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Effect of symbiotic supplemented diet on innate-adaptive immune response, cytokine gene regulation and antioxidant property in *Labeo rohita* against *Aeromonas hydrophila*

Gunapathy Devi[a], Ramasamy Harikrishnan[b], Bilal Ahmad Paray[c,*], Mohammad K. Al-Sadoon[c], Seyed Hossein Hoseinifar[d], Chellam Balasundaram[e]

[a] Department of Zoology, Nehru Memorial College, Puthanampatti, 621 007, Tamil Nadu, India
[b] Department of Zoology, Pachaiyappa's College for Men, Kanchipuram, 631 501, Tamil Nadu, India
[c] Zoology Department, College of Science, King Saud University, PO Box 2455, Riyadh, 11451, Saudi Arabia
[d] Department of Fisheries, Faculty of Fisheries and Environmental Sciences, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran
[e] Department of Herbal and Environmental Science, Tamil University, Thanjavur, 613 005, Tamil Nadu, India

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ABSTRACT

Administration of probiotic, prebiotic or symbiotic supplemented diets boosts the antioxidant property, pro and/or anti-inflammatory cytokine gene transcription, innate-adaptive immunity, growth rate and feed digestibility with very low or no mortality in healthy and infected (both groups) in *Labeo rohita* against *Aeromonas hydrophila* is reported. The probiotic diet increased the white blood cell (WBC) count and globulin (GB) level significantly on or after 6th week whereas with the symbiotic diet the increase was noted two weeks earlier in both groups; the total protein (TP) level also increased significantly when fed with probiotic diet on weeks 6 and 8, whereas with symbiotic diet the significant increase manifested earlier at 4th week itself. The serum phagocytic activity (PA), respiratory burst activity (RBA), complement C3 (CC3) level, alternative complement pathway (ACP), lysozyme activity (LA), and immunoglobulin M (IgM) levels in head kidney (HK) leucocytes increased significantly ($P < 0.05$) in both groups fed with probiotic diet on weeks 6 and 8; with symbiotic diet from weeks 2–8; but with prebiotic diet only on 8th week. With probiotic diet the superoxide dismutase (SOD) and catalase (CAT) activities increased significantly ($P < 0.05$) on weeks 6 and 8; with symbiotic diet from weeks 4–8 but the prebiotics diet only on 8th week. However, glutathione peroxidase (GPx) activity increased significantly ($P < 0.05$) with probiotic diet on weeks 6 and 8 and with symbiotic diet from weeks 4–8. When healthy fish fed with any supplementation diet for a period of 30 days there was no mortality while 5%, 10%, and 10% mortality was observed in infected group fed with symbiotic, probiotic, and prebiotic supplementation diets. In head kidney (HK) leucocytes, the IL-1β, IL-8, TNF-α, and NF-κB gene transcriptions were significantly up-regulation in both groups when fed with probiotic diet on weeks 6 and 8, symbiotic diet from weeks 4–8 while the prebiotic diet only on 8th week. The iNOS expression was up-regulation significantly in both groups fed with probiotic and symbiotic diets on weeks 6 and 8; however, with any diet, the relative IL-10 and TGF-β gene expressions were down-regulated. The present study suggested that dietary administration of symbiotic diet elicited earlier antioxidant activity, innate-adaptive immune response, immune related cytokine gene modulation, and disease protection earlier i.e. on 4th week than with probiotic or prebiotic diets in *L. rohita* against *A. hydrophila*.

1. Introduction

Globally aquaculture is one of the fastest emerging foods producing sectors as well as a significant contributor to economy. India is one of the prominent major carps (Catla, Rohu, and Mrigal) production centers in the world. Among the carps, Rohu (*Labeo rohita*) is the most preferred species cultured in India; it comprised about 35% of the total carp production in the last decade [1]; since rohu is the most economically affordable preferred nutritious and most delicious fish with a high market value. Along with the increasing demand to maximize production the intensive culture is subjected to stress conditions which weaken the fish immune system results increasing the susceptibility to several

* Corresponding author.
E-mail address: bparay@ksu.edu.sa (B.A. Paray).

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bacterial, parasitic, fungal, and viral diseases; thus adversely affecting both the economy and socio-economic status of local people involved in aquaculture in several countries [2–5]. In this regard, the fish farmers are constrained to apply large quantity of antibiotics and chemotherapeutants in disease management. However, over use of these traditional drugs lead to the development of drug-resistant strains of pathogens and environmental issues such as bioaccumulation and public health hazards. Further, the antibiotic often destroys the beneficial microbiota in the gastrointestinal system; besides their bioaccumulation in host tissues renders it unsafe for human consumption. Hence the application of natural immunostimulants is gaining importance as an alternate ecofriendly therapeutic measure.

In this back drop, dietary administration of probiotic, prebiotic, and synbiotic has been reported as an ideal alternative to antibiotics and chemotherapeutants. These are non-digestible safe food supplements and improve the gut microbial balance besides improving the water quality, growth performance, disease resistance, antioxidant activity, and strengthen the specific and non specific immune system of fishes [6–9]. Symbiotic components comprise a mixture of probiotics and prebiotics incorporated in feed conferring a synergistic benign effect in the host triggering the growth metabolism by promoting an ideal population of microbial biota in the digestive tract [10]. However, there is a paucity of comprehensive information on these diets on growth performance, immunomodulation (both innate and adaptive immunity) as well as cytokine gene regulation with reference to fishes against diseases.

The antioxidant enzymes have an important role in countering and attacking the reactive oxygen species (ROS) that are naturally produced and adversely affect the various physiological mechanisms in all living organisms. Besides hypoxia, hyperoxia, and xenobiotic environmental stress also trigger oxidative stress. Therefore, increasing the antioxidant capacity helps to further resistance of an organism against stress or diseases. In aquaculture species like shrimp and fish, prebiotics, probiotics and symbiotic enriched diets have been shown to modulate the antioxidant capacity [11–15]. Galactooligosaccharides (GOS) is produced through the enzymatic conversion of lactose that results in better growth performance in fishes [16–18]. There was no study on the effect of antioxidant activity, innate-adaptive immune response as well as immune related gene modulation of symbiotic supplementation diet in *L. rohita* against pathogens. The present study reports a detailed comparative analysis on the effect of prebiotic, probiotic, and symbiotic supplemented diets on antioxidant activity, innate-adaptive immune immunity as well as pro and/or anti-inflammatory cytokine gene transcription in *L. rohita* against *Aeromonas hydrophila*.

2. Materials and methods

2.1. Experimental diet

The formulated diet comprised rice bran and wheat flour as carbohydrate source; fishmeal and soybean meal, ground nut oil cake, and mustard oil cake as protein source, and fish oil as lipid source. Tapioca powder was used binder; a premix of vitamins and minerals were added as vitamins and minerals source (Table 1). Four experimental pellet diets were prepared such as: (i) basal diet without probiotic/prebiotic/symbiotic, (ii) basal diet with 1 g kg^{-1} probiotic (*Bacillus subtilis*), (iii) basal diet with 1 g kg^{-1} prebiotic, Galactooligosaccharides (GOS), and (iv) basal diet with 1 g kg^{-1} symbiotic (equal quantity of probiotic, *B. subtilis* and prebiotic, GOS). All ingredients except the vitamins and minerals as well as probiotic/prebiotic/symbiotic were mixed well in hot sterile water and steamed for 20 min in a pressure cooker. After cooling at room temperature (RT) the premixed vitamins and minerals as well as probiotic/prebiotic/symbiotic were added by evenly and mixing in respective diet. The paste feeds were extruded manually by passing through a hand pelletizer (0.5 mm diameter size) and air-dried separately for 30 min at RT and then stored in an oven at 60 °C until dry. The dried feeds were tight packed in a container and stored in a freezer at −20 °C until used. The formulated experimental diets were analysed to quantify the proximate compositions using standard methods.

Table 1
Supplementation experimental pellet diet ingredients and proximate composition (g kg^{-1} dry matter).

Ingredients (g kg^{-1})	Concentration
Rice bran	22.0
Wheat flour	10.0
Fishmeal	10.0
Soybean meal	20.0
Ground nut oil cake	10.0
Mustard oil cake	10.0
Fish oil	5.0
Tapioca powder	10.0
Vitamins + minerals premix[a]	1.0
Starch	1.0
Probiotic/prebiotic/symbiotic	1.0
Total	100
Proximate composition (%)	
Crude protein (%)	49.7
Crude lipid (%)	13.5
Crude carbohydrate (%)	18.2
Crude ash (%)	12.4
Crude fiber (%)	5.6

[a] Composition of vitamin–mineral premix (Emix™ plus, Mumbai, Maharastra, India) (quantity/2.5 kg): vitamin A, 5,500,000 IU; vitamin B2, 2000 mg; vitamin B6, 1000 mg; vitamin B12, 6 mg; vitamin D3, 1,100,000 IU; vitamin E, 750 mg; vitamin K, 1000 mg; choline chloride, 150 g; calcium panthothenate, 2500 mg; Co, 450 mg; Ca, 500 g; Cu, 2000 mg; DL-methionine, 10 g; Fe, 7500 mg; iodine, 1000 mg; L-lysine, 10 g; niacinamide, 10 g; Mn, 27,000 mg; Zn, 5000 mg; P, 300 g; Se, 50 ppm; satawari, 2500 mg; Carrier, quantum sufficient; Lactobacillus, 120 million units, and yeast culture, 3000 crore units.

2.2. Aeromonas hydrophila

A. hydrophila (MTCC 1739) was obtained from IMTECH, Chandigarh, India, which was isolated from infected fish and maintained in the laboratory in standard condition. The bacterium was cultured in Tryptone Soya Broth (TSB, Himedia, India) for 24 h at 37 °C. The bacterial culture was then centrifuged at 3000 × *g* for 10 min and the supernatant was discarded. The bacterial pellet was resuspended in phosphate buffered saline (PBS, pH 7.4 ± 0.1) and the bacterial suspension was adjusted to 0.5 at the absorbance or optical density (O.D.) of 456 nm corresponding to 1×10^7 colony forming unit (cfu) using Micro plate Reader (Biotek, Model No: ELX 800, Synergy, HT, NY, USA). The morphological, physiological, and biochemical characteristics of the bacterium were confirmed by standard methods as described by Tonguthai et al. [19]. Further the molecular characterization of the bacterium was conducted using the API-20E kit according to Barrow and Feltham [20].

2.3. Fish

Healthy, *L. rohita* (26.4 ± 1.3 g) was acquired from a local commercial fish farm. The fish were transported to the laboratory and were bath treated for 5 min with 5 ppm potassium permanganate ($KMnO_4$) immediately. Then the fish were acclimatized in cement tanks (500 l capacity) with clean bore-well water for two weeks and provided with basal/control diet (Table 1) at a rate of 4% of their body weight twice a day at 10.00 a.m. and 14.00 p.m. Two-third of the water was replaced to siphon out and the accumulated wastes removed daily.

2.4. Experimental design

After two weeks acclimatization, the fish were randomly distributed into eight groups of 25 each (8 × 25 = 200) in 250 l tanks namely: (1) healthy group fed with basal/control diet without probiotic, prebiotic or symbiotic (0 g kg^{-1}) [H]; healthy groups fed diet supplementation with (2) 1 g kg^{-1} of probiotic, *B. subtilis* [H-PO], (3) 1 g kg^{-1} of prebiotic, GOS [H-PR], and (4) 1 g kg^{-1} of symbiotic [H-SM]; (5) the infected or challenged group fed with basal/control diet without probiotic, prebiotic or symbiotic (0 g kg^{-1}) [I]; the infected groups fed diet supplementation with (6) 1 g kg^{-1} of probiotic, *B. subtilis* [I-PO], (7) 1 g kg^{-1} of prebiotic, GOS [I-PR], and (8) 1 g kg^{-1} of symbiotic [I-SM]. All the groups were kept with three replicates (200 × 3 = 600 fish). Groups 1 to 4 were injected intramuscularly (i.m.) with 50 µl PBS alone whereas groups 5 to 8 were injected or challenge i.m. with 50 µl PBS containing *A. hydrophila* at 1 × 10^7 cfu ml^{-1}. The respective pellet diets were given twice a day at 10.00 a.m. and 14.00 p.m. during the experimental period. The water quality such as temperature (°C), dissolved oxygen, ammonia concentrations, and pH were measured from 26 to 31, 6.1–7.3 mg l^{-1}, 0.03–0.06 ppm, and 7.0 to 8.0 during the experimental period.

2.5. Blood sampling

After post challenge at 2, 4, 6, and 8 weeks, the fish were immediately placed under anaesthesia with 150 ppm buffered MS-222 solution (Sigma-Aldrich, St. Louis, MO, USA). The blood was withdrawn carefully from caudal vasculature (24-gauge syringe needle) in each fish. Each collected blood sample was equally distributed into heparinized and non-heparinized tubes. The non-heparinized blood samples were immediately put under RT for 2 h. Then the samples were spun in a centrifuge at 2700 rpm for 10 min; the sera were separated and stored into new sterile tubes at −20 °C until used for the experiment. The heparinized blood samples were used immediately for the analysis of hematological and biochemical study.

2.6. Hematology and biochemical study

The white blood cell (WBC) count was measured using diluting fluids (Neuberger's counting chamber of haematocytometer) [21]. The total protein (g dl^{-1}) and albumin (g dl^{-1}) content of the serum were estimated using commercial estimation kit (Liquichem Total Protein/Albumin kit, Recorders and Medicare Systems Pvt. Ltd., India) by the Biuret method [22] and BCG method [23]. The amount of total globulin was calculated by deducting the calculated values of albumin from total protein (total serum protein minus total serum albumin) and the albumin minus globulin ratio was calculated of the total serum albumin divided by the total serum globulin.

2.7. Isolation of head kidney (HK) leukocytes

The head kidney (HK) leukocytes were prepared according to Kamilya et al. [24] with some modification. The tissue containing HK leukocytes were harassed with forceps in complete RPMI-1640 medium containing penicillin 100 IU/ml, streptomycin 100 mg ml^{-1}, and 10% fetal calf serum (Hi-media, India) prepared for cell suspensions. The cell suspensions were washed by centrifugation at 1000 rpm for 10 min. The pellet was collected and re-suspended in RPMI-1640 medium and centrifuged against at 1500 rpm for 30 min, then carefully layered on top of the leukocyte isolation medium (HiSep, Hi-media, India). The leukocytes cells were transferred from the HiSep interface medium into sterile tubes and washed twice by centrifugation at 1000 rpm for 10 min. Then the purified leukocytes were collected and counted by using a hemocytometer and the number of leukocytes adjusted to 1 × 10^6 cells/ml in RPMI-1640 medium. The viability of the leukocytes was confirmed by trypan blue exclusion assay.

2.8. Immune assays

2.8.1. Phagocytic activity (PA)

The phagocytic activity was carried out in HK leukocyte by Cai et al. [25] with minor modifications. The blood (500 µl) was taken in small centrifuge tubes (2 ml) mix with 250 µl of 5 × 10^8 CFU ml^{-1} *A. hydrophila* than incubate at 28 °C in a dry bath for 30 min. It was centrifuged at 1500 rpm for 5 min and discarded the supernatant. The upper layer of the precipitate was used to make blood slides for calculating the percentage phagocytic activity (%) through the number of leukocytes that engulfed bacteria as follows: [100 x (phagocytotic leukocyte number)/(Total leukocyte number)].

2.8.2. Respiratory burst activity (RBA)

The RBA produced by leucocytes of the head kidneys was measured by Geng et al. [26] with minor modification. A volume of 100 µl leucocyte suspension (1 × 10^6 cells ml^{-1}) was added in microtiter plate wells previously coated with 100 µl of a poly-L-lysine solution (0.2%) to improve cell adhesion and centrifuged at 700 × *g* for 20 min then the non-adherent cells removed. The plate wells were washed with Hanks' balanced salt solution (HBSS) than 100 µl 0.1% Zymosan (0.1% in HBSS) added in each wells and incubated RT for 30 min. The zymosan was discarded after incubation than the leucocytes were washed at least three times with 100 µl HBSS and the cells were stained with 100 µl of 0.3% nitroblue tetrazolium (NBT) solution for 30 min at RT. The NBT solution was discarded and the reaction was stopped by adding 100 µl of 100% methanol. The formazan was dissolved by mixing of 120 µl of 2 M KOH and 140 µl of DMSO and the O.D. was measured at 630 nm in triplicate using an ELISA plate reader. The RBA was calculated by the stimulated activity (SA) minus basal activity (BA). The SA is the RBA caused by stimulation with zymosan, and BA is the RBA without stimulation by zymosan.

2.8.3. Alternative complement pathway (ACP)

ACP activity was measured and calculated according to Yano [27] method by using rabbit red blood cells (RaRBC). The RaRBC was adjusted to 2 × 10^8 cells ml^{-1} with 0.01 M ethylene glycol tetraacetic acid–magnesium–gelatin veronal buffer (EGTA–Mg–GVB). The 100% lysis value was obtained by lysing of 100 ml of RaRBC with 3.4 ml of distilled water by measuring the O.D. at 414 nm against blank distilled water. The test serum was diluted and different volumes ranging from 0.1 to 0.25 ml and made up to 0.25 ml total volume before being allowed to react with 0.1 ml of RaRBC in test tubes than incubation at 20 °C for 90 min with occasional shaking. After incubation, a volume 3.15 ml of saline solution was added in each tube, centrifuged at 1600 × *g* for 10 min at 4 °C than read O.D. at 414 nm. The volume of serum that produces 50% haemolysis (ACH50) was determined and the number of ACH50 U ml^{-1} was calculated for each group.

2.8.4. Serum complement C3 (CC3) assay

The serum CC3 level was measured by using a C3 kit (Biocompare, CA, USA) according to Thomas [28]. Briefly, take 25 µl of complement C3 standard or sample in each well and add 25 µl in the top of the standard or sample of Biotinylated complement C3 in each well then mix gently. All wells covered with a sealing tape and incubate for 2 h and washed five times invert the plate with 200 µl of washing buffer manually or six times washed with 300 µl of washing buffer and then invert the plate if can use in machine and hit it 4–5 times with absorbent paper towel of the plate to completely remove the liquid. Then each well mixed 50 µl of Streptavidin-Peroxidase Conjugate and incubate for 30 min and washed as described above. After 50 µl of Chromogen Substrate was added in each well and incubate for 20 min or until developed the optimal blue color to ensure thorough mixing and break the bubbles in the well with pipette tip. A volume of 50 µl of stop solution was added in each well and the color will change from blue to yellow then read O.D. at 450 nm. To calculate complement C3

the unknown sample concentration from the standard curve and multiply the value by the dilution factor.

2.8.5. Lysozyme assay

The lysozyme activity was measured through turbidometric assay according to Ellis [29] with minor modifications. Briefly, 0.03% lyophilized *Micrococcus lysodeikticus* in 0.05 mM sodium phosphate buffer (pH 6.2) was used for bacteria suspension. A volume of 10 µl of fish serum was added to 250 µl of bacterial suspension in duplicate wells of a "U" bottom microtitre plate and measured O.D. at 490 nm as determined after 0.5 and 4.5 min of incubation at 22 °C using a microtiter plate reader (Biorad, USA). One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 min^{-1}.

2.8.6. Immunoglobulin M (IgM) assay

Serum total IgM levels were measured according to Sharma et al. [30] with the following protocols. A volume of 0.1 ml of serum was placed into a sterile serum vial and mixed 0.1 ml of 12% polyethylene glycol (PEG) which suspended in de-ionized water and then incubated for 2 h under continuous mixing in RT. After incubation the suspension was centrifuged at 5000 × *g* for 10 min and collect supernatant for determination the amount protein by absorption to PEG. Total immunoglobulin (Ig) was expressed (unit mg ml^{-1}) by total protein in individual sample serum minus total protein obtained by the absorption of PEG.

2.9. Antioxidant assays

The superoxide dismutase (SOD) assay was measured according to Zhang et al. [31] by enzymatic reaction using a commercially kit (Randox, Crumlin, UK), and the malondialdehyde (MDA) content was measured using barbituric acid reaction chronometry according to Draper et al. [32]. The catalase (CAT) activity was measured by the rate of decrease in H_2O_2 absorbance at 240 nm by using a commercially available kit (Sigma-Aldrich, USA). The glutathione peroxidise (GPx) activity was measured using a commercial kit (RanselRS-504, Randox), following the manufacturer's guidelines.

2.10. Disease resistance

In each experiment, a group of 20 fish were maintained separately as mentioned previously to observe for cumulative mortality (CM) for a period of 30 days. The bacterial culture, challenge study, and the concentration were same as above. CM (%) = [(Number of surviving fishes after challenge)/(Number of fish challenged with fungus)] x 100.

2.11. Immune genes

The immune gene expression was studied in HK leukocytes. The total RNA was isolated from head kidney tissue using TRIZOL reagent (Invitrogen, USA) following the manufacturer's guidelines. The concentration and purity of RNA were measured by using a spectrophotometer (Thermo Scientific, USA). The quality of RNA was confirmed by 1% agarose gel with 0.5 µg ml^{-1} ethidium bromide. The RNA was reverse-transcribed by cDNA using SuperScript® cDNA synthesis kit (Life Technologies) following the manufacturer's guidelines. The real-time PCR analysis of immune genes such as IL-1β, IL-8, IL-10, iNOS, TNF-α, TGF-β, NF-κB, and a housekeeping gene (β-actin) were done by using CFX96™ Real-Time PCR (Bio-Rad, Laboratories, Inc.), following standard protocols with primer sequences conditions (Table 2). The accuracy of each amplicon was confirmed by melt curve analysis after amplification. All samples were run in parallel with the housekeeping gene; the housekeeping gene using to normalize cDNA loading. The results of the gene expression were performed by using the $2^{-\Delta\Delta CT}$ method after verifying that the primers were amplified with an efficiency of approximately 100% [33], where $\Delta\Delta C_T$ = [(C_T gene of interest − $C_{T\beta}$-actin) treatment group − (C_T gene of interest − $C_{T\beta}$-actin) control group]. The C_T is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold.

2.12. Statistical analysis

The data were analysed by one-way analysis of variance (ANOVA) and the multiple comparisons between treatments using Tukey's test using the OriginPro software (version 8; Origin-Lab Corporation, Northampton, USA). The significance level of all parameters was set at $P < 0.05$, and the results are expressed as mean ± SEM.

3. Results

3.1. Hematology and biochemistry

Fish fed with probiotic supplementation diet increased the WBC counts and GB levels significantly ($P < 0.05$) in healthy and infected *L. rohita* after 6th week while the fish fed with the symbiotic supplementation diet the increase was noted two weeks earlier (i.e.) on 4th week. However, the total protein (TP) level was significant ($P < 0.05$) in both groups fed with probiotic diet between weeks 6 and 8, whereas it manifested with the symbiotic supplementation diet in both groups earlier at 4th week. The AB level increased significantly ($P < 0.05$) in both groups with probiotic diet on 8th week, but this was not significant ($P > 0.05$) between weeks 2–6 as compared to control. Interestingly, though the AB level differed significantly ($P < 0.05$) in both groups with symbiotic diet between weeks 4–6 the variation was not significant ($P > 0.05$) on 2nd week. However when fed with prebiotic supplementation diet, the AB level and AB/GB ratio with any supplementation diet increased it was not statistically significant ($P > 0.05$) as compared with control (Table 3).

3.2. Immune modulation

The observed immune parameters such serum phagocytic activity (PA), respiratory burst activity (RBA), alternative complement pathway (ACP) activity, serum complement C3 (CC3) level, lysozyme activity (LA), and immunoglobulin M (IgM) level of HK leucocytes were not significant ($P > 0.05$) in both groups fed with probiotic, prebiotic or symbiotic diets on 2nd week. All immune parameters were significantly ($P < 0.05$) modulated in both groups fed with symbiotic diet from weeks 4–8 whereas with probiotic or prebiotic diets the increase was not significant ($P > 0.05$) in both groups on 4th week. However, the healthy and infected groups administered with probiotic or symbiotic diets all the tested immune parameters were significantly modulated ($P < 0.05$) between weeks 6 and 8 (Figs. 1–6).

3.3. Antioxidant protection

The superoxide dismutase (SOD) and catalase (CAT) activities increased significantly ($P < 0.05$) in both groups fed with probiotic diet on weeks 6 and 8, but not between weeks 2 and 4. The SOD and CAT activities differed significantly ($P < 0.05$) in both groups when fed with symbiotic diet from weeks 4–8 as compared with control. However, in both groups fed with prebiotic diet the values became significant ($P < 0.05$) level only on 8th week; though it increased between week 2–6 were not significant ($P > 0.05$) as compared with control. The glutathione peroxidise (GPx) activity significantly ($P < 0.05$) increased between weeks 6 and 8 in the healthy group fed with probiotic diet, but not in the infected group at any time. Both groups fed with symbiotic diet significantly ($P < 0.05$) enhanced GPx activity from weeks 4–8. In groups fed with prebiotic diet, the GPx activity increased; however it was not significant ($P > 0.05$) at any time. The malondialdehyde (MDA) content decreased in both groups

Table 2
Real-time primer sequences for the experiment.

Gene name	Primer sequence (5′ to 3′)	Temperature	Accession number
IL-1β	F: ATCTTGGAGAATGTGATCGAAGAG R: GATACGTTTTTGATCCTCAAGTGTGAAG	61.5 °C	AM932525
IL-8	F: GGGTGTAGATCCACGCTGT R: AGGGTGCAGTAGGGTCCA	60.5 °C	HM363518
IL-10	F: AAGGAGGCCAGTGGCTCTGT R: CCTGAAGAAGAGGCTCTGT	61.1 °C	AB010701
TNF-α	F: CCAGGCTTTCACTTCACG R: GCCATAGGAATCGGAGTAG	61.1 °C	FN543477
iNOS	F: GGAGGTACGTCTGCGAGGAGGCT R: CCAGCGCTGCAAACCTATCATCCA	61.1 °C	AM932526
TGF-β	F: ACGCTTTATTCCCAACCAAA R: GAAATCCTTGCTCTGCCTCA	60.5 °C	AF136947
NF-κB	F: TATTCAGTGCGTGAAGAAG R: TATTAAAGGGGTTGTTCTGTC	61.5 °C	LN590704
β-actin	F: AGACCACCTTCAACTCCATCATG R: TCCGATCCAGACAGAGTATTTACGC	60.5 °C	AY531753

IL: interleukin, TNF: tumor necrosis factor, iNOS: inducible nitric oxide synthase, TGF: Transforming growth factor, NF-κB: nuclear factor kappa beta.

fed with any supplementation diet as compared to control (Table 4).

3.4. Disease resistance

There was no mortality in healthy group fed with or without probiotics, prebiotics or symbiotic supplementation diets; however, the mortality was 5%, 10, and 10% in the infected fish fed with symbiotic, probiotics, and prebiotics supplementation diets. The mortality was high as 80% in the infected fish fed with control diet (Fig. 7).

3.5. Immune genes transcription

The IL-1β and IL-8 transcription level of immune genes in HK leucocytes was not up-regulation in healthy or infected groups fed with probiotic on weeks 2 and 4, but its expressions were significantly up-regulated ($P < 0.05$) when fed with probiotic diet on weeks 6 and 8 as compared with control. In groups fed with symbiotic diet the IL-1β and IL-8 transcription level was significantly up-regulated ($P < 0.05$) between 4 and 8 weeks; it was however up-regulated in the healthy or infected groups fed with prebiotic diet only on 8th week but not between weeks 2 and 6 as compared to control (Fig. 8a and b). In the healthy or infected group any supplementation diet resulted in the down-regulation of IL-10 transcription level in HK leucocytes (Fig. 8c).

The TNF-α and NF-κB gene transcription level was not up-regulation ($P > 0.05$) in groups fed with any enriched diet on 2nd week. But on being fed with symbiotic diet there was a significant up-regulation ($P < 0.05$) of TNF-α and NF-κB gene transcription from week 4–8, probiotic diet on weeks 6 and 8 but with the prebiotic diet only on 8th week (Figs. 9 and 10). The relative mRNA expressions of iNOS gene in HK leucocytes did not modulate significantly when both groups fed with probiotic or symbiotic diets between weeks 2 and 4, whereas in healthy group administration of any supplementation diet significantly modulated the relative mRNA expressions of iNOS gene on weeks 6 and 8 as compared to control except in infected fish fed with prebiotic diet (Fig. 11). The relative mRNA expressions of TGF-β gene in HK leucocytes were down-regulated in both groups when fed with or without probiotic, prebiotic or symbiotic supplementation diets (Fig. 12).

Table 3
Hematological and biochemical profile of probiotics, prebiotics or symbiotic enriched diets in *L. rohita* against *A. hydrophila* for 8 weeks.

Indices	Weeks	H	I	H-PO	I-PO	H-SM	I-SM	H-PR	I-PR
WBC (10^3 ml^{-1})	2	2.12 ± 0.04^a	2.08 ± 0.04^a	2.38 ± 0.04^a	2.30 ± 0.02^a	2.67 ± 0.02^a	2.51 ± 0.02^a	2.44 ± 0.02^a	2.41 ± 0.03^a
	4	2.18 ± 0.03^a	2.06 ± 0.03^a	2.42 ± 0.03^a	2.35 ± 0.03^a	3.36 ± 0.03^b	3.28 ± 0.03^b	2.56 ± 0.03^a	2.49 ± 0.03^a
	6	2.20 ± 0.04^a	2.01 ± 0.04^a	3.63 ± 0.04^b	3.52 ± 0.04^b	3.72 ± 0.04^b	3.54 ± 0.04^b	2.72 ± 0.03^a	2.63 ± 0.04^a
	8	2.24 ± 0.04^a	1.96 ± 0.03^a	3.85 ± 0.05^b	3.66 ± 0.05^b	3.84 ± 0.05^b	3.71 ± 0.05^b	3.49 ± 0.04^b	3.32 ± 0.03^b
TP (g dl^{-1})	2	3.18 ± 0.03^a	3.11 ± 0.03^a	3.37 ± 0.03^a	3.27 ± 0.03^a	3.56 ± 0.03^a	3.45 ± 0.03^a	3.38 ± 0.03^a	3.28 ± 0.03^a
	4	3.25 ± 0.03^a	3.13 ± 0.04^a	3.48 ± 0.04^a	3.34 ± 0.03^a	4.98 ± 0.04^b	4.68 ± 0.04^b	3.58 ± 0.04^a	3.43 ± 0.03^a
	6	3.28 ± 0.04^a	3.16 ± 0.03^a	5.67 ± 0.04^b	5.53 ± 0.04^b	5.31 ± 0.05^b	5.11 ± 0.05^b	3.65 ± 0.04^a	3.53 ± 0.04^a
	8	3.33 ± 0.04^a	3.20 ± 0.04^a	5.92 ± 0.05^b	5.21 ± 0.05^b	5.55 ± 0.05^b	5.22 ± 0.05^b	4.98 ± 0.05^b	4.36 ± 0.04^a
AB (g dl^{-1})	2	1.26 ± 0.02^a	1.24 ± 0.02^a	1.37 ± 0.03^a	1.32 ± 0.02^a	1.38 ± 0.02^a	1.30 ± 0.03^a	1.31 ± 0.02^a	1.27 ± 0.03^a
	4	1.29 ± 0.02^a	1.20 ± 0.02^a	1.75 ± 0.02^a	1.52 ± 0.03^a	2.68 ± 0.03^b	2.53 ± 0.03^b	1.34 ± 0.03^a	1.29 ± 0.03^a
	6	1.33 ± 0.03^a	1.25 ± 0.03^a	2.72 ± 0.03^a	1.96 ± 0.04^a	2.83 ± 0.04^b	2.63 ± 0.04^b	1.55 ± 0.03^a	1.46 ± 0.03^a
	8	1.38 ± 0.02^a	1.29 ± 0.03^a	2.85 ± 0.04^b	2.68 ± 0.04^b	3.15 ± 0.05^b	2.97 ± 0.04^b	2.88 ± 0.05^a	1.93 ± 0.04^a
GB (g dl^{-1})	2	1.38 ± 0.03^a	1.36 ± 0.02^a	1.42 ± 0.02^a	1.40 ± 0.03^a	1.50 ± 0.03^a	1.46 ± 0.03^a	1.44 ± 0.03^a	1.41 ± 0.02^a
	4	1.43 ± 0.03^a	1.31 ± 0.03^a	1.65 ± 0.03^a	1.58 ± 0.02^a	2.88 ± 0.03^b	2.65 ± 0.03^b	1.61 ± 0.03^a	1.53 ± 0.03^a
	6	1.56 ± 0.03^a	1.38 ± 0.02^a	2.78 ± 0.05^b	2.52 ± 0.04^b	3.39 ± 0.05^b	3.12 ± 0.04^b	1.85 ± 0.04^a	1.66 ± 0.04^a
	8	1.61 ± 0.04^a	1.45 ± 0.03^a	3.26 ± 0.05^b	2.98 ± 0.05^b	3.54 ± 0.04^b	3.32 ± 0.05^b	3.08 ± 0.05^b	2.87 ± 0.04^b
A/C (g dl^{-1})	2	0.51 ± 0.02^a	0.48 ± 0.01^a	0.54 ± 0.02^a	0.51 ± 0.03^a	0.62 ± 0.02^a	0.59 ± 0.02^a	0.58 ± 0.02^a	0.55 ± 0.02^a
	4	0.54 ± 0.02^a	0.50 ± 0.02^a	0.57 ± 0.02^a	0.55 ± 0.02^a	0.73 ± 0.02^a	0.70 ± 0.02^a	0.71 ± 0.02^a	0.68 ± 0.02^a
	6	0.58 ± 0.02^a	0.53 ± 0.01^a	0.62 ± 0.02^a	0.60 ± 0.03^a	0.93 ± 0.03^a	0.85 ± 0.03^a	0.89 ± 0.03^a	0.85 ± 0.03^a
	8	0.63 ± 0.03^a	0.55 ± 0.02^a	0.66 ± 0.03^a	0.63 ± 0.03^a	1.03 ± 0.04^a	0.97 ± 0.03^a	0.96 ± 0.03^a	0.88 ± 0.03^a

WBC: white blood cell, TP: total protein, AB: albumin, GB: globulin, A/G ratio: albumin/globulin ratio.

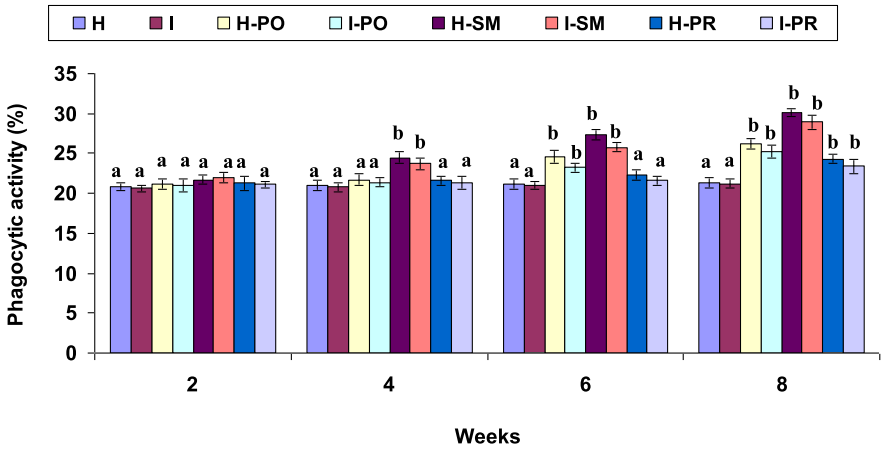

Fig. 1. Serum phagocytic activity (PA) observed in *L. rohita* ($n = 6$) fed with or without probiotics, prebiotics or symbiotic (1 g kg^{-1}) for 8 weeks. Each bars represented by mean ± SEM and the differences represented in letters as indicated levels of significant at $p < 0.05$ within the groups. Note: H: healthy group, I: infected group, H-PO: healthy probiotics diet group, H-PR: healthy prebiotics diet group, H-SM: healthy symbiotic diet group, I-PO: infected probiotics diet group, I-PR: infected prebiotics diet group, I-SM: infected symbiotic diet group.

4. Discussion

In aquaculture oral administration of natural immunostimulants has been successful since besides possessing ideal properties like disease resistance, growth promotion, and promoting better immunity. In this line probiotics, prebiotics, and symbiotics supplemented diet has been explored to enhance several benign properties including improving nutritional maintenance, antagonistic activity towards bacterial multiplication, nutritional competition to pathogens, and modulation of host's immune responses. The oral administration of probiotics or prebiotics supplementation diet positively enhanced growth, immune response, disease protection, maintenance of intestine function, and microbial activity in the intestinal tract of fish [6]. The prebiotic like mannanoligosaccharides (MOS) from yeast (*Saccharomyces cerevisae*) afford mannose substrate upon which pathogenic gut bacteria selectively attach, impairing the adhesion to enterocytes leading to better gut health and villi integrity [34]. Further, prebiotics are food supplements that cannot be digested and provides positive effects to the host by modulating the growth and activity of one or more of the digestive bacteria in the gut [35]. However these beneficial effects do not last longer than the prebiotic supplementation period. Therefore, the application of symbiotics in combination with probiotics and prebiotics need to be investigated to overcome of these problems [36]; since they confer enhanced survival rate and growth of beneficial bacteria in the gut, improving the survival rate by selectively stimulating the growth or by activating the metabolism of one or a limited number of beneficial bacteria [35,37] in the host. Administration of probiotics, prebiotics, and symbiotic supplements promotes better growth, digestion, immunity, and disease resistance in fish by increasing the composition of the benign gut microbiota [7,18,38–41]. Thus the probiotics, prebiotics, and symbiotics as immunostimulants, definitely improve the innate immune system of cellular and humoural components, comprising physical barriers, have the potential to be used as alternatives to antibiotics and chemicals. This study presents a detailed comparative analysis on the effect of oral administration of probiotic, prebiotic, and symbiotic supplemented diet on hematology, innate-adaptive immune response, antioxidant activity, disease resistance, and immune gene regulation in *L. rohita* against *A. hydrophila*.

The WBC and GB concentration are the first line of defense in fish. The significant increase in the total WBC counts comprising neutrophil, large lymphocytes, and monocytes indicate that the innate immunity of the fish is stimulated to facilitate the fight against pathogens by enhancing the primary immune defense. The administration of probiotic diet increased WBC counts and GB level significantly in healthy and infected fish after 6th week but with prebiotics the increase manifested only on 8th week. However, with symbiotic diet the increase in WBC and GB levels were noted earlier i.e. 4th week. These results are in line with earlier studies in *Schizothorax zarudnyi*, *Acipenser stellatus*, and *Cyprinus carpio* after being fed with 2–30 g kg^{-1} of prebiotics and probiotics supplementation diets [42–46]. In Caspian brown trout fed with a synbiotic (basal diet + 2 g kg^{-1} isomaltooligosaccharides, IMOS + 1 g kg^{-1} *Bacillus* spp., BetaPlus®) diet fed for 7 weeks increased

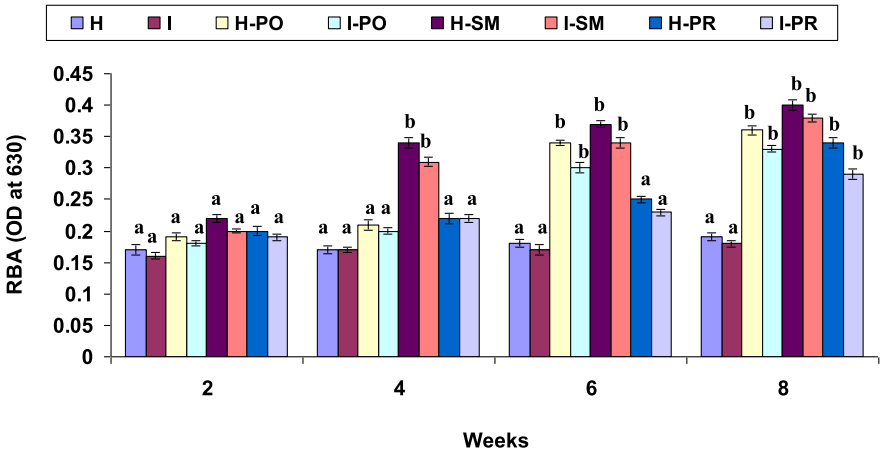

Fig. 2. Serum respiratory burst activity (RBA) observed in *L. rohita* ($n = 6$) fed with or without probiotics, prebiotics or symbiotic (1 g kg^{-1}) for 8 weeks. Details of statistical information and experimental groups were seen in Fig. 1.

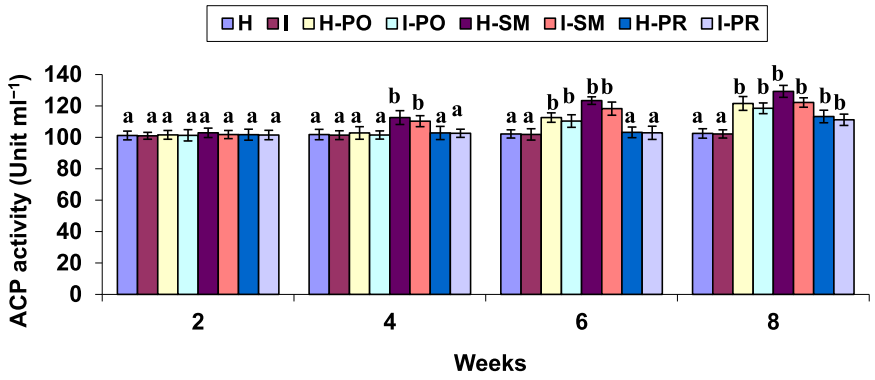

Fig. 3. Alternative complement pathway (ACP) activity observed in *L. rohita* (n = 6) fed with or without probiotics, prebiotics or symbiotic (1 g kg^{-1}) for 8 weeks. Details of statistical information and experimental groups were seen in Fig. 1.

the WBC and albumin/globulin ratio significantly [47].

The high level of serum TP and GB in moribund fish challenged with pathogen indicates that the antibody production was enhanced [48] stimulating the innate immune function against pathogens [49]. Further, GB is the main resource for Ig production; hence its enhancement in serum affords immune-stimulatory activity. The significantly high TP level and GB content was observed in both groups fed with probiotic diet on weeks 6 and 8, whereas in the healthy and infected groups fed with symbiotic diet significantly increased earlier at 4th week. The AB level was high in healthy and infected groups fed with probiotics diet on 8th week but not from weeks 2–6 whereas the AB level significantly increased in both groups when fed with symbiotic diet earlier at 4th week, though with any supplementation diet the albumin-globulin ratio did not increase at any time. The present result is in line with the findings in *Cirrhinus mrigala*, *Oreochromis niloticus*, and *Ctenopharyngodon idella* fed with probiotics and prebiotics supplemented diets against pathogens [46,50,51]. There is little information available on the effect of symbiotic diet on TP, GB, and AB in fishes. In Nile tilapia fed with 0.2% mannan-oligosaccharides (MOS) the TP and GB significantly increased after 2 weeks [52]. Grass Carp fed with probiotic and synbiotic incorporated diets the serum TP, AB, and GL levels indicated positive effects [53]. Nile tilapia fed diet supplemented with fructooligosaccharide (FOS) at 20 g kg^{-1}, the TP and GB levels significantly increased after challenged with *A. hydrophila* [54]. In Caspian brown trout a synbiotic diet containing 2 g kg^{-1} IMOS + 1 g kg^{-1} BetaPlus® significantly increased the TP, AB, albumin/globulin ratio for 7 weeks [47].

The PA provides initial stimulation of the inflammatory response before the antibody development [55] and it associated with RBA which is considered as a very important indicator of innate immune function in fish [56]. In order to attack pathogens the respiratory bursts (RB) are produced by phagocytic cells during phagocytosis; its estimation is considered in the evaluation of immunity against diseases in fish [56]. The serum complement system contains essential constituents of the innate immune defense and plays an important role in alerting and clearing the pathogens on host [57]. In the present study the PA, RBA, and CC3 level in HK leucocytes did not vary significantly ($P > 0.05$) with any diet on 2nd week. In groups fed with prebiotic or probiotic diets (1 g kg^{-1}) enhancement of immune response was manifested on 6th or 8th week but feeding with symbiotic diet (1 g kg^{-1}) the immune response resulted earlier i.e. 4th week. The present results are in agreement with the observations in *Aanabas testudineus*, *Acipenser stellatus*, *C. carpio*, *O. niloticus*, and *L. rohita* fed with 2–30 g kg^{-1} probiotics and prebiotics diets significantly enhancing PA, RBA, and CC3 level [43,44,58–64]. Nile tilapia fed with 0.2% MOS enriched diet for two weeks significantly elevated the non-specific immune parameters such as PA, RBA, and LA in Nile tilapia [52]. However, in the infected tilapia fed with symbiotic diet at 2 g kg^{-1} (whole yeast and MOS) had higher PA, RNS, ROS, LA, and ACH50 activities [65].

The serum comprise a number of peptides such as lysozymes, antibodies, complement and lytic components which indicate the first line of immune defense that prevent the adherence and colonization of

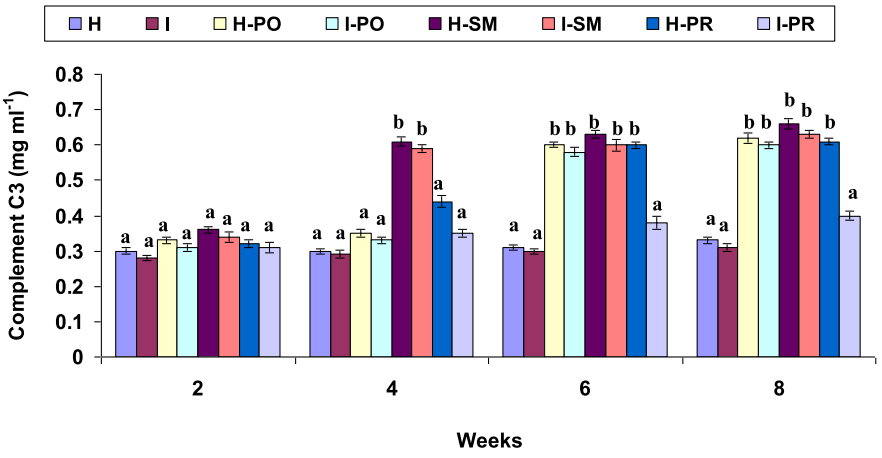

Fig. 4. Serum complement C3 (CC3) level observed in *L. rohita* (n = 6) fed with or without probiotics, prebiotics or symbiotic (1 g kg^{-1}) for 8 weeks. Details of statistical information and experimental groups were seen in Fig. 1.

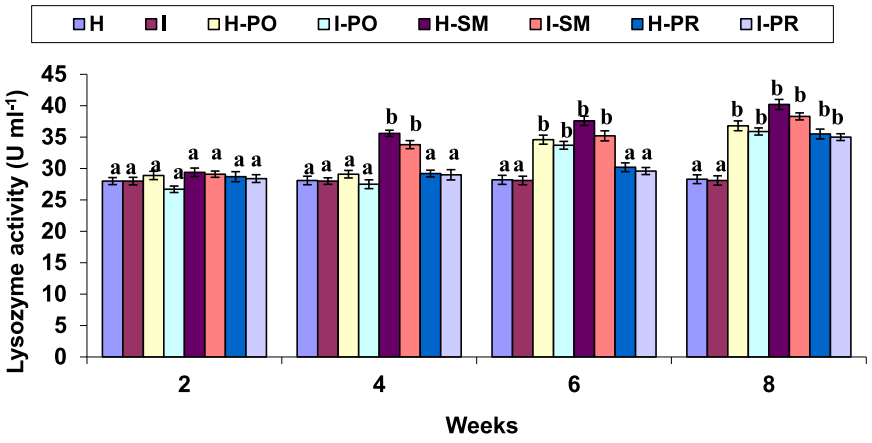

Fig. 5. Serum lysozyme activity (LA) observed in *L. rohita* ($n = 6$) fed with or without probiotics, prebiotics or symbiotic (1 g kg^{-1}) for 8 weeks. Details of statistical information and experimental groups were seen in Fig. 1.

microorganisms [66]. Among the various types of antibody types in fish, the IgM is used to identify and neutralize the pathogenic bacteria and viruses [67]. The serum ACP, LA, and IgM level were not enhanced significantly ($P > 0.05$) with any diet on 2nd week. It was significantly enhanced in groups fed with prebiotic on 8th week, probiotic diet between weeks 6–8 or symbiotic diet from weeks 4–8. Several recent studies report that the dietary supplementation of probiotics, prebiotics or symbiotic diets between 3 and 30 g kg^{-1} enhance ACP, LA, and IgM level as in *A. testudineus*, *Schizothorax zarudnyi*, *A. stellatus*, *Cirrhinus mrigala*, *C. carpio*, *O. niloticus*, *O. mykiss*, *Dicentrarchus labrax*, *L. rohita*, and *Rutilus rutilus* [42,43,46,58–63,68–74], in line with our results. When fed with symbiotics diet with *Entercoccus faecium* and fructooligosaccharide (FOS) fed to the Caspian roach at 2 g kg^{-1} resulted in increased levels of Ig levels, LA, and ACH50 [75]; a similar effect was observed in snow trout resulting in a significantly increased levels of Ig, LA, and ACP in serum when fed with 10–30 g kg^{-1} FOS enriched diet [76]. In Climbing Perch fed prebiotic (FOS) enriched diet at 2% level significantly enhanced the RBA and LA [77]; at 3% level the dietary FOS increased the serum IgM and LA after 3 and 6 weeks of feeding [78]. In line with these findings, the present study clearly shows that the symbiotic diet at 1 g kg^{-1} modulated Ig, LA, and ACP in *L. rohita* against *A. hydrophila*. In Caspian brown trout the synbiotic diet enriched with 2 g kg^{-1} IMOS + 1 g kg^{-1} BetaPlus®) for 7 weeks significantly increased IgM level [47]. *Labeo rohita* fingerlings intraperitoneally injected with 0.1 ml of PBS containing purified *Bacillus licheniformis* (VS16) biosurfactant at 55–330 µg ml^{-1} significantly enhanced PA, LA, and IgM level against *A. hydrophila* [79].

Fish have a complex system of numerous antioxidants including SOD, GPx, and CAT and important biochemical parameters as the first line of antioxidant enzymatic defense. The measurement of SOD, GPx, and CAT antioxidant may provide a hint of the antioxidant status and is considered as biomarkers for the oxidative stress in fish [13,80]. The SOD, CAT, and GPx activities observed in healthy and infected groups fed with probiotics diet were significant ($P < 0.05$) on weeks 6 and 8 and with symbiotic diet between weeks 4–8. A similar antioxidant potential was in *O. niloticus*, *O. mykiss*, *D. labrax*, *C. carpio*, and *L. rohita* fed with probiotics, prebiotics or symbiotic supplementation diets in various concentration [10,13,60,63,68,72,81,82]. Our results reveal that the symbiotic supplementation diet could significantly activate the antioxidant activity in earlier stages against pathogen, which can efficiently eliminate excess free radicals and regulate the balance of free radical in the body, resulting in improved antioxidant potential [13]. The lower MDA content observed in the present study indicates a strengthened higher antioxidant potential of probiotics, prebiotics or symbiotic, because MDA has strong bio-toxicity and can damage cell structure and function [83]. This study confirms that the symbiotic supplementation diet at low level i.e. 1 g kg^{-1} significantly enhanced antioxidant activity and modulate innate-adaptive immune response as early as 4th week than that of probiotics or prebiotics. A recent study, reports that in rainbow trout a combination or symbiotic of GOS (1%) and *P. acidilactici* (7.57 log CFU g^{-1}) has resulted in a high antioxidant capacity against *Streptococcus iniae* [84]. Nile tilapia when fed with FOS enriched diet at a concentration of 20 g kg^{-1}, had a significant decrease in MDA, SOD, and GPx [54]. However, the present study could not find

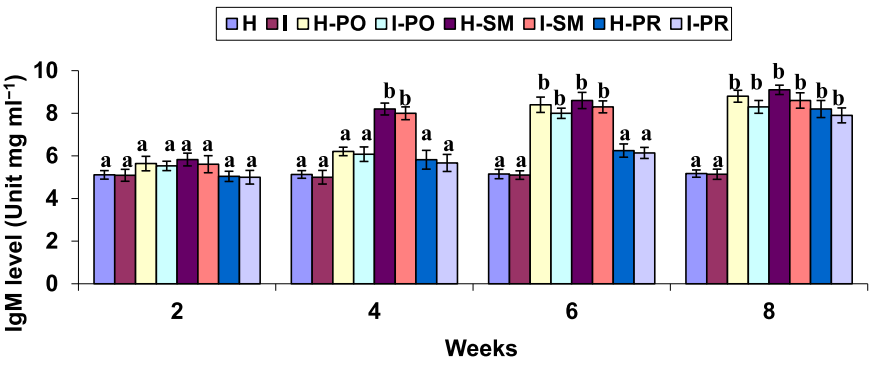

Fig. 6. Immunoglobulin (IgM) level observed in *L. rohita* ($n = 6$) fed with or without probiotics, prebiotics or symbiotic (1 g kg^{-1}) for 8 weeks. Details of statistical information and experimental groups were seen in Fig. 1.

Table 4
Antioxidant activity of probiotics, prebiotics or symbiotic enriched diets in *L. rohita* against *A. hydrophila* for 8 weeks.

Indices	Weeks	H	I	H-PO	I-PO	H-SM	I-SM	H-PR	I-PR
SOD activity (unit ml^{-1})	2	42.8 ± 2.21^a	42.1 ± 1.76^a	43.1 ± 1.86^a	42.8 ± 1.90^a	43.8 ± 1.88^a	43.5 ± 1.72^a	43.0 ± 1.62^a	42.5 ± 1.73^a
	4	43.2 ± 2.12^a	42.3 ± 1.96^a	43.6 ± 2.34^a	42.9 ± 1.66^a	47.3 ± 2.36^b	46.3 ± 2.04^b	43.5 ± 1.92^a	42.9 ± 1.93^a
	6	43.8 ± 1.82^a	42.5 ± 2.06^a	46.9 ± 2.18^b	45.3 ± 2.21^b	49.8 ± 2.52^b	48.4 ± 1.87^b	43.8 ± 2.37^a	43.3 ± 2.09^a
	8	44.0 ± 2.81^a	42.8 ± 1.61^a	49.5 ± 2.44^b	47.5 ± 2.37^b	53.1 ± 2.43^b	51.5 ± 2.37^b	46.9 ± 2.46^b	45.3 ± 2.27^b
MDA activity (mmol mg^{-1})	2	9.11 ± 0.32^a	9.34 ± 0.38^a	9.06 ± 0.26^a	9.23 ± 0.36^a	9.03 ± 0.27^a	9.16 ± 0.31^a	9.07 ± 0.34^a	9.13 ± 0.44^a
	4	9.02 ± 0.28^a	9.67 ± 0.34^a	9.02 ± 0.30^a	9.18 ± 0.28^a	8.88 ± 0.33^a	8.96 ± 0.38^a	8.87 ± 0.39^a	8.96 ± 0.35^a
	6	8.94 ± 0.34^a	11.2 ± 0.38^b	8.86 ± 0.36^a	8.96 ± 0.37^a	8.73 ± 0.36^a	8.63 ± 0.35^a	8.71 ± 0.25^a	8.85 ± 0.33^a
	8	8.86 ± 0.30^a	11.8 ± 0.41^b	8.72 ± 0.38^a	8.89 ± 0.34^a	8.35 ± 0.25^a	8.58 ± 0.39^a	8.63 ± 0.29^a	8.74 ± 0.36^a
CAT activity (unit ml^{-1})	2	13.7 ± 0.58^a	13.1 ± 0.45^a	14.5 ± 0.53^a	14.1 ± 0.43^a	14.9 ± 0.47^a	14.2 ± 0.52^a	14.7 ± 0.40^a	14.4 ± 0.51^a
	4	14.1 ± 0.46^a	13.6 ± 0.48^a	14.9 ± 0.64^a	14.5 ± 0.60^a	16.3 ± 0.57^b	16.0 ± 0.50^b	15.1 ± 0.41^a	14.8 ± 0.42^a
	6	14.7 ± 0.51^a	13.9 ± 0.53^a	16.1 ± 0.65^b	16.0 ± 0.68^b	17.1 ± 0.74^b	16.7 ± 0.43^b	15.8 ± 0.52^a	15.1 ± 0.51^a
	8	15.0 ± 0.49^a	14.3 ± 0.46^a	16.8 ± 0.55^b	16.2 ± 0.53^a	17.8 ± 0.55^b	17.0 ± 0.65^b	16.5 ± 0.68^b	16.2 ± 0.55^b
GPx activity (unit mg^{-1}) protein	2	15.2 ± 0.71^a	14.9 ± 0.63^a	15.9 ± 0.83^a	15.2 ± 0.57^a	16.7 ± 0.76^a	16.2 ± 0.61^a	16.3 ± 0.65^a	16.0 ± 0.82^a
	4	15.8 ± 0.65^a	15.3 ± 0.55^a	16.5 ± 0.83^a	15.8 ± 0.60^a	18.7 ± 0.61^b	16.9 ± 0.53^b	16.6 ± 0.55^a	16.4 ± 0.63^a
	6	16.2 ± 0.76^a	15.8 ± 0.60^a	18.4 ± 0.87^b	16.2 ± 0.65^a	20.5 ± 0.83^b	17.3 ± 0.73^a	17.0 ± 0.73^a	16.9 ± 0.69^a
	8	16.5 ± 0.58^a	16.0 ± 0.67^a	19.4 ± 0.61^b	16.8 ± 0.73^a	22.3 ± 0.67^b	19.8 ± 0.85^b	17.5 ± 0.88^a	17.3 ± 0.59^a

SOD: superoxide dismutase, MDA: malondialdehyde, CAT: catalase, GPx: glutathione peroxidase.

any significant effect on liver MDA level with any of the enriched diets.

There was no mortality in healthy fish fed with any supplementation diet while it was 5%, 10, and 10% in the infected group fed with symbiotic, probiotics, and prebiotics supplementation diets for 30 days. A number of studies indicate that 2–30 g kg^{-1} probiotics, prebiotics or symbiotic supplementation diets produced lower CV in *C. mrigala*, *O. mykiss*, and *L. rohita* against pathogens [10,46,62,64]. In Nile tilapia fed basal diet supplemented with 20 g kg^{-1} FOS supplemented diet had higher survival after being challenged with *A. hydrophila* [54]. Recently, in Caspian brown trout and Caspian salmon also the synbiotic diet is reported to enhance the survival rate [47,85]. In the present study no mortality was observed when healthy fed with 1 g kg^{-1} prebiotics, probiotics, and symbiotic supplementation diets. However, *A. hydrophila* challenged groups fed with 1 g kg^{-1} prebiotics, probiotics, and symbiotic supplementation diets suffered 10%, 10%, and 5% mortality against *A. hydrophila*. A detailed comparative study is required with various levels of prebiotics, probiotics, and symbiotic supplementation diets in other fish against pathogens.

Generally the expression levels of IL-β, IL-8, and TNF-α were considered as an indicator of inflammatory immune function [86] which regulates the production of other cytokine secretion [87]. However, co-expression of IL-1β IL-8, and TNF-α is not unusual but it share similar function in the initiation of immune response [88]. The present study indicate that IL-1β IL-8, and TNF-α gene transcription level in HK leucocytes are significantly up-regulated in the healthy or infected groups fed with symbiotic diet from weeks 4–8; however, transcription level of IL-1β IL-8, and TNF-α genes were observed on or after 6th or 8th week. These results are in agreement with previous studies in *L. rohita* against *A. hydrophila* [64,89–91]. Similarly, the dietary administration of *R. glutinosa* increased the IL-1β and TNF-α gene expression in *C. carpio* against *A. hydrophila* [87]. IL-1β and TNF-α gene expression was high in Nile tilapia fed at 0.2% MOS after 2 weeks [52]. However, another recent study reports that the TNF-α gene expression was high in the infected *Oreochromis niloticus* fed with synbiotic diet containing probiotic (whole yeast cell) and prebiotic (MOS) enriched diet at 2 g kg^{-1} [65]. *Labeo rohita* fingerlings injected intraperitoneally with 0.1 ml of PBS containing purified *Bacillus licheniformis* (VS16) biosurfactant at 55–330 μg ml^{-1} concentrations had significantly enhanced IL-1β and TNF-α gene expression against *A. hydrophila* [92].

The iNOS and NF-κB gene expression in HK leucocytes were not modulated significantly on 2nd week, whereas in healthy or infected groups fed with prebiotic diet there was a significant modulation on 8th week, with probiotic diet between weeks 6 and 8, and symbiotic diet from weeks 4–8. Surprisingly, the iNOS and NF-κB expression in infected group fed with symbiotic diet can modulate significantly earlier at 4th week. The iNOS and NF-κB are important immune-regulatory factors in the fish defense against various pathogens [89–91]. In contrast, *C. carpio* fed with *R. glutinosa* supplementation diet had resulted in

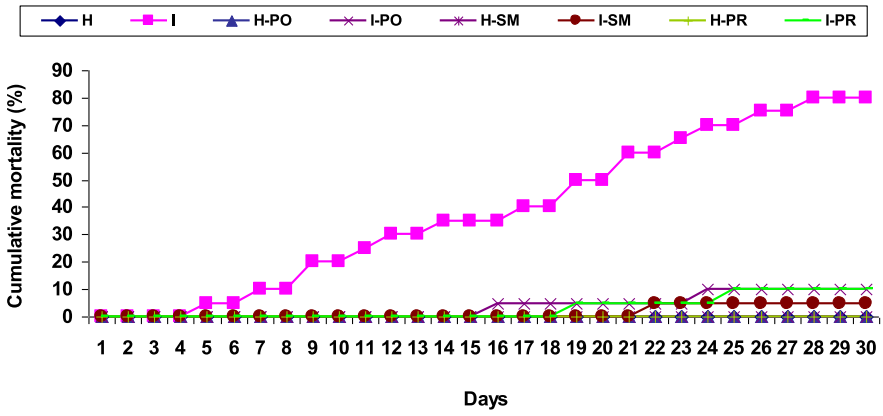

Fig. 7. Cumulative mortality (CM) observed in *L. rohita* (n = 6) fed with or without probiotics, prebiotics or symbiotic (1 g kg^{-1}) for 30 days. Details of experimental groups were seen in Fig. 1.

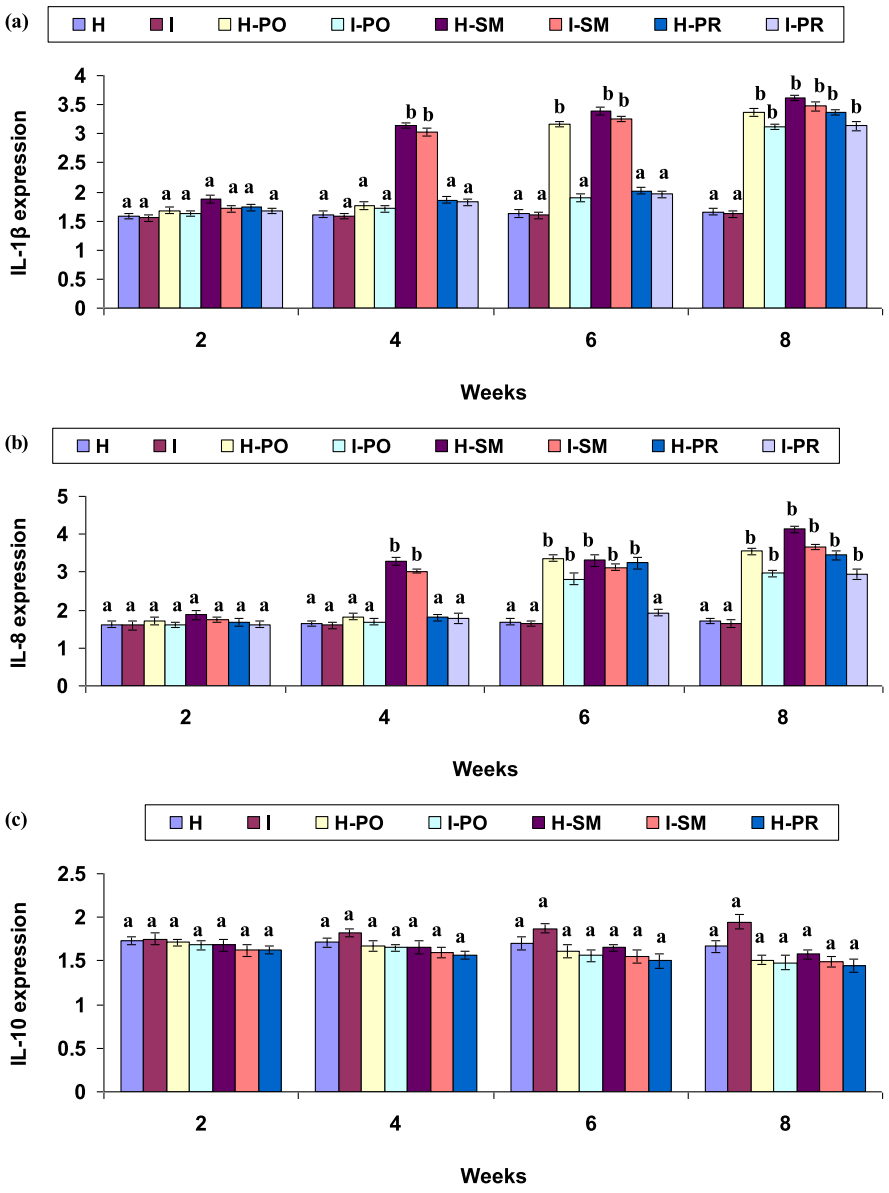

Fig. 8. The relative mRNA expressions of (a) IL-1*β*, (b) IL-8, and (c) IL-10 genes in HK leucocytes of healthy and infected *L. rohita* fed with or without probiotics, prebiotics or symbiotic (1 g kg^{-1}) for 8 weeks. Details of statistical information and experimental groups were seen in Fig. 1.

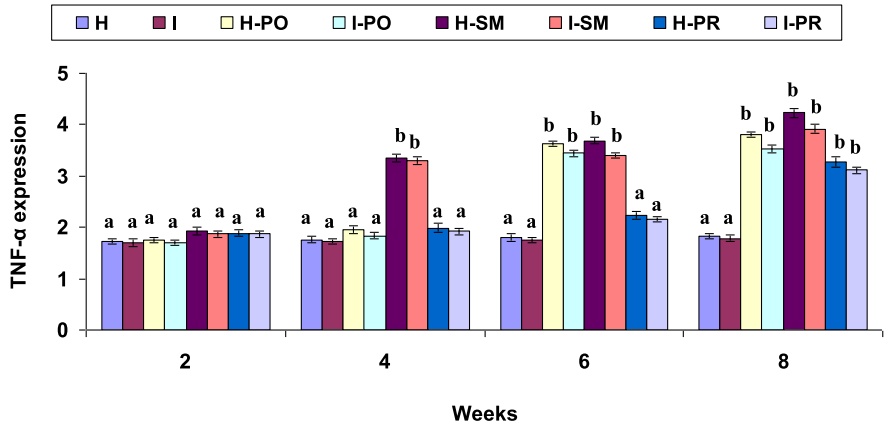

Fig. 9. The relative mRNA expressions of TNF-α gene in HK leucocytes of healthy and infected *L. rohita* fed with or without probiotics, prebiotics or symbiotic (1 g kg^{-1}) for 8 weeks. Details of statistical information and experimental groups were seen in Fig. 1.

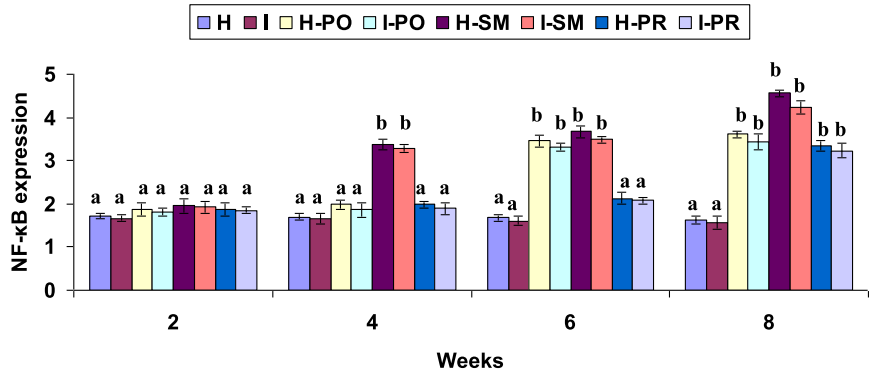

Fig. 10. The relative mRNA expressions of NF-κB gene in HK leucocytes of healthy and infected *L. rohita* fed with or without probiotics, prebiotics or symbiotic (1 g kg^{-1}) for 8 weeks. Details of statistical information and experimental groups were seen in Fig. 1.

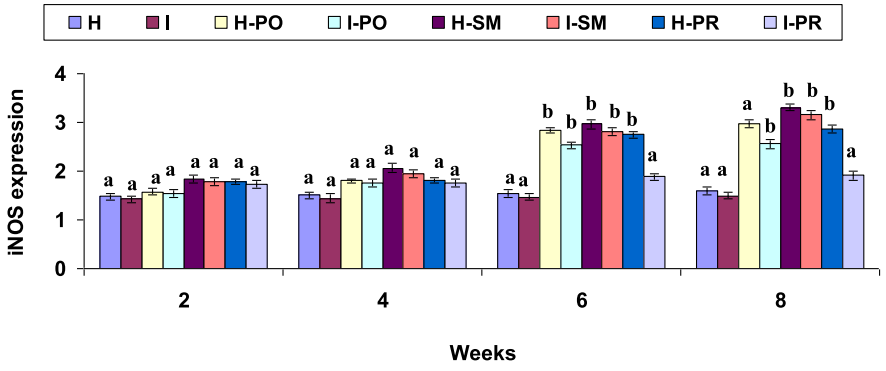

Fig. 11. The relative mRNA expressions of iNOS gene in HK leucocytes of healthy and infected *L. rohita* fed with or without probiotics, prebiotics or symbiotic (1 g kg^{-1}) for 8 weeks. Details of statistical information and experimental groups were seen in Fig. 1.

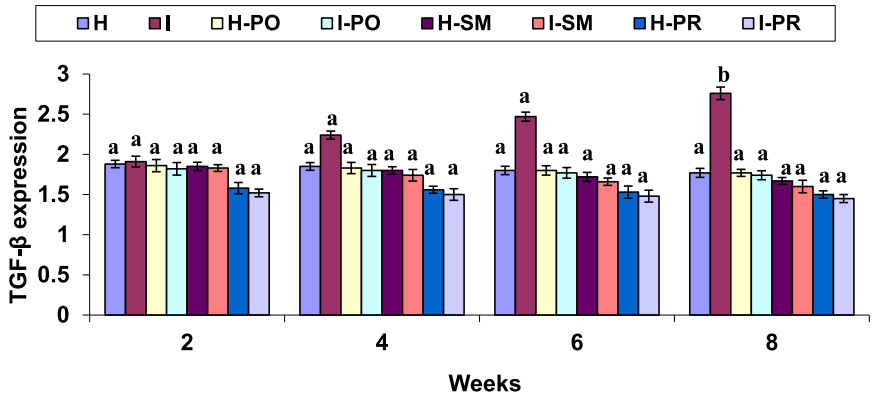

Fig. 12. The relative mRNA expressions of TGF-β gene in HK leucocytes of healthy and infected *L. rohita* fed with or without probiotics, prebiotics or symbiotic (1 g kg^{-1}) for 8 weeks. Details of statistical information and experimental groups were seen in Fig. 1.

a significant increase in iNOS gene expression in HK leucocytes [87]. The mechanism by which probiotics, prebiotics or symbiotic diets in not up-regulating iNOS or NF-κB expression between weeks 2 and 4 is yet to be elucidated.

The IL-10 and TGF-β are important regulatory cytokines with multifunctional roles in the immune system. IL-10 limits the magnitude of immune responses to foreign pathogens [90]. The relative IL-10 and TGF-β mRNA expression decreased with any diet during the experiment. These results correspond with the earlier studies in *C. carpio* and *L. rohita* against *A. hydrophila* [87,89–91]. Swain et al. [92] reported that the mechanism of IL-10 induction could be by blocking NF-κB signaling in *Catla catla*. Generally, TGF-β inhibits B- and T-cell propagation and differentiation, antagonises pro-inflammatory cytokines (IL-1 β, TNF-α, and IFN-γ), and blocks expressions of IL-1 β and IL-12 receptors [93]. This study confirms that symbiotic supplementation at low level i.e. 1 g kg^{-1} significantly enhanced antioxidant activity, modulate innate-adaptive immune response, and the immunological parameters and pro and/or anti-inflammatory cytokine gene transcription in earlier i.e. 4th week than that of probiotics or prebiotics. Further detailed study is required on the effect of symbiotic

supplementation on antioxidant capacity, innate-adaptive immune response, and pro and/or anti-inflammatory cytokine gene transcription level in other fish in different concentration of probiotics, prebiotics and symbiotics against pathogens.

5. Conclusion

The present study reveals that dietary administration of 1 g kg^{-1} symbiotic supplementation provide better antioxidant activity, innate-adaptive immune response, decreased mortality, and modulated immune related cytokine gene expression earlier i.e. on 4th week in *L. rohita* against *A. hydrophila*. The healthy and infected groups administrated with probiotic diet enhancement were noted only on 6th week, but the prebiotic diet it was noted only on 8th week. The present study indicates that the symbiotic diet could result in better antioxidant, innate-adaptive immunity, and higher modulation of immune gene expression when compared to probiotic and prebiotic diets and hence can be a better feed additive in aquaculture to improve production, growth, disease protection. Further, the ingredients are inexpensive and easily available anywhere which may represent a viable alternative to other prophylactics such as antibiotics, chemical disinfection, and vaccine. A detailed study is needed in different level of prebiotic, probiotic or symbiotic on immune related gene performance on the antioxidant, immune function, and immune aspects with reference to the mechanisms underlying symbiotic diet in other fish against various pathogens.

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