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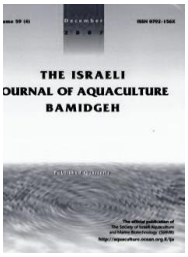

Effect of *Bacillus circulans* and fructooligosaccharide supplementation on growth and haemato-immunological function of *Labeo rohita* (Hamilton, 1822) fingerlings exposed to sub-lethal nitrite stress

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Abstract

The present study was undertaken to evaluate the effect of a probiotic strain, *Bacillus circulans* (BC) and prebiotics fructooligosaccharide (FOS) supplementation in *Labeo rohita* fingerlings subjected to sub-lethal nitrite stress. Earlier a sixty days feeding trial was conducted using eight experimental diets viz. T1 (10^6 cfu/g BC+0%FOS), T2 (10^8 cfu/g BC+0% FOS), T3 (0BC+1%FOS), T4 (0BC+2%FOS), , T5 (10^6 cfu/g BC + 1% FOS), T6 (10^6 cfu/g BC+2% FOS), T7 (10^8 cfu/g BC+1%FOS), T8 (10^8 cfu/g BC+2%FOS) & control (0BC+0%FOS) assigned to triplicate groups. After the initial experiment, fishes were subjected to sub-lethal nitrite (1/5 96 h LC_{50} = 2.08 mg/L) for another 30 days. At the end of the experiment growth and feed utilization parameters viz. weight gain, specific growth rate (SGR), feed conversion ratio (FCR) & survival percentage were calculated. Haemato-immunological parameters viz. total leukocyte count (TLC), total erythrocyte count (TEC), hemoglobin (Hb), hematocrit (Hct) values, lysozyme and respiratory burst activity were assayed after exposure to stress. Growth and feed utilization was significantly ($p < 0.05$) better in probiotic fed groups at all level of supplementation whereas prebiotics supplementation at 2% level had negative impact. Percentage survival was also better in the supplemented groups with highest survival observed in T4 group. Similarly, post challenge haemato-immunological parameters were better in supplemented group compared to control and higher levels were observed in combination groups (T4 and T5). From the present investigation, it can be concluded that probiotic *B. circulans* and prebiotics FOS supplementation improves immune status and thereby conferring better protection of *L. rohita* under sub-lethal nitrite stress.

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Introduction

Carp farming is the backbone of Indian aquaculture contributing more than 85% of the total production. In recent years, technological advancement and modification of culture practice from traditional to semi-intensive and intensive system has led to various environmental related stressors in the culture species. One serious concern among the stressors is the nitrite induced stress. In intensive culture of carps, management of nitrite concentration in water is often ignored, giving more importance to the management of ammonia. Studies in a number of fishes have established that higher nitrite levels in water are one of the important factors causing considerable stress in fish. An elevated nitrite level can be a problem in habitats receiving nitrogenous effluents and in various hypoxic environments (Eddy and Williams, 1987). It is also a matter of great concern for aquaculture, which has become a growing industry in recent years (Naylor *et al.,* 2000). The high density of fish in aquaculture ponds is associated with a large production of waste products, including ammonia excreted by the fish, with the potential accumulation of ammonia and nitrite to toxic levels (Hargreaves, 1998). The influx of nitrite into erythrocytes inevitably leads to oxidation of functional haemoglobin (Hb, with haem irons as Fe^{2+}) to methaemoglobin, (with haem irons as Fe^{3+}), which cannot bind oxygen.

The goal of immunostimulation in fish is not only a greater effective immune response to infectious agent but also to overcome the immunosuppressive effect of stress (Ganguly *et al.* 2010). A mitigation strategy through nutritional intervention provides one promising tool to counteract environmental related stressors in carps. Immune enhancement or immuno-modulation through dietary supplementation of L-tryptophan for mitigation of crowding stress (Tejpal *et al.,* 2009) and pyridoxine for mitigating endosulphan related toxicity (Akhtar *et al.,* 2010) has been successful in Indian Major Carps. Probiotics which are beneficial micro-organisms play an important role in enhancing the immune and health status of cultured fish species. Probiotic *Bacillus subtilis* E20 supplementation in white shrimp, *Litopenaeus vannamei* larvae improved survival and stress tolerance in relation to reduced temperatures (Ganguly *et al.* 2010); Liu *et al.,* 2010). Another probiotic strain pdp 11 was able to improve stress tolerance to high stocking density in gilthead seabream, *Sparus auratus* (Varela *et al.,* 2010). Unlike the probiotics, prebiotics are non-digestible carbohydrates that are utilised by specific health-promoting bacteria, which improve the nutrition and health of the host (Ringø *et al.,* 2010). Among the prebiotics evaluated for use in aquafeed industry, prebiotic fructooligosaccharide along with mannanoligosaccharides are well established (Ganguly *et al.* 2010). Research into stress ameliorating properties of prebiotics in aquaculture is very recent and is limited. Supplementation of prebiotic, fructooligosaccharide (FOS) at 3% inclusion level was able to ameliorate salinity stress in Caspian roach, *Rutilus rutilus* fry (Soleimani *et al.,* 2012).

In view of the earlier research on effect of nitrite induced stress in fin fishes and potential application of probiotics and prebiotics for ameliorating such stressor in the culture environment, the present research was undertaken to evaluate the potential of probiotic strain *B. circulans* and prebiotic fructooligosaccharide as stress mitigating agent against sub lethal nitrite stress in *L. rohita* fingerlings in culture.

Materials and Methods

Experimental fish and maintenance

Labeo rohita fingerlings (*n*=324, average weight = 11.50 ± 0.05 g) were procured from a commercial fish farm (Prem Fisheries Consultancy, Gujarat, India) and transported live to the aquaculture facility of the Central Institute of fisheries Education

(CIFE), Mumbai. Fishes were first given a prophylactic treatment (0.15 ppm $KMnO_4$ dip treatment) and acclimatised to the laboratory condition for 15 days and were fed daily with a commercially available feed (30% protein; Godrej Agrovet Pvt. Ltd., Andhra Pradesh, India) during acclimation. Rectangular fibre reinforced plastic (FRP) tubs of 75 L capacity were used for the experiment. Round the clock aeration was given to maintain the water quality.

Probiotic strain and culture

Probiotic strain *Bacillus circulans,* which was isolated from intestine of *Catla catla* was supplied in lyophilized form from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. Bacteria were inoculated into nutrient broth (Himedia), incubated in BOD incubator for 24 h at 30 °C and colonies were cultured on nutrient agar (Himedia). Colonies from the nutrient agar were sub cultured in nutrient broth. The purity of the isolates of *B. circulans* was confirmed using biochemical test kits (HiBacillus ™ Identification Kit, Himedia, Mumbai, India). Pure culture of *B. circulans* was mass cultured for subsequent experimental use. For inclusion in the feed, the bacterial culture was centrifuged at 10,000 × *g* for 20 min at 4°C, the pellet were washed three times with phosphate buffer saline (PBS, pH 7.2), suspended in PBS and quantified by spread plate technique. The bacterial suspension was diluted with PBS to the required concentrations and added to 100 g of feed.

Quantification of inoculums

To determine the concentrations of the microbial probiotic inoculums to be added into the feed for the experiments, *B. circulans* was streaked in nutrient agar plates and incubated for 12 h at 30 °C. One freshly grown colony was picked up and transferred into 50 mL broth and incubated under the same conditions for 4 h. A third transfer for each bacterium was carried out into 100 ml under same conditions. Then, optical density (OD) of the microbial samples was recorded at 600 nm. Simultaneously, the serial dilutions were performed for each hour. The dilutions were plated onto the agar by spread plate technique. After 12 h of incubation at 28 °C, the colonies were counted. The data were related in graphs, obtaining the relationship cfu versus OD_{600} versus time. Based on this, the required probiotic microorganisms were added to the feed at different concentrations.

Feed ingredients

The prebiotic used in this study (fructooligosaccharide) was obtained from DPO Foods Specialities Private Limited, Thane, Mumbai, India. The minimum level of fructans guaranteed by the manufacturer was 93% and the other components mainly consist of glucose, fructose and sucrose. Other ingredients used in the experimental feed preparation were supplied by Himedia, Mumbai, India.

Experimental diet preparation

Nine iso-caloric (1674.52 - 1702.029 kcal 100 g^{-1}) and iso-nitrogenous (30.07-30.58% crude protein) purified diets were prepared with graded levels of probiotic *B. circulans* and prebiotic fructooligosaccharide (FOS). The diets were control (0% BC or FOS), T1 (10^6 cfu/g BC + 0% FOS), T2 (10^8 cfu/g BC), T3 (1% FOS) T4 (2% FOS), T5 (10^6 cfu/g BC + 1% FOS), T6 (10^6 cfu/g BC + 2% FOS) , T7 (10^8 cfu/g BC + 1% FOS) and T8 (10^8 cfu/g BC + 2% FOS) (Table 1). Diets were prepared using a single screw pelletizer. The required ingredients were mixed with water and oil to make dough followed by cooking in an autoclave at 15 psi for 20 min. After cooling, vitamins and minerals were added. For incorporation of prebiotics, required amount of FOS was dissolved in distilled water and blended with the mixed ingredient. Finally, the dough was pressed through a hand pelletizer to get uniform size pellets (2 mm) and sun dried for 4 h. For incorporation of probiotics, suspended culture of *B. circulans* was sprayed over the extruded pellets (with or without FOS). The probiotic was sprayed over the feed at two

concentrations of 1×10^6 cfu/g and 1×10^8 cfu/g. The pellets were dried under aseptic conditions for 24 h and stored at 4 °C.

Table 1. Formulation and composition of the experimental diets (100 g feed) using graded levels of *Bacillus circulans* and fructooligosaccharide (FOS)

Ingredient	Treatment (T) no. (*B.circulans*/FOS supplementation level expressed in cfu. g^{-1} / %)								
	Control (0/0)	T1 (0/1)	T2 (0/2)	T3 (10^6/0)	T4 (10^6/1)	T5 (10^6/2)	T6 (10^8/0)	T7 (10^8/1)	T8 (10^8/2)
Casein	42	42	42	42	42	42	42	42	42
Dextrin	15	15	15	15	15	15	15	15	15
Cellulose	10	9	8	10	9	8	10	9	8
Starch	10	10	10	10	10	10	10	10	10
Gelatin	8	8	8	8	8	8	8	8	8
Cod liver oil	5	5	5	5	5	5	5	5	5
Sunflower oil	3	3	3	3	3	3	3	3	3
CMC	2	2	2	2	2	2	2	2	2
Mineral Premix [a]	2	2	2	2	2	2	2	2	2
Vitamin Premix [b]	2	2	2	2	2	2	2	2	2
BHC	1	1	1	1	1	1	1	1	1
Prebiotic[c]	0.00	1.00	2.00	0.00	1.00	2.00	0.00	1.00	2.00
Probiotic[d]	0.00	0.00	0.00	$1x10^6$	$1x10^6$	$1x10^6$	$1x10^8$	$1x10^8$	$1x10^8$
BHT	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Proximate composition (expressed in % wet weight basis)									
Moisture	8.34±0.32	8.11±0.20	8.67±0.26	8.08±0.20	8.33±0.27	8.25±0.25	8.42±0.19	8.49±0.22	8.52±0.21
Crude protein	30.07±0.34	30.45±0.28	30.24±0.18	30.58±0.31	30.33±0.21	30.26±0.22	30.04±0.12	30.34±0.24	30.05±0.11
Ether extract	8.21±0.34	8.26±0.23	8.31±0.33	8.36±0.39	8.58±0.31	8.48±0.42	8.55±0.39	8.38±0.41	8.29±0.26
Carbohydrate	52.34±0.53	53.02±0.36	52.76±0.63	53.25±0.72	54.02±0.48	53.32±0.52	53.21±0.69	52.54±0.38	53.65±0.66

[1] g/kg: $KAl(SO4)_2$ 0.159; $CaCO^3$ 18.101; $CoCl_2$; 0.07; KCl 16.553; Ferric citrate ($5H_2O$) 1.338; $MnSO_4$.H2O 0.07; KI 0.014; $ZnSO_4$ 0.192; NaH_2PO_4 13.605; $CuSO_4.5H_2O$ 0.075

[2] per kg: thiamine hydrochloride 10 mg; riboflavin 20 mg; calcium pantothenate 40 mg; nicotinic acid 50 mg; pyridoxine hydrochloride 10 mg; folic acid 5 mg; inositol 400 mg; choline chloride 2000 mg; menadione 10 mg; cholecalciferol 1500 IU; biotin 1 mg; vitamin B12 0.02 mg; vitamin A 3000 IU; vitamin E 50 IU; vitamin C 200 mg

[3] *Bacillus circulans* obtained as lyophilized cell from MTCC, Chandigarh, India

[4] Prebiotic fructooligosaccharide obtained from DPO Foods, Thane, India

Proximate composition of experimental diets

The proximate composition of the experimental diets was performed as prescribed by AOAC 1995. Proximate compositions of different experimental diets are given in Table 1.

Experimental design

The experimental design consisted of nine treatment groups (control, T1, T2, T3, T4, T5, T6, T7 and T8) each with three replicates following a completely randomized design (CRD). Twelve healthy and uniformly sized *L. rohita* fingerlings were introduced into each of different experimental tanks and were fed with the prepared diets for 60 days. From the 60^{th} day onwards the fishes were exposed to sub lethal nitrite stress (1/5 LC_{50}=2.08 mg/L) for a period of 30 days. A positive (control with stressor) C (P) and negative (control without stressor) control C (N) was used in the study to compare the stress ameliorating effect of both probiotic *B. circulans* and prebiotic FOS used singly or in combination. The 96 hr LC_{50} value for this species was found to be 10.4 mg/L in an earlier experiment conducted. The required nitrite level in the tanks was maintained with sodium nitrite (Qualigens Fine Chemicals, India) from a stock solution (based on methods

by Das *et al.,* 2004) that was prepared earlier. The chloride levels in the experimental tanks were checked before the experiment to ensure that the water is chloride free.

Feeding trial

The triplicate groups of fishes were fed with the test diets twice daily (once at 0900 hr and 1700 hr). Feeding rate was done at 3% of body weight. Water temperature in the tanks was maintained at 26 ± 1^0C. Water exchange was done once in three days while daily siphoning was carried out. Water quality parameters were monitored once every three days.

Growth parameters and percentage survival

At the end of the experiment growth and survival of the fishes were analysed. Growth and feed utilization were monitored and survival was recorded as percentage survival (Table 2).

Table 2. Growth, survival, feed conversion ratio, pre and post hematological parameters, serum lysozyme and respiratory burst activity (NBT) of immune enhanced *Labeo rohita* fed *Bacillus circulans* and fructooligosaccharide (FOS) and subsequent exposure to sub-lethal nitrite stress (means ± SE; n = 6).

	Diet (Bacillus circulans/fructooligosaccharide)									
	0/0	*0/0**	*0/1*	*0/2*	*10^6/0*	*10^6/1*	*10^6/2*	*10^8/0*	*10^8/1*	*10^8/2*
Weight gain (g)	4.518±0.12^{a}	4.034±0.17^{a}	5.488±0.09^{b}	4.367±0.24^{a}	6.483±0.28^{c}	6.445±0.13^{c}	6.547±0.22^{c}	6.335±0.13^{c}	5.413±0.19^{b}	5.583±0.07^{b}
Specific Growth Rate (SGR)	0.781±0.01^{a}	0.781±0.01^{a}	0.781±0.01^{a}	0.781±0.01^{a}	0.781±0.01^{a}	0.781±0.01^{a}	0.781±0.01^{a}	0.781±0.01^{a}	0.781±0.01^{a}	0.781±0.01^{a}
Feed Conversion Ratio (FCR)	2.973±0.02^{ab}	2.973±0.02^{ab}	2.973±0.02^{ab}	2.973±0.02^{ab}	2.973±0.02^{ab}	2.973±0.02^{ab}	2.973±0.02^{ab}	2.973±0.02^{ab}	2.973±0.02^{ab}	2.973±0.02^{ab}
Survival (%)	93.33±4.21^{c}	93.33±4.21^{c}	93.33±4.21^{c}	93.33±4.21^{c}	93.33±4.21^{c}	93.33±4.21^{c}	93.33±4.21^{c}	93.33±4.21^{c}	93.33±4.21^{c}	93.33±4.21^{c}
TLC (x10^3µl^{-1})										
Pre-challenge	13.41±0.47^{a}	13.81±0.17^{b}	13.41±0.47^{a}	13.81±0.17^{b}	13.41±0.47^{a}	13.81±0.17^{b}	13.41±0.47^{a}	13.81±0.17^{b}	13.41±0.47^{a}	13.81±0.17^{b}
Post-challenge	13.45±0.36^{aA}	15.33±0.16^{aB}	13.45±0.36^{aA}	15.33±0.16^{aB}	13.45±0.36^{aA}	15.33±0.16^{aB}	13.45±0.36^{aA}	15.33±0.16^{aB}	13.45±0.36^{aA}	15.33±0.16^{aB}
TEC (x10^4 µl^{-1})										
Pre-challenge	60.72±0.62^{b}	60.34±0.62^{bc}	60.72±0.62^{b}	60.34±0.62^{bc}	60.72±0.62^{b}	60.34±0.62^{bc}	60.72±0.62^{b}	60.34±0.62^{bc}	60.72±0.62^{b}	60.34±0.62^{bc}
Post-challenge	60.34±0.57^{aB}	47.45±0.57^{aA}	60.34±0.57^{aB}	47.45±0.57^{aA}	60.34±0.57^{aB}	47.45±0.57^{aA}	60.34±0.57^{aB}	47.45±0.57^{aA}	60.34±0.57^{aB}	47.45±0.57^{aA}
Hb (g dl^{-1})										
Pre-challenge	4.20±0.03^{b}	4.24±0.03^{b}	4.20±0.03^{b}	4.24±0.03^{b}	4.20±0.03^{b}	4.24±0.03^{b}	4.20±0.03^{b}	4.24±0.03^{b}	4.20±0.03^{b}	4.24±0.03^{b}
Post-challenge	4.22±0.05^{aB}	3.57±0.04^{aA}	4.22±0.05^{aB}	3.57±0.04^{aA}	4.22±0.05^{aB}	3.57±0.04^{aA}	4.22±0.05^{aB}	3.57±0.04^{aA}	4.22±0.05^{aB}	3.57±0.04^{aA}
Hct (%)										
Pre-challenge	17.38±0.62^{b}	17.04±0.31^{d}	17.38±0.62^{b}	17.04±0.31^{d}	17.38±0.62^{b}	17.04±0.31^{d}	17.38±0.62^{b}	17.04±0.31^{d}	17.38±0.62^{b}	17.04±0.31^{d}
Post-challenge	17.43±0.34^{aB}	13.36±0.38^{aA}	17.43±0.34^{aB}	13.36±0.38^{aA}	17.43±0.34^{aB}	13.36±0.38^{aA}	17.43±0.34^{aB}	13.36±0.38^{aA}	17.43±0.34^{aB}	13.36±0.38^{aA}
Serum lysozyme (U.mL^{-1})										
Pre-challenge	6.33±0.41^{a}	6.41±0.38^{a}	6.33±0.41^{a}	6.41±0.38^{a}	6.33±0.41^{a}	6.41±0.38^{a}	6.33±0.41^{a}	6.41±0.38^{a}	6.33±0.41^{a}	6.41±0.38^{a}
Post-challenge	6.34±0.21^{a}	7.23±0.32^{a}	6.34±0.21^{a}	7.23±0.32^{a}	6.34±0.21^{a}	7.23±0.32^{a}	6.34±0.21^{a}	7.23±0.32^{a}	6.34±0.21^{a}	7.23±0.32^{a}
Respiratory burst activity (NBT) (OD_{540})										
Pre-challenge	0.288±0.00^{a}	0.293±0.00^{a}	0.288±0.00^{a}	0.293±0.00^{a}	0.288±0.00^{a}	0.293±0.00^{a}	0.288±0.00^{a}	0.293±0.00^{a}	0.288±0.00^{a}	0.293±0.00^{a}
Post-challenge	0.289±0.00^{a}	0.312±0.00^{a}	0.289±0.00^{a}	0.312±0.00^{a}	0.289±0.00^{a}	0.312±0.00^{a}	0.289±0.00^{a}	0.312±0.00^{a}	0.289±0.00^{a}	0.312±0.00^{a}

Data expressed as mean±SE, Mean values (n = 3) in a column under each category bearing different superscript (lower case) vary significantly ($p < 0.05$). Mean values (n = 3) in a row under each parameter bearing different superscript (capital) vary significantly ($p < 0.05$).

Weight gain = Final weight- Initial weight
Specific growth rate = [*ln* (final weight)-*ln* (initial weight)/experimental days] × 100
Feed conversion ratio (FCR) = Feed given (dry weight)/body weight gain (wet weight)
Survival (%) = [Total number of animal harvested/ Total number stocked] × 100

Haematological parameters assay

Blood and serum collection

Six fish were randomly collected from each treatment tanks and the control. The fishes were anesthetized by immersion in clove oil emulsion. Whole blood was collected from the caudal vein of each fish using a 1 mL syringe and 28-guage needles that were rinsed in 2.7% EDTA solution, to determine the various parameters. Blood was pooled by tank thus yielding three replicates (each pooled from six fish) per treatment. For serum preparation, blood was collected without anti-coagulant and allowed to clot for another 2 h and centrifuged at 3,000 x *g* for 5 min and stored at -80°C for further analysis.

Haematological parameters

Total erythrocyte and leucocyte were counted in a haemocytometer using erythrocyte and leucocyte diluting fluids (Qualigens, India), respectively. Twenty microlitres of blood were mixed with 3980 µL of diluting fluid in a clean glass test tube. The mixture was 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:

Number of cells/mL = (Number of cells counted x dilution)/ (Area counted x depth of fluid

The haemoglobin percentage was determined by estimating cyanmethaemoglobin using Drabkin's fluid (Qualigens, India). Five millilitres of Drabkin's working solution was taken in a clean and dry test tube and 20 ml of blood were added to it. The absorbance was measured using a spectrophotometer (MERCK, Nicolet, evolution 100) at a wavelength of 540 nm. The final concentration was calculated by comparing with standard cyanmethaemoglobin (Qualigens, India). For hematocrit assay, hematocrit capillary tubes were two-third filled with the whole blood and centrifuged in a hematocrit centrifuge for 5 min and the percentage of the packed cell-volume was determined by the hematocrit tube reader (Smith, 1967).

Respiratory burst activity

Nitroblue tetrazolium assay (as a function of respiratory burst) was done by the method of Secombes (1990) as modified by Stasiack and Baumann (1996). 50 µL of blood was placed into the wells of 'U' bottom microtitre plates and incubated at $37^{0\ C}$ for 1 h to facilitate adhesion of cells. Then the supernatant was removed and the loaded wells were washed three times in PBS. After washing, 50 µL of 0.2% NBT was added and plate was incubated for further 1 h. The cells were then fixed with 100% methanol for 2-3 minutes and again washed thrice with 30% methanol. The plates were then air dried. 60 µL of 2N potassium hydroxide and 70 µL of dimethyl sulphoxide (DMSO) were added into each well to dissolve the formazon blue precipitate formed. The OD of the turquoise blue colored solution was then read in a plate reader at 540 nm.

Serum lysozyme activity

The lysozyme activity was measured using the turbidity assay. Chicken egg lysozyme (Sigma) was used as a standard and 0.2 mg/mL lyophilized *Micrococcus lysodeikticus* in 0.04 M sodium phosphate buffer (pH 5.75) was used as substrate. Fifty µL of serum was added to 2ml of the bacterial suspension and the reduction in the absorbance at 540 nm was determined after 0.5 and 4.5 min incubation at 22^{0}C. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 min^{-1}.

Statistical analysis

All the data were analysed using SPSS version 16.0 for windows. One-way analysis of variance was used to compare all the treatments. Significant difference between two means was measured by Duncan's multiple range test. All the differences were considered significant at $p < 0.05$ and the results are presented as mean ± standard error (SE).

Results

Growth performance and feed utilization parameters of immune enhanced *Labeo rohita* fingerlings after exposure to 30 days sub-lethal nitrite stress are presented in Table 2. Highest weight gain and SGR were observed in T5 (6.547±0.22; 0.982±0.01) and lowest in C (P) (4.034±0.17; 0.725±0.01) which was significantly different ($p < 0.05$). Highest FCR was observed in C (P) (3.782±0.04) and lowest in T5 (2.336±0.02). Survival percentage was highest in T4 (93.33±2.38) and lowest in C (P) (71.25±1.34).

Pre and post challenge haematological parameters viz. total leucocyte count (TLC), total erythrocyte count (TEC), haemoglobin (Hb) and hematocrit (Hct) value of immune enhanced *L.rohita* exposed to sub-lethal nitrite stress is presented in Table 3. TLC increased following exposure and was significant ($p < 0.05$) in groups C (P) and T2. Pre and post challenge TLC was highest in T4 (15.73±0.56; 16.34±0.18) and lowest in C (N) (13.41±0.47; 13.81±0.17). TEC decreased following exposure and was significant ($P<0.05$) in groups C (P), T2, T7 & T8. Pre challenge TEC was highest in T5 (67.23±0.55) and lowest in C (P) (60.34±0.57) where post challenge TEC was highest in T5 (62.22±0.89) and lowest in C (P) (47.45±0.57). Blood Hb decreased following exposure and was significant in groups C (P) and T2. Pre and post challenge Hb was highest in T4 (5.44±0.05; 5.31±0.04) and lowest in C (P) (4.20±0.05; 3.57±0.04). Following exposure blood Hct value decreased and was significant ($p < 0.05$) in groups C (P), T2, T6, T7 & T8. Pre challenge value was highest in T4 (19.13±0.39) and lowest in C (N) (17.38±0.62) whereas post challenge value was highest in T4 (16.78±0.37) and lowest in C (P) (13.36±0.38).

Pre and post challenge respiratory burst activity of *L. rohita* before and after exposure to 30 days sub-lethal nitrite stress is presented in Table 4. Respiratory burst activity increased following stress exposure and was significant in all groups except C (N), C (P), T1 & T2. Pre and post activity was highest in T4 (0.413±0.00; 0.436±0.00) and lowest in C (N) (2.88±0.00; 0.293±0.00). Pre and post challenge serum lysozyme activity of *L. rohita* after challenge with sub-lethal nitrite stress is presented in Table 4. Serum lysozyme increased post challenge with stressor and was significant in all groups except C (N), C (P), T1 & T2. Pre challenge lysozyme activity was highest in T4 (9.45±0.42) and lowest in C (N) (6.41±0.38) whereas post challenge activity was highest in T5 (11.51±0.15) and lowest in C (N) (6.41±0.38).

Discussion

As reported earlier by many workers, fishes under high environmental nitrite levels suffer from physiological stress leading to poor growth affecting the overall biomass production. In the present study, fishes exposed to sub-lethal nitrite stress were able to withstand the stressful condition and no significant retardation in growth was observed. Fishes fed a combination of 10^6 cfu/g and 2% FOS exhibited best growth. The result of the present study shows that both probiotics and prebiotics are able to enhance growth of fish under nitrite stress. Whole cell yeast or yeast subcomponent supplementation in diet of *Ictalurus punctatus* exhibits better growth of fish after

challenge with nitrite induced stressor (Welker *et al.,* 2007) which was in agreement to this study. Survival percentage in the present study was higher in fishes fed FOS, *B. circulans* and in combination which strongly exerts a positive correlation (r^2=0.876). Lower survival in 2% FOS group may be due to higher stress exhibited at this level of FOS supplementation. The combined effect of probiotic and prebiotics is defined as synbiotics (Cerezuela *et al.,* 2011). There are no earlier report of stress ameliorating effects of both probiotics and prebiotics in combination in terms of survival of fishes. However, Ren *et al.* (2010) better survival of red sea bream, *Pagrus major* was observed in salinity induced stressor after feeding vitamin C and phospholipid that worked synergistically. Possible explanation to this finding may be due to immune enhancement through dietary supplementation.

Haematological variables viz. TLC, TEC, Hb and Hct value were enhanced following dietary supplementation of *B. circulans* and FOS. RBC count decreased following challenge in all groups. Increase and decrease in total WBC and RBC after nitrite stress is in agreement with earlier studies (Madison *et al.,* 2006). Marked variation in WBC and RBC count before and after challenge in control, FOS fed group (T1 & T2) and 10^6 cfu/g *B. circulans* fed along with 1% and 2% FOS (T7 & T8) indicates elevated stress in these groups. Reduce Hct level was due to shrinkage in RBC due to nitrite exposure as reported by Siikavuopio and Saether, 2006). Significant ($p < 0.05$) individual effects of both probiotics and prebiotics were observed. However, in this study dietary interaction between *B. circulans* and FOS was not significant. This suggests that there was no synergistic effect due to combined use of these two supplements. The effect observed may an additive effect due to individual components. In the present study, higher respiratory burst activity was observed in all treatment groups except 2% FOS (T2) group before and after sub-lethal nitrite exposure. Increase in respiratory burst activity post challenge was observed in all groups which were in agreement with previous study (Andrews *et al.,* 2011). Increased activity can be correlated with increased bacterial pathogen killing activity of phagocytes (Sharp and Secombes, 1993). The respiratory burst activity of phagocytes, measured by reduction of NBT by intracellular superoxide radicals produced by leucocytes, was higher in fish fed combination of both probiotics and prebiotics. Higher doses of FOS (2%) show lesser activity which explains immunosuppressive when given in higher doses. Lysozyme activity of serum before and after acid water exposure was significantly higher ($p < 0.05$) in T4 (10^6 cfu/g *B.circulans* +1% FOS) group conferring better response against stressor. 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 (Alexander and Ingram, 1992). Our finding was well supported by those of Chand *et al.* (2006) who reported enhanced immune function of *Macrobrachium rosenbergii* exposed to nitrite stress. The possible mechanism involved in ameliorating nitrite induced stress in the present investigation implies to better health of the fish species through synbiotic application in both indoor and outdoor culture systems.

Conclusion

From the overall study, it can be concluded that dietary supplementation of probiotic *Bacillus circulans* and prebiotic fructooligosaccharide used singly or in combination was able to confer immuno-protection to *Labeo rohita* fingerlings after subsequent exposure to sub-lethal nitrite stress paving ways for nutritional intervention for better health management under severe stress in the aquaculture species.

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Declaration of Interest

There are no conflicts related to the research study and preparation of the manuscript. Authors are solely responsible for any kind of conflict of interest.

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