

Research Article

Synergistic Effect of Dietary Inactivated *Lactobacillus plantarum* and Berberine Supplementation on Growth Performance, Antioxidant Capacity, and Immune Function of Juvenile Black Sea Bream (*Acanthopagrus schlegelii*)

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The interactive effect of dietary inactivated *Lactobacillus plantarum* and berberine on black sea bream, *Acanthopagrus schlegelii*, was investigated with three diets designated as D1 (Con: basal diet), D2 (LP: basal diet + 400 mg/kg *L. plantarum*), and D3 (LPBB: basal diet + 400 mg/kg *L. plantarum* + 50 mg/kg berberine) and fed to juvenile black sea bream (5.67 ± 0.05 g) for 56 days. The growth performance and feed utilization parameters, as well as intestinal trypsin activity, were significantly improved in the LP and LPBB groups ($P < 0.05$). Fish fed the LPBB diet showed better serum and hepatic antioxidant capacity, whilst the LP group had better hepatic antioxidant capacity, than the control fish ($P < 0.05$). Intestinal IgM and C3 levels significantly increased in the LPBB fish than the rest of the groups ($P < 0.05$). *NF- κ B* was significantly upregulated in the LP group ($P < 0.05$). *Nrf2* and *IL-10* were significantly upregulated, whilst *Keap1b* and *NF- κ B* were significantly downregulated in the LPBB group ($P < 0.05$). These findings show that a combination of inactivated *L. plantarum* and berberine in the diet of black sea bream can improve the immune response and antioxidant capacity than a diet with only the inactivated probiotic, whilst both diets can equally improve growth performance.

1. Introduction

Global aquaculture production has been growing consistently over the past decades than all the other food sectors partly due to continuous research and innovation [1]. The use of functional feed additives to alleviate losses to stress, pests, and disease outbreak has been an essential research focus. With the use of antibiotics becoming less accepted due to the detrimental effects of their abuse on aquatic environments, animal natural immune function, and risk of consumers developing super bugs [2, 3], there has been copious

research into alternative functional additives such as probiotics [4, 5], plant extracts [6, 7], prebiotics [8, 9], and organic acids [10, 11], which are considered more environmentally friendly and less likely to develop resistant bacteria.

Berberine is an organic compound present in herbs like *Coptis chinensis*, *Berberis thunbergii*, and *Hydrastis canadensis*, with huge popularity in traditional Chinese medicine. This isoquinoline alkaloid has a long history dating back to ancient China in its use against proliferation, inflammation, and hypertension [12, 13]. Natural materials like hydrochloride present in berberine give it the ability to ameliorate the

impairment of hepatocyte function and excessive lipid in the blood [14]. The potential of berberine to help improve productivity in aquaculture through its prophylactic effects has been demonstrated in different fish species, including grass carp, *Ctenopharyngodon idella* [15]; blunt snout bream, *Megalobrama amblycephala* [16]; and black sea bream, *Acanthopagrus schlegelii* [17].

According to the FAO/WHO, probiotics are live microorganisms that have beneficial effects on the host when administered in ample amount [18]. Although probiotics are expected to be viable in order to perform their functions, there is evidence of “dead” probiotics and their metabolites eliciting similar responses as their live forms albeit with potential differences [19, 20]. Inactivated probiotics are considered a safer alternative to live forms owing to the risk of live microorganisms distorting the ecosystem of the rearing environs, especially in open waters. Furthermore, inactivated bacteria are easier to transport and store [21], and can withstand the harsh conditions associated with feed processing than live forms, making them more suited for aquafeed. *Lactobacillus plantarum* belongs to a group of microorganisms called lactic acid bacteria (LAB), which generate lactic acid as the main product of carbohydrate fermentation, with a wide range of application in nutrition [22, 23]. Inactivated *L. plantarum* was shown to have the potential to improve growth performance and/or health condition in different fish species, including red sea bream, *Pagrus major* [24]; Nile tilapia, *Oreochromis niloticus* [25]; bighead catfish, *Clarias macrocephalus* [26]; snakehead, *Channa striata* [27]; and black sea bream [28].

Black sea bream is a very important commercial fish species in many parts of Asia and is widely distributed along the Northwest Pacific, from the coasts of Japan and Korea to the South China Sea [29]. The commercial and recreational importance of this species has led to depletion of wild stocks due to pressure from overfishing [30]. Aquaculture production of black sea bream is therefore essential to reduce pressure on the wild stocks. Several studies have investigated the effect of combining dietary probiotics with other functional additives on different fish species: *L. plantarum* was combined with beta-glucan in diets of red sea bream [31]; *Bacillus subtilis* was combined with mannanoligosaccharide for Japanese eel, *Anguilla japonica* [32]; and the interactive effect between *L. plantarum* and pineapple peel powder was investigated on Nile tilapia [33]. Drawing inference from the earlier reports that explored the possible synergies between dietary probiotics and other functional additives and previous studies in our laboratory, which separately demonstrated positive effects of inactivated *L. plantarum* (100 mg/kg \leq 400 mg/kg), and berberine (50 mg/kg) on black sea bream [17, 28], this study further explored the possible interactive effects of *L. plantarum* and berberine on growth performance, antioxidant capacity, and immune response of this species. With no previous studies on a combination between the two additives in the diet of any farmed animal to the best of our knowledge, the study focused on whether adding berberine to *L. plantarum* could achieve a better impact than the probiotic-only diet. This will add to the alternatives for producing

functional feeds that can boost productivity in black sea bream farming.

2. Materials and Methods

2.1. Diet Preparation. The inactivated probiotic (Immuno-LP20) is a product of House Wellness Foods Corp. (Itami, Japan). The procedure for preparation was as described previously [34]. It is an off-white powder with 2×10^{11} cfu g⁻¹ inactivated *L. plantarum* (L-137) (20%) and dextrin hydrolysed from tapioca (80%). Berberine (HPLC \geq 98) was procured from Spring and Autumn Biotechnology Company (Nanjing, China). The basal diet was formulated to contain the same crude protein and crude lipid levels (Table 1). The main protein ingredients (fishmeal, fermented soybean meal, and soybean protein concentrate) were finely ground and sieved with a 180 μ m mesh, and samples were taken from each ingredient for proximate composition analysis. Fish oil, soy lecithin, and corn oil were used as the main lipid sources. The diets were designated as D1 (Con: basal diet, control), D2 (LP: basal diet + 400 mg/kg *L. plantarum*), and D3 (LPBB: basal diet + 400 mg/kg *L. plantarum* + 50 mg/kg berberine). Methionine and lysine were supplemented, and the basal diet was formulated based on earlier reports on black sea bream [35, 36], whilst the *L. plantarum* and berberine inclusion levels were based on our previous studies [17, 28]. The ingredients were mixed and homogenized to form a dough, which was extruded via a 2.5 mm mould feed machine (HKJ-218, HUARUI, Wuxi, China). The pellets were then steamed for about 10 minutes and dried at 24°C for about 72 hours in a ventilated room and stored at -20°C. Representative samples were used for proximate composition analysis.

2.2. Experimental Setup and Feeding. The experimental procedures in this study were according to the Guidelines of the Care and Use of Laboratory Animals in China, and the study was approved by the Committee on the Ethics of Animal Experiments of Zhejiang University (Ethics code: ZJU20200076). All fish were handled with care during all the stages of this experiment.

Black sea bream fingerlings were obtained from a hatchery close to the rearing facility and acclimated with a commercial feed (42% crude protein, Ming Hui Co. Ltd.) for a 14-day period. The rearing experiment took place at the Zhejiang University Test Base for Marine Science and Ocean Engineering located on Zhai Ruo Shan Island, East China Sea (Zhoushan, China). After a successful acclimation, the fish were starved for 24 hours, and healthy ones in good physical condition without ragged fins, swollen abdomen, hemorrhages, and exophthalmia were selected (5.67 ± 0.05 g) and stocked in 9 cylindrical tanks. Each tank had 40 fish in 700 L seawater, and they were randomly divided into triplicates of three treatments. The tanks were connected to a flow-through system with continuous supply of sea water filtered with biofilter and drum filter (2 L/min flow rate). The tanks were continuously aerated with air stones for optimum dissolved oxygen (7.1–9.5 ppm). Temperature (26.5–28.2°C), pH (8.1–8.3), and salinity (27–29 g/L) were also monitored

TABLE 1: Formulation and proximate composition of the experimental diets.

Ingredient (g/kg)	D1 (Con)	D2 (LP)	D3 (LPBB)
Fishmeal	340	340	340
Fermented soybean meal	150	150	150
Soy protein concentrate	110	110	110
Fish oil	50	50	50
Corn oil	50	50	50
Soy lecithin	20	20	20
Alpha-starch	160	160	160
Ca (H ₂ PO ₄) ₂ ·H ₂ O	25	25	25
Vitamin mix ¹	3	3	3
Mineral mix ²	5	5	5
Betaine	20	20	20
Phytase	0.5	0.5	0.5
Y ₂ O ₃	1	1	1
Carrageenan	5	5	5
Methionine	6	6	6
Lysine	3	3	3
HKLP ³	0	0.4	0.4
Berberine	0	0	0.05
Alpha-cellulose	21.5	21.1	21.05
Zeolite powder	30	30	30
Sum	1000	1000	1000
Proximate composition (% dry matter)			
Crude protein	40.42	40.43	40.41
Crude lipid	15.05	15.06	15.05
Carbohydrate ⁴	21.26	21.25	21.26
Moisture	11.64	10.81	11.00
Ash	11.64	12.45	12.28
Gross energy (kJ/g) ⁵	19.15	19.15	19.15
P/E ratio	21.10	21.10	21.10

¹Vitamin mix (mg/kg): α -tocopherol, 80; retinyl acetate, 40; cholecalciferol, 0.1; menadione, 15; niacin, 165; riboflavin, 22; pyridoxine, 40; thiamin mononitrate, 45; D-Ca pantothenate, 102; folic acid, 10; vitamin B-12, 0.9; inositol, 450; ascorbic acid, 150; Na menadione bisulphate, 15; thiamin, 5; choline chloride, 320 and p-aminobenzoic acid, 50; thiamin, 5; choline chloride, 320 and p-aminobenzoic acid, 50. ²Mineral mix (mg/kg): Na₂SiO₃, 0.4; CaCO₃, 544.9; NaH₂PO₄·H₂O, 200; KH₂PO₄, 200; MgSO₄·7H₂O, 10; MnSO₄·H₂O, 4; CuCl₂·2H₂O, 2; ZnSO₄·7H₂O, 12; FeSO₄·7H₂O, 12; NaCl, 12; KI, 0.1; CoCl₂·6H₂O, 0.1; Na₂MoO₄·2H₂O, 0.5; AlCl₃·6H₂O, 1; and KF, 1. ³HKLP: Heat-killed *Lactobacillus plantarum* (immuno-LP20) House Wellness Foods Corp., Itami, Japan. ⁴Carbohydrate (%) = 100 - (%crude protein + %crud lipid + %moisture + %ash). ⁵Calculated based on 17.2 kJ/g carbohydrate, 23.6 kJ/g protein, and 39.5 kJ/g lipid.

every day. The fish were fed to satiation twice daily (8:00 and 15:00) during the eight-week rearing period. Each tank was observed regularly to remove dead fish.

2.3. Sampling and Biochemical Analysis. After the feeding period, the fish were anesthetized with 60 mg/L MS-222 after a 24-hour starvation. Thereafter, they were individually counted and measured for weight and length. Blood was drained from the caudal vein with 27-gauge needle and 1 mL syringe, allowed to settle for 2 hours and centrifuged

at 10,000 g for 20 minutes (4°C) to obtain the serum. The liver, viscera, and intraperitoneal fat were weighed and recorded after dissection to determine body condition indices, and dorsal muscle, foregut, and liver samples were stored at -80°C with whole body and serum for proximate analysis and assay. The intestine samples were also frozen in liquid nitrogen for gene analysis.

Proximate analysis of fish and feed samples were conducted using the methods of the Association of Official Analytical Chemists [37]. Supernatants of the intestine and liver samples for assays were separated following the process used in Sagada et al. [38]. The diagnostic reagent kits for chemical analyses were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Glutathione S-transferase (GST) activity was assayed as described by Elia et al. [39]. Malondialdehyde (MDA) content and catalase (CAT), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) activities were determined as described by Han et al. [40]. Concentrations of immunoglobulin (IgM) and complement (C3 and C4) proteins were analysed using the enzyme-linked immunosorbent assay (ELISA) method [41]. Intestinal digestive enzymes (trypsin, amylase, and lipase) were determined according to the assay protocols described by Li et al. [42].

2.4. Quantitative PCR Assay. Total RNA extracted from four replicates of each intestine sample (TaKaRa MiniBEST Universal RNA Extraction Kit, Takara, Japan) were used for reverse transcription with PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time, Takara, Japan). The primer pairs for the various genes were designed by Primer Premier 5.0 using RNaseq data previously obtained in our lab (Table 2). SYBR Premix Ex Taq™ II Kit (Takara, Japan) was used for the real-time qPCR with the following process: 95°C for 30 s, 40 cycles of 95°C for 5 s, followed by 60°C for 30 s. β -Actin was used as the reference gene, and the fold change was calculated with the $2^{-\Delta\Delta CT}$ method [43].

2.5. Statistical Analysis. All the data are shown as the mean \pm standard deviation (SD) ($n = 3$). SPSS 20.0 (IBM SPSS, Chicago, IL, USA) was used to conduct one-way analysis of variance (ANOVA), which tested the effects of the diets on the various parameters. Tukey's multiple range test was used to check for differences between the means ($P < 0.05$), normality was determined with the Kolmogorov-Smirnov test and homogeneity of variance checked by Levene's test.

3. Results

3.1. Growth, Body Condition, and Feed Utilization. According to the growth parameters shown in Table 3, fish fed the diets with inactivated *L. plantarum*-only (LP), and a combination of the inactivated probiotic and berberine (LPBB) had significantly higher final body weight (FBW), weight gain (WG), and specific growth rate (SGR) than the control group ($P < 0.05$), and the growth parameters did not differ significantly between both of the supplemented groups ($P > 0.05$). The body condition indicators (viscerosomatic index, condition factor, hepatosomatic index, and

TABLE 2: Gene-specific primers used in this study.

Gene ¹	Nucleotide sequence	Size (bp)	References
<i>IFN-γ</i>	Forward: CACATACAACCTCCAGAGCCCATAC	163 bp	EU699438.1
	Reverse: ACTGGGTGTAGGTGGCAGGTAGGTC		
<i>IL-10</i>	Forward: TGTCAAACGGTTCCTTGCAG	172 bp	MK922542
	Reverse: GGCATCCTGGGCTTCTATCT		
<i>NF-κB</i>	Forward: AGCCCAAGGCACTCTAGACA	154 bp	MK922543
	Reverse: GTTCTGGGCAGCTGTAGAGG		
<i>Nrf2</i>	Forward: GATGTTACCCTCAAGAAGTTCCCGC	106 bp	
	Reverse: GAGGCTGGGAACATTCTTTGATTG		
<i>Keap1b</i>	Forward: AATGTTCTGCTGTGAGTCTGAAGTCT	111 bp	
	Reverse: ATGGCAGCGGACAGCCTGGAGTAAG		
<i>β-Actin</i>	Forward: TATCGTCATGGACTCCGGTG		
	Reverse: TGATGTCACGCACGATTTCC		

¹*IFN-γ*: interferon γ ; *IL-10*: interleukin 10; *NF-κB*: nuclear factor-kappa B; *Nrf2*: nuclear factor erythroid-2-related factor 2; *Keap1b*: Kelch-like ECH-associated protein 1b.

TABLE 3: Growth performance and morphological indices of juvenile black sea bream (*Acanthopagrus schlegelii*) fed the experimental diets.

Parameters	Diets		
	D1 (Con)	D2 (LP)	D3 (LPBB)
SR ¹	99.17 ± 1.4	99.17 ± 1.4	99.17 ± 1.4
FBW ²	27.30 ± 0.71 ^b	35.00 ± 2.76 ^a	35.95 ± 1.23 ^a
WG ³	382.91 ± 14.32 ^b	516.76 ± 51.55 ^a	532.86 ± 21.11 ^a
SGR ⁴	2.81 ± 0.05 ^b	3.24 ± 0.15 ^a	3.29 ± 0.06 ^a
VSI ⁵	7.12 ± 0.29	7.93 ± 1.23	7.81 ± 0.99
HSI ⁶	1.85 ± 0.33	2.02 ± 0.21	2.15 ± 0.38
IPF ⁷	1.65 ± 0.23	2.13 ± 0.42	1.92 ± 0.41
CF ⁸	2.33 ± 0.63	2.53 ± 0.66	2.72 ± 0.83

Data are presented as the mean ± SD ($n = 3$); values with different superscripts in the same row differ significantly ($P < 0.05$). ¹SR: % (survival rate) = $100 \times (\text{final number of fish}/\text{initial number of fish})$. ²FBW: g (final average body weight). ³WG: % (weight gain) = $100 \times (\text{final body weight} - \text{initial body weight})/\text{initial body weight}$. ⁴SGR: % (specific growth rate) = $100 \times (\ln \text{ final body weight} - \ln \text{ initial body weight})/\text{days}$. ⁵VSI: % (viscerosomatic index) = $100 \times (\text{viscera weight}/\text{body weight})$. ⁶HSI: % (hepatosomatic index) = $100 \times (\text{liver weight}/\text{body weight})$. ⁷IPF: % (intra-peritoneal fat ratio) = $100 \times (\text{intra-peritoneal fat weight})/(\text{body weight})$. ⁸CF: g/cm³ (condition factor) = $100 \times \text{body weight}/(\text{total length, cm})^3$.

intra-peritoneal fat ratio) and survival rate were not significantly affected by the dietary treatments ($P > 0.05$). The impacts of the diets on feeding rate (FR), protein productive value (PPV), protein efficiency ratio (PER), daily feed intake per fish (DFI), total feed intake (TFI), and feed conversion ratio (FCR) are shown in Table 4. FR, PPV, and PER were not significantly affected by the dietary treatments ($P > 0.05$). DFI and TFI significantly increased, whilst FCR significantly decreased in fish fed LP and LPBB in comparison with the control fish ($P < 0.05$). There was however no significant difference in the feed intake and utilization parameters between the LP and LPBB groups ($P > 0.05$).

TABLE 4: Feed utilization of juvenile black sea bream (*Acanthopagrus schlegelii*) fed the experimental diets.

Parameters	Diets		
	D1 (Con)	D2 (LP)	D3 (LPBB)
TFI ¹	1161.43 ± 46.28 ^b	1285.78 ± 29.21 ^a	1312.58 ± 44.31 ^a
DFI ²	0.52 ± 0.02 ^b	0.57 ± 0.02 ^a	0.59 ± 0.09 ^a
FR ³	3.17 ± 0.14	2.83 ± 0.29	2.84 ± 0.10
FCR ⁴	1.36 ± 0.06 ^a	1.11 ± 0.14 ^b	1.10 ± 0.05 ^b
PER ⁵	1.82 ± 0.08	2.26 ± 0.31	2.26 ± 0.11
PPV ⁶	32.96 ± 0.76	39.49 ± 4.80	40.02 ± 20.04

Data are presented as the mean ± SD ($n = 3$); values with different superscripts in the same row differ significantly ($P < 0.05$). ¹TFI: g (total feed intake, dry weight basis). ²DFI: (daily feed intake, g/fish/day) = feed intake (dry weight)/final number of fish/no. of days. ³FR: (feeding rate) = feed intake (dry weight) × 100/[days × (final body weight + initial body weight)/2]. ⁴FCR: (feed conversion ratio) = dry weight of feed (g)/weight gain (g). ⁵PER: (protein efficiency ratio) = weight gain/protein intake. ⁶PPV: % (protein productive value) = $100 \times \text{protein gain (g)}/\text{total protein intake (g)}$.

3.2. Proximate Body Composition. The muscle and whole-body moisture, crude protein, ash, and crude lipid contents of fish fed the test diets were not significantly impacted ($P > 0.05$) as shown in Table 5.

3.3. Serum and Liver Antioxidant Parameters. As shown in Table 6, hepatic GSH-Px activity, serum CAT and GST activities, and serum MDA content were not significantly impacted by the dietary treatments ($P > 0.05$). Fish fed LPBB had significantly higher Serum SOD activity than the control fish and significantly higher GSH-Px activity than the rest of the groups ($P < 0.05$). The hepatic SOD activity was significantly higher in fish fed LP than the control group, and the GST activity significantly higher than the rest of the groups ($P < 0.05$). There was significantly higher liver CAT activity

TABLE 5: Proximate composition (wet weight) of juvenile black sea bream (*Acanthopagrus schlegelii*) fed the experimental diets.

Parameters	Diets		
	D1 (Con)	D2 (LP)	D3 (LPBB)
<i>Whole body</i>			
Moisture	65.73 ± 0.25	65.49 ± 0.19	65.27 ± 0.20
Crude protein	17.44 ± 0.39	17.17 ± 0.26	17.35 ± 0.07
Crude lipid	12.35 ± 0.39	13.18 ± 0.33	12.98 ± 0.49
Ash	4.78 ± 0.30	4.63 ± 0.12	4.69 ± 0.03
<i>Muscle</i>			
Moisture	73.54 ± 0.13	73.21 ± 0.61	72.94 ± 0.32
Crude protein	20.91 ± 0.07	20.93 ± 0.35	20.75 ± 0.48
Crude lipid	4.02 ± 0.17	4.24 ± 0.19	4.38 ± 0.09
Ash	2.13 ± 0.19	2.05 ± 0.14	2.03 ± 0.10

Data are presented as the mean ± SD ($n=3$); values with different superscripts in the same row differ significantly ($P < 0.05$).

in the LP and LPBB groups than fish fed the control diet ($P < 0.05$).

3.4. Intestinal Digestive Enzyme Activity and Immune Parameters. The activities of intestinal lipase (Figure 1(b)) and amylase (Figure 1(c)) in black sea bream were not significantly impacted by the dietary treatments ($P > 0.05$), but trypsin activity (Figure 1(a)) was significantly higher in the LP and LPBB groups than the control fish ($P < 0.05$). C4 content (Figure 2(c)) of the test fish was not significantly affected by the dietary treatments ($P > 0.05$), but the IgM and C3 protein contents (Figures 2(a) and 2(b)) were significantly higher in fish fed the LPBB diet than the rest of the groups ($P < 0.05$).

3.5. Intestinal Gene Expression. The relative expression levels of interferon γ (*IFN- γ*) (Figure 3(a)), interleukin-10 (*IL-10*) (Figure 3(b)), nuclear factor-kappa B (*NF- κ B*) (Figure 3(c)), nuclear factor erythroid-2-related factor 2 (*Nrf2*) (Figure 3(d)), and Kelch-like ECH-associated protein 1b (*Keap1b*) (Figure 3(e)) of black sea bream were assessed. *IFN- γ* was not significantly affected by the dietary treatments ($P > 0.05$). The LP fish had significantly upregulated *NF- κ B* than the rest of the groups and significantly downregulated *Keap1b* than the control fish ($P < 0.05$). The LPBB fish had significantly upregulated *Nrf2* and *IL-10* and significantly downregulated *Keap1b* and *NF- κ B* than the rest of the groups ($P < 0.05$).

4. Discussion

The interactive effect of dietary probiotics in combination with other nonantibiotic functional additives is being investigated in recent times in the quest to develop functional feeds to improve production of different aquaculture species. This study sets out to compare the effects of dietary inactivated *L. plantarum* alone (LP) with that of a combination between *L. plantarum* and berberine (BB) on black sea bream. No berberine-only treatment was included in this

trial because previous studies in our laboratory showed how berberine supplementation in black sea bream diets improved the health condition, evinced in antioxidant function, inflammatory cytokines, and other health-related parameters [17, 44, 45]. The FBW, WG, and SGR results from the study show that the combination (LPBB) significantly improved growth performance, but the synergistic effect of the combination could not significantly improve the growth than the probiotic alone, despite the slightly higher feed intake and feed utilization it produced. The higher growth in fish fed both LP and LPBB can be attributed to better appetite and higher feed intake due to the significantly less feed eaten by the control fish. The ability of dietary probiotics to improve growth by increasing nutrient availability and utilization through synthesizing extracellular enzymes, fatty acids, vitamins, and amino acids [46, 47] was shown in the growth and FCR values of this study. Similar to these results, previous reports showed growth-promoting effects of inactivated probiotics on different fish species, including grouper by *Bacillus pumilus* [48], tilapia by *L. plantarum* [49], red sea bream by *L. plantarum* [24], and black sea bream by *L. plantarum* [28]. Berberine supplementation did not improve the growth of black sea bream in our previous study [17], and combining *L. plantarum* with berberine produced only slightly higher growth than the LP diet in the current result. However, berberine supplementation promoted growth and feed utilization in other fish [12, 50, 51], possibly through modulation of intestinal microbiota [52, 53]. In terms of interactive effect, this study is similar to an earlier one in which dietary *Lactobacillus rhamnosus* GG combined with Jerusalem artichoke, *Helianthus tuberosus*, improved the growth performance of red tilapia, *Oreochromis spp.*, than the nonsupplemented control diet, but there was no significant difference when compared with fish fed the diet with only the probiotic [54]; and this contradicts another study in which dietary *L. plantarum*+Jerusalem artichoke significantly improved growth performance than both the control and probiotic-only diets administered to *Pangasius catfish*, *Pangasius bocourti* [55]. The current result is also dissimilar to our earlier study, in which a combination of selenomethionine and inactivated *L. plantarum* caused a decrease in growth of black sea bream [56]. The inability of the combination of both *L. plantarum* and berberine to improve growth than the LP diet could be because similar to berberine, dietary probiotics can also improve the growth of fish by modulating the intestinal microbiota [57, 58]. The similarity in their growth-promoting mechanisms could have inhibited additivity of their separate effects since they both could have conferred the same benefit in this regard. Considering the additivity of the impacts of various feed supplements could help establish the possibility of their synergy.

Oxidation and antioxidant parameters were measured vis-à-vis the dietary treatments to ascertain the ability of the various diets to provide protection against potential oxidative damage. Hepatic SOD, CAT, and GST activities, as well as serum GSH-Px and SOD activities, were significantly impacted. Interestingly, only the LPBB diet caused significantly increased serum antioxidant enzyme activities than

TABLE 6: Serum and liver antioxidant parameters of juvenile black sea bream (*Acanthopagrus schlegelii*) fed the experimental diets.

Parameters	Diets		
	D1 (Con)	D2 (LP)	D3 (LPBB)
<i>Serum</i>			
SOD (U mL ⁻¹)	155.95 ± 7.48 ^b	177.22 ± 20.20 ^{ab}	191.69 ± 6.26 ^a
GSH-Px (U mL ⁻¹)	116.52 ± 5.48 ^b	114.51 ± 4.39 ^b	144.25 ± 5.77 ^a
GST (U mL ⁻¹)	19.95 ± 1.51	19.72 ± 2.42	19.25 ± 1.76
CAT (U mL ⁻¹)	3.77 ± 1.34	3.80 ± 1.04	3.52 ± 0.50
MDA (nmol mL ⁻¹)	25.68 ± 7.67	25.26 ± 2.32	26.43 ± 4.69
<i>Liver</i>			
SOD (U mgprot ⁻¹)	871.54 ± 128.83 ^b	1103.39 ± 76.53 ^a	892.41 ± 30.97 ^{ab}
GSH-Px (U mgprot ⁻¹)	8.04 ± 2.32	10.95 ± 1.40	10.52 ± 1.23
GST (U mgprot ⁻¹)	27.73 ± 2.10 ^b	37.62 ± 2.65 ^a	28.18 ± 2.95 ^b
CAT (U mgprot ⁻¹)	18.77 ± 1.12 ^b	23.94 ± 0.65 ^a	22.83 ± 1.67 ^a
MDA (nmol mgprot ⁻¹)	2.84 ± 1.01	2.53 ± 0.68	2.99 ± 0.95

Data are presented as the mean ± SD ($n = 3$); values with different superscripts in the same row differ significantly ($P < 0.05$). SOD: superoxide dismutase; GSH-Px: glutathione peroxidase; GST: glutathione S-transferase; CAT: catalase; MDA: malondialdehyde.

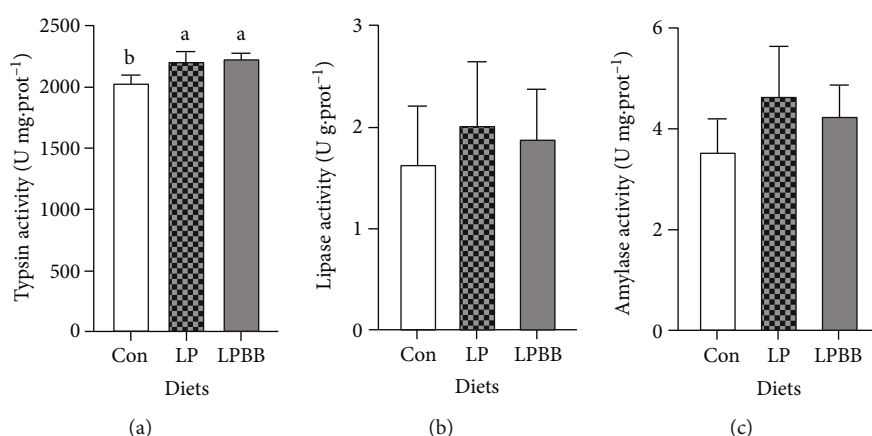


FIGURE 1: Foregut digestive enzyme activity of black sea bream fed the experimental diets. Different letters on bars show significant difference ($P < 0.05$). (a) Trypsin activity; (b) lipase activity; (c) amylase activity. Con, fish fed D1: basal diet; LP, fish fed D2: basal diet +400 mg/kg *L. plantarum*; LPBB, fish fed D3: basal diet+400 mg/kg *L. plantarum*+50 mg/kg berberine.

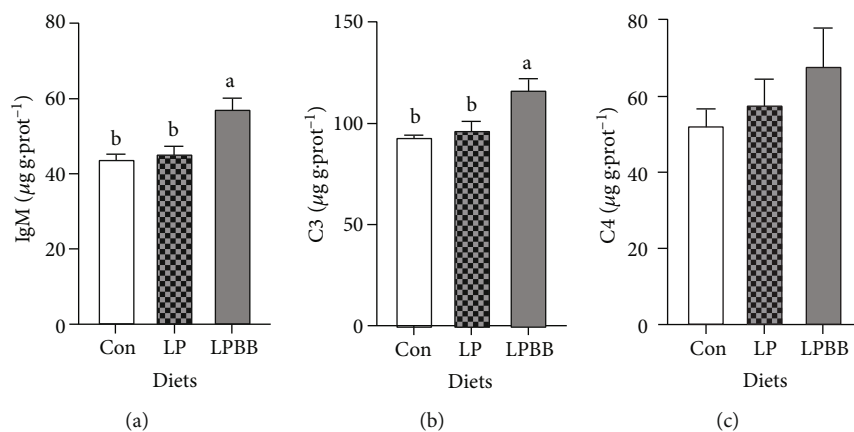


FIGURE 2: Foregut immune parameters of black sea bream fed the experimental diets. Different letters on bars show significant difference ($P < 0.05$). (a) Immunoglobulin M (IgM); (b) complement 3 (C3); (c) complement 4 (C4). Con, fish fed D1: basal diet; LP, fish fed D2: basal diet+400 mg/kg *L. plantarum*; LPBB, fish fed D3: basal diet+400 mg/kg *L. plantarum*+50 mg/kg berberine.

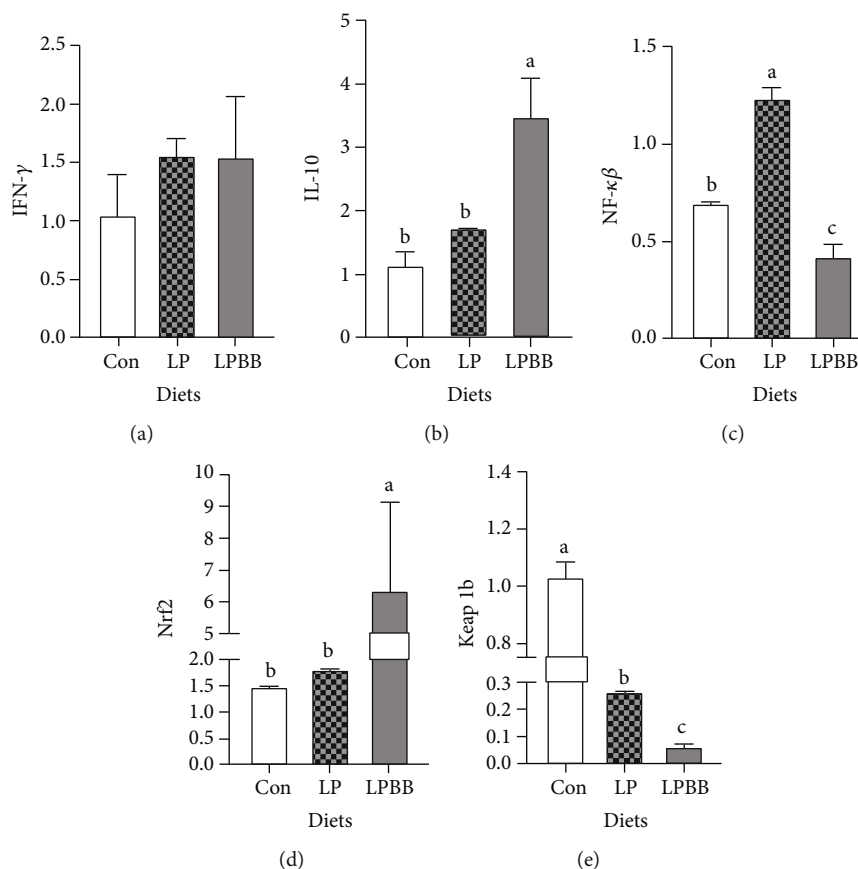


FIGURE 3: Relative expression levels of immune and antioxidant-related genes in foregut of black sea bream fed the experimental diets. Different letters on bars show significant difference ($P < 0.05$). (a) *IFN- γ* : Interferon γ ; (b) *IL-10*: interleukin-10; (c) *NF- κ B*: nuclear factor-kappa B; (d) *Nrf2*: nuclear factor erythroid-2-related factor 2; (e) *Keap1b*: Kelch-like ECH-associated protein 1b. Con, fish fed D1: basal diet; LP, fish fed D2: basal diet+400 mg/kg *L. plantarum*; LPBB, fish fed D3: basal diet+400 mg/kg *L. plantarum*+50 mg/kg berberine.

the control fish, but both the LP and LPBB diets conferred significantly higher antioxidative protection on the liver. The improved antioxidant parameters in both the liver and serum of the LPBB fish compared to improvement in only the liver in the LP group could be an indication of better protection by the combination of *L. plantarum* and berberine against oxidative stress than the probiotic alone, but it is not clear why the LP diet could not improve the parameters in the serum as well. In line with this result, dietary *Pseudomonas aeruginosa* VSG2 and *L. plantarum* VSG3 could not significantly affect the serum SOD activity in rohu, *Labeo rohita* [59, 60]. The antioxidant enzymes GSH-Px, SOD, CAT, and GST all provide antioxidative defence via different mechanisms. The antioxidant defence system of fish works to prevent the buildup of reactive oxygen species (ROS) such as superoxides, hydroxyl radicals, and peroxides, which cause oxidative stress [61]. Comparable to the current results, *Bacillus* supplementation in diets boosted the antioxidant capacity of grass carp, *Ctenopharyngodon idellus* [62, 63]; dietary berberine enhanced the antioxidant defence system in blunt snout bream [50] and black sea bream [44]; whilst *B. licheniformis* and fructooligosaccharide supplemented together in a diet improved the antioxidant defence ability of triangular bream, *Megalobrama terminalis* [64].

Probiotics are believed to boost the antioxidant defence system by enhancing the secretion of antioxidant enzymes [63, 64], whilst berberine was shown to attenuate oxidative stress through improving mitochondria density and increasing activities of complex I and complex II by upregulating *Sirt3* expression [65], or via the p38 mitogen-activated protein kinase (MAPK) pathway [66]. Furthermore, probiotics and berberine can both regulate antioxidant capacity through the Nrf2/Keap1 signaling pathway. *Nrf2* is a transcription factor, which binds with antioxidant response element (ARE) sequences to produce antioxidant enzymes, and *Keap1* is a molecular switch that holds *Nrf2* from translocating into the nucleus to bind with ARE [67]. *L. plantarum* modulated the Nrf2-Keap1-ARE pathway in mice [68], whilst berberine was also shown to activate the Nrf2 antioxidant pathway in rats [69]. In this study, the LPBB diet significantly upregulated *Nrf2* and downregulated *Keap1b* in the intestine of black sea bream than the rest of the diets, and the LP diet significantly downregulated *Keap1b* than the control diet. This indicates the tendency of a diet with both *L. plantarum* and berberine to modulate the Nrf2/Keap1 signaling pathway than *L. plantarum* alone. Interestingly, this bears similarity to the antioxidant enzyme activity in this study, since fish fed the LPBB

diet had significantly higher antioxidant indices in both the serum and liver, whilst the LP fish only had significantly higher hepatic antioxidant parameters, than the control fish. From the results of the antioxidant indices, as well as the expression of *Nrf2* and *Keap1b* genes, it can be posited that supplementing both berberine and *L. plantarum* in the diet of black sea bream could confer better protection against oxidative stress than the inactivated probiotic alone.

The tendency of probiotics to increase digestive enzyme activity possibly through secretion of extracellular enzymes [70] has been well documented in different fish species: *B. subtilis* supplementation in diets of Nile tilapia increased protease and amylase activities [71]; *B. subtilis* and *Lactococcus lactis* improved trypsin activity in Nile tilapia [72]; and *Enterococcus faecalis* inclusion in diets increased protease and lipase activities in Javanese carp, *Puntius gonionotus* [73]. The ability of berberine to modulate intestinal microbiota could somewhat influence digestive enzymes, but there is paucity of empirical evidence to corroborate that. The significantly higher trypsin activity in fish fed both the LP and LPBB diets correlates with the growth and feed utilization results and can be attributed to enzyme-secreting ability of *L. plantarum* and the influence of the probiotic and berberine on intestinal microbiota and antioxidative status, which are beneficial to intestinal integrity and digestive enzymes. Continuous exposure to probiotics could inhibit endogenous enzyme activity [58] and may have affected lipase and amylase activities in this study.

The interaction between ingested feed and the enteric sensory neurons highly influences intestinal health, which encompasses immune function, structure, and microbiome. The intestine in animals is relatively large in comparison to other major immune organs; hence, it plays a vital role in helping fish resist pathogen invasion. The IgM and C3 protein contents in the foregut of black sea bream in this study were significantly increased by inclusion of inactivated *L. plantarum* and berberine together. The immunoglobulin and complement system are essential parts of an animal's immune system. As the predominant antibody in fish, the IgM helps provide protection against different pathogens [64]. C3 is part of the complement cascade system in teleost fish and can cause the death of pathogens by perforating their surface membrane [74]. The higher levels of C3 and IgM in fish fed the LPBB diet than both the control and LP groups means *L. plantarum* and berberine could be functionally synergistic in modulating immune response in black sea bream. Similarly, inactivated *L. plantarum* supplementation did not significantly impact intestinal IgM and C4 in our previous study on the same fish species [28] and is attributable to immunity fatigue from the probiotic supplementation [60, 75], which may have been ameliorated by berberine in this study. The immunostimulant effect of berberine in this study is similar to earlier reports on blunt snout bream [12, 50], and Nile tilapia [51]. The immunostimulating ability of berberine is attributable to its influence on enteric glial cells (EGC), bone marrow-derived dendritic glial cells (BMDCs), T cells, and gut epithelial cells (IEC) [76]. Another mechanism by which berberine modulates immune response could be by attenuating the TLR4/NF-

κ B-p65 signaling pathway and activating the adenosine monophosphate- (AMP-) activated protein kinase (AMPK) signaling [77].

As a first line of defense against pathogens, IECs crucially sense signals from different sources of stimuli, including beneficial bacteria, which it in turn uses to produce cytokines and chemokines for maintenance of gut homeostasis [78, 79]. The integrity of IECs can be compromised by proinflammatory cytokines, leading to disorders, and the anti-inflammatory cytokines serve to counteract this effect. *IFN- γ* , a proinflammatory cytokine, was not significantly affected by the dietary treatments in this study. Fish fed the LPBB diet had significantly downregulated *NF- κ B* and upregulated *IL-10*, an anti-inflammatory cytokine than the rest of the groups, an indication of the ability of the combination of berberine and *L. plantarum* to confer higher anti-inflammatory protection on black sea bream than the probiotic alone. Furthermore, *IL-10* improves immune function by aiding in differentiating immune cells like natural killer (NK) cells, dendritic cells, B cell, and granulocytes [80]. This is in line with the intestinal IgM and C3 contents in this study, which indicated that the LPBB diet conferred higher immunity than *L. plantarum* alone. *NF- κ B* is a transcription factor for proinflammatory cytokines, and fish fed the LP diet had the highest *NF- κ B* expression, indicating the possibility of inactivated *L. plantarum* to confer inflammatory response against pathogen invasion as seen in slightly higher *IFN- γ* expression than the control fish [81]. This is consistent with reports where *Bacillus* spp. [48, 82], and *L. plantarum* [83] increased the expression of proinflammatory cytokines. Berberine has been proven to have anti-inflammatory tendencies and can influence *NF- κ B* pathways [84–86]. These results indicate that inactivated *L. plantarum* alone could increase intestinal proinflammatory response, and berberine inclusion together with *L. plantarum* can help ameliorate the possibility of excessive inflammation damage to IECs and maintain tissue homeostasis.

5. Conclusions

This study determined that combining berberine with inactivated *L. plantarum* can improve the growth, feed utilization, and intestinal trypsin activity of juvenile *A. schlegelii*, albeit not better than the probiotic-only diet. However, the synergy between the functional additives could have a better influence on the immune response as seen in higher intestinal IgM and C3 levels, as well as upregulated *IL-10* and downregulated *NF- κ B*. Combining the additives also had better impact on antioxidant capacity, which was shown in higher serum SOD and GSH-Px activities, as well as upregulated *Nrf2* and downregulated *Keap1b* in the intestine.

Data Availability

All data for this study are available from the corresponding author on reasonable request.

Conflicts of Interest

No conflict of interest exists in this paper.

Acknowledgments

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