Fish and Shellfish Immunology 91 (2019) 358–368

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Effect of dietary synbiotic supplementation on growth, immune and physiological status of *Labeo rohita* juveniles exposed to low pH stress

Soibam Khogen Singh[a,b,*], V.K. Tiwari[a], N.K. Chadha[a], Sukham Munilkumar[c], Chandra Prakash[a], Nilesh A. Pawar[a]

[a] *Aquaculture Division, ICAR-Central Institute of Fisheries Education, Mumbai-400061, Maharashtra, India*
[b] *Department of Aquaculture, College of Fisheries, Central Agricultural University (Imphal), Lembucherra, Tripura West-799210, India*
[c] *ICAR-Central Institute of Fisheries Education, Kolkata Centre, Salt Lake, Kolkata, 70091, West Bengal, India*

ARTICLE INFO

Keywords:
Bacillus circulans PB7
Fructooligosaccharide
Synbiotic
Antioxidant
pH stress
Stress biomarkers

ABSTRACT

In this study, we investigated the effect of dietary *Bacillus circulans* PB7 (BCPB7) and fructoligosaccharide (FOS), used singly or in combination for evaluation of growth, immune and physiological status of *Labeo rohita* (rohu) juveniles reared under low pH and normal pH for 60 days. Experimental fishes were distributed in two sets such as one set continuously exposed to low pH (5.5) and other reared under normal pH (7.0), and fed with four iso-nitrogenous diets viz. basal (control), *Bacillus circulans* PB7 (BCPB7, 10^6 cfug^{-1}), 1% fructooligosaccharide (FOS) and their combination. The effect of such pre, pro and synbiotics dietary treatments on growth performance (weight gain, specific growth rate, feed conversion ratio and protein efficiency ratio), immune response (hematological indices, serum biochemistry, lysozyme, NBT activity), antioxidative status in the form of antioxidant enzyme (catalase, superoxide dismutase, glutathione-S-transferase), acetylcholine esterase (AChE), Na^+ K^+ ATPase and stress bio-markers (cortisol, glucose and HSP-70) were examined. The group treated with low pH and fed with control diet (without supplementation) was found to be inhibited ($p < 0.05$) in growth and immuno-physiological function. However, supplementation of BCPB7 and FOS was non-significant ($p < 0.05$) on growth performance and physiological process but their concurrent feeding remarkably improved ($p < 0.05$) growth and immune-physiological function when exposed to low pH. Overall results indicate that dietary combination of BCPB7 and FOS can be considered an effective synbiotic formula against low pH stress in culture practices of *L. rohita* juveniles.

1. Introduction

Global aquaculture industry has witnessed progressive growth over the years, however, at the expense of diseases emergence and environmental deterioration. The growth in human population demands for rapid fish production through culture system intensification. In due course of time, abrupt changes in pH levels of water, on account of excessive use of supplementary feed, manures and inorganic fertilisers has caused severe stress to the cultured fishes, leading to stressful situations in fishes. In addition, many geographical regions, especially the north-eastern corners of India are facing severe problems relating to low water pH, which has impaired further growth in aquaculture [1]. In freshwater systems, a pH level of 7.0–8.5 is optimal for culture of commercial fish species including carps [2,3]. It is proven that fishes live in a very narrow range of pH near neutrality, and short and long term exposure to acidic water impairs many physiological functions [4,5]. At a low pH, a rapid increase in H^+ causes toxic conditions and disturbs the ion regulatory mechanism by ways of inhibiting ionic (Ca^{2+} and Na^+) influx, and further loss through the gills [6]. In addition, under such prolonged acidic stress, blood pH decreases because of flux of H^+ ions across gill membranes into the blood [7]. The aquatic ecosystem is totally unpredictable and unable to adjust sudden changes in water quality parameters such as pH and other factors. The change in pH cause either acute or chronic stress to aquatic organisms. Sometime, fishes may adapt the changes in long term but only to some extent [8]. The change in pH of more than 1.5 points below or above recommended levels always have a negative effect over time and should never be considered acceptable [9]. Young fish and immature stages of aquatic insects are extremely sensitive to pH levels below 5 and may die at these low pH values. High pH levels (9–14) can harm fish by denaturing cellular membranes.

Labeo rohita (commonly known as rohu) is the most preferred

* Corresponding author. Department of Aquaculture, College of Fisheries, Central Agricultural University (Imphal), Lembucherra, Tripura West-799210, India.
E-mail address: gengang@gmail.com (S.K. Singh).

https://doi.org/10.1016/j.fsi.2019.05.023
Received 21 January 2019; Received in revised form 4 May 2019; Accepted 10 May 2019
Available online 11 May 2019
1050-4648/

freshwater fish and contributes hugely to Indian aquaculture industry. Fishes respond to the varied environmental stressors through a chain of adaptive biochemical and physiological alterations to overcome the stressful situations encountered [10,11]. Among the several indicators of stress in aquatic animals, plasma cortisol, which is released as the main metabolites during stressful condition, is regarded as the most established parameter [12,13]. In addition, blood glucose, which is released further with an elevated plasma cortisol to cope up with the energy demands in tissues during stress, serves as another viable indicator [14]. Stress usually causes a reduction in the immune system of animals which is related to decrease in anabolic processes under elevated cortisol levels [15]. Thus, various immune related blood parameters can possibly indicate the level of stress in the fishes. Several environmental related stressors including acid stress also trigger a rapid increase in the release of reactive oxygen species (ROS) in aquatic animals [16]. Thus, antioxidant enzymes (catalase, superoxide dismutase, glutathione –S – transferase) can also help us determine the level of stress encountered in aquatic animals. In addition, HSP70 remains as a sensitive biomarkers, and their levels are elevated upon exposure to environmental contamination [17].

Stress that the animal encounter has a strong relation to the immune status and strategies to ameliorate it through nutritional approach is quite promising. Hence, we used probiotics, prebiotic and synbiotics for the mitigation of pH stress in fish. Probiotics are live microbial feed supplement such as *Bacillus, Lactobacillus*, and *Pediococcus* which beneficially affects the host animal by improving its microbial balance [18] and has proved as a major discovery towards disease resistance in fishes for a long time [19]. The probiotics strain, *Bacillus circulans* PB7 is reported to play a beneficial role on growth and immune status of fishes [20]. On the other hand, prebiotics which are indigestible substances that allow specific changes in the composition and/or activity of gastrointestinal microbiota has a positive effect on the nutrition and health status of the host [21]. Among the several prebiotics evaluated in fishes, the functional roles of fructooligosaccharide (FOS) are very well established. For quite a few years, work on synbiotic formulations (probiotics and prebiotics in synergism) is also intensively undertaken but focussed only on few selected species which needs to be evaluated for other fishes.

Practically, control over the abrupt change of water pH is difficult and expensive, especially when fish farming is practiced in larger water area and therefore, nutritional approach using bio-therapeutic agents such as synbiotics would be a practical way to improve fish health under stressful conditions. To the best of our knowledge, there is no work reported pertaining to synbiotic application for acidic stress amelioration in carps. Therefore, we tried to delineate immune enhancing and stress mitigating potential of synbiotics in the present work through evaluation of growth, immune and physiological status of *L. rohita* juveniles exposed to low pH water.

2. Materials and methods

2.1. Ethics statement

The use of experimental animals conforms to the existing laws/acts in India. The care and treatment of the fish used in the research work were in accordance with the guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA), Ministry of Environment & Forests (Animal Welfare Division), Government of India on the care and use of animals in scientific research. The study protocol and experimental endpoints were also approved by the research advisory committee of the ICAR-Central Institute of Fisheries Education, Mumbai, India.

2.2. Experimental fish and maintenance

Healthy *L. rohita* (Avg. weight = 11.50 ± 0.05 g) were procured and transported from a commercial fish farm (Prem Fisheries Consultancy, Gujarat, India). Fish was treated with prophylactic in salt solution and potassium permanganate and acclimatized to the laboratory condition for 15 days and were fed *ad libitum* daily with a commercially available feed (Godrej Agrovet Pvt. Ltd., Andhra Pradesh, India). Rectangular fibre reinforced plastic (FRP) tubs (water capacity 75 L) available at Aquaculture Research Facility of ICAR-CIFE, Mumbai, India were used for experimental tanks. Out of the procured stock, 288 uniformly sized fingerlings were randomly distributed in eight treatment groups operated in triplicates (12 fish per tank) following a completely randomized design (CRD). All the experimental tanks were well aerated using a centralized air blower and manual water exchange to almost 75% of volume was done every three days interval for 60 days.

2.3. Experimental design and trial

The experimental design comprises of two groups, one with normal water pH of 7.0 and other with a pH of 5.5. For preparation of acidic water, stock solution of glacial acetic acid was used after standardization following methods described by Das et al. [5]. Water pH was monitored using a digital pH meter (Elico LI-120). Prior to preparation, the chloride level of the source water was checked to avoid presence of chloride. Further, these groups are fed with diets supplemented with either probiotic {*B. circulans* PB7, MTCC no. 4998, lyophilized cells, Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India}, prebiotic (FOS, supply by DPO Foods Specialities Private Limited, Thane, Mumbai, India) alone and their combination. Thus, we used a total of eight distinct groups as follows:

Group 1: Normal pH and fed with the control diet (Normal pH/C diet)
Group 2: Low pH exposure and fed control diet (Low pH/C diet)
Group 3: Normal pH and fed FOS diet (Normal pH/Pre diet)
Group 4: Low pH exposure and fed FOS diet (Low pH/Pre diet)
Group 5: Normal pH and fed *B. circulans* PB7 diet (Normal pH/Pro diet)
Group 6: Low pH exposure and fed *B. circulans* PB7 diet (Low pH/Pro diet)
Group 7: Normal pH and fed *B. circulans* PB7 and FOS diet (Normal pH/Syn diet)
Group 8: low pH exposure and fed *B. circulans* PB7 and FOS diet (Low pH/Syn diet).

2.4. Diet preparation

The basal diet used in our study was formulated and approximately contain 30% crude protein, which is optimal for grow-out rearing of carps (Table 1). The required ingredients were thoroughly mixed with water and oil to prepare dough which follows cooking in an autoclave (15 psi for 20 min). After cooling, required vitamins and minerals were added. Further, the basal diet was supplemented with 1% FOS by manual mixing. The oil (cod liver oil and sunflower oil) was added gradually to assure the homogeneity of the mixture. Finally, the dough was made to pass through a hand pelletizer to obtain uniform pellet size of 2 mm and air dried for 4 h. Then, probiotic was sprayed over the feed at concentrations of 1×10^6 cfu/g, based on our previous trial using two doses of the supplements [22]. The pellets were dried under aseptic conditions for 24 h and stored at 4 °C. Following storage at 4 °C, viable count of *B. circulans* PB7 in the feed was accessed for four weeks following standard methods [23].

2.5. Experimental feeding

Fish were fed test feed twice daily (09:00 and 17:00 h) at 3–5% of their total biomass for 60 days. Fishes were weighed every 15 days in

Table 1
Ingredient composition of experimental diets supplemented with *B. circulans* PB7 and fructooligosaccharide (FOS) and fed to *L. rohita* juveniles for 60 days.

Ingredients	Control	Prebiotic	Probiotics	Synbiotic
Casein	42	42	42	42
Dextrin	15	15	15	15
Cellulose	10	9	10	9
Starch soluble	10	10	10	10
Gelatin	8	8	8	8
Cod liver oil	5	5	5	5
Sunflower oil	3	3	3	3
CMC	2	2	2	2
Mineral Premix[a]	2	2	2	2
Vitamin Premix[b]	2	2	2	2
BHC	1	1	1	1
Prebiotic[c]	0.00	1.00	0.00	1.00
Probiotic	0.00	0.00	1×10^6	1×10^6
Total	100	100	100	100
BHT	0.01	0.01	0.01	0.01
Attractant[d]	0.25	0.25	0.25	0.25

[a] Mineral premix (%): $KAl(SO4)_2$, 0.159; CaCO3, 18.101; $Ca(H_2PO_4)_2$, 44.601; $MgSO_4$, 5.216; $CoCl_2$, 0.07; KCl, 16.553; ferric citrate ($5H_2O$), 1.338; sodium selenite, 0.004; $MnSO_4$.H2O, 0.07; KI, 0.014; $ZnSO_4$, 0.192; NaH_2PO_4, 13.605; $CuSO_4.5H_2O$, 0.075.
[b] Vitamin premix: thiamine hydrochloride, 10 mg kg^{-1}; riboflavin, 20 mg kg^{-1}; calcium pantothenate, 40 mg kg^{-1}; nicotinic acid, 50 mg kg^{-1}; pyridoxine hydrochloride, 10 mg kg^{-1}; folic acid, 5 mg kg^{-1}; inositol, 400 mg kg^{-1}; choline chloride, 2000 mg kg^{-1}; menadione, 10 mg kg^{-1}; cholecalciferol, 1500 IU; biotin, 1 mg kg^{-1}; vitamin B12, 0.02 mg kg^{-1}; vitamin A, 3000 IU; vitamin E, 50 IU; vitamin C, 200 mg kg^{-1},[c] FOS from DPO Foods, Thane, Mumbai, India,[d] Attractant: glycine and betaine.

order to adjust the feeding rate. Excess feed was siphoned after each feeding. During the experiment, optimum water quality was maintained except for the intended pH values of 5.5 and 7.0 in the designated treatments. Recorded values as measured using standard methods described by APHA [24]. The water quality parameters such as temperature (25.0 ± 0.1 °C), dissolved oxygen (5.8–6.9 mg/l), free carbon dioxide (1.9–2.7 mg/l), total hardness (156–185 mg/l), ammonia-N (0.14–0.37 mg/l), nitrite-N (0.06 ± 0.13 mg/l) and nitrate-N (0.03 ± 0.14 mg/l) were recorded.

2.6. Growth performance study

The growth performance of *L. rohita* in terms of weight gain, specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER) evaluated at the end of 60 days experimental trial were recorded using the following formulae:

Weight gain = Final body weight (FBW) - Initial body weight (IBW)

SGR = 100 (*ln* FBW - *ln* IBW)/ number of days

FCR = Total dry feed intake (g)/wet weight gain (g)

PER = Total wet weight gain (g)/crude protein intake (g)

2.7. Blood collection

After 60 days feeding trial, three fishes ($n = 3$) were randomly collected from each treatment tanks and anesthetized using clove oil (50 µg/L). Blood samples were carefully drawn from the caudal peduncle using 1 mL hypodermal medical syringe (24 guage needles) previously rinsed in a 2.7% EDTA solution. Pooled blood samples were transferred immediately to a test tube thinly coated with small amount of EDTA powder (as an anticoagulant) and shaken gently to prevent haemolysis of blood cells. For serum assays, blood was collected in eppendorf tubes, but without anticoagulant and allowed to clot for 2 h and further centrifuged at 3000 *g* for 15 min. The supernatant serum was collected and stored at −20 °C deep freezer until use.

2.8. Hematological parameters

RBC and WBC diluting fluids (Toission's, Turk's solution) were used for determining total erythrocyte and leukocyte counts (TEC, TLC) respectively. Blood (20 µl) was mixed with 3980 µl of corresponding diluting fluid (Qualigens Diagnostics, Mumbai, India) in a clean test tube and shaken well to suspend the cells uniformly in the solution. Then, the cells were counted using a haemocytometer. The following formula was used to calculate the number of erythrocytes and leucocytes per ml of the blood sample:

$$\text{Number of cells ml}^{-1}\,(10^3\ \text{cell/mm}^{-3}) = \frac{[\text{Number of cells counted} \times \text{dilution}]}{[\text{Area counted} \times \text{depth of fluid}]}$$

The haemoglobin level of was analyzed by estimating cyanmethaemoglobin using Drabkins fluid (Qualigens Diagnostics, Mumbai, India). 5 ml of Drabkin's working solution was taken in a clean, dry test tube and added with 20 µl of blood. The absorbance was measured using a spectrophotometer (540 nm wavelength) and the final concentration calculated by comparing with the standard cyanmethaemoglobin (Qualigens Diagnostics). The haemoglobin concentration was then calculated by using the following formula:

$$\text{Haemoglobin (g/dl)} = [\text{OD (T)} / \text{OD (S)}] \times [251 / 1000] \times 60$$

where, OD (T) represents absorbance of the samples; OD (S) the absorbance of standard.

For haematocrit determination, the haematocrit capillary tubes were two-third filled with the blood and centrifuged in a haematocrit centrifuge for 5 min. The percentage of the packed cell-volume was determined by the haematocrit tube reader.

2.9. Blood glucose

Blood glucose was estimated as described by Nelson [25] and Somoyogi [26] and expressed as mg dl^{-1}. Blood was deproteinized with zinc sulphate and barium hydroxide, filtered and then the supernatant was used for glucose estimation. The absorbance was recorded at 540 nm against the blank.

2.10. Respiratory burst activity

The respiratory burst activity of the phagocytes was calculated by Nitro blue tetrazolium (NBT) assay following the method of Secombes [27] subsequently modified by Stasiack and Bauman [28]. The OD of the tursique blue coloured solution was then read using ELISA plate reader. Blood (50 µl) was placed into the wells of 'U' bottomed microtitre plates and incubated (37 °C for 1 h) to allow cell adhesion. Then, the supernatant was removed and wells washed thrice in PBS solution. After washing, 50 µl of 0.2% NBT was added and incubated for another 1 h. The cells are fixed with 100% methanol for 2–3 min and washed thrice with 30% methanol. The plates were air dried and 60 µl 2 N potassium hydroxide and 70 µl dimethyl sulphoxide were added to each well. The OD was recorded at 540 nm in an ELISA plate reader.

2.11. Lysozyme activity

Lysozyme activity of serum was done using ion-exchange chromatography kit (Bangalore Genei, India). Serum samples were diluted with phosphate buffer (pH 7.4) to 1:3 dilution (v/v). 3 ml of *Micrococcus luteus* (Bangalore Genei, India) suspension in phosphate buffer (A_{450} = 0.5–0.7) was taken in a suitable cuvette, to which 50 µl of diluted serum sample was added. The content of the cuvette was further

Table 2
Growth and feed efficiency parameters of *L. rohita* juveniles fed diets supplemented with *B. circulans* PB7 and FOS and exposed to low pH.

Treatment	Weight gain (g)	SGR[a] (%/day)	FCR[b]	PER[c]
Normal pH/C diet	5.324 ± 0.21[b]	0.68 ± 0.02[a]	3.90 ± 0.08[b]	1.05 ± 0.02[b]
Low pH/C diet	3.536 ± 0.03[a]	0.58 ± 0.01[a]	4.40 ± 0.04[c]	0.76 ± 0.00[a]
Normal pH/Pre diet	7.980 ± 0.12[c]	0.86 ± 0.03[b]	2.66 ± 0.07[a]	1.24 ± 0.06[cd]
Low pH/Pre diet	5.410 ± 0.11[b]	0.66 ± 0.01[a]	3.05 ± 0.06[b]	1.15 ± 0.02[b]
Normal pH/Pro diet	8.350 ± 0.08[d]	0.91 ± 0.01[b]	2.43 ± 0.05[a]	1.36 ± 0.03[d]
Low pH/Pro diet	5.776 ± 0.05[b]	0.72 ± 0.01[ab]	2.82 ± 0.02[a]	1.24 ± 0.01[d]
Normal pH/Syn diet	8.820 ± 0.40[cd]	0.94 ± 0.02[b]	2.36 ± 0.09[a]	1.43 ± 0.06[d]
Low pH/Syn diet	7.093 ± 0.43[c]	0.83 ± 0.01[b]	2.78 ± 0.05[a]	1.22 ± 0.02[cd]

[1] Data are means of triplicate. Means in the same column sharing a same superscript are not significantly different (p > 0.05) n = 12.
[a] SGR = Specific growth rate.
[b] FCR = Feed conversion ratio.
[c] PER = Protein efficiency ratio.

mixed well for 15 s and reading recorded at 450 nm using a spectrophotometer exactly after 60 s of addition of serum sample. This absorbance was compared with standard lysozyme of known activity following the same procedure as above. The lysozyme activity was expressed as U $min^{-1}mg^{-1}$ protein of serum.

2.12. Serum biochemical indices

Serum total protein (TP) was estimated by the Biuret and BCG dye binding method [29] using commercial kit (Total protein, Qualigens Diagnostics Ltd., India). Albumin (A) was estimated by bromocresol green binding method [30]. The absorbance of standard and test were measured against blank in a spectrophotometer at 630 nm. Globulin (G) was calculated by subtracting albumin values from serum total protein. A/G ratio was calculated by dividing albumin values by globulin values.

2.13. Tissue collection and homogenate preparation

Fishes were anesthetized and subsequently dissected under aseptic conditions. The tissue of interest for enzymatic assays (liver, gill, brain) were removed carefully and weighed. It was homogenized with chilled sucrose solution (0.25 M, 19:1 v/v) in a glass tube using tissue homogenizer (MICCRA D-9, Digitronic, Germany). The tube was continuously kept in ice bath while homogenizing. The homogenate was centrifuged at 5000 × *g* for 20 min at 4 °C in a cooling centrifuge. The supernatant was kept frozen at −20 °C till further analysis. A 5% homogenate was prepared for all the tissues.

2.14. Estimation of antioxidant enzymes

Superoxide dismutase (SOD) (EC 1.15.1.1) activity in liver and gill tissue was estimated by the method of Misra and Fridovich [31]. The assay is based on the oxidation of epinephrine-adrenochrome transition by the enzyme. Catalase (EC 1.11.1.6) activity was estimated according to the method of Takahara et al. [32]. To 2.45 ml of phosphate buffer (50 mM, pH 7.0), 50 μl of the tissue homogenate was added and the reaction was started by the addition of 1.0 ml of H_2O_2 solution, the decrease in absorbance was measured at 240 nm at 30 s intervals for 2 min. The enzyme activity was expressed as n moles of H_2O_2 catalyzed per min per mg protein. Glutathione-S-transferase (GST) (EC 2.5.1.18) was determined by the method of Habing et al. [33]. The method is based on the principle of formation of adduct of CDNB, S-2, 4- dinitrophenyl glutathione. The S-2, 4- dinitrophenyl glutathione (CDNB) is used as a substrate.

2.15. Estimation of neurotransmitter enzyme activity

The Acetyl cholinesterase enzyme (E.C.3.1.1.7) activity was assayed by the method of Hestrin [34]. The activity was spectrophotometrically measured as the increase in absorbance of the sample at 540 nm OD. Alkaline hydroxylamine was used to terminate the reaction.

2.16. Estimation of Na^+-K^+ ATPase activity

Na^+-K^+ ATPase activity, expressed as μmol Pi liberated/mg protein/h in the gill and was measured by liberating PO_4 from a hydrolysis reaction with ATPase following methods described by Agrahari and Gopal [35].

2.17. Serum cortisol and HSP70 quantification

Cortisol was quantified in the serum of experimental groups through ELISA. The quantification was performed using commercially available Cortisol EIA kit (Catalog no. 500360), procured from Cayman Chemicals, USA. The assay was performed according to the protocol provided along with the kit. The absorbance was read in the ELISA plate reader (Biotek India Private Limited). The expression of HSP-70 (EIA kit) in liver tissue was examined following manufacturer's instructions. The absorbance was read in the ELISA plate reader.

2.18. Statistical analysis

All the data were analysed using statistical package for the social sciences (SPSS) version 16 (SPSS, Chicago, IL). One-way analysis of variance (One-way ANOVA) was used to see treatment effects. Significant difference between means was determined by Duncan's multiple range test. Normality and homogeneity of variance were checked and all percentage data arcsine transformed. Probability levels of 5% were used to find out the significance in all cases. Analyzed data are presented as mean ± S.E.

3. Results

3.1. Growth and feed utilization parameters

Growth performance and feed utilization of *L. rohita* fed biotic supplements and reared under pH stress are presented in Table 2. Weight gain and SGR were lower in low pH/C diet group, whereas, groups in normal pH and fed BCPB7/FOS or both (Normal pH/pre diet, Normal pH/pro diet, Normal pH/syn diet), in addition to low pH/syn diet exhibited significantly higher growth compared to Normal pH/C diet ($p < 0.05$). High FCR and low PER was observed in groups low pH/C diet in compared to Normal pH/C diet ($p < 0.05$). FCR was significantly lower ($p < 0.05$) in all other treatment groups compared to Normal pH/C diet, except Low pH/Pre diet. Similarly, PER values improved in all treatments, except Low pH/Pre diet.

Table 3
Hematological parameters of *L. rohita* juveniles fed diet supplemented with *B. circulans* PB7 and FOS and exposed to low pH.

Treatment	TLC ($\times 10^3$ mm^{-3})	TEC ($\times 10^5$ mm^{-3})	Hb (g dl^{-1})	Hct (%)
Normal pH/C diet	17.42 ± 0.12^b	56.87 ± 1.79^c	4.76 ± 1.06^b	18.55 ± 0.06^b
Low pH/C diet	20.82 ± 0.25^c	42.42 ± 0.59^a	3.18 ± 0.02^a	14.87 ± 0.24^a
Normal pH/Pre diet	15.36 ± 0.24^b	57.00 ± 0.15^c	4.86 ± 0.03^b	19.26 ± 0.41^b
Low pH/Pre diet	15.11 ± 0.12^b	43.97 ± 0.82^a	4.50 ± 0.08^b	15.84 ± 0.09^a
Normal pH/Pro diet	13.83 ± 0.14^b	58.26 ± 0.23^{bc}	5.06 ± 0.12^b	19.23 ± 0.17^b
Low pH/Pro diet	14.16 ± 0.24^b	45.20 ± 0.75^a	4.73 ± 0.04^b	16.71 ± 0.09^a
Normal pH/Syn diet	12.10 ± 0.11^a	64.30 ± 0.70^c	5.40 ± 0.05^b	19.63 ± 0.26^b
Low pH/Syn diet	12.80 ± 0.27^a	58.09 ± 0.55^{bc}	4.79 ± 0.02^b	17.73 ± 0.29^b
P value	< 0.05	< 0.05	< 0.05	< 0.05

Data are means of triplicate. Means in the same column sharing a same superscript are not significantly different ($p > 0.05$) n = 3.
TLC: Total leukocyte count TEC: Total erythrocyte count.

3.2. Hematological parameters

Hematological parameter of *L. rohita* using various treatments recorded during the study is displayed in Table 3. Total Leukocyte Count (TLC) showed a marked elevation ($p < 0.05$) after pH exposure in low pH/C diet group. Values were stable ($p > 0.05$) in pro and pre-biotic fed groups under both Normal pH and low pH exposure. Synbiotic groups (Normal pH/Syn & low pH/syn diet) exhibited reduced TLC value among all groups ($p < 0.05$). Lower EC was observed in groups, Low pH/C diet, Low pH/pre diet and Low pH/pro diet, compared to Normal pH/C diet ($p < 0.05$), whereas, other groups excepting Normal pH/Syn diet did not increase significantly ($p > 0.05$). Hb levels did not show variation ($p > 0.05$) compared to control in all treatment groups, except Normal pH/C diet group where it was significantly lowered ($p < 0.05$). Hct level didn't change in groups Normal pH/pre diet, Normal pH/pro diet, Normal pH/Syn diet and low pH/syn diet compared to Normal pH/C diet ($p > 0.05$), whereas low pH/C diet, low pH/pre diet and low pH/pro diet displayed a marked reduction in Hct levels ($p < 0.05$).

3.3. Immune response

Immunological status of *L. rohita* fed pro-, pre- and syn-biotic diets, and either exposed or non-exposed to low pH stress revealed through lysozyme, nitroblue tetrazolium (NBT) and serum biochemistry are displayed in Tables 4 and 5. Lysozyme activity decreased in low pH/C diet, whereas marked increase in activity was witnessed in other groups. Among all groups, higher activity ($p < 0.05$) was observed in synbiotic groups (Normal pH/syn diet & low pH/syn diet). NBT level was reduced in low pH/C diet, whereas significant increase was observed in Normal pH/pro diet, Normal pH/syn diet and low pH/syn diet. Serum total protein decreased in low pH/C diet, whereas, it increased in other treatment groups as compared to Normal pH/C diet ($p < 0.05$) with highest value observed in Normal pH/syn diet. No change in serum albumin content was observed in low pH/C diet and Normal pH/pro diet compared to Normal pH/C diet ($p > 0.05$). Other groups exhibited higher levels ($p < 0.05$), with low pH/syn diet as highest. Significant lowering and elevation in levels of serum globulin was observed in low pH/C diet and Normal pH/syn diet respectively ($p < 0.05$), with highest value observed in Normal pH/syn diet. Marked increase in A/G ratio was evident in low pH/C diet, whereas highly lowered level was found in Normal pH pro diet ($p < 0.05$). No changes was observed in other groups compared to Normal pH/C diet ($p > 0.05$).

Table 5
Serum lysozyme, blood glucose and NBT activity of *L. rohita* juveniles fed diet supplemented with *B. circulans* PB7 and FOS and exposed to low pH.

Treatments	Lysozyme (U ml^{-1})	NBT (OD_{540})
Normal pH/C diet	6.74 ± 0.30^a	$0.34 \pm 0.\ 01^b$
Low pH/C diet	5.60 ± 0.63^b	0.31 ± 0.00^a
Normal pH/Pre diet	9.30 ± 0.81^c	0.35 ± 0.00^b
Low pH/Pre diet	7.65 ± 0.77^a	0.37 ± 0.00^b
Normal pH/Pro diet	9.29 ± 0.71^c	0.41 ± 0.01^c
Low pH/Pro diet	7.54 ± 0.52^a	0.36 ± 0.01^b
Normal pH/Syn diet	13.49 ± 0.69^d	0.47 ± 0.01^d
Low pH/Syn diet	11.38 ± 0.70^d	0.44 ± 0.01^{cd}
P value	< 0.05	< 0.05

Mean values (n = 3) in a column under each category bearing different superscript (lower case) vary significantly ($p < 0.05$).

3.4. Stress biomarkers

Stress level of rohu juveniles under low pH was examined through important biomarkers viz. glucose, cortisol and HSP70 levels and displayed in Figs. 1–3. Higher glucose level was shown in low pH/C diet and low pH/pro diet, whereas, levels were consistent with Normal pH/C diet group ($p < 0.05$). Similarly, cortisol and HSP70 levels were lower in all treatment groups irrespective of pH exposure, against control ($p < 0.05$).

Table 4
Serum biochemical parameters of *L. rohita* juveniles fed diet supplemented with *B. circulans* PB7 and FOS and exposed to low pH.

Treatments	Total protein (g/l)	Albumin (g/l)	Globulin (g/l)	A/G ratio
Normal pH/C diet	43.84 ± 1.27^a	6.00 ± 0.01^a	37.11 ± 9.38^a	0.170 ± 0.02^a
Low pH/C diet	38.46 ± 1.05^b	6.35 ± 0.12^a	33.70 ± 0.21^b	0.188 ± 0.00^c
Normal pH/Pre diet	47.33 ± 1.20^c	6.66 ± 1.15^b	40.66 ± 1.52^a	0.163 ± 0.01^a
Low pH/Pre diet	45.43 ± 0.36^c	6.71 ± 0.01^b	38.00 ± 0.25^a	0.176 ± 0.00^a
Normal pH/Pro diet	46.66 ± 0.66^c	6.00 ± 1.00^a	40.66 ± 1.52^a	0.147 ± 0.01^b
Low pH/Pro diet	44.27 ± 0.67^c	6.56 ± 0.15^b	38.27 ± 0.43^a	0.171 ± 0.00^a
Normal pH/Syn diet	50.33 ± 1.20^d	7.00 ± 1.00^c	43.33 ± 2.08^c	0.161 ± 0.00^a
Low pH/Syn diet	47.99 ± 0.40^{cd}	7.20 ± 0.06^c	41.87 ± 0.14^{ac}	0.172 ± 0.00^a
P value	< 0.05	< 0.05	< 0.05	< 0.05

Mean values (n = 3) in a column under each category bearing different superscript (lower case) vary significantly ($p < 0.05$).

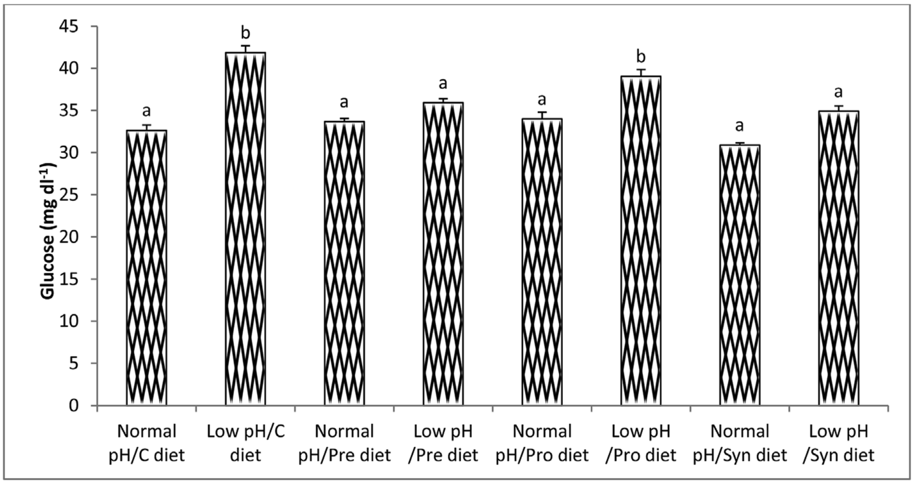

Fig. 1. Blood glucose levels of *L. rohita* fed experimental diets (*B. circulans* PB7, FOS singly or in combination) under both normal and low water pH for 60 days period.

3.5. Antioxidant enzyme status

The oxidative stress in terms of superoxide dismutase (SOD), catalase (CAT), and glutathione-S-transferase (GST) in gill and liver tissues of *L. rohita* juveniles exposed to low pH stress and fed with dietary biotic components are presented in Table 6. Noticeably elevated SOD levels in both tissues were observed in low pH/C diet, compared to Normal pH/C diet ($p < 0.05$) whereas, the dietary synbiotic supplemented groups (Normal pH/syn diet, low pH/syn diet) significantly amended the stressor as evidenced by in SOD levels ($p < 0.05$). CAT levels didn't alter much in both tissues in all supplemented groups ($p < 0.05$). GST level in both tissue were elevated in low pH/C diet group, whereas other treatment groups excepting synbiotic fed group (Normal pH/syn diet, low pH/syn diet) didn't change in levels significantly ($p < 0.05$), eliciting better protection against oxidative stress upon synbiotic feeding.

3.6. Status of neurotransmission and osmoregulation

The neurotransmitter status in brain tissue and Na^+-K^+ ATPase activity in gill tissue to mar as osmoregulatory response to pH changes in *L. rohita* after feeding dietary supplements is depicted in Table 7. Here, we observed no change in AChE activity in synbiotic groups (Normal pH/syn diet, low pH/syn diet), compared to Normal pH/C diet. Marked reduction in levels was noted in low pH/C diet ($p < 0.05$), whereas other groups experienced slight lower level compared to Normal pH/C diet ($p > 0.05$). Ionic disturbances as indicated by lowered Na^+-K^+ ATPase activity in gill was seen due to low pH exposure in group low pH/C diet compared to Normal pH/C diet ($p < 0.05$), whereas, protective role of dietary supplements was noticed in all supplemented groups.

4. Discussion

4.1. Growth and feed utilization

Aquatic animals tend to divert a greater amount of energy to other self-adjusting priority functions under low water pH exposure and this in turn reduces the anabolic activities like body growth [36]. Thus, in order to achieve better growth of fishes in low pH stress, amelioration of the encountered stress through nutritional ways should be given utmost importance. Altered water pH levels is reported to retards growth in three IMC species [5] and was subsequently observed in this study, where dietary non-supplemented groups and further exposed to

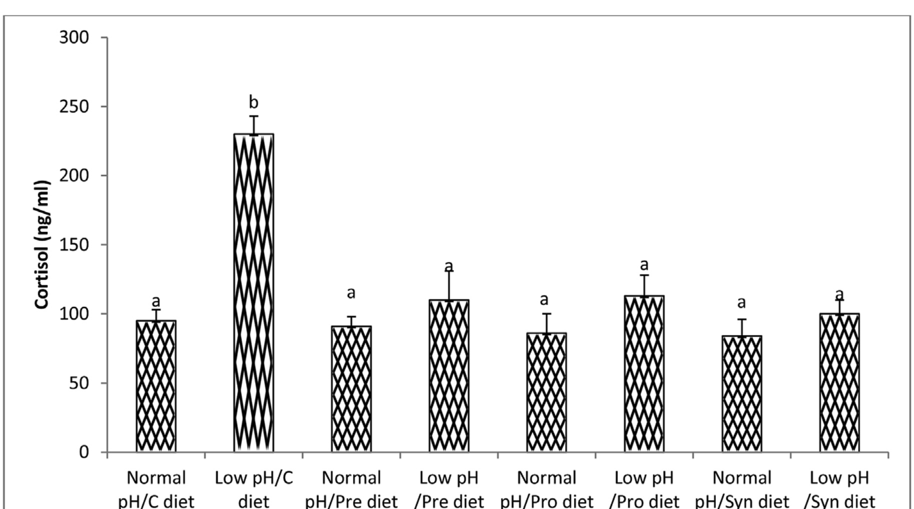

Fig. 2. Serum cortisol levels of *L. rohita* fed experimental diets (*B. circulans* PB7, FOS singly or in combination) under both normal and low water pH for 60 days period.

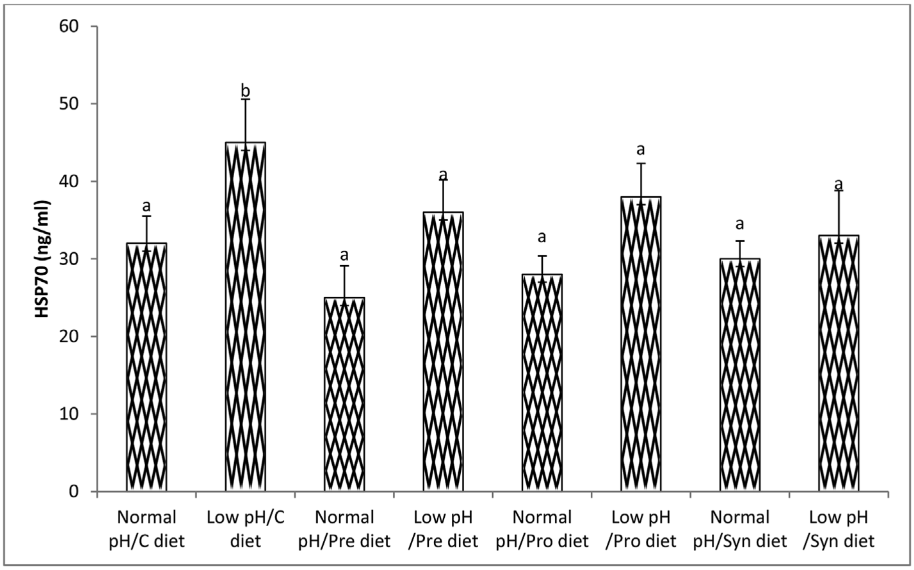

Fig. 3. Changes in HSP70 levels of *L. rohita* fed experimental diets (*B. circulans* PB7, FOS singly or in combination) under both normal and low water pH for 60 days period.

low pH (5.5) showed retarded growth. Such profound effect is reported in other aquaculture species as well [37]. Whereas, unexposed groups fed the supplements (pre-, pro- and synbiotic) demonstrated better growth conferring growth promoting effect as reported by several researchers (see reviews of Nayak, Ringo and Hyunh). The positive growth may relate to the beneficial role of dietary probiotic in maintaining endogenous micro-biota which enhance the immune and growth of host animal under stressor like low pH which is reported to alter the microbial profile in skin and gut of fishes [38]. Further, we speculate that supplementation of *B. circulans* PB7 together with FOS (synbiotics) might have resulted in faster proliferation of the probiotic through substrate provided by FOS in our study. However, further in depth studies pertaining to the speculation made in this study is needed. Parralel to this finding, dietary synbiotic could potentially ameriorate nitrite related stress in *L. rohita* [22]. In line to this, dietary synbiotic enriched *Artemia* fed to angelfish (*Pterophyllum scalare*) showed better resistance to low temperature and higher salinity stress [39]. Stress ameliorating potentials of *Bacillus* sp., MOS, synbiotics [40], MOS [41,42], and β-glucan and synbiotics [43] are also reported. As regards feed utilization as explained by FCR and PER, significantly better performance was observed in treatment groups. Possible explanation to this may link to improved microvilli alignment, as has been reported in MOS fed fish [42] which may increase protective function of the mucin barrier and affect ion regulation [41].

Table 7
Effect of dietary *B. circulans* PB7 and FOS on Acetyl cholinesterase in brain and Na^+ K^+ ATPase in gill tissue of *Labeo rohita* exposed to low pH stress for 60 days.

Treatments	AChE (μmoles hydrolyzed/min/mg protein)	Na^+ K^+ ATPase
Normal pH/C diet	0.71 ± 0.01^c	1.71 ± 0.02^b
Low pH/C diet	0.44 ± 0.01^a	1.35 ± 0.01^a
Normal pH/Pre diet	0.65 ± 0.01^b	1.68 ± 0.01^b
Low pH/Pre diet	0.62 ± 0.01^b	1.64 ± 0.02^b
Normal pH/Pro diet	0.66 ± 0.01^b	1.74 ± 0.01^b
Low pH/Pro diet	0.63 ± 0.01^b	1.73 ± 0.02^b
Normal pH/Syn diet	0.74 ± 0.01^c	1.83 ± 0.03^b
Low pH/Syn diet	0.72 ± 0.01^c	1.76 ± 0.02^b
P value	< 0.05	< 0.05

Mean values (n = 3) in a column under each category bearing different superscript (lower case) vary significantly ($p < 0.05$).

4.2. Immune indices

Hematological variables are generally considered good indicators to better explain the health conditions or metabolic disturbances in fish [44]. In this study, we tried to examine the changes in immune indices in terms of blood parameters in *L. rohita* under both control and pH exposure with and without supplements. The results of the present study noted a significant increase in TEC and reduction in TLC, Hb and

Table 6
Antioxidant enzyme activity of *L. rohita* fed diet supplemented with *B. circulans* PB7 and FOS and exposed to low pH stress.

Treatments	SOD (Units/mg protein/min)		Catalase (Units/mg protein/min)		GST (Units/mg protein/min)	
	Gill	Liver	Gill	Liver	Gill	Liver
Normal pH/C diet	27.01 ± 0.32^a	30.31 ± 0.55^a	1.35 ± 0.01^a	1.56 ± 0.01^a	0.45 ± 0.01^a	0.36 ± 0.00^b
Low pH/C diet	42.96 ± 0.93^b	44.79 ± 0.80^b	1.64 ± 0.01^b	2.23 ± 0.04^b	0.52 ± 0.01^b	0.46 ± 0.01^c
Normal pH/Pre diet	25.21 ± 1.23^a	27.43 ± 0.92^a	1.40 ± 0.02^a	1.48 ± 0.05^a	0.44 ± 0.01^a	0.34 ± 0.00^b
Low pH/Pre diet	29.63 ± 0.61^a	31.31 ± 0.49^a	1.39 ± 0.01^a	1.60 ± 0.02^a	0.46 ± 0.01^a	0.38 ± 0.01^b
Normal pH/Pro diet	26.45 ± 0.67^a	26.36 ± 0.65^a	1.42 ± 0.01^a	1.50 ± 0.03^a	0.47 ± 0.01^a	0.36 ± 0.02^b
Low pH/Pro diet	28.48 ± 0.69^a	29.48 ± 0.42^a	1.38 ± 0.01^a	1.48 ± 0.02^a	0.43 ± 0.01^a	0.37 ± 0.01^b
Normal pH/Syn diet	23.32 ± 0.85^c	24.45 ± 0.32^c	1.29 ± 0.01^a	1.42 ± 0.04^a	0.32 ± 0.01^c	0.32 ± 0.01^a
Low pH/Syn diet	24.64 ± 0.82^c	25.52 ± 0.41^c	1.33 ± 0.01^a	1.43 ± 0.00^a	0.34 ± 0.01^c	0.33 ± 0.01^a
P value	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

Mean values (n = 3) in a column under each category bearing different superscript (lower case) vary significantly ($p < 0.05$).

Hct values of rohu under low pH, as revealed in earlier reports [8]. Significant reduction in TEC as noted here might be due to haemolysis and shrinkage of blood cells due to toxic effects of lowered pH beyond desired level as reported in Indian major carp [5]. Whereas, an increased TLC in this study, reflects leukocytosis carried out as an adaptive response to such chemical stressor [45]. All together, these parameters revealed an induction of stressful situation due to low pH. Our attempt to utilize dietary supplements as pH stress ameliorator resulted in no significant changes in these values in pro- and prebiotic fed groups (both Normal pH and exposed), whereas synbiotic (BCPB7 + FOS) groups showed lowered TLC values which contradicts earlier reports on synbiotic application [46] and needs to be investigated further. Although significant effect on TLC appeared to be low at the end of trial, values recorded on 15th day were higher (data not shown). Such changes in level were also reported by Khan et al. [47] wherein TLC values were lower in *L. rohita* after feeding with plant extract of *Melocanna baccifera*). In other studies, immuno-protective role of both dietary β-glucan and synbiotic against ammonia stress is reported in *Litopenaeus vannamei* [43].

As such, serum biochemical parameters remains stable despite of slight external environmental variations and thus, a varied change can assist us in examining health of many organisms [48]. Serum proteins are very important compounds, with albumin and globulin being the major serum proteins and are important components of innate immunity [49]. Serum total protein mostly participate to maintain a normal osmotic pressure, constant pH, and also in lipid acids and bilirubin transport and thus is used as an indicator of stress response of fish [50,51]. Our results demonstrated a decreased serum total protein following pH stress exposure in groups without supplements which is also reported by Misra et al. [52]. As such, the decrease in level as noted here is mostly due to vascular leaking of serum protein because of increased permeability along with impaired synthesis and non-specific proteolysis of serum protein [46,53]. Improvement in serum protein due to dietary supplementation is observed in our investigation and possibly relate to the subsequent rise in the leukocyte count, which produces protein in the blood [52]. Similar results are reported by Mehrabi et al. [54] through dietary synbiotic feeding in rainbow trout, *Oncorhynchus mykiss*. This indicated that dietary BCPB7, FOS in single or combination could enhance the immunity of rohu fish under pH stress.

Lysozyme is known to act as an important innate defence mediator against, bacterial, viral and parasitic infections and in response to infection/stressor, its activity is found to increase in fish blood [55]. In the present work, synbiotic fed group (both normal and stressed) experienced highest level, illustrating immune enhancing properties of the biotic forms coinciding with the remarkably increased leukocytic count, which in turn elevated the lysozyme concentration and activity [56]. In previous reports, dietary probiotic [57,58] and prebiotics [59,60] administration was able to induce significant increase of serum lysozyme activity in fish. In very agreement to elevated serum lysozyme activity as noticed here, dietary synbiotic (FOS and *Bacillus* spp.) application elevated serum lysozyme activity in *P. olivaceus* [61] and *Larimichthys crocea* [62] under normal rearing conditions and under high temperature stress in *Macrobrachium amblycephala* fed diet supplemented with 0.4% anthraquinone extract [63]. This indicates enhanced immune score of the fish fed supplemented diets under low pH rearing conditions. In support to our finding, probiotics, *Lactobacillus fructivorans* and *L. plantarum* [64], herbal plant, *Pimenta dioica* powder [65] and dietary soybean isoflavones [66] are reported as effective supplements for acidic stress amelioration.

Respiratory burst activity can to some extent conclude the non-specific defense system of aquatic animals and can be measured by NBT assay which determines the ability of phagocytes to reduce the free radicals through production of oxygen radicals [67]. In our study, activity was comparable to control group, except in control fed diet (with low pH), where activity was greatly reduced. This implies a greater protective role of the supplement (in both control and low pH) with an increased bacterial pathogen killing activity of phagocytes [68] and may probably correlate to elevation in lysozyme level as noticed in our study. Similar to this, Khan et al. [47] also observed greater activity in *L. rohita* fingerlings after feeding *M. baccifera* against low pH stress. In addition, respiratory burst activity of phagocytes, measured by reduction of NBT by intracellular superoxide radicals produced by leucocytes, was higher in synbiotic group (both control and low pH) among all treatments suggesting synergistic effect and which warrants further in-depth study.

Several environmental contaminants (xenobiotics) induces oxidative stress with generation of reactive oxygen species (ROS) [69], which further induces oxidation of proteins, DNA, and steroid components, as well as peroxidation of unsaturated lipids in cell membranes [70] and alters their physiological function [71]. Superoxide dismutase (SOD), catalase (CAT) and GST are the key antioxidant enzymes which work as first line of defence against free radicals [72] and are therefore are crucial to counteract oxygen toxicity when the supply of other antioxidant compounds is scarce or depleted [73]. SOD represents group of metallo-enzymes actively involved in the antioxidant defence, which catalyze breakdown of superoxide to hydrogen peroxide which is further neutralized to water and molecular oxygen by CAT and GPx [74]. In the present work, we measured the anti-oxidant indices viz. SOD, CAT and GST and demonstrated elevated levels (in both gill and liver) in groups fed non-supplemented diets and under low pH exposure which indicates oxidative stress under low pH regime, probably due to generation of free radicals in the cellular tissue. Whereas, the dietary supplemented groups (both exposed and un-exposed) showed no significant elevation in the levels of these enzymes compared to control. Alterations in the antioxidant enzyme activity due to pH of water and copper toxicity have been demonstrated earlier in *Prochilodus lineatus* [75]. This suggests that dietary pre-, pro- and syn-biotics improves tolerance of *L. rohita* towards low pH stressor, manifested by noticeable reduction in oxidative stress. To substantiate these observations, some probiotics like *L. fermentum* [76], *L. plantarum* [77] are known to possess anti-oxidant properties which are linked to hetero-polysaccharide fractions derived from the probiotics and increased mRNA levels [77] and are positively related with their gene transcriptional levels [78]. Additionally, probiotics *L. rhamnosus* and *L. lactis* are known to improve antioxidant status in *Pagrus major* [79,80]. An important postulate that probiotic bacteria are able to produce various metabolites with antioxidant activity, such as glutathione (GSH), butyrate and folate [81] might have played significant role in improving oxidative status of *L. rohita* as noted in our study. In contrast to our result, FOS supplementation at 1% level did not affect antioxidant enzyme activity, except for G6PD in white seabream, *Diplodus sargus* [82]. Such contradictory results may happen due to the level and type of stressor encountered and also species under concern. Further, it was noted that synbiotic group had lower levels of these enzymes (except CAT) compared to either pre- or pro-biotic alone. In agreement to our finding, Hoseinifar et al. [83] reported increased antioxidant activity in rainbow trout fed on dietary pre-, pro- and synbiotics (GOS + *P. acidilactici*). In another report, Zhang et al. [84] witnessed an increase in liver SOD and CAT, as well as plasma SOD due to FOS and *B. licheniformis* supplementation in *M. terminalis* diet.

Acetylcholine esterase (AChE) is the most important neurotransmitter enzymes, and a key biomarker of the neurotoxicity in fishes [85,86]. Acetylcholine is synthesized in nervous tissue by choline acetylase and degraded further by cholinesterase [87]. Enzyme activities were remarkably inhibited in exposed group fed with control diet which is likely due to acetylcholine accumulation which affected feeding capability [88] signified by higher FCR as discussed earlier. Interestingly, supplementation of BCPB7 and FOS singly could not maintain AChE level at par with control, whereas, their combination (synbiotic) resisted further down surge of the enzyme activity providing clear evidence for protective role against low pH exposure in our study.

Comparatively, Muthappa et al. [89] reported a stabilized AChE activity upon dietary lipotropes supplementation when exposed to low dose endosulphan.

Gill Na^{+}-K^{+} ATPase is a membrane bound enzyme catalyzing active transport of Na^{+} and K^{+} into animal's body across the gill epithelium. Any significant change in water pH is known to affect such ion regulatory mechanism of the fish. We observed a significant reduction in its level, only in low pH exposed group fed with non-supplemented diet, where levels didn't change much in pre-, pro- and syn-biotic groups fed to both exposed and non-exposed. Earlier, Zhang et al. [84] reported improved Na^{+}-K^{+} ATPase activity through dietary *B. licheniformis* and FOS in *M. terminalis*, although the mechanism could not be explained.

For decades, it has been literally established that elevated blood glucose and cortisol levels are often used as precise indicators of stress in fish [90]. In our study, we observed a coherent increased level of both, in groups fed control diet and further exposed to low pH. This probably is due to the fact that under pH stress, the chromaffin cells release catecholamine hormones toward blood circulation [91] which in conjunction with cortisol, mobilize and elevate glucose production in fish through glucogenesis and glycogenolysis pathways [92] to cope with the energy demand produced by the stressor. Dietary supplemented groups (both control and exposed) exhibit a stable level compared to control conferring optimal physiological condition in the midst of low pH induction in *L. rohita*. In very support to our finding, probiotic, *L. plantarum* and *L. ructivorans* supplementation through live feed vectors could ameliorate pH stress in sea bream, *Sparus aurata*, with subsequent reduction in cortisol levels in circulation [64]. They observed an increased level of the probiotic in gut which favour microflora stability due to the probiotic treatment and thus enhanced fish wellness. At cellular level, HSP 70 serves as sensitive biomarkers that guarantees appropriate protection of protein structures, strengthens the immune system and stops apoptotic mechanisms against stressors [93]. In this study, an elevated level of HSP70 was observed in groups fed control and exposed to low pH, which was consistent with levels of glucose and cortisol, explaining cellular disturbances under stress. HSP70 is thought to have a molecular chaperon function and is closely related to stress tolerance in animal [94] and it's over expression enhances anti-apoptotic activity against cellular stress [95,96]. Other treatment groups experienced low levels, and may point towards our hypothesis that dietary supplements protected the cellular response, in terms of HSP70 expression levels. It is known that the expression level of HSP70 depends on species, time elapsed and intensity of stress. Short term acidic stress showed higher expression levels in four fin-fishes (gourami, carp, goldfish and trout) [97] and in sea cucumber [98]. Further, Zhou et al. [66] observed higher expression levels of HSP70 and HSP90 mRNA in groups supplemented with soybean isoflavones compared to control. However, in our study, we observed decreased levels after 60 days in all treated groups (exposed and unexposed) as compared to control and possibly this is due to prolonged stress (chronic) which causes mutation in cell membrane structure and hepatic protein composition, thereby terminating the transcription of HSPs [99]. In support to our findings, protective role of dietary probiotic against pH stress [64], and selenium nanoparticles against multiple stressors [70] evidenced by stable HSP70 are reported. These varied results might also happen due to tissue specificity of different species under trial which needs to be studied further.

5. Conclusion

In conclusion, the definite role of dietary *B. circulans* PB7 and FOS, administered either singly or in combination in successfully ameliorating the low pH is confirmed based on results of growth, immune status, antioxidant enzyme activity and cellular bio-indicators. Further, relied upon some significant parameters, synbiotic formulation works better compared to probiotic and prebiotics alone which necessitates further studies at field level. This study provides avenues for sustainable mitigation strategies for low pH water in carp farming through dietary supplementation of probiotics and prebiotics for an enhanced production under the present carp farming scenario in India.

Declaration of interest

The authors report no conflicts of interest. The authors alone are solely responsible for the content and presentation of the paper.

Acknowledgements

The authors would like to acknowledge Indian Council of Agricultural Research, New Delhi, India for the financial support. The authors further extend sincere words of thanks to the Director, ICAR-Central Institute of Fisheries Education, Mumbai, India for the facilities and support during the study period. The first author would like to place on record the needful advice and support of scholars under the Department of Aquaculture, ICAR- CIFE, Mumbai, India.

References

[1] M. Debnath, D. Kamilya, H. Saha, R.K. Saha, D. Saikia, Effects of water borne iron on spawn of Indian major carps (*Catla catla* (Ham.), *Labeo rohita* (Ham.) and *Cirrhinus mrigala* (Ham.), Bull. Environ. Contam. Toxicol. 89 (2012) 1170–1174.
[2] Z. Qian, T. Liu, Q. Liu, S. He, Y. Liu, F. Hou, X. Wang, X. Mi, C. Cai, X. Liu, p53 is involved in shrimp survival via its regulation roles on MnSOD and GPx in response to acute environmental stresses, Comp. Biochem. Physiol., C 159 (2014) 38–51.
[3] Y.Y. Chen, J.C. Chen, K.C. Tseng, Y.Q. Lin, C.L. Huang, Activation of immunity, immune response, antioxidant ability, and resistance against *Vibrio alginolyticus* in white shrimp *Litopenaeus vannamei* decrease under long-term culture at low pH, Fish Shellfish Immunol. 46 (2015) 192–199.
[4] F.B. Jensen, J. Brahm, Kinetics of chloride transport across fish red blood cell membranes, J. Exp. Biol. 198 (1995) 2237–2244.
[5] P.C. Das, S. Ayyappan, J.K. Jena, Haematological changes in the three Indian major carps, *Catla catla* (Hamilton), *Labeo rohita* (Hamilton) and *Cirrhinus mrigala* (Hamilton) exposed to acidic and alkaline water pH, Aquaculture 235 (2006) 633–644.
[6] R.W.M. Kwong, Y. Kumai, S.F. Perry, The physiology of fish at low pH: the zebra fish as a model system, J. Exp. Biol. 217 (2014) 651–662 https://doi.org/10.1242/jeb.091603.
[7] P.O. Fromm, A review of some physiological and toxicological responses of freshwater fish to acid stress, Environ. Biol. Fish. 5 (1980) 79–93.
[8] M.M. Zahangir, F. Haque, M.G. Mostakim, M.S. Islam, Secondary stress responses of zebrafish to different pH: evaluation in a seasonal manner, Aqua Rep 2 (2015) 91–96.
[9] R.M. Fenner, The Conscientious Marine Aquarist, T. F.H. Publications, Neptune City, N. J., 2001.
[10] S.E.W. Bonga, The stress response in fish, Physiol. Rev. 77 (1997) 591–625.
[11] B.A. Barton, Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids, Integr. Comp. Biol. 42 (2002) 517–525.
[12] B.A. Barton, C.B. Schreck, R.D. Ewing, A.R. Hemmingsen, R. Patiño, Changes in plasma cortisol during stress and smoltification in coho salmon, *Oncorhynchus kisutch*, Gen. Comp. Endocrinol. 59 (1985) 468–471.
[13] G.P. Chrousos, Stressors, stress, and neuroendocrine integration of the adaptive response, Ann. N. Y. Acad. Sci. 851 (1998) 311–335.
[14] J.D. Morgan, G.K. Iwama, Measurements of stressed states in the field, in: G.K. Iwama, A.D. Pickering, J.P. Sumpter, C.B. Schreck (Eds.), Fish Stress and Health in Aquaculture, Cambridge University Press, Cambridge, UK, 1997, pp. 247–270.
[15] Harris and Bird, Modulation of the fish immune system by hormones, Vet. Immunol. Immunopathol. 77 (2000) 163–176.
[16] P. Di Mascio, M.E. Murphy, H. Sies, Antioxidant defense systems: the role of carotenoids, tocopherols, and thiols, Am. J. Clin. Nutr. 53 (1991) 194S–200S.
[17] G.K. Iwama, P.T. Thomas, R.B. Forsyth, M.M. Vijayan, Heat shock protein expression in fish, Rev. Fish Biol. Fish. 8 (1998) 35–56.
[18] R. Fuller, What is a probiotic? Biologist 51 (2004) 232.
[19] S.K. Nayak, Probiotics and immunity: a fish perspective, Fish Shellfish Immunol. 29 (2010) 2–14.
[20] P. Bandyopadhyay, P.K.D. Mohapatra, Effect of a probiotic bacterium *Bacillus circulans* PB7 in the formulated diets: on growth, nutritional quality and immunity of *Catla catla* (Ham.), Fish Physiol. Biochem. 35 (2009) 467–478 http://doi.org/10.1007/s10695-008-9272-8.
[21] E. Ringø, R.E. Olsen, T.Ø. Gifstad, R.A. Dalmo, H. Amlund, G.I. Hemre, A.M. Bakke, Prebiotics in aquaculture: a review, Aquacult. Nutr. 16 (2010) 117–136.
[22] S.K. Singh, V.K. Tiwari, N.K. Chadha, C. Prakash, S. Munilkumar, P. Das, S.C. Mandal, T.I. Chanu, Effect of *Bacillus circulans* and fructooligosaccharide supplementation on growth and haemato-immunological function of *Labeo rohita* (Hamilton, 1822) fingerlings exposed to sub-lethal nitrite stress, Isr. J. Aquacult. Bamidgeh 66 (2013) 1–10.

[23] A. Irianto, B. Austin, Probiotics in aquaculture, J. Fish Dis. 25 (2002) 633–642.
[24] APHA (American Public Health Association, American Water Works Association and Water Environmental Federation, Standard Methods of Examination of Water and Wastewater, twenty-first ed., APHA, Washington, DC, 2005, pp. 20001–23710.
[25] J.W. Nelson, M. Somogyi, B.L. Oser (Ed.), Hawk's Physiological, Chemistry, fourteenth ed., McGraw Hill Publication, New York, USA, 1945, p. 113.
[26] M. Somoyogi, A new reagent for the determination of sugars, J. Biol. Chem. 160 (1945) 61–68.
[27] S.J. Secombes, Isolation of Salmonid macrophage and analysis of their killing activity, in: J.S.T.C. Stolen, D.P. Fletcher, B.S. Anderson, W.B. Winkel (Eds.), Techniques in Fish Immunology, SOS Publication, New Jersey, USA, 1990, pp. 137–152.
[28] A.S. Stasiack, C.P. Bauman, Neutrophil activity as a potent indicator of concomitant analysis, Fish Shellfish Immunol. 37 (1996) 539.
[29] J.G. Reinhold, Manual determination of serum total protein, albumin and globulin fractions by Biuret method, in: M. Reiner (Ed.), Standard Method of Clinical Chemistry, Academic Press, New York, NY, USA, 1953, p. 88.
[30] B.T. Doumas, W. Watson, H.G. Biggs, Albumin standards and measurement of serum albumin with bromocresol green, Clinica Chemica Acta 31 (1971) 87–96.
[31] H.P. Misra, I. Fridovich, The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase, J. Biol. Chem. 247 (1972) 3170–3175.
[32] S. Takahara, B.H. Hamilton, J.V. Nell, T.Y. Kobra, Y. Ogura, E.T. Nishimura, Hypocatalesemia, a new generis Carrier state, J. Clin. Investig. 29 (1960) 610–619.
[33] W.H. Habing, M.N. Pabst, W. Bjacoby, S. Glutathion, Glutathione S-transferases. Transferase, the first enzymatic step in mercatpopunc acid formation, J. Biol. Chem. 249 (1974) 7130–7139.
[34] S. Hestrin, The reaction of acetyl choline and other carboxylic acid derivatives with hydroxylamine and its analytical application, J. Biol. Chem. 180 (1949) 249–261.
[35] S. Agrahari, K. Gopal, Inhibition of Na^{+}–K^{+}-ATPase in different tissues of freshwater fish *Channa punctatus* (Bloch) exposed to monocrotophos, Pestic. Biochem. Physiol. 92 (2008) 57–60.
[36] R.W. Kwong, Y. Kumai, S.F. Perry, The physiology of fish at low pH: the zebrafish as a model system, J. Exp. Biol. 217 (2014) 651–662.
[37] M. Rask, M. Vuorinen, P.J. Vuorinen, Whitefish stocking: an alternative in mitigating acidification effects? Finn. Fish. Res. 9 (1988) 489–495.
[38] F. Sylvain, B. Cheaib, M. Llewellyn, T.G. Correia, D.B. Fagundes, A.L. Val, N. Derome, pH drop impacts differentially skin and gut microbiota of the Amazonian fish tambaqui (*Colossoma macropomum*), Sci. Rep. 6 (2016), https://doi.org/10.1038/srep32032.
[39] M. Azimirad, S. Meshkini, N. Ahmadifard, S.H. Hoseinifar, The effects of feeding with synbiotic (*Pediococcus acidilactici* and fructooligosaccharide) enriched adult *Artemia* on skin mucus immune responses, stress resistance, intestinal microbiota and performance of angelfish (*Pterophyllum scalare*), Fish Shellfish Immunol. 54 (2016) 516–522 https://doi:10.1016/j.fsi.2016.05.001.
[40] C.L. Daniels, D.L. Merrifield, E. Ringø, S.J. Davies, Probiotic, prebiotic and synbiotic applications for the improvement of larval European lobster (*Homarus gammarus*) culture, Aquaculture 416–417 (2013) 396–406.
[41] G. Salze, E. McLean, M.H. Schwarz, S.R. Craig, Dietary mannan oligosaccharide enhances salinity tolerance and gut development of larval cobia, Aquaculture 274 (2008) 148–152.
[42] A. Dimitroglou, D.L. Merrifield, P. Spring, J. Sweetman, R. Moate, S.J. Davies, Effects of mannan oligosaccharide (MOS) supplementation on growth performance, feed utilization, intestinal histology and gut microbiota of gilthead sea bream (*Sparus aurata*), Aquaculture 300 (2010) 182–188.
[43] S. Boonanuntanasarn, U. Wongsasak, T. Pitaksong, S. Chaijamrus, Effects of dietary supplementation with b-glucan and synbiotics on growth, haemolymph chemistry, and intestinal microbiota and morphology in the Pacific white shrimp, Aquacult. Nutr. 22 (2016) 837–845 https://doi:10.1111/anu.12302.
[44] M.J.T. Ranzani-Paiva, C.M. Ishikawa, M.C. Portella, R.J. Ceriberto, Hematology of the carp, *Cyprinus carpio*, infested by *argulus* sp. and after treatment with fosfonate 0,0-dimetil- oxi-2,2,2- tricoloetilo (neguvon), Bol Inst Pesca 14 (1987) 83–92.
[45] P.K. Joshi, D. Harish, M. Bose, Effect of lindane and malathion exposure tocertain blood parameters in a fresh water teleost fish *Clarius batrachus*, Pollut. Res. 21 (2002) 55–57.
[46] P. Kumar, K.K. Jain, P. Sardar, Effects of dietary synbiotic on innate immunity, antioxidant activity and disease resistance of *Cirrhinus mrigala* juveniles, Fish Shellfish Immunol. 80 (2018) 124–132.
[47] M.I.R. Khan, R.K. Saha, H. Saha, Muli bamboo (*Melocanna baccifera*) leaves ethanolic extract a non-toxic phyto-prophylactic against low pH stress and saprolegniasis in *Labeo rohita* fingerlings, Fish Shellfish Immunol. 74 (2018) 609–619.
[48] M. Abdel-Tawwab, M.H. Ahmad, Y.A.E. Khattab, A.M.E. Shalaby, Effect of dietary protein level, initial body weight, and their interaction on the growth, feed utilization and physiological alterations of Nile tilapia, *Oreochromis niloticus* (L.), Aquaculture 298 (2010) 267–274.
[49] F. Qin, M. Shi, H. Yuan, L. Yuan, W. Lu, J. Zhang, J. Tong, X. Song, Dietary nanoselenium relieves hypoxia stress and, improves immunity and disease resistance in the Chinese mitten crab (*Eriocheir sinensis*), Fish Shellfish Immunol. 54 (2016) 481–488.
[50] H.D. Smet, R. Blust, Stress responses and changes in protein metabolism in carp *Cyprinus carpio* during cadmium exposure, Ecotoxicol. Environ. Saf. 48 (2001) 255–262.
[51] M.M. Refaey, D. Li, Transport stress changes blood biochemistry, antioxidant defense system, and hepatic HSPs mRNA expressions of Channel catfish, *Ictalurus punctatus*, Front. Physiol. 9 (2018), https://doi:10.3389/fphys.2018.01628.
[52] C.K. Misra, B.K. Das, S.C. Mukherjee, P. Pattnaik, Effect of multiple injections of beta-glucan on non-specific immune response and disease resistance in *Labeo rohita* fingerlings, Fish Shellfish Immunol. 20 (2006) 305–331.
[53] A.E. Ellis, T.S. Hastings, A.L.S. Munro, The role of *Aeromonas salmonicida* extracellular products in the pathology of furunculosis, J. Fish Dis. 4 (1981) 41–52.
[54] Z. Mehrabi, F. Firouzbakhsh, A. Jafarpour, Effects of dietary supplementation of synbiotic on growth performance, serum biochemical parameters and carcass composition in rainbow trout (*Oncorhynchus mykiss*) fingerlings, Anim Physiol Anim Nutr 96 (2012) 474–481.
[55] J.B. Alexander, G.A. Ingram, Non-cellular non-specific defence mechanisms of fish, Annu. Rev. Fish Dis. 2 (1992) 249–279.
[56] T. Yano, Non-specific immune system: humoral defense, in: G. Iwama, T. Nakanishi (Eds.), The Fish Immune System, Academic Press, 1996, pp. 106–159.
[57] J.L. Balcazar, I. de Blas, I. Ruiz-Zazuela, D. Cunningham, D. Vendrell, J.L. Muzquiz, The role of probiotics in aquaculture, Vet. Microbiol. 114 (2006) 173–186.
[58] D.L. Merrifield, A. Dimitroglou, A. Foey, S.J. Davies, R.T.M. Baker, J. Bøgwald, M. Castex, E. Ringø, The current status and future focus of probiotic and prebiotic applications for salmonids, Aquaculture 302 (2010) 1–18.
[59] Y. Staykov, P. Spring, S. Denev, J. Sweetman, Effect of amannan oligosaccharide on the growth performance and immune status of rainbow trout (*Oncorhynchus mykiss*), Aquacult. Int. 15 (2007) 153–161.
[60] J.A. Buentello, W.H. Neill, D.M. Gatlin III, Effects of dietary prebiotics on the growth, feed efficiency and non-specific immunity of juvenile red drum, *Sciaenops ocellatus* fed soybean-based diets, Aquacult. Res. 41 (2010) 411–418.
[61] J.D. Ye, K. Wang, F.D. Li, Y.Z. Sun, Single or combined effects of fructo- and mannan oligosaccharide supplements and *Bacillus clausii* on the growth, feed utilization, body composition, digestive enzyme activity, innate immune response and lipid metabolism of the Japanese flounder *Paralichthys olivaceus*, Aquacult. Nutr. 17 (2011) 902–911.
[62] O. Ai, H. Xu, K. Mai, W. Xu, J. Wang, W. Zhang, Effects of dietary supplementation of *Bacillus subtilis* and fructooligosaccharide on growth performance, survival, non-specific immune response and disease resistance of juvenile large yellow croaker, *Larimichthys crocea*, Aquaculture 317 (2011) 155–161.
[63] K.F. Liu, C.H. Chiu, Y.L. Shiu, W. Cheng, C.H. Liu, Effects of the probiotic, *Bacillus subtilis* E20, on the survival, development, stress tolerance, and immune status of white shrimp, *Litopenaeus vannamei* larvae, Fish Shellfish Immunol. 28 (2010) 837–844.
[64] A. Rollo, R. Sulpizio, M. Nadi, S. Silvi, C. Orpiansei, M. Caggiano, A. Creci, O. Carnevali, Live microbial supplements in aquaculture for improvement of stress tolerance, Fish Physiol. Biochem. 33 (2006) 429–440.
[65] S. Yılmaz, Ü. Acar, O.S. Kesbiç, N. Gültepe, S. Ergün, Effects of dietary allspice, *Pimenta dioica* powder on physiological responses of *Oreochromis mossambicus* under low pH stress, SpringerPlus 4 (2015) 719.
[66] C. Zhou, H. Lin, Z. Huang, J. Wang, Y. Wang, W. Yu, Effects of dietary soybean isoflavones on non-specific immune responses and hepatic antioxidant abilities and mRNA expression of two heat shock proteins (HSPs) in juvenile golden pompano *Trachinotus ovatus* under pH stress, Fish Shellfish Immunol. 47 (2015) 1043–1053.
[67] J.M. Robinson, Reactive oxygen species in phagocytic leukocytes, Histochem. Cell Biol. 130 (2008) 281–297.
[68] G.J.E. Sharp, C.J. Secombes, The role of reactive oxygen species in the killing of the bacterial fish pathogen *Aeromonas salmonicida* by rainbow trout macrophages, Fish Shellfish Immunol. 3 (1993) 119–129.
[69] P. Di Mascio, M.E. Murphy, H. Sies, Antioxidant defense systems: the role of carotenoids, tocopherols, and thiols, Am. J. Clin. Nutr. 53 (1991) 194S–200S.
[70] N. Kumar, K.K. Krishnania, S.K. Gupta, R. Sharma, R. Baitha, D.K. Singh, N.P. Singh, Immuno-protective role of biologically synthesized dietary selenium nanoparticles against multiple stressors in *Pangasinodon hypophthalmus*, Fish Shellfish Immunol. 78 (2018) 289–298.
[71] A. Bhattacharyya, R. Chattopadhyay, S. Mitra, E. Sheila, Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases, Physiol. Rev. 94 (2014) 329–354.
[72] T. Yang, X. Li, W. Zhu, C. Chen, Z. Sun, et al., Alteration of antioxidant enzymes and associated genes induced by grape seed extracts in the primary muscle cells of goats in Vitro, PLoS One 9 (9) (2014) e107670 https://doi:10.1371/journal.pone.0107670.
[73] S. Ahmad, Oxidative Stress and Antioxidant Defenses in Biology, Chapman & Hall, New York, 1995.
[74] B. Halliwell, J.M.C. Gutteridge, Free Radicals in Biology and Medicine, Oxford University Press, Oxford, U. K., 2001, p. 936.
[75] C.S. Carvalhoa, V.A. Bernussob, M.N. Fernandes, Copper levels and changes in pH induce oxidative stress in the tissue of curimbata (*Prochilodus lineatus*), Aquat. Toxicol. 167 (2015) 220–227.
[76] E. Persichetti, A. De Michele, M. Codini, G. Traina, Antioxidative capacity of *Lactobacillus fermentum* LF31 evaluated *in vitro* by oxygen radical absorbance capacity assay, Nutrition 30 (2014) 936–938.
[77] D. Gao, Z. Gao, G. Zhu, Antioxidant effects of *Lactobacillus plantarum* via activation of transcription factor Nrf2, Food Funct 4 (2013) 982–989.
[78] Y. Wang, Y. Wu, Y. Wang, H. Xu, X. Mei, D. Yu, Y. Wang, W. Li, Antioxidant properties of probiotic bacteria, Nutrients 9 (2017) 521 https://doi:10.3390/nu9050521.
[79] M.A. Dawood, S. Koshio, M. Ishikawa, S. Yokoyama, M.F. El Basuini, M.S. Hossain, T.H. Nhu, S. Dossou, A.S. Moss, 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 Immunol. 49 (2016) 275–285.
[80] M.A. Dawood, S. Koshio, M. Ishikawa, M. El-Sabagh, M.A. Esteban, A.I. Zaineldin, Probiotics as an environment-friendly approach to enhance red sea bream, *Pagrus*

major growth, immune response and oxidative status, Fish Shellfish Immunol. 57 (2016) 170–178.
[81] Y. Wang, Y. Wu, Y. Wang, H. Xu, X. Mei, D. Yu, Y. Wang, W. Li, Antioxidant properties of probiotic bacteria, Nutrients 9 (2017) 521.
[82] I. Guerreiro, C.R. Serra, P. Pousão-Ferreira, A. Oliva-Teles, P. Enes, Prebiotics effect on growth performance, hepatic intermediary metabolism, gut microbiota and digestive enzymes of white sea bream (*Diplodus sargus*), Aquacult. Nutr. 24 (2018), https://doi.org/10.1111/anu.12543.
[83] S.H. Hoseinifar, S.M. Hoseini, D. Bagheri, Effects of galactooligosaccharide and *Pediococcus acidilactici* on antioxidant defence and disease resistance of rainbow trout, *Oncorhynchus mykiss* Ann, Anim. Sci. 17 (2017) 217–227 https://doi:10.1515/aoas-2016-0024.
[84] C.N. Zhang, X.F. Li, W.N. Xu, D.D. Zhang, K.L. Lu, L.N. Wang, H.Y. Tian, W.B. Liu, Combined effects of dietary fructooligosaccharide and *Bacillus licheniformis* on growth performance, body composition, intestinal enzymes activities and gut histology of triangular bream (*Megalobrama terminalis*), Aquacult. Nutr. 21 (2015) 755–766.
[85] N. Kumar, K.K. Krishnani, P. Kumar, A.K. Jha, S.K. Gupta, N.P. Singh, Dietary zinc promotes immuno-biochemical plasticity and protects fish against multiple stresses, Fish Shellfish Immunol. 17 (2017) 184–194.
[86] V.K. Sahu, S. Karmakar, S. Kumar, S.P. Shukla, K. Kumar, Triclosan toxicity alters behavioral and hematological parameters and vital antioxidant and neurological enzymes in *Pangasianodon hypophthalmus* (Sauvage, 1878), Aquat. Toxicol. 202 (2018) 145–152.
[87] M.A. Prado, R.A. Reis, V.F. Prado, de M.C. Mello, M.V. Gomez, F.G. de Mello, Regulation of acetylcholine synthesis and storage, Neurochem. Int. 41 (2002) 291–299.
[88] M. Banaee, Adverse effects of insecticides on various aspects of fish's biology and physiology, in: S. Soloneski, M. Larramendy (Eds.), Insecticides - Basic and Other Applications, Rijeka, Croatia, 2012, pp. 101–126.
[89] N.A. Muthappa, S. Gupta, S. Yengkokpam, D. Debnath, N. Kumar, A.K. Pal, S.B. Jadhao, Lipotropes Promote immunobiochemical plasticity and protect fish against low-dose pesticide-induced oxidative stress, Cell Stress Chaperones 19 (1) (2014) 61–81.
[90] B.A. Barton, G.K. Iwama, Physiological changes in fish from stress in aquaculture with emphasis on the response in juvenile Chinook salmon (*Oncorhynchus tshawytscha*), Aquaculture 62 (1991) 299–310.
[91] S.G. Reid, N.J. Bernier, S.F. Perry, The adrenergic stress response in fish: control of catecholamine storage and release, Comp. Biochem. Physiol., C 120 (1998) 1–27.
[92] G.K. Iwama, M.M. Vijayan, R.B. Forsyth, P.A. Ackerman, Heat shock proteins and physiological stress in fish, Am. Zool. 39 (1999) 901–909.
[93] L. Ravagnan, S. Gurbuxani, S.A. Susin, C. Maisse, E. Daugas, N. Zamzami, T. Mak, M. Jäättelä, J.M. Penninger, C. Garrido, G. Kroemer, Heat-shock protein 70 antagonizes apoptosis-inducing factor, Nat. Cell Biol. 3 (2001) 839–843.
[94] M. Yamashita, T. Yabu, N. Ojima, Stress protein HSP70 in fish, Aqua-Bioscience Monographs 3 (2010) 111–141.
[95] M.E. Feder, N.V. Cartano, L. Milos, R.A. Krebs, S.L. Lindquist, Effect of engineering Hsp70 copy number on Hsp70 expression and tolerance of ecologically relevant heat shock in larvae and pupae of *Drosophila melanogaster*, J. Exp. Biol. 199 (1996) 1837–1944.
[96] Y.M. Kim, M.E. de Vera, S.C. Watkins, T.R. Billiar, V.L. Gabai, A.B. Meriin, D.D. Mosser, A.W. Caron, S. Rits, V.I. Shifrin, M.Y. Sherman, Nitric oxide protects cultured rat hepatocytes from tumor necrosis factor-alpha-induced apoptosis by inducing heat shock protein 70 expression, J. Biol. Chem. 272 (1997) 1402–1411.
[97] M. Martín, C. Hernandez, G. Bodega, Heat-shock proteins express ion in fish central nervous system and its possible relation with water acidosis resistance, Neurosci. Res. 31 (1998) 97–106.
[98] Y.W. Dong, S.L. Dong, X.L. Meng, Effects of thermal and osmotic stress on growth, osmoregulation and HSP70 in sea cucumber (*Apostichopus japonicas* Selenka), Aquaculture 276 (2008) 179–186.
[99] J. Qiang, H. Yang, H. Wang, P. Xu, Z.L. Qi, J. He, Studies on blood biochemical indices and expression of hepatic HSP70 mRNA of different tilapia strains artificially challenged with *Streptococcus iniae*, J. Fish. China 36 (2012) 958–968.