Dietary lactoferrin supplementation improves growth performance and intestinal health of juvenile orange-spotted groupers (Epinephelus coioides)

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Abstract

A 56-day feeding trial was conducted to investigate the effects of dietary lactoferrin (LF) supplementation on growth performance and intestinal health of juvenile orange-spotted groupers (*Epinephelus coioides*) fed high soybean meal (SBM) diets. The control diet (FM) and high soybean meal diet (SBM60) were prepared to contain 48% protein and 11% fat. Three inclusion levels of 2, 6, and 10 g/kg LF were added into the SBM diets to prepare three experimental diets (recorded as LF2, LF6, and LF10, respectively). The results showed that supplementation of LF in SBM diets increased growth rate in a dose dependent manner. However, feed utilization, hepatosomatic index, whole-body proximate composition and the abundance and diversity of intestinal microbiota did not vary across dietary treatments ($P > 0.05$). After dietary intervention with LF, the contents of intestinal malondialdehyde, endotoxin, and D-lactic acid, as well as plasma low density lipoprotein cholesterol, high density lipoprotein cholesterol and total cholesterol were lower, and the intestinal activities of glutathione peroxidase, lipase, trypsin, and protease were higher in LF2-LF10 groups than that in SBM60 group ($P < 0.05$). Supplementation of LF in SBM diets increased the muscle layer thickness of middle and distal intestine and the mucosal fold length of middle intestine vs SBM60 diet. Furthermore, supplementation of LF in SBM diets resulted in an up-regulation of the mRNA levels for *IL-10* and *TGF-β1* genes and a down-regulation of the mRNA levels of *IL-1β*, *IL-12*, *IL-8*, and *TNF-α* genes vs SBM60 diet ($P < 0.05$). The above results showed that dietary LF intervention could improve the growth and alleviate soybean meal induced enteritis in juvenile orange-spotted groupers. The dietary appropriate level of LF was at 5.8 g/kg, through the regression analysis of percent weight gain against dietary LF inclusion levels.

Introduction

Fish meal (FM) has always been the main important protein source in the feeds of farmed marine fish due to its high-quality protein, balanced amino acids profile, and less anti-nutritional factors (ANFs) in comparison with terrestrial animals and plant protein sources [1]. However, over the past three decades, the stagnant global marine fishery catches have also led to stagnant FM production due to the shift of climate change [2]. At the same time, the rapid expansion of aquaculture industry has stimulated a huge demand FM, resulting in a sustained shortage of FM supply [3]. Therefore, research on FM replacement with other protein sources has always been one of the most priority issues in aquafeeds. Soybean meal (SBM), as the major plant protein source, is widely used in aquafeeds due to its relatively balanced amino acids profile, huge output and availability, and reasonable price [1, 4, 5]. However, when SBM is used in aquafeeds in a large proportion, its side effects are also considerable. It is well known that SBM can cause the reduction of the ability of fish to digest and absorb nutrients, due to many ANFs that induce enteritis [6, 7], the so-called soybean meal induced enteritis (SBMIE)[8]. Therefore, how to prevent and control the widespread SBMIE is the key to maintain normal daily fish culture and reduce disease risk.

In the last two decades, a great deal of research effort has been devoted to alleviating or/and preventing SBMIE. An effective strategy to counteract fish intestinal inflammation is the dietary use of functional substances that exert metabolic regulation, antioxidant properties and immune promotion [5, 9–12]. Lactoferrin (LF), a glycoprotein with a molecular weight of 78–80 kDa is rich in milk [13], and has many biological functions [14, 15]. Previous study showed that dietary LF supplementation could enhance the growth performance of early-weaned piglets by promoting the proliferation of intestinal beneficial bacteria such as lactic acid bacteria, inhibiting the proliferation of *E.coli*, and improving the intestinal mucosal morphological structure and intestinal function [16]. Dietary LF supplementation could improve the growth performance of early-weaned piglets and zebrafish by reducing pathogenic bacteria and diarrhea, enriching beneficial bacteria and affecting intestinal morphology [14, 15, 17]. LF was found to inhibit the activation of TOR signaling pathway induced by lipopolysaccharide (LPS), thereby inhibiting the production of pro-inflammatory factors such as *IL-1β* and *IL-8* or/and indirectly promoting the production of anti-inflammatory factors such as *IL-10* in terrestrial animals [18]. However, little is known about dietary LF intervention effect on the improvement of SBMIE in farmed fish.

Orange-spotted grouper (*Epinephelus coioides*), a marine economic carnivorous fish, which is widely cultured in Southeast Asian countries including China [19, 20]. This fish has become the third largest mariculture fish in China, with an annual output of 192,045 tons in 2020 [21]. Although there are many reports about its nutrition, feed research and development [20, 22–24], there is still a lack of nutritional regulation research on the prevention of SBMIE of the fish species. The recent studies also showed that high SBM diet caused juvenile orange-spotted grouper enteritis [1, 4]. Therefore, in this study, the growth performance, intestinal health and prevention or/and control of SBMIE were investigated to evaluate the effects of dietary LF supplementation in juvenile *E. coioides* fed high SBM diets. For this purpose, five experiment diets were prepared, including the FM diet, SBM60 diet without LF, and three SBM60 diets with LF at inclusion levels of 2, 6, and 10 g/kg. This study provided a new technical reference for the prevention of SBMIE in the fish species.

Materials And Methods

Experimental Diets

A control diet (FM) was formulated using FM, casein, and gelatin as the protein sources and fish oil, soybean oil, and soybean lecithin as the lipid sources to contain 48% crude protein and 11% crude lipid (Table 1). On the basis of the FM diet, SBM was used to replace 60% FM protein to prepare a high SBM diet (SBM60). LF was then added to SBM60 diets at 2, 6, and 10 g/kg to prepare another three experimental diets (LF2, LF6, and LF10 respectively), according to a previous study [16]. The coarse dry feed ingredients were pulverized using a grinder (ZFJ-300, Jiangyin Ruizong Machinery Manufacturing Co., Ltd, Jiangyin, Jiangsu, China) and sifted through a 60-mesh sieve (250 μm particle size), weighed and homogenized. The liquid ingredients (water, soybean oil, fish oil and soy lecithin) were then added to the dry feed ingredients and a mash was prepared. This dough was extruded into strands and pelletized through 2.5 mm and 4 mm die using a double-helix rod feed extruder (F-76, Guangzhou Huagong Optical Mechanical and Electrical Technology Co., Ltd, Guangzhou, Guangdong, China) and a feed pellet shaping machine (GY-500, Changzhou Beicheng Drying Equipment Engineering Co., Ltd, Changzhou, Jiangsu, China). The pellets were dried in a ventilated oven at 55 °C for 24 h to reduce the moisture of the feed to less than 100 g/kg, and then stored at room temperature for 24 h, before being sealed in plastic bags and stored in a refrigerator at -20 °C.

Table 1

Formulations and nutrient levels of the experimental diets (on an as-fed basis, g/kg)

Items	Diets [1]				
	FM	SBM60	LF2	LF6	LF10
Ingredients					
Fish meal 2	520.0	220.0	220.0	220.0	220.0
Casein	119.8	112.7	112.7	112.7	112.7
Gelatin	30.0	28.2	28.2	28.2	28.2
Soybean meal 3	—	470.0	470.0	470.0	470.0
Soybean oil	35.0	35.0	35.0	35.0	35.0
Fish oil	8.2	35.2	35.2	35.2	35.2
Soybean lecithin	20.0	20.0	20.0	20.0	20.0
Lactoferrin 4	—	—	2.0	6.0	10.0
Corn starch	177.2	32.6	30.6	26.6	22.6
Sodium alginate	10.0	10.0	10.0	10.0	10.0
Ca(H2PO4)2	15.0	15.0	15.0	15.0	15.0
Choline chloride	4.0	4.0	4.0	4.0	4.0
Stay-C (350 g/kg)	0.3	0.3	0.3	0.3	0.3
Vitamin premix 5	4.0	4.0	4.0	4.0	4.0
Mineral premix 5	5.0	5.0	5.0	5.0	5.0
Taurine	5.0	8.0	8.0	8.0	8.0
Microcrystalline cellulose	46.5	—	—	—	—
Total	1000.0	1000.0	1000.0	1000.0	1000.0
Nutrient level (analyzed values)					
Dry matter	950.6	957.8	955.6	952.7	954.7
Crude protein	480.5	503.4	517.2	513.3	515.0
Crude lipid	120.6	114.2	116.3	116.4	116.3
Ash	91.7	84.2	84.8	84.0	82.9

[1] FM, fish meal diet (control diet); SBM60, high soybean meal diet with 60% fish meal protein replacement and without LF supplementation; LF2, LF6, and LF10 were added 2, 6, and 10 g/kg LF in SBM60 diets, respectively.

[2] Fish meal was obtained from Austral Group S.A.A.Peru. (crude protein 703.4 g/kg, crude lipid 90.6 g/kg).

[3] Soybean meal was obtained from Jiakang Feed Co.,Ltd. (crude protein 466.0 g/kg, crude lipid 7.2 g/kg).

[4] Lactoferrin (LF, food grade) was obtained from Fujian Furun Pharmaceutical Co.,Ltd.

[5] The vitamin and mineral premixes were obtained from Guangzhou Feixite Aquatic Technology Co.,Ltd. Vitamin premix (per kg diet): VA, 10 mg; VD, 10 mg; VE, 100 mg; VB_1, 10 mg; VB_2, 20mg; VB_6, 20 mg; VB_{12}, 0.05 mg; nicotinic acid, 50 mg; calcium-D-pantothenate, 100 mg; D-biotin, 1 mg; *meso*-inositol, 500 mg; folic acid, 4 mg. Mineral premix (per kg diet): ferric citrate, 497 mg; $CuSO_4 \cdot 5H_2O$, 24 mg; $ZnSO_4 \cdot 7H_2O$, 176 mg; $MnSO_4 \cdot 4H_2O$, 122 mg; $CoCl_2 \cdot 6H_2O$, 0.18 mg; KIO_3, 0.51 mg; Na_2SeO_3, 0.33 mg.

Feeding Trial

This experiment was conducted at Fujian Dabeinong Fisheries Technology Company (Zhaoan County, Zhangzhou city, Fujian, China). Prior to the trial, orange-spotted grouper juveniles were kept in a concrete pond and fed with diet FM for a 2-week acclimatization. At the beginning of the experiment, 450 orange-spotted grouper juveniles (initial average weight of 33.82 ± 0.03 g) were randomly distributed into 15 blue polypropylene tanks (500 L/tank), at a density of 30 fish per tank in a water temperature-controlled recirculating culture system. Groups of triplicate tanks were hand-fed one of the diets to apparent satiation twice daily (8:00 and 17:00) under a natural photoperiod across a feeding period 56-day. Excess feed was collected 30 min after each meal, and dried for 24 h at 65 ℃ and weighed for the calculation of feed intake. Because of the water loss of the aquaculture system caused by daily sewage discharge, fresh sea

water was refilled until the original water level of tanks was reached. During the experimental period, water salinity ranged from 32 to 36, temperature was about 28.5 ℃, and dissolved oxygen level was > 5.7 mg/L.

Sample Collection and Chemical Analysis

At the end of the growth trial, fish in each tank were caught and anaesthetized with a dose of 100 mg/L solution of MS-222 (tricaine methane sulphonate, Sigma-Aldrich Shanghai Trading Co. Ltd., Shanghai, China). Fish weight and number were then recorded for each tank to measure weight gain (WG), feed efficiency (FE), specific growth rate (SGR), and survival. Three fish from each tank were randomly sampled and pooled in plastic bags, and stored at -20 ℃ for whole-body proximate composition determination. Nine fish per tank (27 fish each group) were weighed individually after anaesthesia with MS-222 (100 ml/L) to calculate the hepatosomatic index (HSI) and condition factor (CF). Blood was drawn from the caudal vein, using 1-mL heparinized syringe, and centrifuged at 1027 × *g*, 4 ℃, 10 min. Plasma was then collected, pooled by tank and stored in 1.5-mL Eppendorf tubes at -80 ℃ for the subsequent biochemical analysis. The intestine of nine fish per tank were aseptically removed and pooled into one tube by tank, stored at -80 ℃ for the analysis of biochemical components, microbiota analysis, and gene expression.

Prior to component analysis, whole-fish samples were prepared according to the method described by Ye et al.[25]. The proximate composition of diet and whole-body fish samples were determined according to standard methods [26]. Dry matter was determined by drying the samples in an oven at 105 ℃ to a constant weight. Crude protein was determined by the Kjeldahl method (N × 6.25) using Kjeltec TM 8400 Auto Sample Systems (Foss Teacher AB). The crude lipid content was determined by the Soxtec extraction method by using Soxtec Avanti 2050 (Foss Teacher AB). Ash was measured in the residues of samples burned in a muffle furnace at 550 ℃ for 8 h.

The total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) contents in plasma samples, the contents of diamine oxidase (DAO), d-lactic acid (D-Lac), endotoxin (ET), endothelin-1 (ET-1), glutathion peroxidase (GSH-Px), superoxide dismutase (SOD), total antioxidant capacity (T-AOC), and malondialdehyde (MDA) in the intestinal samples were determined using commercial kits (Nanjing jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) according to the manufacturer's instructions.

Intestinal Histology Observation

One fish was caught from each tank and dissected to obtain the whole gut, then divided into proximal, middle, and distal intestine (i.e. PI, MI, and DI, respectively), according to the method described by Anguiano et al.[27]. All the segments were washed with normal saline, fixed in Bouin's solution for 24 h, rinsed with 70% ethanol solution, and finally immersed in 70% ethanol until histological processing was performed [19]. The fixed gut segments were embedded in paraffin and 5-μm sections were cut by using a rotary microtome (KD-2258S, China). The serial histological sections were then mounted on glass slides, and stained with hematoxylin and eosin for morphometric analysis. Pictures were examined under a light microscope (Leica DM5500B, Germany), and digital images were taken and processed with a digital camera (Leica DFC450) equipped with the image program LAS AF (Version 4.3.0 Leica). Five slides were prepared for each gut segment sample and 30 measurements were made to determine the number of mucosal folds, muscle layer thickness, and length of the complete mucosal fold.

Intestinal Microbiota Analysis

The total DNA in the distal intestine (DI) of juvenile orange-spotted groupers were extracted using a DNA extraction kit (Omega Bio-teK, Norcross, GA, USA) according to the manufacturer's instructions. The integrity and quality, purity and quantity of DNA samples were assessed by electrophoresis on a 1% (w/v) agarose gel and spectrophotometer method (NanoDrop 2000, Wilmington, DE, U.S. 260nm/280nm optical density ratio), respectively. The V3-V4 region of the 16S rDNA gene of DI bacterial was amplified by polymerase chain reaction (PCR) using the forward primer 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and the reverse primer 806R (5'-GGACTACNNGGGTATCTAAT-3'). The PCR reaction system included pre-denaturation at 95 ℃ for 5 min; denaturation at 95 ℃ for 45 s, annealing at 55 ℃ for 50 s, and extension at 72 ℃ for 45 s, 32 cycles; extension at 72 ℃ for 10 min. Subsequently, high-throughput sequencing was performed using Illumina Miseq PE300 at Beijing Allwegene technology Co., Ltd (Beijing, China). A library of small fragments was constructed using paired-end for sequencing, and the data was passed through QIIME (v1.8.0) for removal low-quality sequences and chimeras. Based on 97% sequence similarity, similar sequences were assigned to the same operational taxonomic units (OTU). Species classification information corresponding to each OTU was obtained by comparing with the sliva database, and alpha diversity analysis (Shannon, ACE, and Chao1) was performed using Mothur software (version 1.31.2). Based on the weighted unifrace distance, the pheatmap of R (v3.1.1) software package was used for clustering analysis. After the UniFrac algorithm, the information of system evolution was used to compare the difference of species communities among samples and Beta diversity analysis was performed.

RNA Extraction and Gene Expression

The total RNA was extracted from the intestinal samples using TRIzol® reagent (Takara Co., Ltd., Japan) according to the manufacturer's instructions. Isolated RNA was quantified using the NanoDrop ND-2000 Spectrophotometer, and its integrity was confirmed by agarose gel electrophoresis. The cDNA was generated from 1 μg DNase-treated RNA and synthesized by a PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara Co., Ltd., Japan). Real-time PCR was employed to determine mRNA levels based on the TB Green™ Premix Ex Taq™ Ⅱ (Tli RNaseH Plus) (Takara Co., Ltd, Japan) using a QuantStudio™ Real-Time PCR System (ABI) quantitative thermal cycler. The fluorescent quantitative PCR solution consisted of 10 μL TB Green Premix Ex Taq™ Ⅱ (Tli RNaseH Plus) (2×), 0.8 μL PCR forward primer (10 μM), 0.8 μL PCR reverse primer (10 μM), 2.0 μL RT reaction (cDNA solution), and 6 μL dH_2O. The thermal program included 30 s at 95 ℃, 40 cycles at 95 ℃ for 5 s, and 60 ℃ for 30 s. The sequences of primers are showed in Table 2. All amplicons were initially separated by agarose gel eletrophoresis to ensure that they were of the correct size. β-actin served as the internal reference gene to normalize cDNA loading. The gene expression levels of the target genes were analyzed by the $2^{-\Delta\Delta Ct}$ method [28] after verifying that the primers were amplified with an efficiency of approximately 100% [29], and the data for all treatment groups were compared with the data for the control group.

Table 2

Primers sequences for lipid related genes and reference genes used for real-time PCR of juvenile orange-spotted groupers.

Genes [1]	Primer sequence (5' to 3') [2]	E-value (%)	Accession number
IL-8	F: AAGTTTGCCTTGACCCCGAA	94.0	FJ913064.1
	R: TGAAGCAGATCTCTCCCGGT		
IL-1β	F: GCAACTCCACCGACTGATGA	116.0	EF582837.1
	R: ACCAGGCTGTTATTGACCCG		
IL-10	F: GTCCACCAGCATGACTCCTC	99.0	KJ741852.1
	R: AGGGAAACCCTCCACGAATC		
TGF-β1	F: GCTTACGTGGGTGCAAACAG	102.0	GQ503351.1
	R: ACCATCTCTAGGTCCAGCGT		
IL-12	F: CCAGATTGCACAGCTCAGGA	115.0	KC662465.1
	R: CCGGACACAGATGGCCTTAG		
TNF-α	F: GGATCTGGCGCTACTCAGAC	91.0	FJ009449.1
	R: CGCCCAGATAAATGGCGTTG		
β-actin	F: TGCTGTCCCTGTATGCCTCT	104.0	AY510710.2
	R: CCTTGATGTCACGCACGAT		

[1] IL-8, interleukin-8; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; IL-12, interleukin-12; TGF-β1, transforming growth factor-β1; IL-10, interleukin-10.

[2] F, forward; R, reverse.

Statistical Analysis

All data were presented as mean and standard error of the mean (SEM). The data were analyzed using a one-way analysis of variance (ANOVA) to test for differences between treatments and then the Student-Neuman-Keuls multiple comparison test was performed after confirming the normality and homogeneity of variance using the Kolmogorov-Smirnov test and Levene's test in SPSS Statistics 25.0 (SPSS, Michigan Avenue, Chicago, IL, USA). The data expressed as percentages or ratios were subjected to data conversion prior to statistical analysis. P-values < 0.05 was deemed as significant difference.

Results

Growth Performance and Whole-body Proximate Composition

The results of growth performance and whole-body proximate composition are presented in Table 3. The diets SBM60 had lower WG and SGR compared with diets FM ($P < 0.05$), but diets LF2-LF10 showed improved WG and SGR vs diets SBM60 ($P < 0.05$), and returned to the level of diets FM ($P > 0.05$). The WG and FE were in a dose-dependent relationship with the dietary LF inclusion levels (Fig. 1). The maximum WG and FE were observed for the diet LF6 and diet LF10, respectively. However, there were no differences in FE, HSI, CF, survival and whole-body proximate composition among dietary treatments ($P > 0.05$).

Intestinal Antioxidant Capacity

As shown in Table 4, FM group had higher intestinal GSH-Px activity and lower MDA content compared with SBM60 group ($P < 0.05$), but the values of the two parameters were not different between LF2-LF10 groups and FM group ($P > 0.05$). The MDA content showed a negative quadratic response to increasing dietary LF inclusion levels, a minimum value was observed for diet LF6. However, dietary treatments did not affect intestinal SOD and T-AOC activities ($P > 0.05$).

Table 3

Effects of lactoferrin (LF) supplementation in high SBM diets on growth performance and whole-body proximate composition of juvenile orange-spotted groupers in a 56-d feeding period. [1]

Items	Diets [2]				
	FM	SBM60	LF2	LF6	LF10
Growth performance					
IBW (g/fish) [3]	33.82±0.10	33.76±0.06	33.74±0.05	33.80±0.07	33.98±0.01
FBW (g/fish) [3]	113.24±0.66[b]	95.47±2.59[a]	107.49±4.28[b]	109.85±3.46[b]	106.65±2.96[b]
WG (%) [3]	234.81±2.54[b]	182.81±7.24[a]	218.56±12.96[b]	224.98±9.89[b]	208.68±5.73[ab]
SGR (%/d) [3]	2.16±0.01[b]	1.86±0.05[a]	2.07±0.07[b]	2.10±0.05[b]	2.04±0.05[b]
FE [3]	0.98±0.00	0.95±0.00	0.96±0.12	0.99±0.07	1.00±0.12
Survival (%) [3]	100.00±0.00	97.78±1.11	100.00±0.00	100.00±0.00	98.89±1.11
HSI (%) [4]	1.31±0.09	1.24±0.04	1.28±0.07	1.24±0.05	1.17±0.02
CF (g/cm^3) [4]	3.16±0.07	3.05±0.11	3.19±0.02	2.93±0.12	2.94±0.03
Proximate composition (%)					
Moisture	67.05±0.21	67.27±0.22	67.56±0.37	67.42±0.34	68.26±0.39
Crude protein	18.01±0.49	17.95±0.27	17.93±0.90	19.20±0.33	17.75±0.42
Crude lipid	8.25±0.17	7.90±0.29	8.18±0.40	7.85±0.32	7.49±0.11
Ash	5.00±0.15	4.96±0.07	4.90±0.06	4.87±0.22	4.93±0.09

[1]Statistical analysis was performed by one-way ANOVA, followed by S-N-K test.

[2] FM, fish meal diet (control diet); SBM60, high soybean meal diet with 60% fish meal protein replacement and without LF supplementation; LF2, LF6, and LF10 were added 2, 6, and 10 g/kg LF in SBM60 diets, respectively.

[3] Values are presented as the means ± SEM (n = 3 tanks)

[4] Values are presented as the means ± SEM (n = 27 fish)

Values in the same row with different superscripts indicate significant differences ($p < 0.05$), while that with the same letter or no letter superscripts indicate no significant differences ($p > 0.05$).

WG, weight gain (%) = 100 × (FBW - IBW)/IBW.

SGR, specific growth rate (%/d)= 100 × (lnFBW - lnIBW)/days.

FE, feed efficiency = 100 × (FBW - IBW)/FI (as fed basis, g/fish).

Survival (%) = 100 × FN/IN.

HSI, hepatosomatic index (%) = 100 × LW/BW.

CF, condition factor (g/cm^3) = 100 × $BW/(BL)^3$

Abbreviations: IBW, initial body weight (g/fish); FBW, final body weight (g/fish); FI, feed intake (g/fish); FN, final number; IN, initial number; LW, liver weight (g/fish); BW, body weight (g/fish); BL, body length (cm/fish).

Table 4

Effect of lactoferrin (LF) supplementation in high SBM diets on intestinal antioxidant indices of juvenile orange-spotted groupers in a 56-day feeding period. [1]

Items	Diets [2]				
	FM	SBM60	LF2	LF6	LF10
SOD (U/mg protein)	71.67±5.50	68.92±1.76	62.16±5.85	64.26±3.23	60.70±2.15
GSH-Px (U/mg protein)	79.58±3.31^{bc}	65.29±2.97^{a}	82.52±1.76^{bc}	72.99±1.35^{b}	86.76±4.00^{c}
T-AOC (U/mg protein)	0.19±0.01	0.19±0.01	0.21±0.01	0.19±0.02	0.18±0.01
MDA (nmol/mg protein)	3.00±0.28^{a}	4.56±0.88^{b}	1.97±0.21^{a}	1.86±0.12^{a}	2.55±0.05^{a}

$Y_{MDA} = 0.0783X^2 - 0.9351X + 4.2006$, $R^2 = 0.8297$, X = LF supplementation levels (g/kg)

[1] Data were presented as means ± SEM (n = 3 tanks). Statistical analysis was performed by one-way ANOVA, followed by S-N-K test.

[2] FM, fish meal diet (control diet); SBM60, high soybean meal diet with 60% fish meal protein replacement and without LF supplementation; LF2, LF6, and LF10 were added 2, 6, and 10 g/kg LF in SBM60 diets, respectively.

Values in the same row with different superscripts indicate significant differences ($P < 0.05$), while that with the same letter or no letter superscripts indicate no significant differences ($P > 0.05$).

Abbreviations: T-AOC, total antioxidant capacity; SOD, superoxide dismutase; MDA, malondialdehyde; GSH-Px, glutathion peroxidase.

Plasma Components

Table 5 shows that plasma HDL-C content had an irregular change with increasing dietary LF inclusion levels, and the value in LF6 group was similar ($P > 0.05$) to that of SBM60 and FM groups but was higher than that of LF2 and LF10 groups ($P < 0.05$). FM group had higher plasma LDL-C content ($P < 0.05$) and comparable plasma TC content compared with SBM60 group ($P > 0.05$), but plasma TC content was not different between LF2-LF10 groups and SBM60 group ($P > 0.05$), while SBM60 and LF2 groups had higher plasma LDL-C contents ($P < 0.05$) vs LF6 and LF10 groups. Both plasma LDL-C and TC contents showed a linear decreasing trend with the dietary increase of LF inclusion levels, and reached the minimum values at LF6 and LF10, respectively. However, plasma TG content did not differ across all dietary treatments ($P > 0.05$).

Intestinal Digestive Enzyme Activity

As shown in Table 6, intestinal lipase and protease activities were comparable and intestinal trypsin activity was lower in SBM60 group than that in FM group ($P < 0.05$). The intestinal trypsin and lipase activities were higher in LF2-LF10 groups than that in SBM60 group ($P < 0.05$) and returned to the level of FM group and even higher than that of FM group. The intestinal activities of lipase, trypsin, and protease showed a linear increasing trend with increasing dietary LF inclusion levels. However, the intestinal amylase activity was not affected by dietary treatments ($P > 0.05$).

Intestinal Permeability

Table 7 shows that intestinal D-Lac and ET contents were higher in SBM60 group than that in FM group ($P < 0.05$). However, intestinal D-Lac and ET contents were reduced when SBM60 diet with LF supplementation ($P < 0.05$), and the values in fish receiving SBM60 diets with LF supplementation returned to the level and even lower than that of FM group. Intestinal D-Lac and ET contents did not differ with dietary LF levels from 2 to 10 g/kg ($P > 0.05$). Both intestinal ET-1 and DAO contents were not affected by dietary treatments ($P > 0.05$).

Table 5

Effect of lactoferrin (LF) supplementation in high SBM diets on plasma components of juvenile orange-spotted groupers in a 56-day feeding period. [1]

Items	Diets [2]				
	FM	SBM60	LF2	LF6	LF10
HDL-C (mmol/L)	1.06±0.05^{b}	1.00±0.03^{b}	0.81±0.05^{a}	1.03±0.03^{b}	0.83±0.09^{a}
LDL-C (mmol/L)	0.28±0.01^{c}	0.19±0.01^{b}	0.18±0.01^{b}	0.12±0.01^{a}	0.12±0.01^{a}
TC (mmol/L)	3.77±0.21^{b}	3.49±0.23^{ab}	3.35±0.19^{ab}	3.09±0.09^{ab}	2.90±0.09^{a}
TG (mmol/L)	1.61±0.17	1.36±0.08	1.25±0.06	1.57±0.10	1.55±0.07

$Y_{LDL\text{-}C} = -0.0079X + 0.188$, $R^2 = 0.8573$, X = LF supplementation levels (g/kg)

$Y_{TC} = -0.0592x + 3.4741$, $R^2 = 0.9931$, X = LF supplementation levels (g/kg)

[1] Data were presented as means ± SEM (n = 3 tanks). Statistical analysis was performed by one-way ANOVA, followed by S-N-K test.

[2] FM, fish meal diet (control diet); SBM60, high soybean meal diet with 60% fish meal protein replacement and without LF supplementation; LF2, LF6, and LF10 were added 2, 6, and 10 g/kg LF in SBM60 diets, respectively.

Values in the same row with different superscripts indicate significant differences ($P < 0.05$), while that with the same letter or no letter superscripts indicate no significant differences ($P > 0.05$).

Abbreviations: TG, triglyceride; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

Table 6

Effect of lactoferrin (LF) supplementation in high SBM diets on activities of intestinal digestive enzymes of juvenile orange-spotted groupers in a 56-day feeding period. [1]

Items (U/mg protein)	Diets [2]				
	FM	SBM60	LF2	LF6	LF10
Lipase	0.68±0.00[a]	0.61±0.00[a]	0.84±0.05[b]	0.86±0.02[b]	0.95±0.05[bc]
Amylase	0.76±0.06	0.73±0.11	0.90±0.04	0.73±0.07	0.82±0.06
Trypsin	256.07±17.23[b]	175.55±17.55[a]	238.95±17.46[b]	235.03±9.36[b]	283.57±8.83[bc]
Protease	20.54±0.87	15.91±2.04	17.37±2.91	23.54±2.56	26.54±2.53

$Y_{Lipase} = 0.0283X + 0.6876$, $R^2 = 0.7515$, X = LF supplementation levels (g/kg)

$Y_{Trypsin} = 8.8954X + 193.25$, $R^2 = 0.7917$, X = LF supplementation levels (g/kg)

$Y_{Protease} = 1.1231X + 15.786$, $R^2 = 0.9775$, X = LF supplementation levels (g/kg)

[1] Data were presented as means ± SEM (n = 3 tanks). Statistical analysis was performed by one-way ANOVA, followed by S-N-K test.

[2]FM, fish meal diet (control diet); SBM60, high soybean meal diet with 60% fish meal protein replacement and without LF supplementation; LF2, LF6, and LF10 were added 2, 6, and 10 g/kg LF in SBM60 diets, respectively.

Values in the same row with different superscripts indicate significant differences ($P < 0.05$), while that with the same letter or no letter superscripts indicate no significant differences ($P > 0.05$).

Table 7

Effect of lactoferrin (LF) supplementation in high SBM diets on the biochemical indices of intestinal mucosal permeability of juvenile orange-spotted groupers in a 56-day feeding period. [1]

Items	Diets [2]				
	FM	SBM60	LF2	LF6	LF10
DAO (U/L)	19.75±1.39	20.59±1.05	16.09±1.22	17.77±2.40	17.72±1.47
D-Lac (nmol/mL)	2.03±0.20[a]	4.05±0.23[b]	1.41±0.18[a]	1.59±0.21[a]	1.41±0.07[a]
ET-1 (ng/L)	1.91±0.07	2.12±0.09	2.27±0.10	1.92±0.13	2.36±0.20
ET (EU/L)	1.51±0.03[b]	1.70±0.10[c]	1.23±0.01[a]	1.25±0.01[a]	1.25±0.04[a]

[1] Data were presented as means ± SEM (n = 3 tanks). Statistical analysis was performed by one-way ANOVA, followed by S-N-K test.

[2] FM, fish meal diet (control diet); SBM60, high soybean meal diet with 60% fish meal protein replacement and without LF supplementation; LF2, LF6, and LF10 were added 2, 6, and 10 g/kg LF in SBM60 diets, respectively.

Values in the same row with different superscripts indicate significant differences ($P < 0.05$), while that with the same letter or no letter superscripts indicate no significant differences ($P > 0.05$).

Abbreviations: DAO, diamine oxidase; D-lac, d-lactic acid; ET, endotoxin; ET-1, endothelin-1.

Intestinal Histomorphology

Table 8 shows the effects of dietary treatments on the mucosal fold number (nMF), muscle layer thickness (tML), and mucosal fold length (lMF) in the three intestinal segments (PI, MI, and DI). The nMF of the PI, MI, and DI, the lMF of PI and DI, and the tMF of PI remained unaffected by dietary treatments ($P > 0.05$), but the tML of DI showed a positive quadratic response to increasing dietary LF inclusion levels, with a maximum value observed for diet LF6. Diets LF2 to LF10 displayed higher ($P < 0.05$) tML of DI vs diet SBM60, and the value returned to the level of diet FM ($P > 0.05$). The lMF and tML of MI had an irregular change in response to the dietary LF inclusion levels, but the maximum values observed all for diet LF2.

Table 8

Effect of lactoferrin (LF) supplementation in high SBM diets on the intestinal morphology of juvenile orange-spotted groupers in a 56-day feeding period. [1]

Items	Diets [2]				
	FM	SBM60	LF2	LF6	LF10
PI					
lMF (μm)	577.30±87.68	489.10±54.31	574.92±35.62	513.26±50.67	737.53±95.20
tML (μm)	63.24±6.74	64.56±8.11	79.05±2.27	86.60±9.31	86.00±2.51
nMF (unit)	42.50±4.25	45.83±3.09	51.67±1.36	50.33±8.62	48.00±3.55
MI					
lMF (μm)	465.12±50.20^{ab}	381.90±42.42^{a}	580.47±9.06^{b}	356.66±9.37^{a}	540.48±47.06^{b}
tML (μm)	53.53±2.44^{ab}	44.96±4.06^{a}	76.61±7.02^{b}	63.13±4.61^{ab}	69.62±7.81^{b}
nMF (unit)	34.33±2.20	31.67±1.01	43.00±4.36	34.83±3.49	39.17±0.33
DI					
lMF (μm)	417.87±63.72	337.13±44.48	437.82±22.32	397.03±4.38	466.67±53.64
tML (μm)	87.58±7.61^{b}	51.53±1.48^{a}	74.80±3.34^{b}	86.49±1.35^{b}	69.78±7.20^{b}
nMF (unit)	32.00±5.20	37.00±4.00	40.83±5.33	35.00±1.04	34.00±3.21

DI: $Y_{tML} = -1.0492X^2 + 12.167X + 52.619$, $R^2 = 0.9884$, X = LF supplementation levels (g/kg)

[1] Data were presented as means ± SEM (n = 3 tanks). Statistical analysis was performed by one-way ANOVA, followed by S-N-K test.

[2] FM, fish meal diet (control diet); SBM60, high soybean meal diet with 60% fish meal protein replacement and without LF supplementation; LF2, LF6, and LF10 were added 2, 6, and 10 g/kg LF in SBM60 diets, respectively.

Values in the same row with different superscripts indicate significant differences ($P < 0.05$), while that with the same letter or no letter superscripts indicate no significant differences ($P > 0.05$).

Abbreviations: PI, proximal intestine; MI, middle intestine; DI, distal intestine; nMF, mucosal fold number; tML, muscle layer thickness; lMF, mucosal fold length.

Abundance and Difference of Intestinal Microbiota

The Firmicutes, Bacteroidetes, and Proteobacteria in DI were the dominant phyla of all the dietary treatments (Fig. 2A). Compared with diets FM, the abundance of Firmicutes was increased and the abundances of Bacteroidetes and Proteobacteria were decreased by diets SBM60. The abundances of Firmicutes and Bacteroidetes generally increased, but the abundance of Proteobacteria decreased with increasing dietary LF levels supplementation in SBM60 diets (Fig. 2A and Supplementary Table 1). However, no significant differences in the abundances of dominant phyla were observed between dietary treatments at the phylum level ($P > 0.05$).

At the genus level, the DI bacteria of all dietary treatments mainly contained genera *Photobacterium, Selenomonas_1*, *Prevotella_1*, *Vibrio*, and *Rikenellaceae_RC9_gut_group* (Fig. 2B and Supplementary Table 1). The decreased abundances of genera *Photobacterium*, *Selenomonas_1*, and *Prevotella_1* and the increased abundances of genera *Vibrio* and *Rikenellaceae_RC9_gut_group* were observed in diets SBM60 vs diets FM. The abundances of genera *Selenomonas_1*, *Prevotella_1*, and *Rikenellaceae_RC9_gut_group* showed an open downward parabola response, but the abundances of genera *Photobacterium* and *Vibrio* showed an open upward parabola in response to increasing dietary LF inclusion levels. However, there was no significant difference in the abundances of dominant genera between dietary treatments at the genus level ($P > 0.05$). .

Expression of Intestinal Inflammatory Factor Genes

As shown in Fig. 3, FM group had lower mRNA levels for *IL-1β*, *IL-12*, *IL-8*, and *TNF-α* in comparison with SBM60 group ($P < 0.05$), and no differences in the value for the *TGF-β1* and *IL-10* were observed between them ($P > 0.05$). Although the intestinal mRNA levels for *IL-1β*, *IL-12*, *IL-8*, *TGF-β1*, and *TNF-α* did not differ across diets from LF2 to LF10 ($P > 0.05$), the values for *IL-1β*, *IL-12*, *IL-8*, and *TNF-α* were lower, but the value for *TGF-β1* was higher in diets LF2 - LF10 than in diet SBM60 ($P < 0.05$), and returned to the level of FM group ($P > 0.05$) and even lower than that of FM group. The mRNA level for *IL-10* was not different in LF2 and LF10 groups ($P > 0.05$) but higher than FM, SBM60, and LF6 groups ($P < 0.05$).

Discussion

Our previous study and other studies have shown that high SBM diets resulted in poor fish growth performance [1, 4, 30, 31], which supported our current results that the growth rate was reduced when juvenile orange-spotted groupers were fed high SBM diets vs FM diets, as evidenced by significant decreased in WG and SGR. Consistent with our previous results [1], no variations between high SBM diets and FM diets in HSI, CF, and FE were observed. In contrast, feeding a high SBM diet led to a reduction in HSI [30, 32, 33], CF [31, 32, 34], and FE [4]. The restricted growth of fish caused by high SBM diets might be due to

the imbalanced amino acids profile in high SBM diets [4, 30] and SBMIE, a resultant low feed utilization [1, 35]. It's clear that dietary LF administration shows a strong effect on improving growth for terrestrial animals. For example, dietary LF administration could enhance growth performance of early-weaned piglets and neonatal calves[36, 37]. Recently, Olyayee et al.[38] reported a growth promotion and carcass yield of broiler chicken when fed a diet with 0.8 g/kg LF. In fish, LF was used as a feed additive to promote the growth of farmed fish [39-41]. However, in other studies, this promoting effect was not observed in Atlantic salmon [42], Nile tilapia [43], gilthead sea bream [44], African cichlid [45], and Siberian sturgeon [46]. This discrepancy may be ascribed to the differences in fish species and physiological status, growth stage, LF dosage, water temperature, etc.

There were no differences in whole-body proximate composition between fish fed high SBM diets and fed FM diets in the present study and previous studies with largemouth bass [30], rockfish [47], and pompano [48]. In contrast, previous studies showed that feeding high SBM resulted in an increase in moisture content [32, 49] and a decrease in crude protein content [32, 49] and crude lipid content [1, 32, 49]. After dietary LF intervention, no notable changes were observed in the whole-body proximate composition in the present study and a previous study on porgy [50], which was inconsistent with [41], who reported that dietary LF increased whole-body protein content and decreased whole-body lipid content of Nile tilapia.

The activities of digestive enzymes reflect the ability to digest feed [51]. In the present study, the intestinal activities of trypsin and protease were decreased in fish fed with high SBM diets vs fish fed FM diets, which supported the results in previous studies with drum fish [52], Japanese seabass [53], hybrid tilapia [54] as well as our previous study [1]. The reduced intestinal trypsin and the protease activities were the result of the presence of trypsin inhibitors or other ANFs in the SBM, resulting in poor growth performance and feed utilization in fish [54-57]. After the dietary intervention of LF, the intestinal activities of lipase, protease, and trypsin showed a linear response to dietary LF inclusion levels, and reached or even exceeded the original levels of FM diet, indicating that LF could promote the digestion of feed. Dietary LF administration alone or in combination with other functional feed additives was also reported to have an enhancement effect on intestinal trypsin and protease activities of silvery-black porgy [58, 59]. Dietary LF administration could promote the proliferation of intestinal epithelial cells, and protect the intestinal crypt and villous structure of piglets [60, 61], reflecting the integrity of the intestinal mucosa and the stabilization of the intestinal brush border. Therefore, it is natural to improve the digestive capacity through the intervention of dietary LF on the enteritis response of fish.

Intestinal tract is not only the place where nutrients are digested and absorbed [62], but also a site of immune defense, as an important barrier against exogenous pathogens [63]. D-Lac and ET are the products secreted by the inherent bacteria of gastrointestinal tract, and increases in their concentrations in the blood reflect dysregulation of the intestinal flora and impaired permeability [64-67]. In this study, intestinal D-Lac and ET contents increased in diets SBM60 vs that in diets FM. This finding was consistent with previous results [1, 53, 68], indicating the intestinal mucosal injury of fish induced by dietary high-SBM. So far, there is no report on the intervention of LF in SBMIE of fish. In the present study, we observed reduced intestinal ET and D-Lac contents in juvenile orange-spotted groupers administrated with dietary LF vs those of fish fed SBM60 diets, and reduced to the level of the control or even lower. This indicated that dietary LF supplementation in high-SBM diet could reduce the intestinal mucosal permeability of the fish species.

Intact intestinal histomorphology is a prerequisite for maintaining good digestion and absorption state and intestinal immune disease resistance [69]. The histomorphological indicators such as lMF, tML, and nMF were used to evaluate intestinal digestion and absorption capacity in previous studies [1, 19]. In the present study, a significant reduction in tML in DI was observed in SBM diets vs FM diets, as evidenced by previous study [1]. Many studies showed that fish fed high-SBM diets had shortened villus height and thinned muscular thickness [4, 53, 70], which indicated potential abnormal intestinal histomorphology caused by dietary high SBM. The intestinal histopathological changes were improved when fish were intervened by dietary LF administration in our current study. We observed an increase of lMF in MI, and tML in MI and DI in a dose-dependent manner with increasing LF levels in high SBM diets. Consistent with results of our current study, dietary LF administration increased the height, width and surface area of intestinal villi, the depth of the crypts, and the thickness of the muscular layer of broiler chickens [38]. The positive effect of dietary LF administration on the intestinal histomorphology of terrestrial animals has been widely recognized [71-73]. Therefore, the intervention effect of dietary LF administration on the SBMIE of juvenile orange-spotted groupers can also be achieved by improving histomorphology.

The intestine also is so-called the "second brian" that links between intestinal microbiota and diseases. Dysbacteriosis of the intestinal flora increases the susceptibility to intestinal pathogens, and in severe cases, it will further develop into intestinal infection and reduce immune function [74, 75]. In the present study, fish fed high-SBM diets showed increased abundance of phylum Firmicutes and decreased abundance of phylum Bacteroidetes and Proteobacteria vs fish fed FM diets. At genus level, fish fed high-SBM diets exhibited decreased abundance of *Photobacterium*, *Selenomonas_1*, and *Prevotella_1* and increased abundance of *Vibrio* and *Rikenellaceae_RC9_gut_group* vs fish fed FM diets. Nevertheless, high-SBM substitution for FM did not alter intestinal microbial abundance and diversity of groupers at either the phylum level or the genus level, which was consistent with previous studies in largemouth bass [76], gilthead sea bream [77], and large yellow croaker [78]. The difference in relative abundance of intestinal microbiota at the phylum and genus levels may be directly or indirectly influenced by external environmental conditions such as the living environment, fish species, growth stage, and feed composition. In the present study, the phylum Firmicutes, Bacteroidetes, and Proteobacteria were identified as the dominant phyla of the intestine in fish fed high SBM diets with LF administration. The relative abundances of Firmicutes and Bacteroidetes were generally promoted, while the abundance of Proteobacteria was reduced by the intervention of dietary LF administration. At genus level, the intestinal microbiota diversity did not vary, but the relative abundance of genus bacteria varied with dietary LF inclusion levels. As mentioned above, thus far, there are few reports regarding the intervention of LF on fish enteritis.

The presence of an inflammatory response is a complex pathophysiological process, which is mediated by the activation of a variety of cytokines and complement factors secreted by macrophages and leukocytes [79]. LF can bind specifically B-lymphocytes and macrophages, thus inhibiting the production of pro-inflammatory factors such as *IL-1β*, *IL-8*, and *TNF-α*, or/and indirectly promoting the production of anti-inflammatory factors such as *IL-10* through preventing the activation of TOR signaling pathway induced by lipopolysaccharide [18, 80, 81]. LF was used to modulate the inflammatory response, with an emphasis on protection against intestinal infections and inflammatory bowel diseases of mammals [81-83]. Like mammals, fish have pro-inflammatory cytokines included *IL-1β*, *TNF-α*, *IL-8*, and *IL-12*, and the anti-inflammatory ones are *IL-10* and *TGF-β1* in fish immune responses [1, 84, 85]. It is

clear that up-regulation of pro-inflammatory gene expression and down-regulation of anti-inflammatory gene expression are caused by SBMIE [86, 87]. In the present study, fish fed with high-SBM diets promoted the expression of such pro-inflammatory genes as *IL-1β*, *TNF-α*, *IL-8*, and *IL-12* vs fish fed with FM diets. The findings agreed with what has been reported in previous studies with SBM substitution for FM in different fish species [1, 4, 76]. In the present study, the mRNA levels of *IL-1β*, *TNF-α*, *IL-8*, and *IL-12* genes were down-regulated, while the mRNA levels of *IL-10* and *TGF-β1* genes up-regulated after fish fed high SBM diets administrated with LF. This finding indicated that dietary LF intervention could alleviate the SBMIE of fish through promoting the production of anti-inflammatory factors and preventing the production of pro-inflammatory factors in this study.

Antioxidant capacity reflects the physiological status of aquatic animals [88]. It is well known that the antioxidant enzyme SOD catalyzes the dismutation of superoxide anion into O_2 and H_2O_2. H_2O_2 is subsequently degraded into H_2O by antioxidant enzyme GSH-Px in the cytosol. These enzymes are easily induced by oxidative stress and their activity is usually used to reflect the ability of cleaning free radicals in cells [89]; T-AOC reflects the protective capacity of non-enzymatic antioxidant defense system [90]; MDA as one of the end-products of lipid peroxidation reflects the degree of lipid peroxidation caused by free radicals and indirectly reflect the degree of cell damage [91]. In the present study, the intestinal GSH-Px activity was decreased and MDA content was increased in fish fed high SBM diets vs those fed FM diets. Similarly, fish showed decreased activities of SOD [68, 76, 92] and GSH-Px [47], T-AOC [76] as well as increased MDA content [68, 76] when fed the diets with FM replacement by high SBM. This indicated that fish suffered from oxidative stress. The decline of immunity and antioxidant capacity of animals is associated with the presence of ANFs in SBM [93, 94]. After dietary intervention with LF, fish had higher intestinal GSH-Px activity and lower MDA content vs high SBM diets. Furthermore, the intestinal GSH-Px activity showed an increasing trend with increasing dietary LF inclusion levels and even returned to the level of diet FM. The findings suggest dietary LF intervention could promote the intestinal antioxidant capacity and prevent the intestinal lipid peroxidation in juvenile orange-spotted groupers with SBMIE, through influencing both enzymatic and non-enzymatic antioxidants. Consistent with our results, a reduction of liver MDA content on day 15 and an increase in liver T-AOC on day 30 were observed after *Aeromonas veronii*-induced Nile tilapias were administrated with LF at 0.8 g/kg diet [95]; Increased SOD activity and decreased MDA content in the hepatopancreas were observed in shrimp receiving the diets with 1.5-2.5 g/kg LF [96]; Similar effects occurred for weaned piglets when they were fed diets with LF [36]. The above results validated that dietary LF intervention has a protective effect against oxidative stress resulting from different sources of stress. However, higher dietary LF inclusion levels did not improve the oxidative stress of the fish species. This was also reflected in growth, that is, higher dietary LF inclusion levels did not further enhance growth performance.

Besides antioxidant capacity, another important indicator to measure the health status of fish is the plasma biochemical components [25, 97]. HDL-C participates in the transportation of lipids from peripheral tissues to the liver for catabolism, whereas LDL-C transports cholesterol from the liver to peripheral tissues [24]. High SBM diets could decrease plasma TC, TG, LDL-C, and/or HDL-C contents vs the control (FM diet) in many previous studies [1, 68, 98-100], as observed in our current study. This indicates inferior nutritional status of fish caused by high SBM inclusion. Not as expected, plasma contents of TC, HDL-C and LDL-C were still lower in high SBM diets with LF intervention than that in FM diets. As a result, dietary intervention of LF on malnutrition of grouper caused by high SBM diets is limited, though improved overall antioxidant capacity and growth.

Conclusion

Supplementation of LF in high SBM diets not only improves the growth performance and intestinal morphology, but also reduces the permeability of intestinal mucosal cells and attenuates the intestinal inflammatory response in juvenile orange-spotted groupers, the improvement of nutritional status under the intervention of LF. The optimal appropriate supplementation level of LF was 5.8 g/kg based on quadratic regression analysis of the percent weight gain against dietary LF inclusion levels. This is the first report on the intervention effect of dietary LF on grouper enteritis induced by dietary SBM. Our current study will provide a basis for LF use as a functional feed additive to alleviate fish SBMIE.

Declarations

Author Contributions: Tao Song and Yingmei Qin conducted the experiments and analyzed the data, and wrote the manuscript; Liner Ke and Kun Wang contributed to data analysis and curation; Xuexi Wang and Yunzhang Sun contributed to the formal analysis and the writing review; Jidan Ye supervised the experiments and reviewed the manuscript.

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Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Institutional Review Board Statement: Experimental design and procedures in this study were reviewed and approved by the Animal Ethics Committee of Jimei University, Xiamen, China (Approval number: 2011-58).

Declaration of Competing Interest: The authors declare on conflict of interest.

Supplementary Materials: The original contributions presented in the study are included in the article/Supplementary Materials.

References

1. Zhao X, Wang Y, Wang X, Ye J (2021) Growth performance, plasma components, and intestinal barrier in grouper (*Epinephelus coioides*) are altered by dietary fish meal replacement with extruded soybean meal. Aquacult Rep 21:100863

2. Ghosh K, Ray AK, Ringø E (2019) Applications of plant ingredients for tropical and subtropical freshwater finfish: possibilities and challenges. Rev Aquacult 11:793-815
3. Shao J, Zhao W, Liu X, Wang L (2018) Growth Performance, Digestive Enzymes, and TOR Signaling Pathway of Litopenaeus vannamei Are Not Significantly Affected by Dietary Protein Hydrolysates in Practical Conditions. Front Physiol 9:998
4. Wang Y, Wang L, Zhang C, Song K (2017) Effects of substituting fishmeal with soybean meal on growth performance and intestinal morphology in orange-spotted grouper (*Epinephelus coioides*). Aquacult Rep 5:52-57
5. Bravo-Tello K, Ehrenfeld N, Solís CJ, Ulloa PE, Hedrera M, Pizarro-Guajardo M, Paredes-Sabja D, Feijóo CG, Martínez-Llorens S (2017) Effect of microalgae on intestinal inflammation triggered by soybean meal and bacterial infection in zebrafish. Plos One 12:e187696
6. Chen Y, Ma J, Huang H, Zhong H (2019) Effects of the replacement of fishmeal by soy protein concentrate on growth performance, apparent digestibility, and retention of protein and amino acid in juvenile pearl gentian grouper. Plos One 14:e222780
7. Dam C, Ventura T, Booth M, Pirozzi I, Salini M, Smullen R, Elizur A (2020) Intestinal transcriptome analysis highlights key differentially expressed genes involved in nutrient metabolism and digestion in Yellowtail kingfish (*Seriola lalandi*) fed terrestrial animal and plant proteins. genes 11:621
8. Bai N, Gu M, Xu X, Xu B, Krogdahl Å (2017) Protective effects of mannan oligosaccharides on turbot *Scophthalmus maximus* suffering from soy enteropathy. Aquaculture 476:141-151
9. Grammes F, Reveco FE, Romarheim OH, Landsverk T, Mydland LT, Overland M (2013) *Candida utilis* and *Chlorella vulgaris* counteract intestinal inflammation in Atlantic salmon (*Salmo salar L.*). Plos One 8:e83213
10. Romarheim OH, Hetland DL, Skrede A, Overland M, Mydland LT, Landsverk T (2013) Prevention of soya-induced enteritis in Atlantic salmon (*Salmo salar*) by bacteria grown on natural gas is dose dependent and related to epithelial MHC II reactivity and CD8alpha+ intraepithelial lymphocytes. Br J Nutr 109:1062-1070
11. Wu N, Xu X, Wang B, Li X, Cheng Y, Li M, Xia X, Zhang Y (2020) Anti-foodborne enteritis effect of galantamine potentially via acetylcholine anti-inflammatory pathway in fish. Fish Shellfish Immun 97:204-215
12. Xie J, Li M, Ye W, Shan J, Zhao X, Duan Y, Liu Y, Unger BH, Cheng Y, Zhang W, Wu N, Xia X (2021) Sinomenine hydrochloride ameliorates fish foodborne enteritis via α7nAchR-Mediated anti-Inflammatory effect whilst altering microbiota composition. Front Immunol 12:766845
13. Rascón-Cruz Q, Espinoza-Sánchez EA, Siqueiros-Cendón TS, Nakamura-Bencomo SI, Arévalo-Gallegos S, Iglesias-Figueroa BF (2021) Lactoferrin: A Glycoprotein involved in immunomodulation, anticancer, and antimicrobial processes. Molecules 26:205
14. Wang Y, Shan T, Xu Z, Feng J, Wang Z (2007) Effects of the lactoferrin (LF) on the growth performance, intestinal microflora and morphology of weanling pigs. Anim Feed Sci Tech 135:263-272
15. Ulloa PE, Solis CJ, De la Paz JF, Alaurent TG, Caruffo M, Hernandez AJ, Dantagnan P, Feijoo CG (2016) Lactoferrin decreases the intestinal inflammation triggered by a soybean meal-based diet in Zebrafish. J Immunol Res 2016:1639720
16. Li M, Fang C, Zhang K, Li Y, Zhang R (2012) Effects of lactoferrin supplementation on growth performance, intestinal microflora and mucosal morphology of early weaned piglets. Chinese J Anim Nutr 24:111-116
17. Wang Y, Shan T, Xu Z, Feng J, Wang Z (2007) Effects of the lactoferrin (LF) on the growth performance, intestinal microflora and morphology of weanling pigs. Anim Feed Sci Tech 135:263-272
18. Drago-Serrano ME, de la Garza-Amaya M, Luna JS, Campos-Rodriguez R (2012) Lactoferrin-lipopolysaccharide (LPS) binding as key to antibacterial and antiendotoxic effects. Int Immunopharmacol 12:1-9
19. Niu X, Qian X, Feng H, Yi K, Li D, Chen W, Ye J (2021) Growth and metabolic responses of grouper juveniles (*Epinephelus coioides*) fed diets containing varying levels of leucine. Aquaculture 534:736281
20. Shapawi R, Abdullah FC, Senoo S, Mustafa S (2019) Nutrition, growth and resilience of tiger grouper (*Epinephelus fuscoguttatus*)×giant Grouper (*Epinephelus lanceolatus*) hybrid- a review. Rev Aquacult 11:1285-1296
21. China Fishery Statistics Yearbook (2021) Bureau of Fisheries, Ministry of Agriculture. China Agriculture Press, Beijing, China
22. Qin Y, He L, Wang Y, Li D, Chen W, Ye J (2022) Growth performance, fatty acid composition, and lipid metabolism are altered in groupers (*Epinephelus coioides*) by dietary fish oil replacement with palm oil. Anim Nutr 8:102-113
23. Zhuo L, Chen C, Lin Y (2021) Dietary supplementation of fermented lemon peel enhances lysozyme activity and susceptibility to Photobacterium damselae for orange-spotted grouper, *Epinephelus coioides*. Fish Shellfish Immun 117:248-252
24. Bai F, Niu X, Wang X, Ye J (2021) Growth performance, biochemical composition and expression of lipid metabolism related genes in groupers (*Epinephelus coioides*) are altered by dietary taurine. Aquacult Nutr 27:2690-2702
25. Ye J, Liu X, Wang Z, Wang K (2011) Effect of partial fish meal replacement by soybean meal on the growth performance and biochemical indices of juvenile Japanese flounder *Paralichthys olivaceus*. Aquacult Int 19:143-153
26. AOAC (1995) Official Methods of Analysis of AOAC International, 16th ed. Association of Official Analytical Chemists, Arlington, VA, USA
27. Anguiano M, Pohlenz C, Buentello A, Gatlin DM (2013) The effects of prebiotics on the digestive enzymes and gut histomorphology of red drum (*Sciaenops ocellatus*) and hybrid striped bass (*Morone chrysops× M. saxatilis*). Brit J Nutr 109:623-629
28. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3:1101-1108
29. Hanaki K, Ike F, Kajita A, Yasuno W, Yanagiba M, Goto M, Sakai K, Ami Y, Kyuwa S (2014) A broadly reactive one-step SYBR Green I real-time RT-PCR assay for rapid detection of murine norovirus. Plos One 9:e98108

30. Yang H, Bian Y, Huang L, Lan Q, Ma L, Li X, Leng X (2022) Effects of replacing fish meal with fermented soybean meal on the growth performance, intestinal microbiota, morphology and disease resistance of largemouth bass (*Micropterus salmoides*). Aquacult Rep 22:100954
31. Jahan H, Tumpa IJ, Qasem WA, Moniruzzaman M, Pervin MA, Akter R, Omri A, Min T, Hossain Z (2021) Evaluation of the partial replacement of dietary fish meal with fermented or untreated soybean meal in juvenile silver Barb, *Barbonymus gonionotus*. Front Nutr 8:733402
32. Liang XF, Hu L, Dong YC, Wu XF, Qin YC, Zheng YH, Shi DD, Xue M, Liang XF (2017) Substitution of fish meal by fermented soybean meal affects the growth performance and flesh quality of Japanese seabass (*Lateolabrax japonicus*). Anim Feed Sci Tech 229:1-12
33. He M, Li X, Poolsawat L, Guo Z, Yao W, Zhang C, Leng X (2020) Effects of fish meal replaced by fermented soybean meal on growth performance, intestinal histology and microbiota of largemouth bass (*Micropterus salmoides*). Aquacult Nutr 26:1058-1071
34. Lim S, Kim S, Ko G, Song J, Oh D, Kim J, Kim J, Lee K (2011) Fish meal replacement by soybean meal in diets for Tiger puffer, *Takifugu rubripes*. Aquaculture 313:165-170
35. Abdel-Warith AA, Younis EM, Al-Asgah NA, Mahboob S (2020) Effect of replacing fish meal by full fat soybean meal on growth performance, feed utilization and gastrointestinal enzymes in diets for African catfish *Clarias gariepinus*. Braz J Biol 80:535-543
36. Tang Z, Yin Y, Zhang Y, Huang R, Sun Z, Li T, Chu W, Kong X, Li L, Geng M, Tu Q (2009) Effects of dietary supplementation with an expressed fusion peptide bovine lactoferricin-lactoferrampin on performance, immune function and intestinal mucosal morphology in piglets weaned at age 21 d. Br J Nutr 101:998-1005
37. Prenner ML, Prgomet C, Sauerwein H, Pfaffl MW, Broz J, Schwarz FJ (2007) Effects of lactoferrin feeding on growth, feed intake and health of calves. Arch Anim Nutr 61:20-30
38. Olyayee M, Javanmard A, Janmohammadi H, Kianfar R, Alijani S, Mir Ghelenj SA (2022) Supplementation of broiler chicken diets with bovine lactoferrin improves growth performance, histological parameters of jejunum and immune-related gene expression. J Anim Physoil An N:Early Access
39. Kakuta I (1996) Effects of orally administrated bovine lactoferrin on growth and blood properties of goldfish. Suisan Zoshoku 44:419-426
40. Esmaeili A, Sotoudeh E, Morshedi V, Bagher D, Dorafshan S (2019) Effects of dietary supplementation of bovine lactoferrin on antioxidant status, immune response and disease resistance of yellowfin sea bream (*Acanthopagrus latus*) against Vibrio harveyi. Fish Shellfish Immun 93:917-923
41. El-Sayed BT, Al-Kenawy D (2013) Assessment of immune response supplemental immunoton and bovine lactoferrin as alternatives to antibiotics in Nile tilapia (*Oreochromis niloticus*). J Arabian Aquacult Soc 8:341-356
42. Lygren B, Sveier H, Hjeltnes B, Waagbø R (1999) Examination of the immunomodulatory properties and the effect on disease resistance of dietary bovine lactoferrin and vitamin C fed to Atlantic salmon (*Salmo salar*) for a short-term period. Fish Shellfish Immun 9:95-107
43. Welker TL, Lim C, Yildirim-Aksoy M, Klesius PH (2007) Growth, immune function, and disease and stress resistance of juvenile Nile tilapia (*Oreochromis niloticus*) fed graded levels of bovine lactoferrin. Aquaculture 262:156-162
44. Esteban MA, Rodríguez A, Cuesta A, Meseguer J (2005) Effects of lactoferrin on non-specific immune responses of gilthead seabream (*Sparus auratus L.*). Fish Shellfish Immun 18:109-124
45. Moradian AM, Dorafshan S, Heyrati FP, Ebrahimi E (2017) Effects of dietary bovine lactoferrin on growth, haemato-biochemical parameters, immune functions and tolerance to air exposure stress in the African cichlid *Sciaenochromis fryeri*. Aquacult Nutr 24:392-399
46. Falahatkar B, Eslamloo K, Yokoyama S (2014) Suppression of stress responses in Siberian sturgeon, *Acipenser baeri*, juveniles by the dietary administration of bovine lactoferrin. J World Aquacult Soc 45:699-708
47. Lee S, Mohammadi Azarm H, Chang KH (2016) Effects of dietary inclusion of fermented soybean meal on growth, body composition, antioxidant enzyme activity and disease resistance of rockfish (*Sebastes schlegeli*). Aquaculture 459:110-116
48. Lin H, Chen X, Chen S, Zhuojia L, Huang Z, Niu J, Wu K, Lu X (2012) Replacement of fish meal with fermented soybean meal in practical diets for pompano *Trachinotus ovatus*. Aquac Res 44:151-156
49. García-Ortega A, Kissinger KR, Trushenski JT (2016) Evaluation of fish meal and fish oil replacement by soybean protein and algal meal from *Schizochytrium limacinum* in diets for giant grouper *Epinephelus lanceolatus*. Aquaculture 452:1-8
50. Morshedi V, Agh N, Noori F, Jafari F, Ghasemi A, Mozanzadeh MT (2020) Effects of single and combined supplementation of dietary probiotic with bovine lactoferrin and xylooligosaccharide on hemato-immunological and digestive enzymes of silvery-black porgy (*Sparidentex hasta*) fingerlings. Ann Anim Sci 20:137-155
51. Li J, Li J, Li W, Sun Y, Liu X, Liu M, Cheng Y (2019) Juvenile Procambarus clarkii farmed using biofloc technology or commercial feed in zero-water exchange indoor tanks: A comparison of growth performance, enzyme activity and proximate composition. Aquac Res 50:1834-1843
52. Fuentes-Quesada JP, Viana MT, Rombenso AN, Guerrero-Rentería Y, Nomura-Solís M, Gomez-Calle V, Lazo JP, Mata-Sotres JA (2018) Enteritis induction by soybean meal in Totoaba macdonaldi diets: Effects on growth performance, digestive capacity, immune response and distal intestine integrity. Aquaculture 495:78-89
53. Zhang C, Rahimnejad S, Wang Y, Lu K, Song K, Wang L, Mai K (2018) Substituting fish meal with soybean meal in diets for Japanese seabass (*Lateolabrax japonicus*): Effects on growth, digestive enzymes activity, gut histology, and expression of gut inflammatory and transporter genes. Aquaculture 483:173-182
54. Lin S, Luo L (2011) Effects of different levels of soybean meal inclusion in replacement for fish meal on growth, digestive enzymes and transaminase activities in practical diets for juvenile tilapia, *Oreochromis niloticus×O. aureus*. Anim Feed Sci Tech 168:80-87
55. Dias J, Alvarez MJ, Arzel J, Corraze G, Diez A, Bautista JM, Kaushik SJ (2005) Dietary protein source affects lipid metabolism in the European seabass (*Dicentrarchus labrax*). Comp Biochem Phys Part A: Mol Integr Physiol 142:19-31

56. Santigosa E, Sánchez J, Médale F, Kaushik S, Pérez-Sánchez J, Gallardo MA (2008) Modifications of digestive enzymes in trout (*Oncorhynchus mykiss*) and sea bream (*Sparus aurata*) in response to dietary fish meal replacement by plant protein sources. Aquaculture 282:68-74
57. Yaghoubi M, Mozanzadeh MT, Marammazi JG, Safari O, Gisbert E (2016) Dietary replacement of fish meal by soy products (soybean meal and isolated soy protein) in silvery-black porgy juveniles (*Sparidentex hasta*). Aquaculture 464:50-59
58. Pagheh E, Marammazi JG, Agh N, Nouri F, Sepahdari A, Gisbert E, Mozanzadeh MT (2017) Growth performance, hemato-immunological responses, and digestive enzyme activities in Silvery-black porgy (*Sparidentex hasta*) fed dietary bovine lactoferrin. Probiotics Antimicro 10:399-407
59. Morshedi V, Agh N, Marammazi JG, Noori F, Mohammadian T, Mozanzadeh MT (2019) Combined effects of dietary bovine lactoferrin, lactobacillus plantarum, and xylooligosaccharide on hemato-immunological and digestive enzymes of silvery-black porgy (*Sparidentex hasta*) fingerlings. Comp Clin Path 28:731-736
60. Li Q, Hu W, Zhao J, Wang J, Dai Y, Zhao Y, Meng Q, Li N (2014) Supplementation transgenic cow's milk containing recombinant human lactoferrin enhances systematic and intestinal immune responses in piglets. Mol Biol Rep 41:2119-2128
61. Nguyen DN, Li Y, Sangild PT, Bering SB, Chatterton DE (2014) Effects of bovine lactoferrin on the immature porcine intestine. Br J Nutr 111:321-331
62. Ray AK, Ghosh K, Ringø E (2012) Enzyme-producing bacteria isolated from fish gut: a review. Aquacult Nutr 18:465-492
63. Liu C, Chi K, Yang M, Guo N (2022) Staphylococcal enterotoxin a induces intestinal barrier dysfunction and activates NLRP3 inflammasome via NF-κB/MAPK signaling pathways in mice. Toxins 14:29
64. Peng P, Chen J, Yao K, Yin Y, Long L, Fang R (2019) The effects of dietary supplementation with porous zinc oxide on growth performance, intestinal microbiota, morphology, and permeability in weaned piglets. Anim Sci J 90:1220-1228
65. Zhao Y, Qin G, Sun Z, Che D, Bao N, Zhang X (2011) Effects of soybean agglutinin on intestinal barrier permeability and tight junction protein expression in weaned piglets. Int J Mol Sci 12:8502-8512
66. Ruan P, Gong ZJ, Zhang QR (2004) Changes of plasma D(-)-lactate, diamine oxidase and endotoxin in patients with liver cirrhosis. Hepatobiliary Pancreat Dis Int 3:58-61
67. Han R, Wang L, Zhao Z, You L, Pedisić S, Kulikouskaya V, Lin Z (2020) Polysaccharide from *Gracilaria Lemaneiformis* prevents colitis in Balb/c mice via enhancing intestinal barrier function and attenuating intestinal inflammation. Food hydrocolloid 109:106048
68. Rahimnejad S, Zhang J, Wang L, Sun Y, Zhang C (2021) Evaluation of Bacillus pumillus SE5 fermented soybean meal as a fish meal replacer in spotted seabass (*Lateolabrax maculatus*) feed. Aquaculture 531:735975
69. He Y, Yang Y, Dong Y, Yan C, Zhang B (2019) The effects of flavomycin and colistin sulfate pre-treatment on ileal bacterial community composition, the response to salmonella typhimurium and host gene expression in broiler chickens. Microorganisms 7:574
70. Zhou Z, Yao W, Ye B, Wu X, Li X, Dong Y (2020) Effects of replacing fishmeal protein with poultry by-product meal protein and soybean meal protein on growth, feed intake, feed utilization, gut and liver histology of hybrid grouper (*Epinephelus fuscoguttatus* ♀× Epinephelus lanceolatus ♂) juveniles. Aquaculture 516:734503
71. Shimizu H (2004) Development of an enteric-coated lactoferrin tablet and its application. Biometals 17:343-347
72. Wang Y, Shan T, Xu Z, Liu J, Feng J (2006) Effect of lactoferrin on the growth performance, intestinal morphology, and expression of PR-39 and protegrin-1 genes in weaned piglets. J Anim Sci 84:2636-2641
73. Blais A, Fan C, Voisin T, Aattouri N, Dubarry M, Blachier F, Tomé D (2014) Effects of lactoferrin on intestinal epithelial cell growth and differentiation: an in vivo and in vitro study. Biometals 27:857-874
74. Xu J, Li Y, Yang Z, Li C, Liang H, Wu Z, Pu W (2018) Yeast probiotics shape the gut microbiome and improve the health of early-weaned piglets. Front Microbiol 9:2011
75. Fu R, Li Z, Zhou R, Li C, Shao S, Li J (2021) The mechanism of intestinal flora dysregulation mediated by intestinal bacterial biofilm to induce constipation. Bioengineered 12:6484-6498
76. Li C, Zhang B, Liu C, Zhou H, Wang X, Mai K, He G (2020) Effects of dietary raw or enterococcus faecium fermented soybean meal on growth, antioxidant status, intestinal microbiota, morphology, and inflammatory responses in turbot (*Scophthalmus maximus L.*). Fish Shellfish Immun 100:261-271
77. Estruch G, Collado MC, Penaranda DS, Tomas VA, Jover CM, Perez MG, Martinez-Llorens S (2015) Impact of fishmeal replacement in diets for Gilthead sea bream (*Sparus aurata*) on the gastrointestinal microbiota determined by pyrosequencing the 16S rRNA gene. Plos One 10:e136389
78. Wang P, Zhou Q, Feng J, He J, Lou Y, Zhu J (2019) Effect of dietary fermented soybean meal on growth, intestinal morphology and microbiota in juvenile large yellow croaker, *Larimichthys crocea*. Aquac Res 50:748-757
79. Ruhee RT, Ma S, Suzuki K (2019) Sulforaphane protects cells against lipopolysaccharide-stimulated inflammation in murine macrophages. Antioxidants 8:577
80. Liu J, Li B, Lee C, Zhu H, Zheng S, Pierro A (2019) Protective effects of lactoferrin on injured intestinal epithelial cells. J Pediatr Surg 54:2509-2513
81. Prgomet C, Prenner ML, Schwarz FJ, Pfaffl MW (2007) Effect of lactoferrin on selected immune system parameters and the gastrointestinal morphology in growing calves. J Anim Physoil An N 91:109-119
82. Valenti P, Berlutti F, Conte MP, Longhi C, Seganti L (2004) Lactoferrin functions: Current status and perspectives. J Clin Gastroenterol 38:S127-S129
83. Wakabayashi H, Takakura N, Yamauchi K, Tamura Y (2006) Modulation of immunity-related gene expression in small intestines of mice by oral administration of lactoferrin. Clin Vaccine Immunol 13:239-245
84. Yang Y, Wang Y, Lu Y, Li Q (2010) Effect of replacing fish meal with soybean meal on growth, feed utilization and nitrogen and phosphorus excretion on rainbow trout (*Oncorhynchus mykiss*). Aquacult Int 19:405-419

85. Lin S, Zhou X, Zhou Y, Kuang W, Chen Y, Luo L, Dai F (2020) Intestinal morphology, immunity and microbiota response to dietary fibers in largemouth bass, *Micropterus salmoide*. Fish Shellfish Immun 103:135-142
86. Urán PA, Gonçalves AA, Taverne-Thiele JJ, Schrama JW, Verreth JAJ, Rombout JHWM (2008) Soybean meal induces intestinal inflammation in common carp (*Cyprinus carpio L.*). Fish Shellfish Immun 25:751-760
87. Gu M, Bai N, Zhang Y, Krogdahl Å (2016) Soybean meal induces enteritis in turbot *Scophthalmus maximus* at high supplementation levels. Aquaculture 464:286-295
88. Tusi SK, Manesh TE, Fathollahi MS, Bagherian A (2017) Can tert-butylhydroquinone improve the healing of extracted tooth socket in rats? J Dent Res 14:8-12
89. van der Oost R, Beyer J, Vermeulen NPE (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ Toxicol Phar 13:57-149
90. Wan J, Zhang J, Chen D, Yu B, Huang Z, Mao X, Zheng P, Yu J, He J (2018) Alginate oligosaccharide enhances intestinal integrity of weaned pigs through altering intestinal inflammatory responses and antioxidant status. Rsc Adv 8:13482-13492
91. Aguiló A, Tauler P, Fuentespina E, Tur JA, Córdova A, Pons A (2005) Antioxidant response to oxidative stress induced by exhaustive exercise. Physiol Behav 84:1-7
92. Lin YH, Mui JJ (2017) Comparison of dietary inclusion of commercial and fermented soybean meal on oxidative status and non-specific immune responses in white shrimp, *Litopenaeus vannamei*. Fish Shellfish Immun 63:208-212
93. Baeverfjord GT, Krogdahl A (2010) Development and regression of soybean meal induced enteritis in Atlantic salmon, *Salmo salar L.*, distal intestine: a comparison with the intestines of fasted fish. J Fish Dis 19:375-387
94. Olsvik PA, Torstensen BE, Hemre GI, Sanden M, Waagbo R (2011) Hepatic oxidative stress in Atlantic salmon (*Salmo salar L.*) transferred from a diet based on marine feed ingredients to a diet based on plant ingredients. Aquacult Nutr
95. Hashem NMA, El Son MAM, Ateya AI, Saleh RM (2022) Impact of lactoferrin supplementation on oxidative stress, gene expression and immunity dysfunction induced by *Aeromonas veronii* in Nile tilapia (*Oreochromis niloticus*). Aquac Res 53:2392-2407
96. Zhuang Y, Huang H, Liu X, Wang N, Zhong G (2022) Effect of bovine lactoferricin on the growth performance, digestive capacity, immune responses and disease resistance in Pacific white shrimp, *Penaeus vannamei*. Fish Shellfish Immun 123:282-289
97. Jiang M, Wen H, Gou GW, Liu TL, Lu X, Deng DF (2018) Preliminary study to evaluate the effects of dietary bile acids on growth performance and lipid metabolism of juvenile genetically improved farmed tilapia (*Oreochromis niloticus*) fed plant ingredient-based diets. Aquacult Nutr 24:1175-1183
98. Ye H, Xu M, Chen L, Tan X, Chen S, Zou C, Sun Z, Liu Q, Ye C, Wang A (2019) Effects of dietary plant protein sources influencing hepatic lipid metabolism and hepatocyte apoptosis in hybrid grouper (*Epinephelus lanceolatus*♂ × *Epinephelus fuscoguttatus*♀). Aquaculture 506:437-444
99. Yamamoto T, Iwashita Y, Matsunari H, Sugita T, Furuita H, Akimoto A, Okamatsu K, Suzuki N (2010) Influence of fermentation conditions for soybean meal in a non-fish meal diet on the growth performance and physiological condition of rainbow trout *Oncorhynchus mykiss*. Aquaculture 309:173-180
100. Dossou S, Koshio S, Ishikawa M, Yokoyama S, Dawood MAO, El Basuini MF, El-Hais AM, Olivier A (2018) Effect of partial replacement of fish meal by fermented rapeseed meal on growth, immune response and oxidative condition of red sea bream juvenile, *Pagrus major*. Aquaculture 490:228-235

Figures

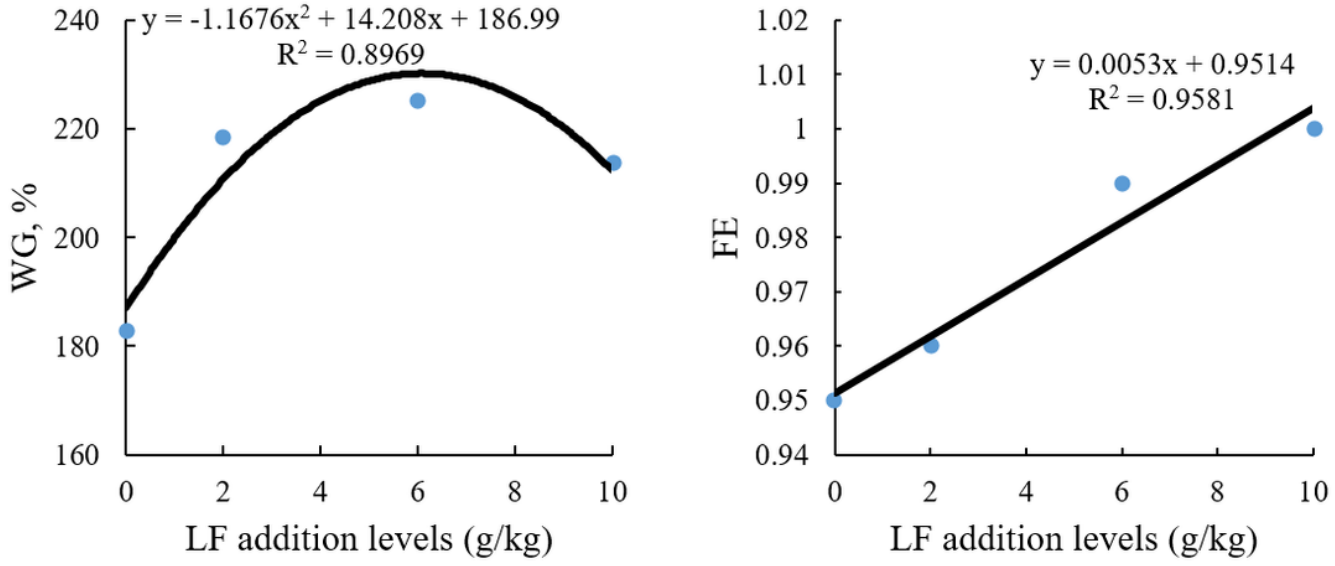

Figure 1

The relationship between weight gain (WG) and feed efficiency (FE) of juvenile orange-spotted groupers and the lactoferrin (LF) inclusion levels in SBM60 diets in a 56-d feeding period. Values are means of 3 triplicates per dietary treatment. SBM60, high soybean meal diet with 60% fish meal protein replacement and without LF supplementation; LF2, LF6, and LF10 were added 2, 6, and 10 g/kg LF in SBM60 diets, respectively.

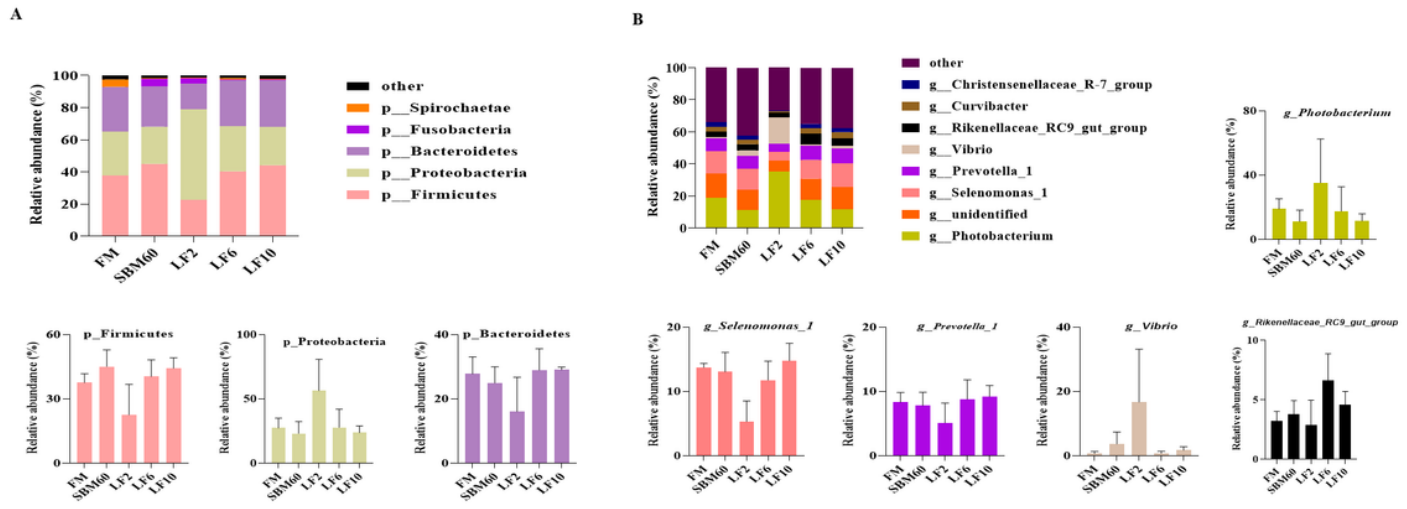

Figure 2

Relative abundances of the dominant bacterial at phylum (A) and genus (B) in distal intestine (DI) of juvenile orange-spotted grouper fed different diets in a 56-day feeding period.

Bars bearing the different letters are significantly different ($P < 0.05$).

FM, fish meal diet (control diet); SBM60, high soybean meal diet with 60% fish meal protein replacement and without LF supplementation; LF2, LF6, and LF10 were added 2, 6, and 10 g/kg LF in SBM60 diets, respectively.

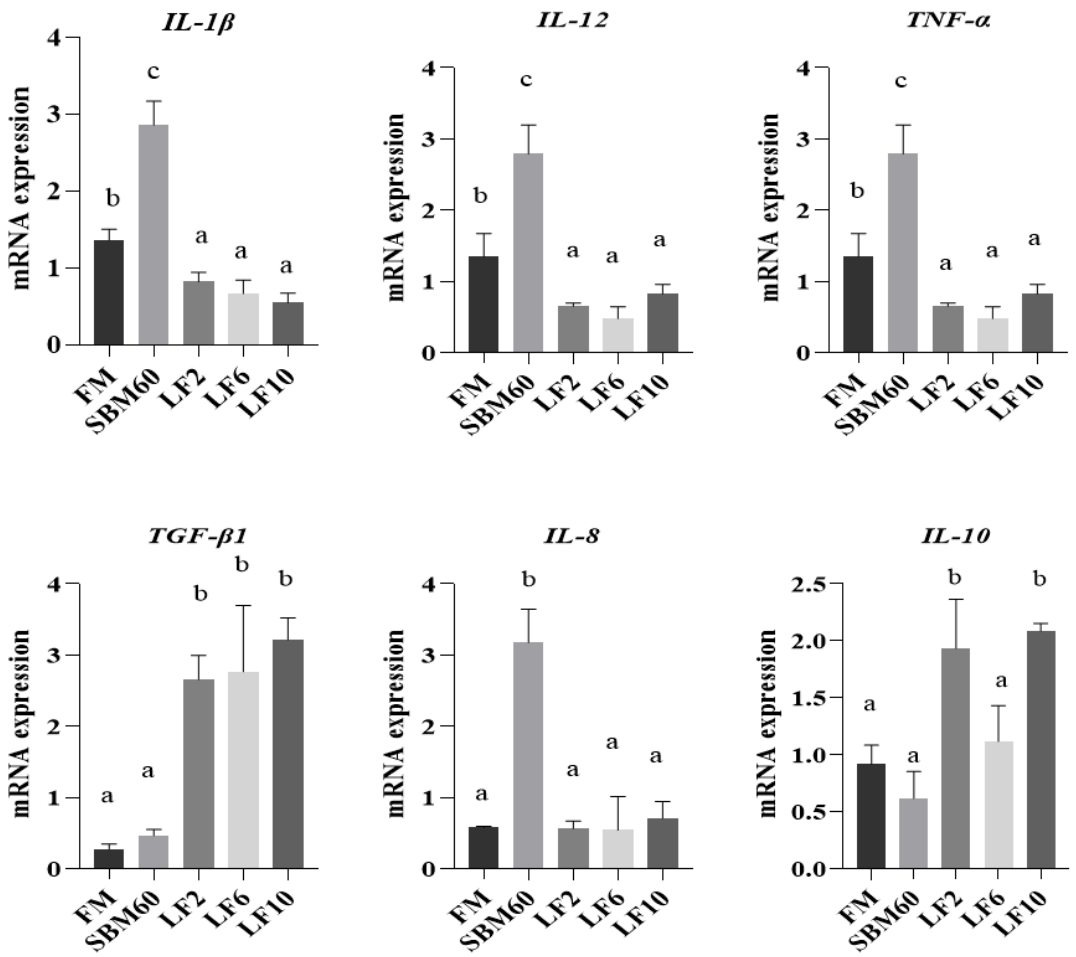

Figure 3

Effect of lactoferrin (LF) inclusion levels in SBM60 diets on mRNA levels of intestinal inflammatory factor genes of juvenile orange-spotted groupers in a 56-day feeding period.

Data are presented as means ± SEM (n = 3 tanks). Statistical analysis was performed by one-way ANOVA, followed by S-N-K test.

Bars bearing the different letters are significantly different ($P < 0.05$).

FM, fish meal diet (control diet); SBM60, high soybean meal diet with 60% fish meal protein replacement and without LF supplementation; LF2, LF6, and LF10 were added 2, 6, and 10 g/kg LF in SBM60 diets, respectively.

Abbreviations: IL-8, interleukin-8; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; IL-12, interleukin-12; TGF-β1, transforming growth factor-β1; IL-10, interleukin-10.

Supplementary Files

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