.48 ± 0.06^{a}	0.54 ± 0.03^{a}	1.26 ± 0.19^{bc}
Liver							
IGF-I	1.49 ± 0.08^{b}	1.19 ± 0.10^{ab}	1.02 ± 0.09^{a}	1.02 ± 0.08^{a}	0.98 ± 0.12^{a}	1.08 ± 0.09^{ab}	1.11 ± 0.13^{ab}
TOR	1.39 ± 0.19	1.19 ± 0.13	0.92 ± 0.08	0.94 ± 0.07	0.92 ± 0.12	0.95 ± 0.06	1.04 ± 0.07

Values are mean values of each group of hybrid grouper (three replicates) ± SE. Means in each row without superscript do not differ significantly ($p > 0.05$), while those with superscript differ significantly ($p < 0.05$). IGF-I, insulin-like growth factor; TOR, target of rapamycin.

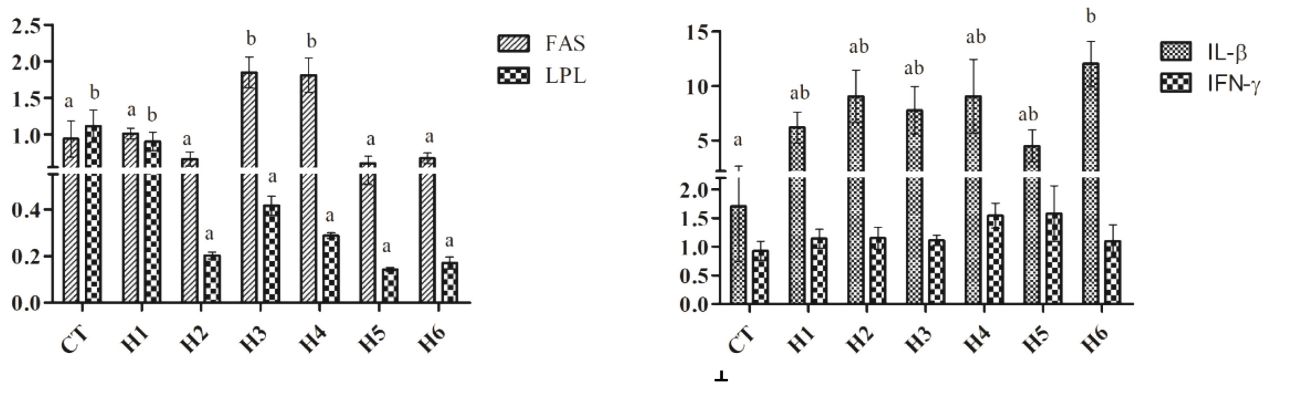

FIGURE 2 | Effect of HFP on the expression of *FAS*, *LPL*, *IL*-β, and *IFN*-γ in the hepatopancreas of juvenile pearl gentian hybrid grouper. *FAS*, fatty acid synthase; *LPL*, lipoprotein lipase; *IL*-β, interleukin beta; *IFN*-γ, interferon-gamma.

groups ($p > 0.05$) but significantly higher than CT and H1 groups ($p < 0.05$). There were no significant differences in the level of ALB and GSH-PX activities in the serum in all the groups ($p > 0.05$). Serum SOD was significantly higher in H5 and H6 groups in comparison with groups H2 and H3 ($p < 0.05$) but similar to the remaining groups ($p > 0.05$). Serum MDA in the H6 group was significantly lower but highest in H3 and CT groups ($p < 0.05$). No significant difference in MDA and SOD activity in the liver was observed for all groups ($p > 0.05$).

The highest level of GH was observed in the H5 group compared to CT and H6 groups ($p < 0.05$) but did not differ significantly from H1, H2, and H3 groups ($p > 0.05$). For serum INS levels, the CT group was significantly lower than H2, H5, and

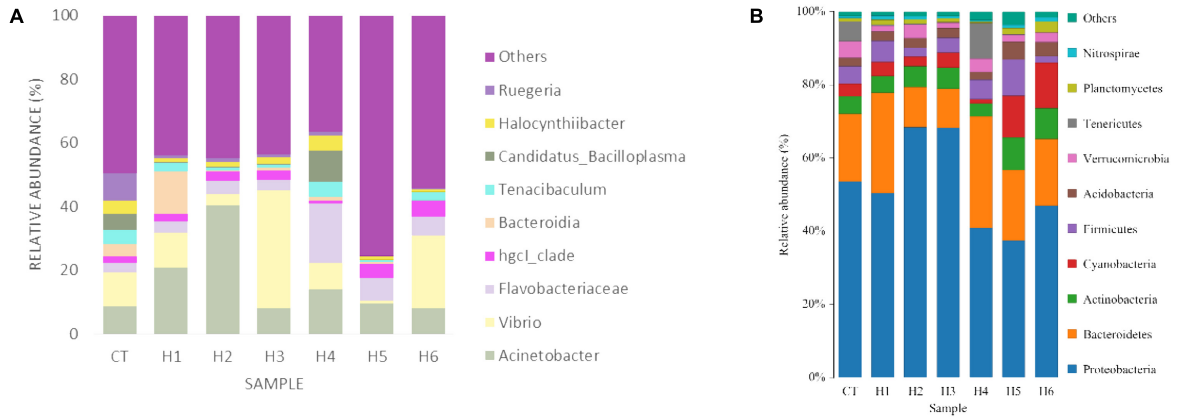

FIGURE 3 | Effect of HFP on genus **(A)** and phylum **(B)** diversity in the intestine of juvenile pearl gentian hybrid grouper.

H6 groups ($p < 0.05$) but did not have any significant difference with H1, H3, and H4 groups ($p > 0.05$).

mRNA Gene Expression in the Intestine, Muscle, and Liver

In the fish intestine, insulin-like growth factor (*IGF-I*) expression in H1, H2, H3, H4, and H6 groups was significantly higher than that in the CT and H5 groups ($p < 0.05$). The target of rapamycin (*TOR*) expression level in the intestine was significantly higher in the H2 group ($p < 0.05$), which was not significantly different from the H3 group ($p > 0.05$).

The *IGF-I* expression level in the muscle was significantly lower in the H4 group than the H1 group ($p < 0.05$) but was not significantly different from CT, H2, H3, H5, and H6 groups ($p > 0.05$). *TOR* expression level in the muscle was significantly higher in the H1 group ($p < 0.05$) but was not significantly different when compared to CT, H2, and H6 groups ($p > 0.05$).

The *IGF-I* expression level in the liver was significantly higher in the CT group compared to H2, H3, and H4 groups ($p < 0.05$), but no significant difference in liver *TOR* expression was observed ($p > 0.05$), as shown in **Table 6**. As shown in **Figure 2**, fatty acid synthase (*FAS*) expression levels in the liver were significantly higher in H3 and H4 groups than those in other groups ($p < 0.05$). The higher lipoprotein lipase (*LPL*) levels were observed in CT and H1 groups compared to the remaining groups ($p < 0.05$). In the liver, the interleukin beta (*IL*-β) expression level in the H6 group was significantly higher than that in CT group ($p < 0.05$). No significant difference in interferon-gamma (*IFN*-γ) expression levels was observed in all the groups ($p > 0.05$).

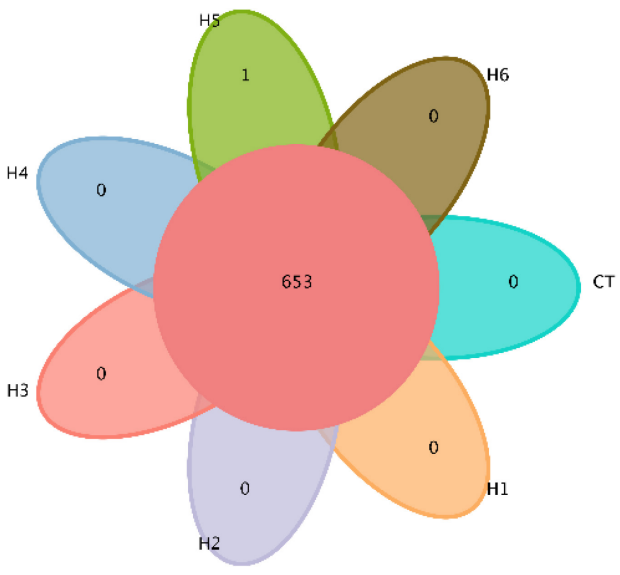

FIGURE 4 | Venn diagram demonstrating the distribution of operational taxonomic units (OTUs) shared by juvenile pearl gentian hybrid grouper fed with different levels of HFP.

Intestinal Microbiota

In the gut of juvenile hybrid grouper, the most abundant bacteria found at the genus level are *Acinetobacter*, *Vibrio,* and *Flavobacteriaceae* (**Figure 3A**). *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* were observed to be the most abundant bacteria at the phylum level, as shown in **Figure 3B**. As shown in **Figure 4**, a total of 653 operational taxonomic units (OTUs) were shared among all the treatments, and only H5 group had 1 unique OTU. *Vibrio*, *Flavobacteriaceae*, and *Acinetobacter* were significantly higher in H3, H4, and H2 groups, respectively, according to the heatmap (**Figure 5**). From the beta diversity distance matrix presented in **Figure 6**, the unweighted pair group method with arithmetic mean (UPGMA) tree showed a distinct dissociation from the control group. This indicates that HFP modified the overall structure of intestinal microbiota in hybrid grouper.

Intestinal Morphology

The VL was significantly longer in the H2 group compared with the CT group ($p < 0.05$). Compared to the other groups, the villus was significantly wider in H5 and H6 groups ($p < 0.05$), and the least VW was recorded in the H4 group. The intestinal muscle layer in CT and H1 groups was significantly thicker than that of

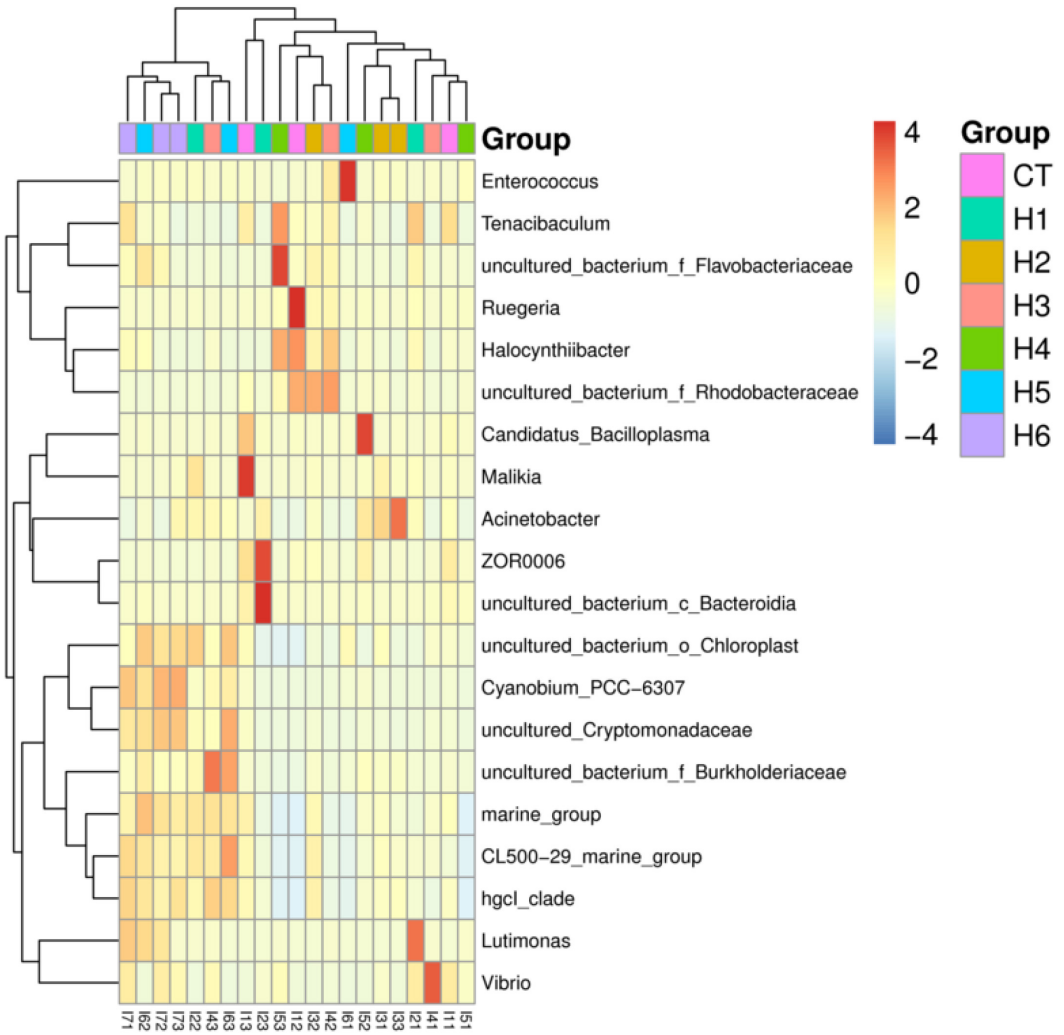

FIGURE 5 | Heatmap of the abundance of intestinal bacteria at the genus level of juvenile pearl gentian hybrid grouper fed with different levels of HFP. Color intensity indicates the relative enrichment of OTUs.

the H2 group but significantly thinner than that of the three other groups ($p < 0.05$), as summarized in **Table 7** and **Figure 7**.

DISCUSSION

The HFP is a promising ingredient used in the diet of aquatic animals due to its potential function to improve growth as well as immune status (Siddik et al., 2019b). HFP used to partially replace FM in the experimental diets in this study contains about 78.5% of small peptides less than 1,000 Da (**Figure 8**). No significant difference and high SR were observed in this study, which showed that all nutritional requirements were met in all the diets and experimental conditions were suitable for hybrid grouper in this study (Wei et al., 2016). The growth performance of hybrid grouper fed with different levels of HFP was slightly reduced, but there was no significant difference to the control group. This reduction in growth performance was also observed by Oliva-Teles et al. (1999) and Khieokhajonkhet and Surapon (2020) when HFP was added to the diet of Nile tilapia (*Oreochromis niloticus*), and juvenile turbot (*Scophthalmus maximus*), respectively. However, results observed in this study were not in accordance with the study conducted in juvenile barramundi (*Lates calcarifer*) fed with different levels of HFP which contained peptides with a molecular size of <3,000 Da (Siddik et al., 2019b). It might be a result of an increase in the catabolism of small peptides and amino acids *via* the gut wall due to the limited availability of peptide transporters (Bakke-McKellep et al., 2000). This can be related to reduce growth rate performance in this study. It is also possible that the macronutrients' requirements by hybrid grouper were met by FM, hence, masking the profitable effect of HFP (Wei et al., 2016).

At various inclusion levels of HFP, whole-body ash and crude protein contents were altered similar to the results observed by Zheng et al. (2012) in Japanese flounder (*Paralichthys*

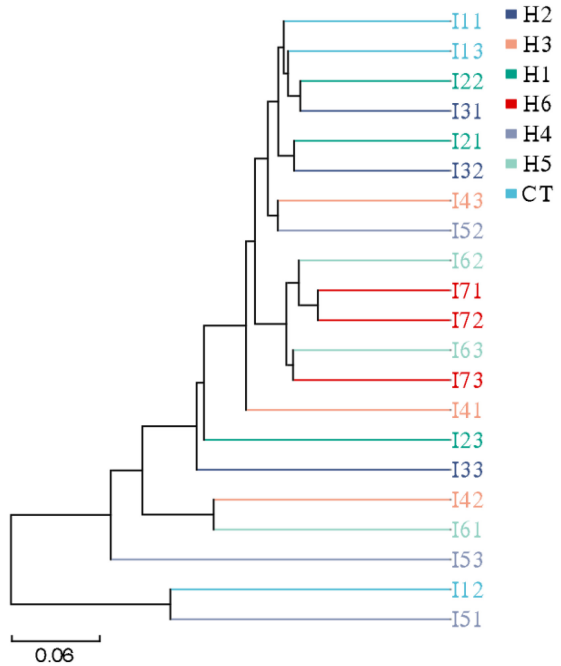

FIGURE 6 | Unweighted pair group method with arithmetic mean-clustering tree of juvenile pearl gentian hybrid grouper fed with different levels of HFP.

olivaceus) when fed with feed containing HFP with about 66.4% small peptides with a size between 100 and 1,000 Da. Sources of hydrolysates and small peptide-sized molecules may have influenced these results. Fish fed with different levels of HFP obtained significantly higher crude protein levels compared to those fed the control diet. In aquatic animal nutrition, levels of TP have been regarded as an indicator of the health and physiological condition of an aquatic organism (Harikrishnan et al., 2003).

The *IGF-I* and *TOR* levels have been evaluated to correlate with growth in different aquatic animals, such as European sea bass (*Dicentrarchus labrax*) (Carnevali et al., 2006), tilapia (*O. niloticus*) (Yan et al., 2013), juvenile turbot (*S. maximus*) (Wang et al., 2016), and pacific white shrimp (Liu et al., 2018). In this study, it was observed that hybrid grouper fed with different levels of HFP obtained a significantly higher total crude protein level compared to the control. A higher level of *IGF-I* and *TOR* mRNA gene expression in the intestine of hybrid grouper was also observed in fish fed with different levels of HFP compared to the control. Wei et al. (2020) also observed a higher *IGF-I* mRNA gene expression when FM was replaced with HFP in the diet of turbot. This could be due to better digestion and absorption of hydrolysate protein and signifies an improvement in general fish health (Khosravi et al., 2015).

The addition of suitable amounts of ingredients with small-sized peptides in feed increased digestive enzyme activity in the intestine of hybrid grouper (Yang et al., 2021), turbot (Jia et al., 2019), and sea bass (Zambonino Infante et al., 1997). Small-size peptide in HFP may play essential roles in regulating enzyme activity by promoting the secretion of digestive enzymes in aquatic animals (Madeira and Paula-Barbosa, 1999). In this study, intestinal trypsin and chymotrypsin were significantly high in the H5 group. These results show that 3% HFP in the diet of hybrid grouper could efficiently increase trypsin and chymotrypsin activity; hence, the presence of rich small-sized peptides could be linked to high levels of digestive enzymes.

The immunity and health of fish are greatly linked to the antioxidant defense system (Ahmadifar et al., 2019). Small-sized molecular peptides can improve and stimulate the capacity and activity of antioxidants in fish (Moure et al., 2006; Wu et al., 2018), and resistance in oxide damage is performed by antioxidant capacity (Lopes et al., 2001). The highest SOD activity in the serum was observed in the H6 group which contained 4% HFP. This shows that a 35% replacement ratio of dietary FM with HFP can increase the level of SOD in the serum (Moure et al., 2006).

Cytokines, which are small glycoprotein messengers, help in intercellular communication to support adaptive and innate immune responses against parasites, bacteria, and viruses (Bruce and Brown, 2017). Kotzamanis et al. (2007) stated that bioactive peptides with antibacterial and immunostimulating properties are produced during the procedure of hydrolysis. HFP used in this study is assumed to contain these peptides. *IL*-β is a pro-inflammatory cytokine in fish, which enhances lysozyme synthesis and defense mechanism with regard to bacterial colonization (Kim and Austin, 2006; Giri et al., 2015). *IL*-β was significantly higher in groups fed with different levels of HFP compared to the control. This was in agreement with Siddik et al. (2019a) who observed an upregulation of *IL*-β when HFP was included in the diet of juvenile barramundi. Results observed

TABLE 7 | Effect of hydrolyzed fish protein powder on the body composition of juvenile pearl gentian hybrid grouper.

Group	Moisture	Crude protein	Crude lipid	Ash	PPV (%)
CT	56.21 ± 0.94	66.17 ± 0.78^{ab}	21.03 ± 0.71	9.95 ± 0.74^{a}	35.26 ± 0.29
H1	56.75 ± 1.56	64.91 ± 0.39^{a}	20.19 ± 1.03	12.98 ± 0.58^{b}	37.29 ± 3.23
H2	53.11 ± 2.26	66.55 ± 1.01^{abc}	20.45 ± 0.36	12.96 ± 1.21^{b}	34.50 ± 0.12
H3	57.60 ± 1.65	68.50 ± 1.38^{abc}	21.96 ± 0.74	18.60 ± 0.38^{c}	35.15 ± 0.85
H4	56.92 ± 1.83	71.12 ± 1.03^{abc}	22.83 ± 0.99	13.60 ± 0.44^{b}	31.89 ± 0.91
H5	57.25 ± 1.16	72.51 ± 1.68^{bc}	20.90 ± 1.18	13.33 ± 0.29^{b}	27.82 ± 6.73
H6	55.25 ± 0.49	72.88 ± 3.03^{c}	20.38 ± 1.31	12.18 ± 0.06^{ab}	32.77 ± 0.51

Values are mean values of each group of hybrid grouper (three replicates) ± SE. Means in each row without superscript do not differ significantly ($p > 0.05$), while those with superscript differ significantly ($p < 0.05$). PPV, protein production value.

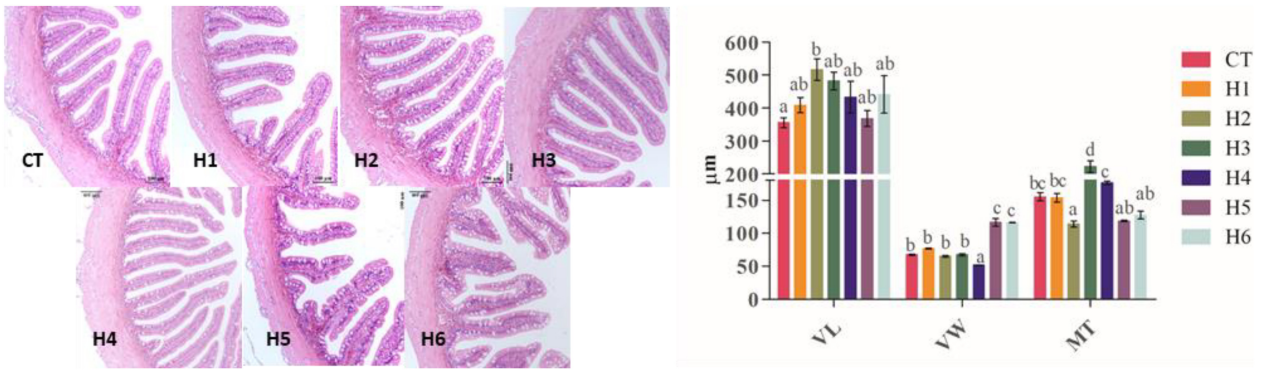

FIGURE 7 | Effect of HFP on the intestinal morphology of juvenile pearl gentian hybrid grouper (H&E, 20×). VL, villus length; VW, villus width; MT, muscle thickness.

in this study could be due to the sufficient content of bioactive peptides in HFP used (Tang et al., 2008; Bui et al., 2014).

Fish gut comprises different groups of microbial communities, including virus, protists, fungi, and bacteria. The bacterial community, which is the dominant group found in the intestine, affects the immune system, metabolism, and health (Wardwell et al., 2011; Tran et al., 2018). The intestinal bacteria community can be influenced by feed composition (Merrifield et al., 2009). The results of this study were not entirely different from Kuebutornye et al. (2020) and Amoah et al. (2021) in tilapia (*O. niloticus*) and northern whiting fish (*Sillago sihama*), respectively. They noted that prevalent bacteria phyla found in the gut of fishes include *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria*. At this level, *Proteobacteria* were the most abundant bacteria which increased and later decreased with increasing HFP inclusion in this study.

In this study, VL was significantly affected by HFP with the highest value observed when 1.5% HFP was used to replace FM. Improvement in the intestinal morphology, thus VL, is a positive indication of the fish's ability to digest feed and absorb nutrients in the digestive canal (Dimitroglou et al., 2009; Tan et al., 2018). Villi growth and intestinal digestive enzymes are effectively stimulated by small peptides (Zhang et al., 2017; Jia et al., 2019). The intestinal surface available for nutrient absorption can be expanded by VL and VW, while the efficiency of nutrient absorption is determined by MT in the intestine (Geda et al., 2012; Lauriano et al., 2016).

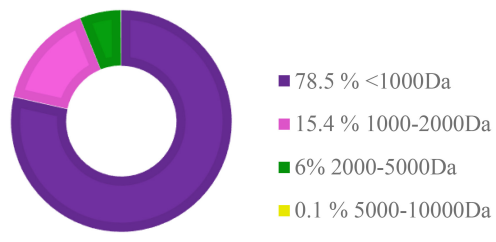

FIGURE 8 | The molecular weight distribution of peptides in hydrolyzed fish protein powder (HFP). Da, Dalton.

CONCLUSION

The addition of HFP to a low FM diet of hybrid grouper will increase the activity of intestinal trypsin and chymotrypsin and the deposition of crude protein and ash in hybrid groupers. An improvement in antioxidant capacity and the development of the intestine in hybrid grouper fed with different levels of HFP was observed. A 5% replacement ratio using 1% HFP is suggested in the diet of hybrid grouper due to a higher WGR compared to the other groups containing HFP.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Review Board of Guangdong Ocean University.

AUTHOR CONTRIBUTIONS

SC aided in the experimental design, fund acquisition, supervision of the project, and review and editing of the manuscript. VH conducted the study, analyzed the data, and drafted the original manuscript. BT and TL aided in funding acquisition. HL, QY, XD, SZ, JW, and ZC aided in the experimental design. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: JW, TL, and ZC were employed by Guangdong XIPU Biotechnology Co., Ltd.

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