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Research Article

Effects of Single and Mixture Probiotic Supplements on Growth, Digestive Activity, Antioxidative Status, Immune and Growth-Related Genes, and Stress Response of Juvenile Red Sea Bream (*Pagrus Major*)

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A 50-day feeding trial was conducted to evaluate the efficiency of single and mixed strains of probiotic bacteria supplements on juvenile red sea bream (*Pagrus major*). The study investigated the growth, digestibility, hemato-biochemistry, antioxidant, immune, immune, and growth gene expression and stress responses of *P. major*. Three hundred juvenile *P. major* (21.56 ± 0.09 g) were randomly distributed into fifteen 200-L polyethylene tanks (20 fish per tank) in triplicate of 5 treatments designated as D1 = the basal diet; *Streptococcus faecalis* (SF) T – 110 5 × 106 cfu/g diet and *Bacillus amyloliquefaciens* (BA) TOA 5001 5 × 105 cfu/g diet in the second group (D2) at 0.2%; mix SF T – 110 1 × 106 cfu/g diet, *Lactobacillus plantarum* (LP) TO – A 4 × 105 cfu/g diet, *Bacillus mesentericus* (BM) TO – A 1 × 102 cfu/g diet, BA TOA5001 5 × 105 cfu/g diet in the third group (D3) at 1%; mix SF T – 110 1 × 106 cfu/g diet, LP TO – A 4 × 105 cfu/g diet, and BM TO – A 1 × 102 cfu/g diet in fourth group (D4) at 0.5%; and single strain BA TOA5001 5×105 cfu/g diet in the fifth group (D5) at 0.5% of dietary proportion. Results showed that D2 and D3 fish groups exhibit better performance, followed by D4, D5, and control group D1. This finding demonstrated that the use of BA in mix strain probiotic bacteria diet (D2, D3) had improved immune response, antioxidant enzymes, immunity (*TNF-a*, *IL-1b*), and growth-related (*IGF-1*, *IGF-2*) mRNA expression of juvenile *P. major* compared to the mix strain D4, single strain D5, and the control D1. Furthermore, single strain D5 and mix strain D4 also exhibit relatively better immune responses in *P. major* than in control D1. Considering the overall fish performances, mix strain SF+BA (D2) and mix strain SF+BM+LP+BA (D3) were the recommended potential mix strain probiotic bacteria supplement for *P. major* and may be useful also for other related aquatic species.

1. Introduction

Historically, marine aquaculture has been a tradition in the Southeast Asia region and has contributed significantly to the provision of animal protein in human diets [1]. Due to increased demand for finfish in domestic and global markets, aquaculture systems are intensified to increase fish production. The increased intensive aquaculture systems encounter ongoing problems with fish subjected to stress conditions resulting in weakening of fish immune system and high susceptibility to pathogens and are still an ongoing problem [2, 3]. Previous studies have highlighted the use of probiotics and prebiotics as alternative best approaches to improve growth performance, fish health condition, tolerance ability to environmental stressors, improve immune responses, and disease resistance [4–6]. Probiotic bacteria have been approved as effective feed supplements for boosting growth performance, immune function, and resistance to pathogens [7]. Common and safe probiotics used in aquaculture feeds include lactic acid bacteria, *Bacillus*, and *Saccharomyces* species [8]. The supplementation of *Bacilli* probiotic (*Bacillus subtilis* and *Bacillus licheniformis*) in white leg shrimp, *Litopenaeus vannamei*, improved growth performance parameters and increased total protein, lysozyme, and hemocyte cell count [9]. The dietary inclusion of *Bacillus amyloliquefaciens* (BA) significantly improves condition factor and relative condition factor in amberjack, *Seriola dumerili* [10]; improves specific growth rate (SGR) and feed conversion ratio (FCR) in Nile tilapia, *Oreochromis niloticus* [11, 12]; improves disease resistance against *Aeromonas hydrophila* infection in eels [13]; improves immune responses in catla fish (*Catla catla*) and channel catfish, *Ictalurus punctatus* [14, 15]; and improves the survival rate of *L. vannamei* challenged with *Vibrio parahaemolyticus* [16]. The individual strain of probiotic bacteria such as *Lactobacillus plantarum* (LP) was reported to improve feed intake (FI), SGR, weight percent, oxidative status, and immune enzyme in koi carp (*Carassius auratus*) [17]. Dietary supplementation of *Lactobacillus casei* in common carp (*Cyprinus carpio*) mitigated exposure to iron oxide nanoparticles [18]. Mix probiotic bacteria (*Streptococcus faecalis* (SF), *Lactobacillus plantarum* (LP), and *Bacillus mesenteric* (BM)) numerically improves final weight gain (WG%), SGR, and relative condition factor in juvenile amberjack, *S. dumerili* [10]. The combination of probiotic bacteria strains may complement or improve the health of an individual strain [8, 19]. A single probiotic may not be suitable for certain host species due to dissimilarity in the physiological and physiochemical status of a host or the surrounding environment [20]. The inclusion of multistrain probiotic bacteria in diets of olive flounder reduces the potential adverse effects of low fishmeal diets [8]. Prior studies on the use of multistrain probiotics in aquaculture have indicated significant results that serve as benchmark information vital for further studies on its application in aquatic animals [21]. Shadrack et al. [10] concluded that using single strain BA and mixed probiotic SF+LP+BM improves the physiological condition and weight gain in *S. dumerili*. Meanwhile, the present study was designed to investigate the potential effects of mix strain SF+BA, mix strain SF+LP+BM+BA, mix strain SF+LP+BM, and single strain BA on growth, digestive activity, blood hemato-biochemistry, immune and growth-related gene, antioxidant, and stress response of juvenile red sea bream, *Pagrus major*. The single strain BA and mix strain SF+LP+BM are commercial probiotic products in Japan. Thus, it is important to evaluate the effect of combining commercial strains BA and SF+LP+BM or commercial strain BA with SF to determine a potential mix probiotic bacteria supplement for fish.

2. Material and Methods

2.1. Bacteria Strain and Ethics. The bacteria strains (BA, SF, LP, and BM) were generously supplied by TOA Pharmaceutical Company (Japan), and the concentration of the dry products were 2×10^8 cfu/g for SF T-110 strain, 8×10^7 cfu/g for LP TO-A strain, 2×10^4 cfu/g for BM TO-A strain, and 1×10^8 cfu/g for BA TOA5001 strain. These probiotic products were sealed in polypropylene bags and stored at -20°C for further use, according to Dawood et al. [22].

Rules of animal experiment in Kagoshima University were applied to fish in an integrated manner on handling and husbandry of cultured species incorporated in the Japanese higher education system. Thus, the experimental protocol of this study was due consideration for animal care (number of fish, fish handling, etc.) according to Japanese animal care guidelines. The experiment was not regarded as harmful to the experimental animals and therefore did not need approval.

2.2. Experimental Design and Diet Formulation. The experiment was conducted based on the design outlined in Shadrack et al. [10] with five diets designated as treatment. The formulation and proximate composition of the experimental diets are shown in Table 1. The bacteria cell powder was supplemented following the percentage proportion of the experimental diets: 0% (control diet = D1), 0.2% (D2 = the basal diet + SF 5×10^6 cfu/g diet, BA 5×10^5 cfu/g diet), 0.5% (D3 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet + BA 5×10^5 cfu/g diet), 1% (D4 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet), and 0.5% (D5 = the basal diet + BA 5×10^5 cfu/g diet). The weight of the dietary proportion was adjusted to 100% by adding cellulose powder. The dry bacteria cells were combined with lipid ingredients, including Pollack liver oil and soybean lecithin, prior to adding water. The ingredients were gently stirred with a spatula for 5 minutes and then in an automated food mixer for 15 minutes. Based on the total weight of the ingredient, 30-40% water was added and stirred for an additional 10 minutes. Pellets were produced using a meat grinder with a 1.2 mm diameter opening and then dried in a convection oven at 45°C to less than 10% of the moisture content. Finally, the dry pellets were packed in polypropylene bags and stored in a freezer at -28°C until use.

The proximate composition of the diets is presented in Table 1. The viability of the bacteria cells incorporated in feed was assessed by spreading onto 3 M™ Petri film aerobic

Table 1: Experimental diets ingredients and proximate composition.

Ingredients	Experimental diets (g/kg)				
	CD1	D2	D3	D4	D5
Brown fish meal[1]	900	900	900	900	900
Soybean meal[2]	300	300	300	300	300
Wheat flour	300	300	300	300	300
Pollack liver oil[3]	80	80	80	80	80
Soybean lecithin[4]	40	40	40	40	40
n-3 HUFA[5]	10	10	10	10	10
Methionine[6]	2.2	2.2	2.2	2.2	2.2
Lysine[7]	8	8	8	8	8
Taurine[8]	1.4	1.4	1.4	1.4	1.4
Vitamin mix[9]	80	80	80	80	80
Mineral mix [10]	80	80	80	80	80
Probio EP [11]	0	4	0	0	0
Toaraze for aquaculture[12]	0	0	10	10	0
Design[13]	0	0	10	0	10
Vitamin C[14]	6	6	6	6	6
Activated gluten[15]	100	100	100	100	100
CMC[16]	20	20	20	20	20
α–Cellulose[17]	72.4	68.4	52.4	62.4	62.4
Total	2000	2000	2000	2000	2000
proximate composition					
Crude protein (g/kg)	406.8 ± 1.6	427.4 ± 48.5	403 ± 6.2	408.4 ± 2.8	401.7 ± 1.1
Crude lipid (g/kg)	127.1 ± 1	141.6 ± 2.01	129.9 ± 1.1	131.3 ± 1.6	130.7 ± 3.4
Crude ash (g/kg)	118 ± 0.3	116.6 ± 1.1	119.2 ± 1	118.4 ± 0.7	117.8 ± 1
Carbohydrate (g/kg)[18]	293.9 ± 0.18	261.6 ± 2.26	303 ± 4.5	294.1 ± 0.4	297.9 ± 2.9
Gross energy (KJ/g)[19]	190.8 ± 1	195.2 ± 1	192 ± 0.7	192.7 ± 0.2	191.5 ± 0.9

D1 = the basal diet; D2 = the basal diet + SF 5×10^6 cfu/g diet + BA 5×10^5 cfu/g diet; D3 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet + BA 5×10^5 cfu/g diet; D4 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet; D5 = the basal diet + BA 5×10^5 cfu/g diet. [1]Nihon Suisan Co. Ltd (Tokyo, Japan); [2]J. Oil Mills, Japan; [3,4]Riken Vitamines, Tokyo, Japan; [5]Highly unsaturated fatty acid n-3: (eicosapentaenoic acid) EPA 0.25 g and (docosahexaenoic acid) DHA 0.25; [6,7,8]Nacalai Tesque, Inc. (Kyoto, Japan); [9]vitamin mixture (g kg^{-1} diet): ?-carotene, 0.10; vitamin D3, 0.01; menadione $NaHSO_3 \cdot 3H_2O$ (K3), 0.05; DL-α-tocopherol acetate (E), 0.38; thiamine-nitrate (B1), 0.06; riboflavin (B2), 0.19; pyridoxine-HCl (B6), 0.05; cyanocobalamin (B12), 0.0001; biotin, 0.01; inositol, 3.85; niacin (Nicotic acid), 0.77; Ca pantothenate, 0.27; folic acid, 0.01; choline chloride, 7.87; ?-aminobenzoic acid, 0.38; cellulose, 1.92; [10]mineral mixture (g kg^{-1} diet): $MgSO_4$, 5.07; Na_2HPO_4, 3.23; K_2HPO_4, 8.87; Fe citrate, 1.10; Ca lactate, 12.09; Al $(OH)_3$, 0.01; $ZnSO_4$, 0.13; $CuSO_4$, 0.004; $MnSO_4$, 0.03; Ca $(IO3)_2$, 0.01; $CoSO_4$, 0.04; [11]Probio EP; *Streptococcus faecalis* and *Bacillis amyloliquefaciens* made by Toa Biopharma Co., Tokyo, Japan;[12,13]*Streptococcus faecalis*, *Lactobacillus plantarum*, *Bacillis amyloliquefaciens*, and *Bacillus mesentericus* (Toa Biopharma Co., Tokyo, Japan); [14]L-ascrobil-2 phosphates-Mg; [15]Glico Nutrition Company Ltd., Osaka, Japan. Commercial name: "A-glu SS"; [16]Nippon paper chemicals, Tokyo, Japan; [17]values are means of triplicate groups ± SEM of the mean; [18]carbohydrate (g/kg): 100 – (crude protein + crude lipid + crude ash); [19]gross energy: calculated using combustion values for protein, lipid, and carbohydrate of 23.6, 39.5 and 17.2 kJ/g, respectively.

count plates (Thomas Scientific, USA) in triplicate per treatment. The lactic acid bacteria (LAB) content in the feed was determined by spreading onto 3MTM Petri film anaerobic count plate (Thomas Scientific, USA) in triplicate per treatment. Briefly, after the diets were prepared, 1 g of the test diet was homogenized in 10 ml PBS (0.05 m, pH 7.4). Then, 1 ml of the solution was serially diluted in 10 ml PBS buffer to the fourth dilution. Finally, 1 ml of each dilution was spread over the 3MTM Petri film plate and incubated at 26°C for 3-5 days. The bacteria colony-forming unit (CFU g^{-1}) was counted using a colony counter (ACK-3 AS ONE, Japan) as described in Ren et al. [23]. Similarly, the total bacteria count and LAB count were determined from intestinal fish content.

2.3. Feeding and Experimental Conditions. The experiment was conducted at the Kamoike Marine Research facility, Faculty of Fisheries, Kagoshima University, Japan. The experiment was maintained at similar conditions described in Shadrack et al. [10]. The juveniles of *P. major* were purchased from a commercial hatchery (Miyazaki prefecture, Japan). Juvenile fish were stocked in 100-L polyethylene tanks filled with 80 L of seawater in a flow-through seawater system at 1.51 L per minute, with continuous aeration. The juvenile fish were fed with commercial feed for seven-day acclimation period. The rearing water condition was maintained as follows: temperature (26.1 ± 1.2°C), pH (8.1 ± 0.5), salinity (33.1 ± 0.5 PSU), and dissolved oxygen (6.1 ± 0.5 mg/L).

Table 2: Forward (F) and Reverse (R) primers used for growth and immune mRNA quantification with quantitative real-time PCR.

Primer name	Primer sequence (5′-3′)	Accession number
ß-actin-F	TCTGTCTGGATCGGAGGTC	JN226150.1
ß-actin-R	AAGCATTTGCGGTGGACG	
TNF-a-F	CCAAACAGAAGCACTAACC AAGA	AY314010.1
TNF-a-R	CTAAATGGATGGCTGCCTTG	
IL-1b-F	CGAGTACCAAACAGCATGGA	AY257219.1
IL-1b-R	GTGTAGGGGGCAGGTAGGTC	
IGF-1-F	TAAACCCACACCGAGTGACA	AB050670.1
IGF-1-R	GCGATGSSGAAAAGCTACGG	
IGF-2-F	CGGCAAACTAGTGATGAGCA	AB360966.1
IGF-2-R	CAGTGTCAAGGGGGAAGTGT	

ß-actin: housekeeping gene; *TNF-a*: tumor necrosis factor; *IL-1b*: interleukim-1b; *IGF-1*: insulin-like growth factor 1; *IGF-2*: insulin-like growth factor 2.

After the acclimation period, juvenile fish ($n = 300$) of 21.56 g average initial body weight were stocked into the rearing tanks. Each fish was assigned randomly into five experimental groups at 20 fish per tank (triplicate tanks per treatment). Feeding was conducted twice daily at 8 am and 4 pm over the 50-day trial period. Uneaten feed was siphoned after one hour of feeding and then dried using a freeze drier. The weight was used later to calculate feed intake (FI).

2.4. Sampling. Sampling was conducted following the methods described in Shadrack et al. [10]. At the end of the 50-day feeding trial, fish were starved for 24 hours [4] prior to sampling to digest all food material so that the weight taken was accurate. During sampling, eugenol (4-allylomethoxyphenol, 50 ml/Ml) was used to anesthetize the fish for weight and length data collection. Five juvenile fish were collected per tank and stored at -20°C for the final whole-body analysis.

Blood was collected using heparinized ($n = 5$) and non-heparinized ($n = 3$) syringes. A small fraction of the heparinized blood was used to determine hematocrit by following the microhematocrit technique [24]. The heparinized blood was centrifuged at 3000×*g* for 15 minutes using a high-speed refrigerated microcentrifuge, and the plasma obtained was kept in a freezer at -80°C until used. The nonheparinized blood was kept at room temperature for 2 hours and then centrifuged at 3000×*g* for 15 minutes to collect serum which was then stored at -80°C until use.

A total of 3 fish per tank were dissected, and liver and viscera weight were taken to calculate viscerasomatic index (VSI) and hepatosomatic indices (HSI). Liver samples per tank were pooled together and stored at -80°C for further analysis. VSI and HSI were calculated according to the following equations:

$$\text{VSI} = \left(\frac{\text{viscera weight}}{\text{fish body weight}}\right) \times 100, \quad (1)$$

$$\text{HSI} = \left(\frac{\text{liver weight}}{\text{fish body weight}}\right) \times 100. \quad (2)$$

Nine fish (3 fish per tank) were collected, and the skin was washed with PBS and distilled water, followed by gentle rubbing with a sterilized piece of cotton over 200 mm^2 of the body surface according to the protocol described in [25]. The cotton containing the mucus was transferred into a 1.5-ml tube and suspended in 1 ml PBS (pH = 7.4). Then, the samples were centrifuged at 2000×*g*, 4°C for 10 min. The supernatant was collected and transferred into new 1.5-ml tubes and stored at -80°C for further analysis.

2.5. Performance and Feed Utilization. The growth performance and feed utilization indices are calculated following the equations described in Kader et al. [26] and Shadrack et al. [10].

$$\text{Weight gain (WG\%)} = \left(\frac{\text{final weight} - \text{initial weight}}{\text{initial weight}}\right) \times 100, \quad (3)$$

$$\text{Specific growth rate (SGR\%)} = \left(\frac{\text{Ln (final weight)} - \text{Ln (initial weight)}}{\text{duration of feeding (days)}}\right) \times 100, \quad (4)$$

$$\text{Survival (\%)} = \left(\frac{\text{final no.of fish at 50 days}}{\text{total no.of fish at stocking}}\right) \times 100, \quad (5)$$

$$\text{Feed conversion ratio (FCR)} = \left(\frac{\text{dry feed intake (g)}}{\text{final wet weight gain (g)}}\right), \quad (6)$$

$$\begin{aligned}&\text{Protein gain (PG, g/kg weight gains)}\\ &= (\text{(final weight, g x final whole body protein content/100)}\\ &\quad - (\text{initial weight gain, g} \times \text{initial whole} - \text{body protein/100}))\\ &\quad \times 1000/\text{weight gain (g)}.\end{aligned} \quad (7)$$

2.6. Biochemical Proximate Analysis. Diet and whole-body proximate composition were determined following the standard procedures outlined in AOAC [27]. A mechanical convection oven (Dk400, Yamato Scientific CO., Tokyo, Japan) was used to dry the samples at 105°C to constant, and the weight loss represents moisture content. Crude protein was quantified following the Kjeldahl nitrogen method (Kjeltec System 1002 tecator, Sweden) and evaluating the protein content. Ash content was quantified by burning at 550°C in a muffle furnace for 4 hours, and the final product represents ash content. Crude lipid content was determined following the Soxhlet extraction method. Gross energy was calculated using combustion values for protein, lipid, and carbohydrate.

2.7. Blood and Antioxidant Activity. Blood plasma chemical parameters were measured using commercial kits with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Array, Inc. Kyoto, Japan) as described in Shadrack et al.

Table 3: Red sea bream juvenile performance variables (growth performance, feed utilization, survival rate, and biometric indices) and whole-body proximate analysis after 50-day feeding period.

Items	Experimental diets				
	CD1	D2	D3	D4	D5
IBW, g/fish[1]	21.6 ± 0.69	21.38 ± 0.47	21.45 ± 0.46	21.66 ± 0.03	21.72 ± 0.07
FBW, g/fish[2]	45.48 ± 0.23	46.39 ± 1.44	45.84 ± 2.05	45.01 ± 3.12	44.5 ± 3.16
WG, %[3]	110.47 ± 13.73	116.97 ± 3.98	113.65 ± 6.42	107.78 ± 14.68	104.84 ± 13.96
SGR[4]	2.48 ± 0.22	2.58 ± 0.06	2.53 ± 0.1	2.53 ± 0.1	2.38 ± 0.23
FI, g/fish/60 days[5]	31.51 ± 0.51	28.8 ± 4.43	32.83 ± 2.48	28.94 ± 3.89	33.09 ± 4.05
FCR[6]	1.34 ± 0.22	1.16 ± 0.23	1.36 ± 0.19	1.25 ± 0.2	1.46 ± 0.07
PER[7]	1.87 ± 0.28	2.07 ± 0.37	1.86 ± 0.27	1.99 ± 0.3	1.71 ± 0.08
PG[8]	656.3 ± 53.2	642.43 ± 19.92	597.32 ± 26.71	604.5 ± 41.95	587 ± 41.7
PR[9]	51.25 ± 4.88	53.1 ± 8.79	45.41 ± 5.42	51.7 ± 6.97	44.38 ± 2.75
SR, %[10]	76.92 ± 15.38	79.49 ± 8.88	84.62 ± 13.32	79.49 ± 4.44	82.05 ± 16.01
HSI[12]	0.95 ± 0.23	1.2 ± 0.43	1.12 ± 0.27	0.99 ± 0.21	0.87 ± 0.15
VSI[13]	7.35 ± 1.04	7.33 ± 1.03	6.54 ± 1.13	6.12 ± 0.35	6.51 ± 0.56
Whole-body proximate analysis[14]					
Moisture	68.74 ± 1.23	69.2 ± 1.63	69.19 ± 1.42	69.82 ± 0.5	69.62 ± 0.62
Crude protein	14.43 ± 0.73	13.85 ± 0.21	13.08 ± 0.16	13.43 ± 0.3	13.19 ± 0.33
Crude lipid	7.98 ± 1.17	7.99 ± 0.39	7.95 ± 0.28	8.37 ± 0.06	8.15 ± 0.12
Crude ash	5.63 ± 0.13	5.08 ± 0.02	4.66 ± 0.13	4.73 ± 0.02	5.23 ± 0.05

D1 = the basal diet; D2 = the basal diet + SF 5×10^6 cfu/g diet + BA 5×10^5 cfu/g diet; D3 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet + BA 5×10^5 cfu/g diet; D4 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet; D5 = the basal diet + BA 5×10^5 cfu/g diet. Values are the means of triplicate groups ± SEM of the mean. The absence of superscript letters indicates no significant difference in means between groups ($P > 0.05$). [1]IBW (g/fish): initial body weight; [2]FBW (g/fish/50days): final body weight; [3]WG (%): weight gain percentage per fish; [4]SGR (%/day): specific growth rate; [5]FI (g/fish/50 days): feed intake per fish per 50 day; [6]FCR: feed convention ratio; [7]PER: protein efficiency ratio; [8]PG: protein gain; [9]PR: protein retention; [10]SR: survival rates %; [11]CF: condition factor, [12]HSI: hepatosomatic index (%); [13]VSI: viscerasomatic index; [14]whole-body proximate analysis are expressed on a wet weight basis.

[10]. The biological antioxidant potential (BAP) and derivative of reactive oxygen metabolites (d-ROM) from blood serum were determined by an automated analyzer using a commercial kit (FRAS4, Diacron international s.r.l., Grosseto, Italy), according to the manufacture's instruction. The red blood cells (RBCs) were counted with a hemocytometer [28] immediately after dilution with Natt and Herrick's solution as described in Shadrack et al. [10]. The blood hemoglobin (Hb) concentration was determined using a commercial kit with an automated analyzer [29], as described in Shadrack et al. [10]. The hematological indices were calculated as described in Ghafarifarsani et al. [30]. The superoxide dismutase (SOD) activity of blood serum and liver was measured using the SOD assay kit ((Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's instruction. According to the manufacturer's instruction, the malondialdehyde (MDA) in blood serum or liver was measured using the colorimetric TBARS microplate assay commercial kit (Oxford Biomedical Research, Inc., USA). The serum peroxidase (GPx) activity was quantified according to the method described in Salinas et al. [31] and Shadrack et al. [10]. Catalase (CAT) activity of serum was quantified following previous methods [32, 33].

2.8. Nonspecific Immune Responses. The serum lysozyme activity was measured following the turbidometric assay technique described in Lygren et al. [29] and Shadrack et al. [10]. Serum and mucus Immunoglobulin (IgM) activity were measured according to the procedure of Siwicki et al. [34] described in Shadrack et al. [10]. The oxidative radical production of neutrophils during respiratory burst was measured according to the NBT assay in whole blood [35] described in Shadrack et al. [10]. The protease activity of serum was measured following the procedure described in Cordero et al. [36] and Shadrack et al. [10]. The total antiprotease (T-antiprotease) and α-antiprotease of serum were measured following the methods described in Newaj-Fyzul et al. [37] and Shadrack et al. [10]. The amount of mucus as an indicator of immune response was quantified following the method described by Dawood et al. [38] and Shadrack et al. [10]. The serum and mucus bacteria activity was quantified following the method described in Shadrack et al. [10], using *Escherichia coli* (1×10^8) bacteria suspension, and the OD was read at 570 nm (Multiskan Go, Thermo Fisher Scientific K. K., Tokyo, Japan) and was expressed as percentage inhibition of *E. coli* relative to the positive control.

2.9. Real-Time PCR Analysis. The insulin-like growth factor 1 (*IGF-1*) and insulin-like growth factor 2 (*IGF-2*) mRNA were quantified for growth gene expression, and tumor necrosis factor-alpha (*TNF-a*) and interleukin 1 beta (*IL-1b*) mRNA were quantified for immune gene expression

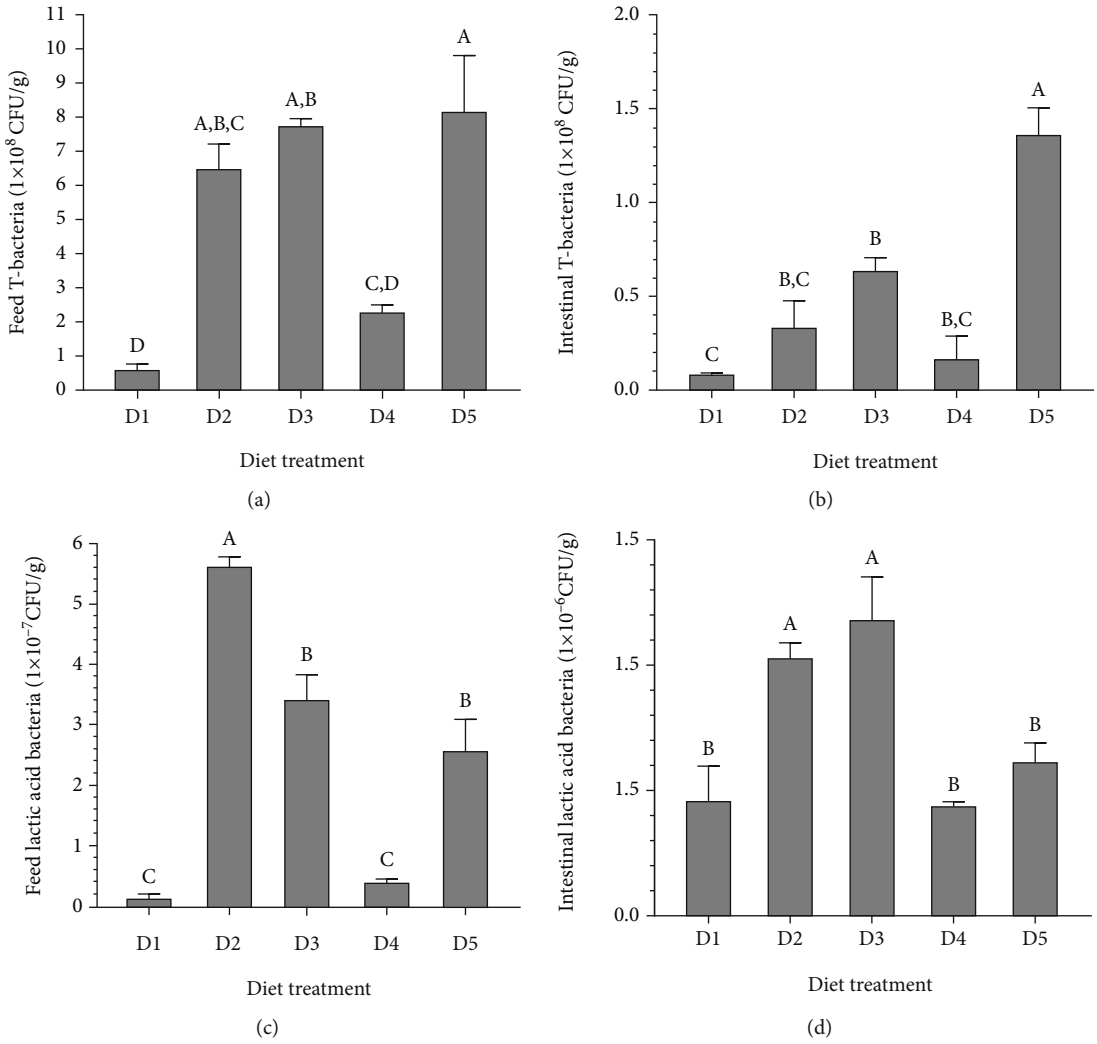

Figure 1: Total and lactic acid bacteria count in test diets and intestine of juvenile red sea bream fed for 50 days. Graphical values represent mean ± SEM of triplicate samples ($n = 3$) constructed with Graphpad prism software. Different superscripts indicate significant difference ($P < 0.05$) between treatment means. Figure 1. (a) Total bacteria in feed; (b) total bacteria in the intestine; (c) lactic acid bacteria in feed; and (d) lactic acid bacteria in the intestine. D1 = the basal diet; D2 = the basal diet + SF 5×10^6 cfu/g diet + BA 5×10^5 cfu/g diet; D3 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet + BA 5×10^5 cfu/g diet; D4 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet; and D5 = the basal diet + BA 5×10^5 cfu/g diet.

using the quantitative real-time polymerase chain reaction (PCR) method. Livers were obtained from dissecting the fish, weighed and placed in fivefold of RNAlater (Invitrogen; Thermo Fisher Scientific K. K., Tokyo, Japan) solution, and stored at -80°C until analysis as described in Mzengereza et al. [39]. The RNA from liver samples was extracted using the RNeasy Mini Kit 50 (Qiagen; Hilden, Ger- 321 many). Briefly, 30 mg liver was placed in a sterilized tube (1.5 ml), homogenized, and centrifuged at 12000 rpm for 15 s. The supernatant was collected and mixed with 70% ethanol. After completing the RNA extraction, the Prime 324 ScriptTM RT Master Mix Kit (Takara Bio Inc. Shiga, Japan) was used to obtain the cDNA following the manufacturer's protocol. Finally, the PCR analysis is performed using the SYBR Master Mix Kit (Thermo Fisher Scientific K. K., Tokyo, Japan) using the primers presented in Table 2. The elongation factor (β-Actin) was tested for stability and used as a housekeeping gene [40]. Amplification of genes was made with CFD-3120 Mini Opticon Real-Time PCR System (BIO-RAD, Singapore) according to the following steps: 2 min denaturation at 95°C, 40 cycles at 95°C for 15 s, and 65°C for 30 s. Each assay was performed in triplicate 0°C for 30 s. The delta delta CT method was used to calculate the relative fold change for the gene of interest [41].

2.10. Low Salinity Stress Assessment. Four juvenile fish per tank were randomly selected and were placed in 20-L transparent glass aquaria containing 18 L of dechlorinated water as described in Shadrack et al. [10]. The test was conducted in triplicate for each experimental treatment fish group. Time taken to reach 50% death was expressed as tolerance limit and

Table 4: Blood status of juvenile red sea bream fed the experimental diets for 50 days.

Parameter	Experimental diets				
	D1	D2	D3	D4	D5
Hematocrit (%)	41.33 ± 8.14	46.0 ± 0.1	43.67 ± 7.51	46.33 ± 0.58	44.0 ± 7.0
Hb (mg/dl)	6.6 ± 1.77	6.4 ± 1.57	7.1 ± 1.73	6.23 ± 1.15	5.6 ± 2.07
Glucose (mg/dl)	123.5 ± 6.36^{b}	54.2 ± 0.01^{a}	88.5 ± 7.78^{b}	72 ± 4.24^{ab}	71 ± 2.83^{ab}
T-Cho (mg/dl)	239.5 ± 10.61^{b}	167.5 ± 12.02^{a}	227 ± 14.14^{ab}	218.5 ± 14.85^{ab}	240.5 ± 4.95^{b}
Bun (mg/dl)	21 ± 2.83	16.5 ± 0.71	22 ± 7.07	18 ± 1.41	18 ± 2.83
T-bill (mg/dl)	0.4 ± 0.14	0.35 ± 0.07	0.4 ± 0.14	0.35 ± 0.07	0.55 ± 0.07
AST (IU/L)	35 ± 1.41^{c}	12.5 ± 0.71^{a}	60 ± 7.07^{bc}	56 ± 1.41^{bc}	55 ± 8.49^{c}
ALT (IU/L)	13 ± 4.24^{a}	12 ± 2.83^{a}	30 ± 5.66^{ab}	53.5 ± 10.61^{b}	21 ± 1.41^{a}
TP (g/dl)	4.9 ± 1.41	3.25 ± 0.07	5.15 ± 1.63	4.15 ± 0.64	4.95 ± 0.64
TG (mg/dl)	126 ± 11.31^{ab}	105 ± 8.49^{b}	138 ± 7.07^{a}	133.5 ± 9.19^{ab}	137.5 ± 6.36^{ab}
RBC (1×10^{6} mm^{3})	6.53 ± 1.05	7.13 ± 2.93	8.17 ± 3.1	7.3 ± 0.22	8.72 ± 3.13
MCH	10.58 ± 4.5	9.41 ± 1.65	6.43 ± 0.52	8.57 ± 1.76	6.52 ± 1.04
MCV	65.58 ± 22.19	72.23 ± 29.08	57.05 ± 14.15	63.55 ± 2.74	53.78 ± 13.92
MCHC	15.82 ± 1.44	13.91 ± 3.42	13.23 ± 3.39	13.44 ± 2.42	12.49 ± 3.01

D1 = the basal diet; D2 = the basal diet + SF 5×10^{6} cfu/g diet + BA 5×10^{5} cfu/g diet; D3 = the basal diet + SF 1×10^{6} cfu/g diet + LP 4×10^{5} cfu/g diet + BM 1×10^{2} cfu/g diet + BA 5×10^{5} cfu/g diet; D4 = the basal diet + SF 1×10^{6} cfu/g diet + LP 4×10^{5} cfu/g diet + BM 1×10^{2} cfu/g diet; D5 = the basal diet + BA 5×10^{5} cfu/g diet. Values are means of triplicates represented as means ± S.E. Different superscript letters indicate significant difference ($P < 0.05$). Absence of letters indicates no significance difference between groups. Hb: hemoglobin; T-Cho: total cholesterol; Bun: blood urea nitrogen; T-Bill: total bilirubin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; TG: total glycerides; TP: total protein; RBC: red blood cells; MCH: mean capsular hemoglobin; MCV: mean capsular volume; MCHC: mean capsular hemoglobin concentration.

was calculated according to the long-rank 50% mortality (LT_{50}) equation of Moe et al. [42] outlined as follows:

$$Y = aX + b\,(8), \tag{8}$$

where Y is the log10 (survival) and X is the time to individual death of fish (min). LT50 is (X) obtained when $Y = 1.7$ as $\log 10\,(50) = 1.7$.

2.11. Digestibility Assessment. The digestibility of crude protein, crude lipid, and dry matter was measured indirectly using chromium oxide as an inert marker [43]. Fish were fed with a diet containing chromium oxide for 5 days to be accustomed to the feed. After that, feeding to satiation was conducted twice per day, and feces were collected 3 hours after each feeding session using a siphon and a fine mesh nylon net. Feces were freeze-dried and milled to powder form. The quantification of chromium oxide in diet and feces was made following Furukawa and Tsukahara [43]. The following formula was used to determine the apparent digestibility coefficients (ADC).

$$\begin{aligned}&\text{ADC nutrient}\,(\%)\\&\quad = 100 - (\%Cr_2O_3\text{diet}/\%Cr_2O_3\text{faeces} \times \%\text{nutrient faeces}/\%\text{nutrient diet}),\end{aligned} \tag{9}$$

$$\text{ADC dry matter}\,(\%) = 100 - \left(100 - \left(\frac{\%Cr_2O_3\text{diet}}{\%Cr_2O_3\text{faeces}}\right)\right). \tag{10}$$

2.12. Statistical Analysis. The Kolmogorov-Smirnov and Shapiro-Wilk tests were performed to verify the normality of the data, and homogeneity of variance was confirmed using the Levene test. One-way analysis of variance (ANOVA) was performed using palaeontology statistical software version 3.21 [44]. The probability of $P < 0.05$ was considered significant. The significant differences were further evaluated using Tukey-Kramer post hoc test. The principal component analysis (PCA) and circular hierarchical cluster (CHC) analysis using a correlation matrix were employed to show dietary group relatedness based on the coefficient of variances. The single linkage clustering (SLC) used the Bray-Curtis dissimilarity matrix to identify fish groups with similar overall performance. All data were standardized prior to PCA, CHC, and SLC analysis.

3. Results

3.1. Growth and Feed Utilization. The growth parameters, nutrient utilization, and survival rate presented in Table 3 revealed no existing differences ($P > 0.05$) between all dietary groups, although final body weight (FBW), WG%, and SGR were numerically improved in D2 and D3 supplemented diets compared to D4, D5, and the control D1. The comparison of feed conversion ratio revealed that bacteria-supplemented diet D2 exhibits lower FCR values compared to the fish group fed other supplemented diets (D2, D4, D5) and the control diet D1. However, no significant difference was detected ($P > 0.05$). The survival rate was numerically improved in fish-fed bacteria-supplemented diets compared to the control ($P > 0.05$). No significant difference was established between the whole-body proximate composition among the fish group fed the test diets ($P > 0.05$). The LAB in diets were significantly

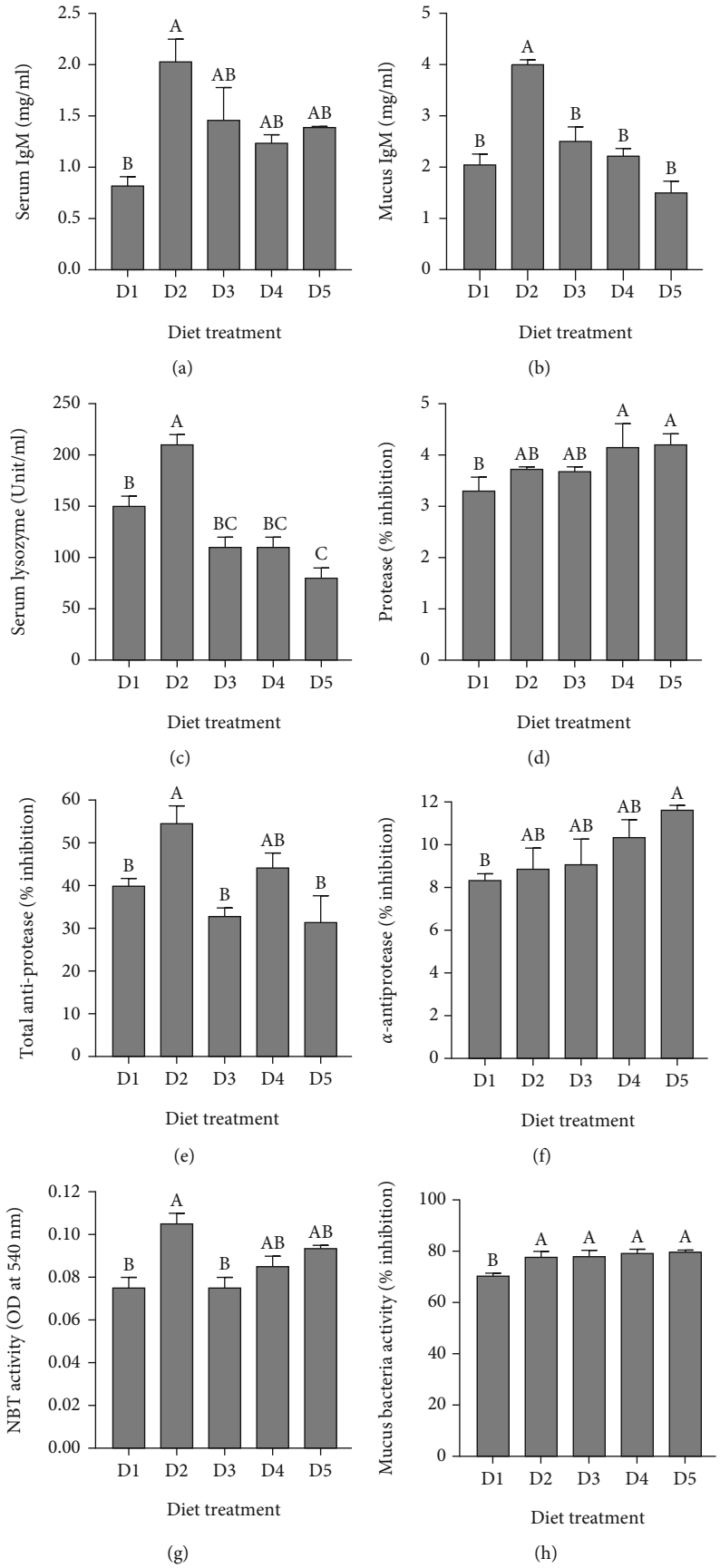

Figure 2: Continued.

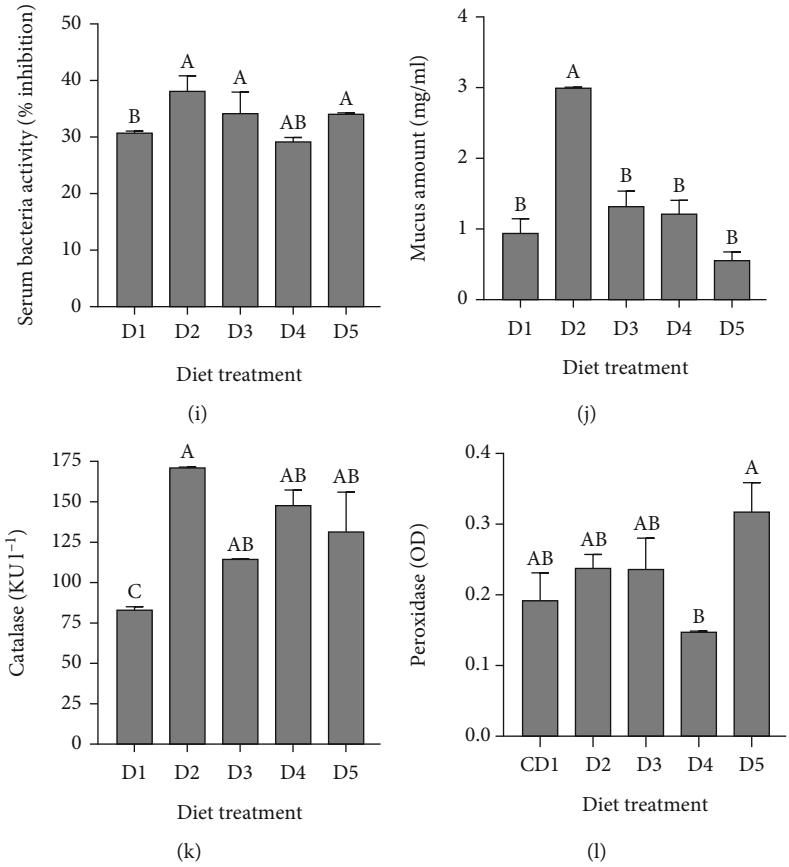

Figure 2: Serum and mucus, immune responses of juvenile red sea bream fed test diets for 50 days. Graphical values represent mean ± SEM of triplicate samples ($n = 3$) constructed with Graphpad prism software. Different superscripts indicate significant difference ($P < 0.05$) between treatment means: (a) serum immunoglobin (mg/ml); (b) mucus immunoglobin (mg/ml); (c) serum lysozyme (unit/ml); (d) serum protease activity (% inhibition); (e) serum total-antiprotease (% inhibition); (f) serum α-antiprotease (% inhibition); (g) nitroblue tretazolium activity in whole blood (optical density); (h) mucus bacteria activity (% inhibition); (i) serum bacteria activity % inhibition; (j) mucus amount (mg/ml); (k) serum catalase activity (kU l^{-1}); and (l) serum peroxidase activity (optical density). D1 = the basal diet; D 2 = the basal diet + SF 5×10^6 cfu/g diet + BA 5×10^5 cfu/g diet; D3 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet + BA 5×10^5 cfu/g diet; D4 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet; and D5 = the basal diet + BA 5×10^5 cfu/g diet.

Table 5: Antioxidant potential of red sea bream juvenile fed test diets for 50 days.

Parameters	Experimental groupings				
	D1	D2	D3	D4	D5
MDA (nmol/ml)	48.51 ± 4.21^{bc}	46.43 ± 2.28^{bc}	37.32 ± 3.98^{ab}	21.95 ± 0.19^{a}	60.98 ± 3.94^{c}
Liver MDA (nmol/mg)	36.59 ± 2.89^{c}	7.76 ± 0.31^{a}	29.93 ± 0.24^{bc}	12.65 ± 1.66^{a}	28 ± 0.01^{b}
Serum SOD (50% inhibition)	48.57 ± 0.23	47.87 ± 1.13	45.3 ± 0.35	41.85 ± 0.81	44.56 ± 1
Liver SOD (50% inhibition)	23.89 ± 0.43	20.87 ± 1.68	26.82 ± 4.01	24.38 ± 5.28	25.09 ± 2.94
d-ROMs (μMol/L)	4.5 ± 0.71^{a}	12.5 ± 7.78^{b}	11.5 ± 6.36^{b}	7 ± 5.66^{ab}	9.5 ± 4.95^{ab}
BAP (U. Carr)	1145 ± 21.21^{b}	1814 ± 8.49^{ab}	1850 ± 15.56^{a}	986.5 ± 6.36^{c}	1003 ± 16.97^{c}

D1 = the basal diet; D2 = the basal diet + SF 5×10^6 cfu/g diet + BA 5×10^5 cfu/g diet; D3 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet + BA 5×10^5 cfu/g diet; D4 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet; D5 = the basal diet + BA 5×10^5 cfu/g diet. Values are means of triplicates represented as means ± S.E.M. Different superscript letters indicate a significant difference ($P < 0.05$) of means. The absence of letters indicates no significant difference between groups.

higher in the fish group fed on D2, D3, and D5 than D1 and D4 (Figure 1). The LAB from intestinal content of fish fed on D2, D3, and D5 were significantly improved compared to fish fed on the control ($P < 0.01$).

3.2. Blood Evaluation. The blood composition of juvenile red sea bream fed the test diets for 50 days is shown in Table 4. Hematocrit (Hrt) and RBC count were numerically higher in the fish group fed the bacteria-supplemented diets compared

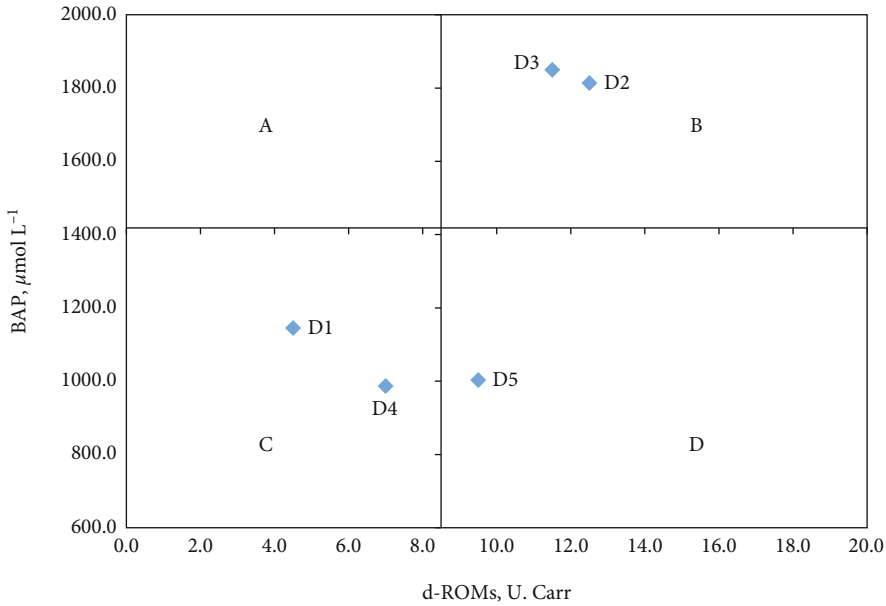

Figure 3: Oxidative condition of red sea bream juvenile fed test diets for 50 days. Graphical values represent the means ($n = 3$) constructed in Microsoft excel. The central axis is the means of both d-ROM and BAP for the dietary groups: zone A: high antioxidant capacity and low reactive oxygen metabolites (good condition); zone B: high antioxidant capacity and low reactive oxygen metabolite (acceptable condition); zone C: low antioxidant potential and low reactive oxygen metabolite (acceptable condition); and zone D: low antioxidant potential and high reactive oxygen metabolite (poor condition). D1 = the basal diet; D2 = the basal diet + SF 5×10^6 cfu/g diet + BA 5×10^5 cfu/g diet; D3 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet + BA 5×10^5 cfu/g diet; D4 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet; and D5 = the basal diet + BA 5×10^5 cfu/g diet.

to the control group. The fish group fed on supplemented diet D2 showed significantly lower values of total cholesterol (T-Cho), glucose (GLU), and aspartate aminotransferase (AST) compared to the fish group fed the control diet ($P < 0.05$). The total glycerides (TG) and alanine aminotransferase (ALT) values showed the lowest peak in the fish group fed D2 compared to the fish group fed other mixes (D3, D5) and single (D4) probiotic-supplemented diets and the control diet D1 ($P > 0.05$). No significant differences were observed in other blood parameters such as total protein (T-Pro), Hb, total bilirubin (T-Bill), blood urea nitrogen (bun), mean capsular hemoglobin (MCH), mean capsular volume (MCV), and mean capsular hemoglobin concentration (MCHC) ($P > 0.05$).

3.3. Immune Response Assessment. Figure 2 displays the first line of defense in the fish body, such as the IgM, lysozyme, protease, t-antiprotease, α-antiprotease, NBT, bacteria activity, mucus amount, CAT, and GPx in fish fed the test diets. Fish-fed bacteria supplement D2 group exhibits high serum and mucus IgM values, serum lysozyme, T-antiprotease, NBT activity, mucus and serum bacteria activity, and mucus amount compared to fish fed the control diets ($P < 0.05$). High values of protease were observed in groups fed D4 and D5, α-antiprotease in D5, and high CAT and mucus bacteria activity in all fish groups fed supplemented diet (D2-D5) compared to the control D1 ($P < 0.05$). The GPx activity was higher in all fish-fed probiotic bacteria-supplemented diets except for D4, which is lower and significantly different from D5 ($P < 0.05$).

3.4. Antioxidant Activity. The antioxidant activity of juvenile red sea bream fed the test diets for 50 days is presented in Table 5 and Figure 3. Fishes fed on D2 and D3 diets exhibited significantly lower values of MDA compared to other bacteria-supplemented groups (D4, D5) and the control group D1 ($P < 0.05$, Table 4). SOD values were not significantly different among fish fed all test diets ($P < 0.05$). The BAP activity was significantly higher ($P < 0.05$) in the D3 group followed by D2 compared to the control group and other bacteria-supplemented groups (Table 5). The d-ROM activity was significantly ($P < 0.05$) lower in the control group compared to supplemented group (D2, D3). The combined pattern of the effect of BAP and d-ROM shown in Figure 3 reflects the balance between antioxidant and oxidative stress. Zone A reflects a good condition with low oxidative stress and high tolerance ability. Zone B shows a balance between oxidative stress and tolerance ability, where D2 and D3 were favored, similarly to zone C, where D1 and D4 were favored. Zone D represents high oxidative stress condition and low tolerance ability, where D5 was favored.

3.5. Relative Growth and Immune Gene Expression. A significantly high hepatic expression of *IGF-1* and *IGF-2* mRNA was found in fish fed D2 and D3 compared to fish fed D4, D5, and the control diet D1 ($P < 0.05$, Figure 4). A significantly high hepatic expression of *TNF-a* and *IL-1b* was found in the fish group fed probiotic-supplemented diets compared to fish fed the control diets ($P < 0.05$, Figure 4). Fish group fed D3 showed the highest expression of *IGF-1*, *IGF-2*, and *TNF-a* compared to all fish groups fed the supplemented diets and control diet diets.

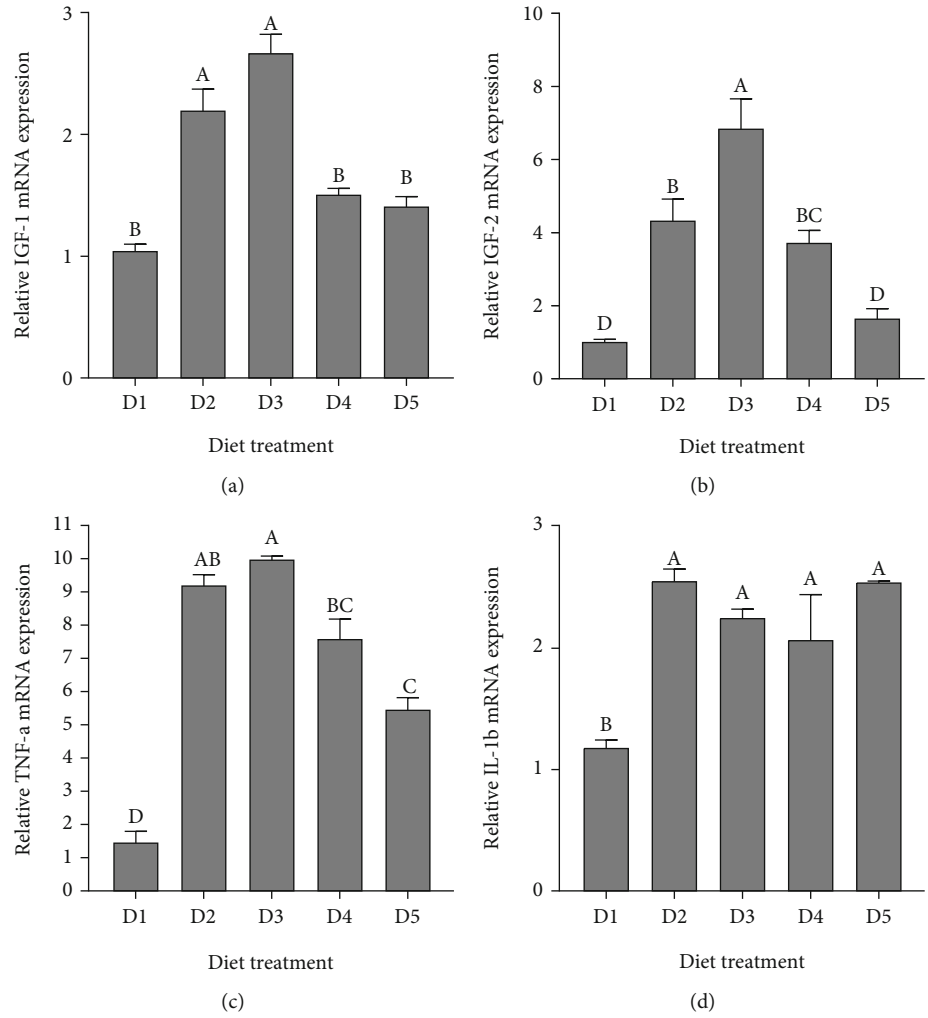

Figure 4: Relative mRNA expression of growth (*IGF-1*, *IGF-2*) and immune (*TNF-a*, *IL-1b*) gene for juvenile red sea bream fed the test diets in 50 days. Graphical values represent mean ± SEM of triplicate samples ($n = 3$) constructed with Graphpad prism software. Different superscripts indicate significant difference ($P < 0.05$) between treatment means. D1 = the basal diet; D2 = the basal diet + SF 5×10^6 cfu/g diet + BA 5×10^5 cfu/g diet; D3 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet + BA 5×10^5 cfu/g diet; D4 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet; and D5 = the basal diet + BA 5×10^5 cfu/g diet.

Table 6: Apparent digestibility coefficient (%) of nutrients in juvenile red sea bream fed the test diets in 50 days.

Parameters	D1	D2	D3	D4	D5
Dry matter	74.07 ± 5.52^{b}	80.48 ± 2.31^{ab}	73.37 ± 0.01^{b}	84.64 ± 0.19^{a}	79.77 ± 0.28^{ab}
Total lipid	87.22 ± 0.35^{c}	94.94 ± 0.02^{a}	88.93 ± 1.04^{b}	94.59 ± 0.5^{a}	91.78 ± 0.56^{ab}
Crude protein	88.54 ± 1.09^{b}	93.53 ± 0.82^{a}	90.3 ± 1.56^{ab}	92.6 ± 0.35^{ab}	92.74 ± 0.88^{ab}

D1 = the basal diet; D2 = the basal diet + SF 5×10^6 cfu/g diet + BA 5×10^5 cfu/g diet; D3 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet + BA 5×10^5 cfu/g diet; D4 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet; D5 = the basal diet + BA 5×10^5 cfu/g diet. Values are the means of triplicate groups ± SEM of the mean. Different superscript letters indicate a significant difference ($P < 0.05$) of means. The absence of letters indicates no significant difference between group.

3.6. Digestibility. The digestibility of nutrients presented in Table 6 shows a significantly high crude protein and crude lipid digestibility coefficient for the D2 group compared to fish fed the control group D1 ($P < 0.05$). A high digestibility coefficient of dry matter was observed in fish groups fed D4 followed by D2 and D5, while D3 and D4 were the least. The crude protein digestibility coefficient was generally higher for fish groups fed all probiotic-supplemented diets (D2-D5) than the control diet D1.

3.7. Salinity Stress. The tolerance ability of juvenile red sea bream exposed to low salinity stress after 50 days of the feeding trial is presented in Figure 5. The LT_{50} between the fish group revealed a significantly higher tolerance ability in fish

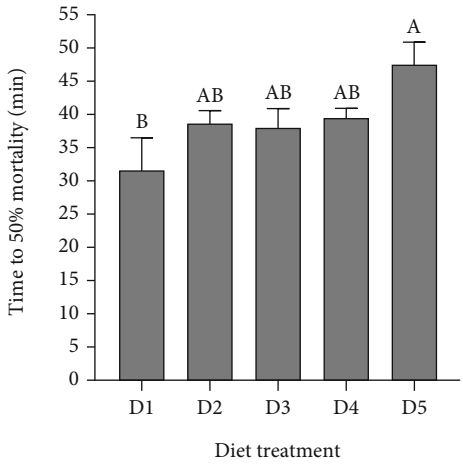

FIGURE 5: Time (min) to 50% mortality (LT_{50}) of juvenile red sea bream exposed to low salinity stress after 50 days feeding test diets. Graphical values represent mean ± SEM of triplicate samples ($n = 3$) constructed with Graphpad prism software. Different superscripts indicate significant difference ($P < 0.05$) between treatment means. D1 = the basal diet; D2 = the basal diet + SF 5×10^6 cfu/g diet + BA 5×10^5 cfu/g diet; D3 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet + BA 5×10^5 cfu/g diet; D4 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet; and D5 = the basal diet + BA 5×10^5 cfu/g diet.

fed D5 supplement compared to the control group D1 ($P < 0.05$). In general, fish-fed bacteria supplement (D2-D4) showed better tolerance ability compared to the fish group fed the control diet D1 ($P > 0.05$).

3.8. PCA and CHC Analysis. The PCA and CHC analysis results of several significant growths, blood, immune, antioxidant, and relative gene expression of juvenile red sea bream fed the test diets in 50 days are shown in Figures 6 and 7. The PC1 and PC2 explained 71.5% (42.46% and 29.04%) of the existing correlation between variables and the test diets. Immune (*TNF-a*) and growth (*IGF-1*, *IGF-2*) relative gene expression and RBC were strongly related to the fish group fed supplemented diet D3. Immune enzymes like IgM, lysozyme, protease, T-antiprotease, CAT, and antioxidant enzyme (BAP) were strongly correlated to the fish group fed diet D2. The blood parameters such as AST, TG, and ALT were strongly correlated to fish-fed diet D4. The fish group fed diet D5 showed a strong correlation with FCR, T-Cho, T-Pro, and GLU, while fish fed the control diet D1 showed a strong correlation with SOD and MDA. The CHC using correlation matrix analysis revealed fish fed D1 in the first but lower cluster, D4 and D5 forming the second cluster but higher than D1, while D2 and D3 form the third cluster, which is furthest from D1, D4, and D5. The fish group fed D2 and D3 form the higher cluster, which is enormously different from D1 (cluster 1), D4, and D5 (cluster 2) (Figure 7). The assessment of similarity index using Bray-Curtis dissimilarity index in SLC algorithm revealed that fish group fed D2 (SF + BA) and D3 (SF, LP, BA+BM) were relatively similar. At the same time, high differences were established compared to D4, D5, and the control group D1 (Figure 8).

4. Discussion

Aquaculture has contributed significantly to meet animal protein demand in the phase of rapid population growth and environmental crises [45]. Challenges associated with culture systems require better strategies to mitigate the effects on production and establish the mechanism for improving culture and production chain [46]. The use of available feeds can boost production by promoting the growth and health status of the cultured animal [47]. Commonly used available feeds in aquaculture include nucleotides [48], fermented feed [24], and probiotic bacteria supplements [5]. Available feed types have specific responses in the animal, such as stimulating immune and growth response and enhancing disease resistance in cultured species [49]. Probiotic bacteria are potential feed additives, and the use of each strain varies in their immunological effects depending on species and host [7]. Multistrain probiotic bacteria supplementation has displayed beneficial effects compared to a single strain because multistrain may promote a better environment or improve the health of individual strain benefiting the host [8]. Hence, this study on juveniles of *P. major* was used to test for the effects of single and mixed probiotic bacteria in modifying the gut microflora and influencing fish's growth and health status.

The common beneficial effect of probiotic bacteria supplement in feed is the stimulation of animal growth by modulation of intestinal microflora for better feed utilization [50]. Single strain BA supplement has increased SGR in *O. niloticus* [11, 12] immune responses and FCR in *C. catla* and *I. punctatus* [14, 15]. In the present study, final body weight (FBW), WG%, and SGR were higher in fish-fed probiotic-supplemented diet D2 and D3 compared to the control group D1 and mix strain (D5) or mix strain (D4) ($P < 0.05$, Table 3). The relative expression of growth genes *IGF-1* and *IGF-1* was significantly higher in fish groups fed D2 and D3 compared to other supplemented groups (D4, D5) and the control group D1 ($P < 0.05$, Figure 4). Probiotic bacteria are a functional growth promoter due to their various functions in the gastrointestinal tract of fish, including producing growth inhibition substances such as bacteriocins, hydrogen peroxides, and diacyl [51]. Supplementation of BA as a mix of strain in SF+BA (D2) and SF+LP+BA+BM (D3) boosted the growth responses, while mix strain SF+LP+BM (D4) or single strain BA (D5) does not show better growth responses. Mix strain may promote the health of individual strain benefiting the host organism, which is in line with previous reports on the use of mix strain probiotic bacteria in aquaculture [8]. A significant growth response may be achieved if the trial period is extended as the gene expression showed favorable growth.

Blood chemistry parameters are valuable indicators of stressors and external stimuli in fish health [52]. The assessment of blood parameters showed significantly lower values for GLU, T-Cho, AST, ALT, and TG in the fish group fed D2 supplement than the control D1 ($P < 0.05$, Table 4),

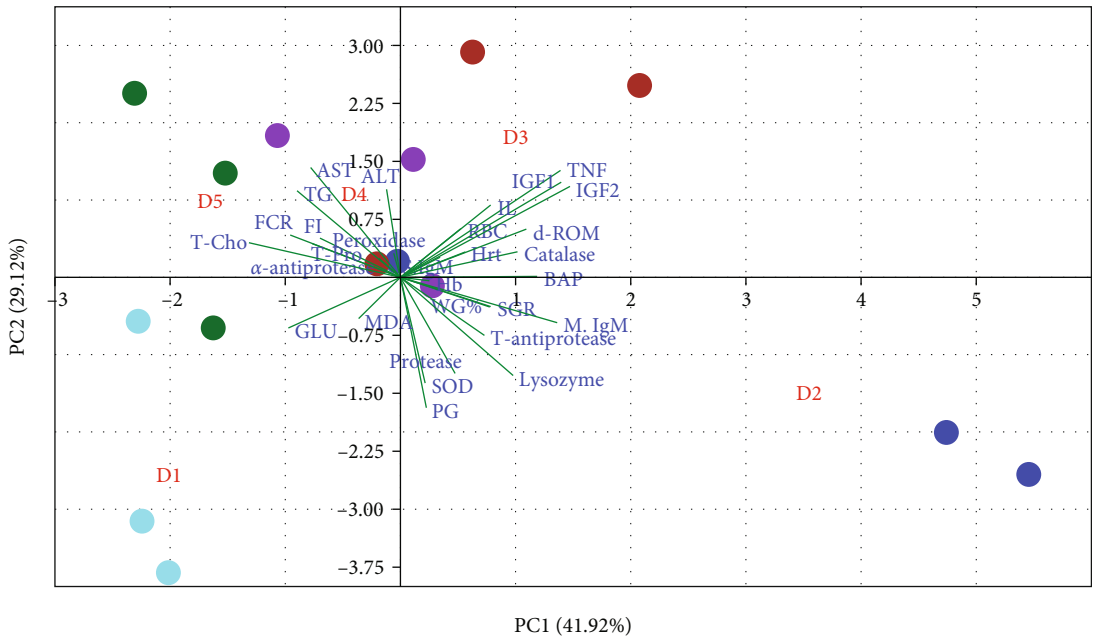

FIGURE 6: The PCA plot of parameters observed in red sea bream fed the dietary groups for 50 days. The convex hull connects the region between triplicate ($n = 3$) samples per group, generated in PAST software. The PC1 and PC2 axis explained 42.46% and 29.04% of the total variation, respectively. The direction of the green lines from the central region of the axis indicates the relationship of each variable in association with the dietary groups. The filled color circles represent replicate per treatment group. D1 = the basal diet; D2 = the basal diet + SF 5×10^6 cfu/g diet + BA 5×10^5 cfu/g diet; D3 = the basal diet + SF 1×10^6 cfu/g diet + LP $\times 10^5$ cfu/g diet + BM 1×10^2 cfu/g diet + BA 5 $\times 10^5$ cfu/g diet; D4 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet; and D5 = the basal diet + BA 5×10^5 cfu/g diet.

implying that the positive alteration in the health status of fish. A numerically high Hrt in the fish group fed the probiotic-supplemented diets suggests that the improved health status as high hematocrit indicates evenly distributed ions without any reduction in hemoglobin synthesis [5]. Hematocrit was improved in amberjack and rainbow trout fed heat kill LP [53] and *Enterococcus feacalis* supplemented diets [54].

Oxidative stress was determined by measuring free radicals using derivatives such as d-ROM and BAP in blood plasma [4]. The measurement was used to reveal the imbalance between oxidants and antioxidants and if the neutralizing capacity of antioxidants is exceeded [5, 55]. In addition, d-ROM and BAP are reliable parameters for determining oxidative stress conditions in fish [56]. The current study concluded that D2 and D3 were in minor oxidative stress conditions compared to the control D1, D4, and D5 groups. The lipid peroxidase MDA and other antioxidant enzymes such as SOD were also essential reflectors of antioxidant defense mechanisms in fish [57]. Furthermore, the results of the present study showed significantly lower SOD and high MDA activity for D2 compared to D1 (control) or D3, D4, and D5 group, suggesting that fish fed D2 group showed better response to antioxidant activity and can be recommended as a functional feed supplement for fish.

The phagocytosis defense mechanism in fish expressed in lysozyme activity and respiratory burst activity (NBT) is used as the first line of defense in fish [22, 58]. The present study results showed a significantly high lysozyme, and NBT in the fish group fed D2 compared to fishes fed all other probiotic-supplemented diets (D3-5) and the control diet D1. The increase in NBT activity demonstrates the immune-stimulating effect of bacteria supplement BA in fish-fed diet D2. Similarly, BA supplements increased NBT in *O. niloticus* [59]. Significantly high CAT activity was observed in fish groups fed all probiotic-supplemented diets (D2-D5) compared to the control diet D1. In contrast, peroxidase (GPx) activity was numerically higher in fish groups fed supplemented diets compared to control diets, except for the D4 group, which is significantly lower than fish fed D5 ($P < 0.05$, Figure 2). Findings on CAT and GPx were in line with the reports of Salinas et al. [31] and Dawood et al. [4] on the beneficial effect of probiotic heat kill bacteria in the diets of *P. major* and *S. dumerili*.

The protease and T-antiprotease were reflectors of the capacity of fish to resist diseases. However, the balance between these two immune enzymes is important for the proper functioning of the immune system [36]. The use of probiotic supplements in fish diets was reported to improve protease, T-antiprotease, and α-antiprotease in fish [37]. The present study showed improved protease and α-antiprotease activities in fish groups fed probiotic-supplemented diets (D2-D5) compared to the fish group fed the control diet D1 (Figure 2, d-f). Fish group fed diet D2 (SF + BA) supplement showed generally better activity of protease, T-antiprotease, and α-antiprotease enzyme compared to fish fed another mix probiotic supplement or single strain (BA) and the control group. Skin mucus is the first line of defense

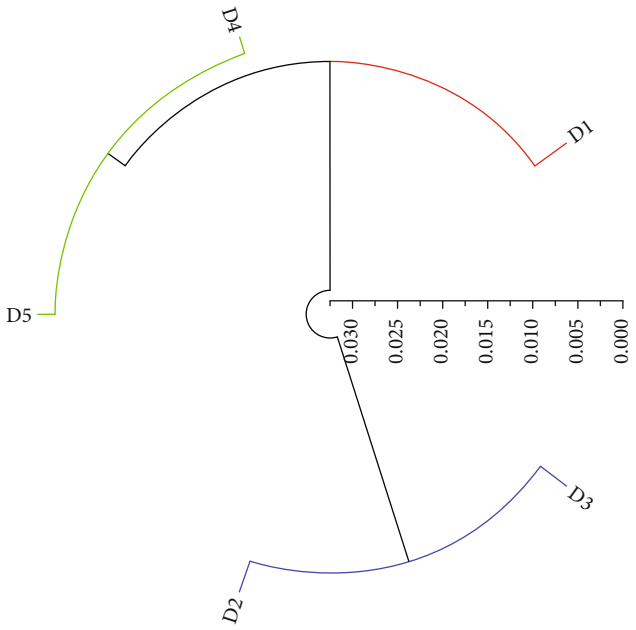

Figure 7: Circular hierarchical clustering dendrogram of growth performance variable, blood, immune, antioxidant enzyme, and gene-related expression (Figure 6) of juvenile red fed the test diets for 50 days. The graph was generated from triplicate samples ($n = 3$) using Origin Pro 2021, software. D1 = the basal diet; D2 = the basal diet + SF 5×10^6 cfu/g diet + BA 5×10^5 cfu/g diet; D3 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet + BA 5×10^5 cfu/g diet; D4 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet; and D 5 = the basal diet + BA 5×10^5 cfu/g diet.

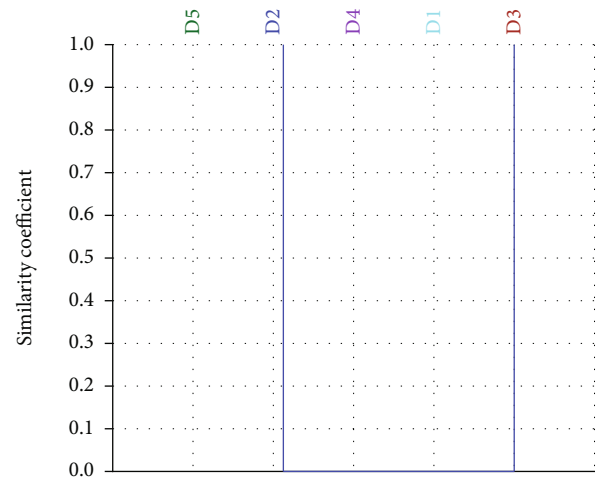

Figure 8: Single linkage Cluster of growth performance parameters, blood health, immune and antioxidant enzyme, and gene-related expression (Figure 6) using Bray-Curtis similarity matrix for fish group fed the test diets in 50 days. The graph was generated from triplicate samples ($n = 3$) per treatment using PAST software. (Dis)similarity coefficient score of zero (0) indicated similarities between groups while score of one (1) indicated differences between groups. D1 = the basal diet; D2 = the basal diet + SF 5×10^6 cfu/g diet + BA 5×10^5 cfu/g diet; D3 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet + BA 5×10^5 cfu/g diet; D4 = the basal diet + SF 1×10^6 cfu/g diet + LP 4×10^5 cfu/g diet + BM 1×10^2 cfu/g diet; and D5 = the basal diet + BA 5×10^5 cfu/g diet.

against microorganisms and a component of innate immunity that protects fish from infection [60]. In the present study, supplementation of probiotic bacteria significantly improved skin mucus amount in the fish group fed D2 diet compared to other probiotic-supplemented diets (D3-D5) and the control diet D1. Bacteria activity in skin mucus and serum is related to the ability of these body fluids to stimulate the production of particular molecules in the innate immune system that affects the antimicrobial responses and kills pathogenic bacteria [60]. In the present study, the evaluation of mucus bacteria activity against *E. coli* revealed significantly better response in the fish group fed probiotic-supplemented diets (D2-D5) compared to the control diet D1 (Figure 2, h). The serum bacteria activity was significantly improved in fish groups fed probiotic bacteria-supplemented diets (D2, D3, D5) except for D4, which was not significantly different from the control group ($P < 0.05$, Figure 2, i). The expression of *TNF-a* and *IL-1b* was significantly improved in the fish group fed probiotic-supplemented diets compared to the control ($P < 0.05$, Figure 4), suggesting the improved lipopolysaccharides binding to endothelial cells to toll-like receptors activating dendritic and macrophage cells, thus increasing inflammatory markers [61].

Probiotic bacteria supplements can stimulate host GI development, digestive function, and mucosal tolerance; stimulate immune responses; and improve disease resistance [7]. The total bacteria count in diet and intestinal content of fish was generally higher in bacteria-supplemented groups (D2-D5) compared to the control D1; however, significant differences were also observed between supplemented groups ($P < 0.05$, Figures 1(a) and 1(b)). A higher quantity of LAB was observed in the feed and intestine content of fish fed D2 and D3 groups than control D ($P < 0.05$, Figure 1(c) and 1(d)). Less amount of LAB was present on the intestinal content of fish fed D4, D5, and the control D1. This result suggests that the inclusion of a single strain BA in D2 and D3 mix probiotic strain has regulated the microflora in favoring the condition that stimulates and improves the growth of LAB, which is consistent with previous findings [62]. The digestibility of nutrients showed that the fish group fed D2 supplement exhibited better digestibility of crude protein and crude lipid than the fish group fed the control diet D1 ($P < 0.05$, Table 6). Additionally, fish fed D3, D4 ,and D5 showed better digestibility of crude protein and crude lipid than the control D1. An investigation of the retention of probiotic bacteria BA in the digestive tract of penaeid shrimp (*L. vannamei* and *Marsupenaeus japonicas*) revealed their presence in all sections of the digestive tract, with the stomach content having a significantly high count [16], suggesting better digestive activity when combine with other strain (D2) as reported in the present study.

Challenge tests have often been used to gauge fish's biological and physical stress responses [63]. The challenge test includes salinity as a physiological indicator of fish tolerance to stress and is usually performed after animal nutritional

feed trials [40]. In the current study, stress tolerance ability LT_{50} was higher in all fish groups fed the bacteria-supplemented diets (D2-D5) (Figure 5). Similarly, *P. major* feed immunostimulant showed greater tolerance to salinity [53] which could be related to improve feed utilization due to improved microvilli alignment. Thus, energy and other nutrients were available to synthesis adrenal steroids to respond to physical stressors [64].

The multivariate analysis helps to explain the correlation of variables with the dietary groups in two-dimensional for a better understanding of the effect of single and mixed probiotic supplements. The PCA analysis revealed that most variables were correlated to fish groups fed on D2 and D3 compared to D5, D4, and the control D1 (Figure 6). Several variables such as AST, ALT, T-Cho, MDA, and TG correlated to D4, D5, and control D1, suggesting a lower performance than D2 and D3 groups. The CHC analysis revealed a noticeable trend where fish groups fed on mix (D2, D3) probiotic diets containing BA supplement had improved performance compared to mix of strain (D4) or single strain (D5) and the control (Figure 7). The single linkage cluster using Brey-Curtis (dis) similarity matrix revealed that fish fed on D2 and D3 had the most improved performance and were different from D1, D4, and D5 (Figure 8), suggesting that BA in the mixed probiotic diet has improved health of other strains resulting in better response in fish.

5. Conclusion

The supplementation of probiotic strains D2, D3, D4, and D5 improved immune responses and antioxidant defense mechanisms in juvenile red sea bream compared to control (D1). The use of mixed strain commercial product (D4) and single strain commercial product (D5) did not show better performance compared to when combining commercial product (BA) as mixed of strain with SF (D2) or SF+LP+BM (D3). Mix strain D2 and D3 supplement improved digestibility of nutrients, growth (*IGF-1*, *IGF-2*), and immune (*TNF-a*, *IL-1b*) mRNA expression in fish. This study concludes that mixed probiotic bacteria strain containing commercial BA TOA5001 strain has significant potential to improve growth performance, feed utilization, and immune response in red sea bream. Further study is recommended over an extended period to detect significant differences in growth performances. Also, the mechanism responsible for the improved performance in mixed supplements with the inclusion of BA may be an exciting topic for further research.

Data Availability

The data supporting the findings of this study are available from the corresponding author and may be provided upon reasonable request.

Conflicts of Interest

The authors declare that there is no conflict of interest.

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References

[1] R. L. Naylor, R. W. Hardy, A. H. Buschmann et al., "A 20-year retrospective review of global aquaculture," *Nature*, vol. 591, no. 7851, pp. 551–563, 2021.

[2] R. S. Shadrack, I. Manabu, S. Koshio, and V. Waqalevu, "Physiological condition, digestive enzyme, blood haemato-biochemistry, antioxidant, immune and stress response of juvenile red sea bream (*Pagrus major*) fed diets containing spent oleaginous yeast," *Aquaculture Reports*, vol. 21, article 100913, 2021.

[3] M. A. O. Dawood, H. G. Abo-Al-Ela, and M. T. Hasan, "Modulation of transcriptomic profile in aquatic animals: probiotics, prebiotics and synbiotics scenarios," *Fish & Shellfish Immunology*, vol. 97, pp. 268–282, 2020.

[4] M. A. O. Dawood, S. Koshio, M. Ishikawa, and S. Yokoyama, "Effects of heat killed *Lactobacillus plantarum* (LP20) supplemental diets on growth performance, stress resistance and immune response of red sea bream, *Pagrus major*," *Aquaculture*, vol. 442, pp. 29–36, 2015.

[5] M. A. O. Dawood, S. Koshio, M. Ishikawa, and S. Yokoyama, "Interaction effects of dietary supplementation of heat-killed *Lactobacillus plantarum* and β-glucan on growth performance, digestibility and immune response of juvenile red sea bream," *Pagrus major, Fish & Shellfish Immunology*, vol. 45, no. 1, pp. 33–42, 2015.

[6] P. Yarahmadi, H. Ghafari Farsani, A. Khazaei, M. Khodadadi, G. Rashidiyan, and M. A. Jalali, "Protective effects of the prebiotic on the immunological indicators of rainbow trout (*Oncorhynchus mykiss*) infected with *Aeromonas hydrophila*," *Fish & Shellfish Immunology*, vol. 54, pp. 589–597, 2016.

[7] E. Ringø, S. H. Hoseinifar, K. Ghosh, H. V. Doan, B. R. Beck, and S. K. Song, "Lactic acid bacteria in finfish an update," *Frontiers in Microbiology*, vol. 9, 2018.

[8] K.-M. Niu, S. Khosravi, D. Kothari et al., "Effects of dietary multi-strain probiotics supplementation in a low fishmeal diet on growth performance, nutrient utilization, proximate composition, immune parameters, and gut microbiota of juvenile olive flounder (*Paralichthys olivaceus*)," *Fish & Shellfish Immunology*, vol. 93, pp. 258–268, 2019.

[9] N. Sadat Hoseini Madani, T. J. Adorian, H. Ghafari Farsani, and S. H. Hoseinifar, "The effects of dietary probiotic bacilli (*Bacillus subtilis* and *Bacillus licheniformis*) on growth performance, feed efficiency, body composition and immune parameters of whiteleg shrimp (*Litopenaeus vannamei*) postlarvae," *Aquaculture Research*, vol. 49, no. 5, pp. 1926–1933, 2018.

[10] R. S. Shadrack, I. Manabu, and S. Yokoyama, "Efficacy of single and mix probiotic bacteria strain on growth indices, physiological condition and biochemical composition of juvenile amberjack (*Seriola dumerili*)," *Aquaculture Reports*, vol. 20, article 100753, 2021.

[11] M. T. Ridha and I. S. Azad, "Preliminary evaluation of growth performance and immune response of Nile tilapia Oreochromis niloticus supplemented with two putative probiotic bacteria," *Aquaculture Research*, vol. 43, no. 6, pp. 843–852, 2012.

[12] R. M. Reda and K. M. Selim, "Evaluation of *Bacillus amyloliquefaciens* on the growth performance, intestinal morphology, hematology and body composition of Nile tilapia, *Oreochromis niloticus*," *Aquaculture International*, vol. 23, no. 1, pp. 203–217, 2015.

[13] H. Cao, S. He, R. Wei, M. Diong, and L. Lu, "*Bacillus amyloliquefaciens* G1: a potential antagonistic bacterium against eel-pathogenic *Aeromonas hydrophila*," *Evidence-Based Complementary Alternative Medicine: eCAM*, vol. 2011, 2011.

[14] C. Ran, A. Carries, M. A. Williams et al., "Identification of Bacillus strains for biological control of catfish pathogens," *PLoS One*, vol. 7, article e45793, 2012.

[15] A. Das, K. Nakhro, S. Chowdhury, and D. Kamilya, "Effects of potential probiotic *Bacillus amyloliquifaciens* FPTB16 on systemic and cutaneous mucosal immune responses and disease resistance of catla (*Catla catla*)," *Fish & Shellfish Immunology*, vol. 35, no. 5, pp. 1547–1553, 2013.

[16] K. Imaizumi, S. Tinwongger, H. Kondo, and I. Hirono, "Analysis of microbiota in the stomach and midgut of two penaeid shrimps during probiotic feeding," *Scientific Reports*, vol. 11, no. 1, p. 9936, 2021.

[17] P. Zhang, F. Yang, J. Hu et al., "Optimal form of yeast cell wall promotes growth, immunity and disease resistance in gibel carp (*Carassius auratus* gibelio)," *Aquaculture Reports*, vol. 18, article 100465, 2020.

[18] S. A. Hedayati, R. Sheikh Veisi, S. P. Hosseini Shekarabi, S. Shahbazi Naserabad, D. Bagheri, and H. Ghafarifarsani, "Effect of dietary *Lactobacillus casei* on physiometabolic responses and liver histopathology in common carp (*Cyprinus carpio*) after exposure to iron oxide nanoparticles," *Biological Trace Element Research*, pp. 1–9, 2021.

[19] S. S. Giri, V. Sukumaran, S. S. Sen, and P. K. Jena, "Effects of dietary supplementation of potential probiotic *Bacillus subtilis* VSG1 singularly or in combination with *Lactobacillus plantarum* VSG3 or/and *Pseudomonas aeruginosa* VSG2 on the growth, immunity and disease resistance of *Labeo rohita*," *Aquaculture Nutrition*, vol. 20, no. 2, pp. 163–171, 2014.

[20] C. C. Lazado, C. M. A. Caipang, and E. G. Estante, "Prospects of host-associated microorganisms in fish and penaeids as probiotics with immunomodulatory functions," *Fish & Shellfish Immunology*, vol. 45, no. 1, pp. 2–12, 2015.

[21] M. A. O. Dawood, S. Koshio, and M. Á. Esteban, "Beneficial roles of feed additives as immunostimulants in aquaculture: a review," *Reviews in Aquaculture*, vol. 10, no. 4, pp. 950–974, 2018.

[22] M. A. O. Dawood, S. Koshio, M. Ishikawa et al., "Effects of dietary supplementation of *Lactobacillus rhamnosus* or/and *Lactococcus lactis* on the growth, gut microbiota and immune responses of red sea bream, *Pagrus major*," *Fish & Shellfish Immunology*, vol. 49, pp. 275–285, 2016.

[23] T. Ren, S. Koshio, M. Ishikawa et al., "Influence of dietary vitamin C and bovine lactoferrin on blood chemistry and non-specific immune responses of Japanese eel, *Anguilla japonica*," *Aquaculture*, vol. 267, no. 1-4, pp. 31–37, 2007.

[24] S. Dossou, S. Koshio, M. Ishikawa et al., "Growth performance, blood health, antioxidant status and immune response in red sea bream (*Pagrus major*) fed *Aspergillus oryzae* fermented rapeseed meal (RM-Koji)," *Fish & Shellfish Immunology*, vol. 75, pp. 253–262, 2018.

[25] M. A. O. Dawood, S. Koshio, S. E. Fadl et al., "The modulatory effect of mannan oligosaccharide on oxidative status, selected immune parameters and tolerance against low salinity stress in red sea bream (*Pagrus major*)," *Aquaculture Reports*, vol. 16, article 100278, 2020.

[26] M. A. Kader, M. Bulbul, S. Koshio et al., "Effect of complete replacement of fishmeal by dehulled soybean meal with crude attractants supplementation in diets for red sea bream, *Pagrus major*," *Aquaculture*, vol. 350-353, pp. 109–116, 2012.

[27] "AOAC, official methods of analysis: changes in official methods of analysis made at the annual meeting," *Assoc Off Anal Chem J*, 1990.

[28] A. Houston, *Blood and Circulation/Methods for Fish Biology*, American Fish Society, NY, 1990.

[29] B. Lygren, B. Hjeltnes, and R. Waagbø, "Immune response and disease resistance in Atlantic salmon (*Salmo salar* L.) fed three levels of dietary vitamin E and the effect of vaccination on the liver status of antioxidant vitamins," *Aquaculture International*, vol. 9, no. 5, pp. 401–411, 2001.

[30] H. Ghafarifarsani, G. Rashidian, T. Bagheri, S. H. Hoseinifar, and H. Van Doan, "Study on growth enhancement and the protective effects of dietary prebiotic inulin on immunity responses of rainbow trout (*Oncorhynchus mykiss*) fry infected with *Aeromonas hydrophila*," *Annals of Animal Science*, vol. 21, no. 2, pp. 543–559, 2021.

[31] I. Salinas, L. Abelli, F. Bertoni et al., "Monospecies and multispecies probiotic formulations produce different systemic and local immunostimulatory effects in the gilthead seabream (*Sparus aurata* L.)," *Fish & Shellfish Immunology*, vol. 25, no. 1, pp. 114–123, 2008.

[32] S. Cecchini, G. Terova, G. Caricato, and M. Saroglia, "Lysozyme activity in embryos and larvae of sea bass (*Dicentrarchus labrax* L.), spawned by broodstock fed with vitamin C enriched diets," *Bulletin-European Association of Fish Pathologists*, vol. 20, no. 3, pp. 120–124, 2000.

[33] L. Góth, "A simple method for determination of serum catalase activity and revision of reference range," *Clinica Chimica Acta*, vol. 196, no. 2, pp. 143–151, 1991.

[34] A. K. Siwicki, D. P. Anderson, and G. L. Rumsey, "Dietary intake of immunostimulants by rainbow trout affects non-specific immunity and protection against furunculosis," *Veterinary Immunology and Immunopathology*, vol. 41, no. 1, pp. 125–139, 1994.

[35] D. P. Anderson and A. K. Siwicki, *Basic Hematology and Serology for Fish Health Programs*, Asian Fisheries Society, Manila, Philippines, Fish Health Section, 1995.

[36] H. Cordero, A. Cuesta, J. Meseguer, and M. Á. Esteban, "Changes in the levels of humoral immune activities after storage of gilthead seabream (*Sparus aurata*) skin mucus," *Fish & Shellfish Immunology*, vol. 58, pp. 500–507, 2016.

[37] A. Newaj-Fyzul, A. A. Adesiyun, A. Mutani, A. Ramsubhag, J. Brunt, and B. Austin, "*Bacillus subtilis* AB1 controls Aeromonas infection in rainbow trout (*Oncorhynchus mykiss*, Walbaum)," *Journal of Applied Microbiology*, vol. 103, no. 5, pp. 1699–1706, 2007.

[38] M. A. O. Dawood, S. Koshio, M. Ishikawa, and S. Yokoyama, "Immune responses and stress resistance in red sea bream, *Pagrus major*, after oral administration of heat-killed

Lactobacillus plantarum and vitamin C," *Fish & Shellfish Immunology*, vol. 54, pp. 266–275, 2016.

[39] K. Mzengereza, M. Ishikawa, S. Koshio et al., "Growth performance, growth-related genes, digestibility, digestive enzyme activity, immune and stress responses of de novo Camelina meal in diets of red seabream (*Pagrus major*)," *Animals*, vol. 11, no. 11, 2021.

[40] M. S. Hossain, S. Koshio, M. Ishikawa et al., "Efficacy of nucleotide related products on growth, blood chemistry, oxidative stress and growth factor gene expression of juvenile red sea bream, *Pagrus major*," *Aquaculture*, vol. 464, pp. 8–16, 2016.

[41] X. Rao, X. Huang, Z. Zhou, and X. Lin, "An improvement of the 2ˆ(-delta delta CT) method for quantitative real-time polymerase chain reaction data analysis," *Biostat Bioinforma Biomath*, vol. 3, no. 3, pp. 71–85, 2013.

[42] Y. Y. Moe, S. Koshio, S.-I. Teshima, M. Ishikawa, Y. Matsunaga, and A. Panganiban, "Effect of vitamin C derivatives on the performance of larval kuruma shrimp," *Marsupenaeus japonicus, Aquaculture*, vol. 242, no. 1, pp. 501–512, 2004.

[43] A. Furukawa and H. Tsukahara, "On the acid digestion method for the determination of chromic oxide as an index substance in the study of digestibility of fish feed," *Bulletin of the Japanese Society of Scientific Fisheries*, vol. 32, no. 6, pp. 502–508, 1966.

[44] Ø. Hammer, D. A. Harper, and P. D. Ryan, "PAST: paleontological statistics software package for education and data analysis," *Palaeontologia Electronica*, vol. 4, no. 1, p. 9, 2001.

[45] S. Maulu, O. J. Hasimuna, L. H. Haambiya et al., "Climate change effects on aquaculture production: sustainability implications, mitigation, and adaptations," *Frontiers in Sustainable Food Systems*, vol. 5, p. 70, 2021.

[46] M. Mugwanya, M. A. O. Dawood, F. Kimera, and H. Sewilam, "Anthropogenic temperature fluctuations and their effect on aquaculture: a comprehensive review," Aquaculture and Fisheries, 2022.

[47] M. C. Leal and R. Calado, "The key role of functional aquafeeds to achieve a more sustainable aquaculture," *Journal of the World Aquaculture Society*, vol. 50, no. 6, pp. 1044–1047, 2019.

[48] M. S. Hossain, N. M. Sony, S. Koshio, M. Ishikawa, T. Lieke, and V. Kumar, "Long term feeding effects of functional supplement 'cytidine monophosphate' on red sea bream, Pagrus major performances," *Aquaculture*, vol. 533, article 736150, 2021.

[49] A. Oliva-Teles, "Nutrition and health of aquaculture fish," *Journal of Fish Diseases*, vol. 35, no. 2, pp. 83–108, 2012.

[50] E. Ringø, R. E. Olsen, T. M. Mayhew, and R. Myklebust, "Electron microscopy of the intestinal microflora of fish," *Aquaculture*, vol. 227, no. 1, pp. 395–415, 2003.

[51] H.-L. Alakomi, E. Skyttä, M. Saarela, T. Mattila-Sandholm, K. Latva-Kala, and I. M. Helander, "Lactic acid Permeabilizes gram-negative bacteria by disrupting the outer membrane," *Applied and Environmental Microbiology*, vol. 66, no. 5, pp. 2001–2005, 2000.

[52] M. F. El Basuini, S. A. Shahin, I. I. Teiba et al., "The influence of dietary coenzyme Q10 and vitamin C on the growth rate, immunity, oxidative-related genes, and the resistance against *Streptococcus agalactiae* of Nile tilapia (*Oreochromis niloticus*)," *Aquaculture*, vol. 531, article 735862, 2021.

[53] M. A. O. Dawood, S. Koshio, M. Ishikawa, and S. Yokoyama, "Effects of partial substitution of fish meal by soybean meal with or without heat-killed *Lactobacillus plantarum* (LP20) on growth performance, digestibility, and immune response of amberjack, *Seriola dumerili* juveniles," *BioMed Research International*, vol. 2015, Article ID 514196, 2015.

[54] U. Rodriguez-Estrada, S. Satoh, Y. Haga, H. Fushimi, and J. Sweetman, "Effects of inactivated *Enterococcus faecalis* and Mannan oligosaccharide and their combination on growth, immunity, and disease protection in rainbow trout," *North American Journal of Aquaculture*, vol. 75, no. 3, pp. 416–428, 2013.

[55] P. Celi, M. Sullivan, and D. Evans, "The stability of the reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) tests on stored horse blood," *The Veterinary Journal*, vol. 183, no. 2, pp. 217-218, 2010.

[56] J. Gao, S. Koshio, M. Ishikawa, S. Yokoyama, R. E. P. Mamauag, and Y. Han, "Effects of dietary oxidized fish oil with vitamin E supplementation on growth performance and reduction of lipid peroxidation in tissues and blood of red sea bream *Pagrus major*," *Aquaculture*, vol. 356-357, pp. 73–79, 2012.

[57] L. Xu, C. Ran, S. He et al., "Effects of dietary yeast nucleotides on growth, non-specific immunity, intestine growth and intestinal microbiota of juvenile hybrid tilapia *Oreochromis niloticus* ♀ × *Oreochromis aureus* ♂," *Animal Nutrition*, vol. 1, no. 3, pp. 244–251, 2015.

[58] S. Saurabh and P. K. Sahoo, "Lysozyme: an important defence molecule of fish innate immune system," *Aquaculture Research*, vol. 39, no. 3, pp. 223–239, 2008.

[59] M. M. Abomughaid, "Isolation and identification of some probiotic bacteria and their potential role in improving immune response and resistance of Nile tilapia (*Oreochromis niloticus*) in comparison with a commercial product," *International Journal of Microbiology*, vol. 2020, 2020.

[60] F. A. Guardiola, A. Cuesta, M. Arizcun, J. Meseguer, and M. A. Esteban, "Comparative skin mucus and serum humoral defence mechanisms in the teleost gilthead seabream (*Sparus aurata*)," *Fish & Shellfish Immunology*, vol. 36, no. 2, pp. 545–551, 2014.

[61] F. Cristofori, V. N. Dargenio, C. Dargenio, V. L. Miniello, M. Barone, and R. Francavilla, "Anti-inflammatory and immunomodulatory effects of probiotics in gut inflammation: a door to the body," *Frontiers in Immunology*, vol. 12, p. 178, 2021.

[62] F. K. A. Kuebutornye, E. D. Abarike, Y. Lu et al., "Mechanisms and the role of probiotic Bacillus in mitigating fish pathogens in aquaculture," *Fish Physiology and Biochemistry*, vol. 46, no. 3, pp. 819–841, 2020.

[63] S. E. Wendelaar Bonga, "The stress response in fish," *Physiological Reviews*, vol. 77, no. 3, pp. 591–625, 1997.

[64] N. Soleimani, S. H. Hoseinifar, D. L. Merrifield, M. Barati, and Z. H. Abadi, "Dietary supplementation of fructooligosaccharide (FOS) improves the innate immune response, stress resistance, digestive enzyme activities and growth performance of Caspian roach (*Rutilus rutilus*) fry," *Fish & Shellfish Immunology*, vol. 32, no. 2, pp. 316–321, 2012.