Indian J. Fish., 68(4): 82-92, 2021
DOI: 10.21077/ijf.2021.68.4.92225-10

Evaluation of the effect of mono or multi-microbe probiotics on growth, serum enzymes and non-specific immune parameters in rohu *Labeo rohita* (Hamilton, 1822) fingerlings

PUSHPA CHOUDHARY[1], RAKESH DAS[1,3], SHAILESH SAURABH[1], SWETA PRADHAN[1], PRIYABRAT SWAIN[1], S. S. MISHRA[1] AND B. K. DAS[2]

[1]*ICAR-Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar - 751 002, Odisha, India*
[2]*ICAR-Central Inland Fisheries Research Institute, Monirampur (Post), Barrackpore Kolkata - 700 120, West Bengal, India*
[3]*Faculty of Veterinary Medicine, Norwegian University of Life Sciences (NMBU), Universitetstunet 3, 1433 As, Norway*
e-mail: pushpafpm@gmail.com

ABSTRACT

An experiment of 60 days duration was conducted to evaluate the effect of mono or multi-microbe probiotics on growth, enzymatic profile and immune response in rohu *Labeo rohita* (Hamilton, 1822) fingerlings. Two hundred-forty numbers of uniformly sized rohu fingerlings were randomly distributed in four treatment groups *viz,* C (control feed without probiotics); T1 (control feed + *Bacillus subtilis* AN11); T2 (control feed + *Clostridium bifermentans* CPSS2) and T3 (control feed + *B. subtilis* AN11 and *C. bifermentans* CPSS2), following a completely randomised design. Probiotic bacterial strains were incorporated in feed separately as well as in combination, at a rate of 10^7 CFU g^{-1} of feed. Results showed that fish fed diet containing a combination of two probiotic bacteria showed significant ($p<0.05$) increase in percent weight gain, specific growth rate, feed utilisation, superoxide dismutase (SOD) activity, total serum protein, albumin, globulin and non-specific immune parameters as compared to other treatment groups and control. Furthermore, a significant reduction ($p<0.05$) in alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) levels were observed in the probiotic fed group as compared to the control fish. Results of the study would be of use in delineating the potential of mono or multi-microbe probiotics for better health management of farmed fish and to boost the overall production in aquaculture systems.

Keywords: Aquaculture, Enzymatic profiles, Indian major carps, Non-specific immunity, Probiotics

Introduction

The Indian major carp *Labeo rohita* (Hamilton, 1822), commonly known as rohu is the most popular species in carp polyculture for its better compatibility with other species, excellent growth, consumer preference and high marketability. The establishment of successful hatchery technology and production of rohu seed in captivity motivated farmers to intensify the farming of this species. However, due to the switching of cultural practices in an intensive manner, the occurrence of diseases in culture ponds increased, causing substantial losses to fish production from aquaculture. The safe application of antibiotics or any chemotherapeutic agents is often advocated for combating the outbreaks of diseases. However, the recent concerns about potential environmental and food contamination and the reported increase in resistance among different pathogens to antibiotics, together with complete banning of antibiotics that promotes the growth of fish in several countries, have enlightened the scientific body to search for substances that can be used as alternatives to antibiotics; while maintaining the productivity of culture system at optimum levels (Seal *et al.,* 2013; Park *et al*., 2016).

Nowadays, the judicious application of probiotics is getting importance for the maintenance of healthy immunity and physiological upkeep in humans including animals like poultry, pigs, cattle and fish (Choi *et al*., 2011; Giannenas *et al*., 2012; Newaj-Fyzul *et al*., 2014; Nordeste *et al*., 2017; Markowlak and Slizewska, 2018; Stefanaki *et al*., 2018). The probiotics which are used in lieu of antibiotics or complementary approach to vaccination or chemotherapy to prevent aquatic animal disease outbreak has generated lots of interest (Mehrim 2009; Merrifield *et al*., 2010; Dawood *et al*., 2015). "Probiotics are single or mixed cultures of live microorganisms that, when supplied in suitable numbers, impart a health benefit on the host" (FAO/WHO, 2001). The application of probiotics and its efficacy for commercially viable aquaculture depends on various factors *viz,* source, dose, type of probiotics, mode of action, strains of probiotics, method and duration of administration (Saurabh *et al*., 2005; De *et al.,* 2014; Dawood *et al*., 2015; Hai, 2015; Banerjee and Ray, 2017). Several studies have demonstrated that supplementation

of *Bacillus* and *Clostridium* in the form of probiotics in the diet could enhance growth, digestibility of nutrients, blood profile, intestinal microbial balance, body immunity and reduce the foul smell of excreta (Min *et al*., 2004; Chen *et al*., 2006; Baruah *et al*., 2016; Lan *et al*., 2016; Soto 2017). Leja *et al*. (2013) had shown the ability of *Clostridium bifermentans* strains to biosynthesise lactic acid in various environmental conditions. Further, there are not enough studies on the use of two or more microorganisms as a probiotic and their effects on body growth, digestibility of nutrients and immunity of fish (Park *et al.,* 2016). Standardisation of an appropriate and efficient strain of probiotic or probiotics consortium is yet to be done for sustainable production of cultured rohu *L. rohita* across the country. Considering all these, the current study has been designed to evaluate the effects of mono or multi-probiotics using the bacterial strains *Bacillus subtilis* AN11 and *C. bifermentans* CPSS2 on growth, enzyme profile and immune responses of rohu, *L. rohita*. Further, this forms the first wet laboratory study or investigation on the probiotic effect of two spore-forming beneficial bacterial strains on fish growth and immune response.

Materials and methods

Experimental fish

Healthy fingerlings of rohu *L. rohita* having no symptoms of disease (evaluated by gross examination of the skin, fins and gills of respective samples) and with a mean body weight of 29±5 g were obtained from Kausalyaganga fish farm, Bhubaneswar, India and maintained in freshwater for 2 weeks in 500 l FRP tanks with continuous aeration. All the fishes were fed basal diet at 3% body weight during the acclimatisation period. About 30% of the water in all FRP tanks was exchanged every day. The basic water quality parameters were measured systematically at seven days interval to maintain optimal levels (pH 7.4-7.8, dissolved oxygen 5.5-6.8 mg l^{-1}, free carbon dioxide 2.3-2.7 mg l^{-1}, total alkalinity 130-155 mg l^{-1}, ammonia-N 0.04-0.05 mg l^{-1} and nitrate-N 0.046 mg l^{-1}) throughout the experimental period. The water temperature in the tanks varied from 28 to 30ºC during the experiment.

Source of probiotic strains

Pure cultures of *Bacillus subtilis* AN11 (GenBank accession no. JX860845) previously isolated from sediment samples of Bhitarkanika mangrove forest and *C. bifermentans* CPSS2 (GenBank accession No. KT367517) isolated from pond sediments of ICAR-Central Institute of Freshwater Aquaculture (ICAR-CIFA) and maintained in soft agar as well at -80^{0}C in glycerol stocks, in the Fish Health Management Division of ICAR-CIFA, were used for the present experiment. Both the strains have shown probiotic potential against common fish pathogenic bacteria in previous *in vitro* laboratory studies (Das *et al*., 2014; Baruah *et al.,* 2016). The bacterial cell density was calculated spectrophotometrically at OD 600 nm using overnight broth cultures (both strains) after centrifugation at 2500 *g* for 10 min and washed twice with phosphate buffered saline (PBS, pH 7.4) and values were correlated with colony-forming unit (CFU) by following standard serial dilution and spread plating on respective agar medium.

Experimental feed preparation

Raw materials (fish meal, soya meal, ground nut oil cake, rice bran, corn flour, oil and vitamin-mineral mixture) were procured from the near-by fish farm and suitably processed aseptically in the feed laboratory of ICAR-CIFA. The composition of basal feed is shown in Table 1. All the ingredients were mixed appropriately to maintain the crude protein level at ~30%. The dough was prepared and pressure cooked for 15-20 mins, cooled to 37ºC) and then the live bacterial cell suspensions as well as vitamin-mineral mixture were added.

Table 1. Composition of experimental and control feeds (g kg^{-1})

Ingredients	Ingredient composition per kg feed (g)	T1	T2	T3
Fish meal	50	50	50	50
Soy meal	280	280	280	280
GNOC	260	260	260	260
Rice bran	200	200	200	200
Corn flour	150	150	150	150
Oil (ml)	40	40	40	40
Vitamin and mineral mixture[a]	20	20	20	20
	Basal feed as control feed	*B. subtilis*AN11 @ 10^7 CFU g^{-1}	*C. bifermentans* CPSS2 @ 10^7 CFU g^{-1}	*B. subtilis*AN11 and *C. bifermentans* CPSS2 @ 10^7 CFU g^{-1}

[a] Vitamin and mineral mixture: Procured from Sarabhai Chemicals Ltd., India.
Composition: Vit. A - 500,000 IU; vit D3 - 100,000 IU; vit B2 - 0.2 g; vit E - 75 U; vit K - 0.1 g; Calcium pantothenate - 0.25 g; Nicotinamide - 1.0 g; vit. B12 - 0.6 g; Choline chloride -15 g; Calcium -75 g; Manganese - 2.75 g; Iodine - 0.1 g; Iron - 0.75 g; Zinc - 1.5 g.

The supplemented bacteria's survival in the diets was checked following storage at 4°C and at room temperature (28-30°C) respectively on weekly basis throughout the feeding trial period (Irianto and Austin, 2002). In brief, 1 g of the prepared diet was sufficiently homogenised in 9 ml of sterile saline solution (NSS) and serial dilutions were carried out down to 10^{-4} from where 0.1 ml (100 µl) was spread onto nutrient agar (HiMedia, India) plate in each time period, in triplicates. The colonies were counted after incubation for 24 h at 30°C. At the end, based on the survivability data, feeds were prepared on weekly basis to ensure the desired probiotic levels in the diet.

Experimental design

Two hundred-forty numbers of uniformly sized rohu fingerlings (average weight : 29±5 g) were randomly distributed in four treatment groups *viz*, C (control feed without probiotics); T1 (control feed + *B. subtilis* AN11 @ 10^7 CFU g^{-1}); T2 (control feed + *C. bifermentans* CPSS2 @ 10^7 CFU g^{-1}) and T3 (control feed + combination of *B. subtilis* AN11 and *C. bifermentans* CPSS2 @ 10^7 CFU g^{-1}) in 200 l FRP tanks containing 20 fish per tank with three replicates following a completely randomised design (CRD). Fish were fed twice daily (at 09.00 and 16.00 hrs) @3% average body weight (ABW). Unconsumed and left-over feed was siphoned out manually on each alternate day and 10-15% of tank water was exchanged.

Growth performance

Fifteen fish were randomly selected from each tank at day 0, day 30 and day 60 respectively during the feeding trial for estimation of growth parameters. The growth performances were assessed in terms of percent weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FCR), employing the following formulae:

Percent weight gain = (Wf - Wi) / Wi*100

SGR (%/day) = 100 X (lnWf - lnWi) / t

FCR = FI / (Wf - Wi)

where Wf and Wi are final and initial weight of fish, respectively; t is the duration of feeding (in days) and FI is the feed intake.

Sampling

Sampling was carried out on the 30th and 60th day of the feeding trial and five fish from each tank was randomly sampled and weighed for assessing growth parameters. The sampled fishes were then anesthetised using clove oil (50 µl l^{-1}) and blood was drawn from the caudal vein using 2 ml disposable syringes rinsed with heparin (50 IU ml^{-1} of blood) as well as without heparin. The blood samples collected with anticoagulants were immediately processed for analysing immune parameters. The non-heparinised blood samples were transferred to microcentrifuge tubes and after coagulation of blood; tubes were kept at 4^0C for 4 h and then centrifuged at 3000 *g* for 5 min to separate serum. The serum samples were collected and stored at -80°C until further use.

Enzyme assays

Super oxide dismutase (SOD) (E.C.1.15.1.1)

The SOD activity in the fish sera samples was assessed as per the manufacturer's instructions mentioned in the SOD assay kit (Sigma-Aldrich, USA), where percentage reaction inhibition rate of the enzyme with WST-1 (water soluble tetrazolium dye) substrate and xanthine oxidase were used. The rate of reaction was monitored by absorbance at 450 nm after 20 min of incubation time at 37°C.

Alkaline phosphatase (ALP) (E.C. 3.1.3.1)

Alkaline phosphatase activity in the fish sera was assessed using the ALP assay kit (BioVision Inc., USA) by strictly following the manufacturer's instructions. Here p-nitophenyl phosphate (pNPP) is used as a phosphatase substrate which turns yellow (λ_{max} = 405 nm) when dephosphorylated by ALP.

Lactate dehydrogenase (LDH) (L-lactate NAD + oxidoreductase; E.C.1.1.1.27)

The lactate dehydrogenase activity in the fish serum samples was analysed using LDH assay kit (Sigma-Aldrich, USA) as per manufacturer's instructions. LDH reduces NAD to NADH, which is specifically detected spectrophotometrically at 340 nm.

Non-specific immune parameters

Total protein, albumin and globulin

The total protein content of serum was measured as per Bradford (1976) and albumin content according to Doumas *et al.* (1971). By subtracting albumin from total protein, the globulin content was calculated.

Respiratory burst activity

The reduction of nitro blue tetrazolium (NBT) by intracellular superoxide radicals was assessed according to Anderson and Siwicki (1994) with slight modification. Briefly, 50 µl of heparinised blood from each experimental group of fish was blended with 50 µl of 0.2% NBT (Sigma, USA) solution. After that, the mixture was incubated for 30 min at 25°C, followed by the addition of 50 µl of above prepared mixture to 1 ml of N, N diethymethylformamide (Qualigens, India). Finally, the entire mixture was centrifuged at 6000 *g* for 5 min and the optical density of the supernatant was read at 540 nm.

Serum lysozyme activity

Lysozyme assay utilising lyophilised *Micrococcus lysodeikticus* (Sigma, USA) was done as per Ellis (1990). Briefly, *M. lysodeikticus* solution was prepared at a concentration of 0.6 mg ml^{-1} (in 0.02 M sodium citrate buffer) from where about 130 µl was added to a mixture containing 10 µl fish serum samples and 10 µl of 0.02 M sodium citrate buffer aseptically. The initial optical density (O.D) was read at 450 nm immediately after the addition of the bacterial solution. After that, samples were incubated at 24°C for 1 h followed by the reading of OD at 450 nm. A standard curve was prepared using a mixture of 20 µl working standard and 130 µl of *M. lysodeikticus* solution. Lysozyme activity was expressed in units ml^{-1} where one unit is defined as the decrease in absorbance of 0.001 min^{-1}.

Myeloperoxidase activity

The total myeloperoxidase activity in fish serum was measured according to Quade and Roth (1997) with slight modification. Briefly, fish serum was diluted by mixing of about 15 µl of fish serum in 135 µl of Hank's balanced salt solution (Ca^{2+}, Mg^{2+} free), after that 50 µl of 20 mM of 3, 3', 5, 5'- tetra methyl benzidine and 5 mM of hydrogen peroxide were added in to it. The prepared mixture was incubated for about 2 min at room temperature, finally, the reaction was stopped by the addition of 4 M H_2SO_4 followed by estimation of optical density at 450 nm.

Serum bactericidal activity (SBA)

The serum bactericidal activity assay was performed as per the method of Rao *et al*. (2006). Briefly, young bacterial culture (18-20 h) of *Aeromonas hydrophila* was centrifuged and the pellet was harvested by washing in phosphate buffered saline (PBS, pH 7.4) and resuspended in PBS .The optical density of the suspension was adjusted to 0.5 at 546 nm. After that, this bacterial suspension was serially diluted (1:10) with PBS five times. The serum bactericidal activity was determined by incubating 2 ml of the diluted bacterial suspension with 20 ml of the serum in a micro-vial for 1 h at 37°C. In the case of control group, PBS replaced the serum. Finally, the number of viable bacteria was determined by plating on nutrient agar plates followed by incubation at 37°C for 24 h.

Statistical analysis

The results generated by conducting series of experiments were statistically analysed by standard statistical tools *i.e.* Statistical Package for the Social Sciences (SPSS version 16.0; SPSS Inc., Chicago, IL, USA) in which data were subjected to one-way ANOVA and Duncan's multiple range test (Duncan, 1955) was used to determine the significant differences between the means. Further, the comparisons were made at 5% probability level. All the assays were performed in triplicate and values are presented as mean±SE.

Results and discussion

Growth performance

The growth performance of *L. rohita* after 30 and 60 days of feeding with control diet or diets containing probiotics alone or in combination is given in Table 2. The final weight, percent weight gain and SGR were significantly higher ($p<0.05$) in all treatment groups (T1, T2, T3) as compared to control at both time points. Among treatment groups, T3 showed significantly higher ($p<0.05$) percent weight gain and SGR followed by T2 and T1. The percent weight gain increased by 101.51 and 127.07% in T3 followed by 98.37 and 120.97% in T2 and 95.52 and 110.16 in T1 respectively after 30 and 60 days of feeding. Further, FCR decreased significantly in all treatments (T1, T2 and T3) compared to the control group both in 30 and 60 days of feeding. At the end of the experiment the SGR and FCR of T3 diet were 1.37 and 1.33 respectively which were maximum and minimum among all the groups. The present findings are in line with several studies where the researchers made observations on increase in growth performance of fish upon multispecies probiotics supplementation in fish feed, some of these are cited here: Wang (2011) for *Ctenopharyngodon idella*; Gupta *et al*. (2014) for *Cyprinus carpio*; Beck *et al.* (2015) for *Paralichthys olivaceus*; Giannenas *et al*. (2015) for *Oncorhynchus mykiss*; Aly *et al*. (2008) and Standen *et al*. (2016) for *Oreochromis niloticus* and Thy *et al*. (2017) for *Pangasianodon hypophthalmus.* Furthermore, Lara-Flores *et al*. (2003) reported that supplementation of combined *Saccharomyces faecium* and *Lactobacillus acidophilus* or *Saccharomyces cerevisiae* singly in tilapia diets containing 27 or 40% crude protein produced higher weight gain and feed utilisation efficiency compared with the control group. In addition to the above findings, a recent study by Xia *et al.* (2018) reported improved growth performance and feed utilisation efficiency of Nile tilapia when fed with *L. rhamnosus* JCM1136 and *L. lactis* subsp. *lactis* JCM5805. The results of this study suggest that different mixture of probiotics when supplemented with diet for 60 days, the respective treatment group showed better weight gain, SGR and low FCR which might be due to better digestion of nutrients and their absorbance in the body as compared to untreated ones.

Enzyme assays

Superoxide dismutase (SOD)

The SOD concentration of serum increased significantly ($p<0.05$) in all the treatment groups as

Table 2. Growth performance of *L. rohita* fingerlings after 30 and 60 days of feeding with the control and experimental diets

		C	T1	T2	T3
0-30 days	Initial weight (g)	29.32 ± 0.31^{a}	29.28 ± 0.35^{a}	29.56 ± 0.26^{a}	29.73 ± 0.41^{a}
	Final weight (g)	56.45 ± 0.21^{a}	57.25 ± 0.26^{ab}	58.64 ± 0.36^{b}	59.91 ± 0.45^{c}
	Weight gain (%)	92.53 ± 0.22^{a}	95.52 ± 0.29^{b}	98.37 ± 0.37^{b}	101.51 ± 0.42^{b}
	SGR %	2.18 ± 0.024^{a}	2.23 ± 0.028^{b}	2.28 ± 0.034^{b}	2.33 ± 0.041^{c}
	FCR	0.97 ± 0.025^{a}	0.95 ± 0.032^{a}	0.93 ± 0.042^{b}	0.92 ± 0.028^{b}
0-60 days	Initial weight (g)	29.32 ± 0.31^{a}	29.28 ± 0.35^{a}	29.56 ± 0.36^{a}	29.73 ± 0.42^{a}
	Final weight (g)	61.62 ± 0.41^{a}	63.54 ± 0.45^{b}	65.32 ± 0.52^{c}	67.51 ± 0.54^{d}
	Weight gain (%)	110.16 ± 0.47^{b}	117.01 ± 0.48^{b}	120.97 ± 0.51^{c}	127.07 ± 0.51^{b}
	SGR %	1.23 ± 0.051^{a}	1.29 ± 0.021^{b}	1.32 ± 0.034^{c}	1.37 ± 0.042^{d}
	FCR	1.57 ± 0.032^{c}	1.48 ± 0.054^{b}	1.42 ± 0.061^{b}	1.33 ± 0.071^{a}

Different superscripts in the same row indicate significance difference ($p<0.05$) amongst different treatments. Values are expressed as mean±SE (n = 15). C - Control; T1 - *B. subtilis*AN11; T2 - *C. bifermentans* CPS222; T3 - *B. subtilis*AN11 and *C. bifermentans* CPSS2

compared to control. It was significantly higher ($p<0.05$) in T3 followed by T1 and T2 (Table 3). The SOD concentration increased by 8.08 and 7.35% as compared to control after 30 and 60 days of feeding respectively. Superoxide dismutase is an important antioxidant enzyme that regulates levels of superoxide anions during defense and has a significant impact on inflammation (Marikovsky *et al*. 2003). It catalyses the dismutation of the highly reactive $^{-}O_2$ to less reactive H_2O_2 and functions in the main antioxidant defense pathways in response to oxidative stress (Fridovich, 1995). The activity of SOD in serum can reflect the competence of clearing the free radicals and the severity of cell impairment. The present findings agree with observations of Cha *et al*. (2013) who found elevated serum SOD activity in *Paralichthys olivaceus* when fed with *Bacillus pumilus* and *Bacillus licheniformis*. Similarly, Giri *et al*. (2014) used probiotic *Bacillus subtilis* VSG1 singly or in combination with *Lactobacillus plantarum* VSG3 or/ and *Pseudomonas aeruginosa* VSG2 for *L. rohita* and found increased SOD activity. On the contrary, Thy *et al.* (2017) reported that SOD activities in striped catfish *Pangasianodon hypopthalmus* were not stimulated by *Bacillus amyloliquefaciens* 54A and *B. pumilus* 47B after 90 days of feeding and also in white shrimp *Penaeus vannamei* fed *B. subtilis* E20 for 98 days (Tseng *et al*., 2009). Such variation of SOD activities might be due to the varied concentration of probiotic bacteria in the feed and duration of probiotic application.

Alkaline phosphatase (ALP) and Lactate dehydrogenase (LDH) activity

Levels of ALP and LDH were found significantly varied ($p<0.05$) with dietary probiotic supplementation (Table 3). The ALP and LDH concentration with respect to all treatment groups (T1, T2 and T3) showed a decreasing trend after 30 and 60 days of feeding trial. Alkaline phosphatase is a key enzyme that may defend the body during the early phases of stress, as well as during skin regeneration and pathogenic infection (Fast *et al*., 2002; Sheikhzadeh *et al.*, 2012). Lactate dehydrogenase (LDH) is an oxidoreductase enzyme which helps in catalysis of the interconversion of pyruvate and lactate (Soetan *et al*., 2010). Upon tissue damage or red blood cell haemolysis, LDH is released into the bloodstream. LDH isozyme profiles were the first isozyme profiles used in clinical veterinary medicine to detect specific organ damage (Kaneko, 1989). Mohapatra *et al*. (2014) also reported less ALP activity upon feeding of probiotic supplemented diet to *L. rohita*. Furthermore, decreased levels of serum ALP activities in multi-species probiotic supplemented group suggested that there was no adverse effect on any of the important internal organs or any kind of pathological processes such as liver impairment, kidney dysfunction and

Table 3. Effect of probiotics supplementation on serum enzymes of *L. rohita* fingerlings

Treatment	30 days			60 days		
	SOD (activity %)	ALP (U l^{-1})	LDH (mU ml^{-1})	SOD (activity %)	ALP (U l^{-1})	LDH (mU ml^{-1})
C	71.46 ± 0.98^{a}	6.32 ± 0.05^{d}	7054.00 ± 19.84^{d}	72.08 ± 0.39^{a}	5.36 ± 0.17^{c}	6600 ± 62.99^{c}
T1	74.04 ± 0.78^{b}	1.83 ± 0.03^{a}	552.04 ± 0.45^{a}	74.90 ± 0.49^{b}	2.10 ± 0.14^{a}	554.80 ± 5.38^{a}
T2	72.32 ± 0.63^{ab}	3.43 ± 0.13^{b}	1568.00 ± 22.80^{b}	73.70 ± 0.30^{b}	2.74 ± 0.02^{b}	5549.80 ± 53.01^{b}
T3	77.24 ± 1.74^{c}	4.18 ± 0.07^{c}	5191.60 ± 98.46^{c}	77.38 ± 0.46^{c}	2.56 ± 0.02^{b}	5390.8 ± 86.52^{b}

Different superscripts in the same column indicate significance difference ($p<0.05$) amongst different treatments. Values are expressed as mean±SE (n = 15). C - control; T1 - *B. subtilis*AN11; T2 - *C. bifermentans* CPS222; T3 - *B. subtilis*AN11 and *C. bifermentans* CPSS2; SOD - Superoxide dismutase; ALP - Alkaline phosphatase; LDH - Lactate dehydrogenase

bone disease. In contrary, Bandyopadhyay and Mohapatra (2009) observed that *B. circulans* PB7 supplemented feed when fed to *L. rohita* juveniles resulted in higher ALP and ACP activity. Similarly, Guardiola *et al*. (2014) found that dietary administration of *B. subtilis* was responsible for the increase of ALP levels in the skin mucus of gilthead sea bream.

Non-specific immune parameters

Total protein, albumin and globulin

Total serum protein increased significantly ($p<0.05$) in T1 and T3 as compared to control. Among the treatment groups, the highest serum protein was shown in T3 followed by T1 and T2 at both the time points. Serum albumin and globulin concentrations were found to be significantly high ($p<0.05$) in all the groups fed with probiotics on both the sampling days (Table 4). After 30 and 60 days, the total protein content, albumin as well as globulin levels of serum from all treatment groups was significantly higher than that of the control group. Similar increment in the serum protein, albumin as well as globulin levels was observed in rohu (*L. rohita*) fed with *B. subtilis* supplemented diet (Nayak *et al*., 2007). Das *et al*. (2013) also reported an increase in serum and mucus protein in catla when fed with *B. amyloliquefaciens* supplemented diets for 4 and 8 weeks. Recently Lin *et al*. (2017) reported significantly higher protein contents in the muscle of Asian seabass *Lates calcarifer* fed mixture of probiotics. In this study, we noticed an increase in serum protein, albumin, and globulin levels, which is thought to be linked to a higher innate response in fishes (Wiegertjes *et al*., 1996). Increase in total protein content in serum validated the fact that these defense molecules were synthesised in ample quantity because of the synergistic effects of two bacterial strains.

Respiratory burst activity

The respiratory burst activity (RBA) was significantly higher ($p<0.05$) in T1, T2 and T3 diet at both time points as compared to control (Fig. 1a). Fish groups fed T3 diet supplemented with a combination of microbes showed highest RBA activity after 30 and 60 days of feeding. Increase in respiratory burst activity by dietary supplementation of multispecies probiotics combination has been reported in *Oreochromis niloticus* (Aly *et al*., 2008; Zhou *et al*., 2010), *C. carpio* (Gupta *et al*., 2014) and *O. mykiss* (Giannenas *et al*., 2015). In another study, Sharifuzzaman *et al*. (2011) reported that subcellular components of probiotics *Kocuria* SM1 and *Rhadococcus* SM2 increased the respiratory burst activity in rainbow trout against *Vibrio anguillarum*. Over the decades, in mammals, the respiratory burst activity which generates reactive oxygen radicals, is well characterised in macrophages/monocytes and granulocytes (Dalmo *et al*., 1997). Furthermore, this process is believed to be important in killing microbes (unspecified manner). In this function, phagocytes produce large quantities of superoxide anion during phagocytosis or upon stimulation. Hence, the NBT reduction product obtained after reaction with superoxide is a very reliable indicator of the health status or the effectiveness of immunisation in fish (Anderson *et al*., 1992). Therefore, the increase in oxygen radical production in the blood of probiotic supplemented fish might be due to gradual activation of phagocytes, in turn leading to enhanced immunity of fish.

Serum lysozyme activity

The serum lysozyme activity was significantly higher ($p<0.05$) in T3 treatment groups supplemented with combination of microbes as compared to control at both 30 and 60 days of feeding (Fig. 1b). This finding is in accordance with the observations made by Thy *et al.* (2017) who found higher lysozyme activity in striped catfish *P. hypophthalmus* fed mixed probiotic spores of *B. amyloliquefaciens* 54A and *B. pumilus* 47B. Similarly, olive flounder when fed probiotic mixture containing *L. lactis* BFE 20 and *L. plantarum* FGL0001 showed enhanced skin mucus lysozyme activity compared to flounder fed a single probiotic agent or control diet (Beck *et al*., 2015). In another study, Ramesh *et al*. (2015) reported that cellular components and viable cells of probiotics *B. licheniformes* and *B. pumilus* increased the activity of lysozyme in *L. rohita* whereas subcellular components of probiotics *Kocuria* SM1 and *Rhadococcus* SM2 increased the lysozyme activity in rainbow trout (*O. mykiss*) (Sharifuzzaman *et al*., 2011). Generally,

Table 4. Effect of probiotics supplementation on total serum protein, albumin and globulin in *L. rohita* fingerlings

Treatment	30 days			60 days		
	Total serum protein (g dl^{-1})	Albumin (g dl^{-1})	Globulin (g dl^{-1})	Total serum protein (g dl^{-1})	Albumin (g dl^{-1})	Globulin (g dl^{-1})
C	2.28 ± 0.07^{a}	0.53 ± 0.01^{a}	1.75 ± 0.07^{a}	2.37 ± 0.03^{a}	0.57 ± 0.03^{a}	1.80 ± 0.19^{a}
T1	2.64 ± 0.02^{b}	0.62 ± 0.03^{b}	2.02 ± 0.04^{b}	2.70 ± 0.03^{b}	0.72 ± 0.14^{b}	1.97 ± 0.04^{b}
T2	2.33 ± 0.02^{a}	0.58 ± 0.01^{ab}	2.09 ± 0.01^{b}	2.45 ± 0.02^{a}	0.62 ± 0.01^{a}	1.82 ± 0.018^{a}
T3	2.80 ± 0.03^{c}	0.71 ± 0.03^{c}	2.12 ± 0.09^{b}	2.83 ± 0.08^{c}	0.78 ± 0.03^{b}	2.04 ± 1.88^{b}

Different superscripts in the same column indicate significance difference ($p<0.05$) amongst different treatments. Values are expressed as mean±SE (n = 15). C - Control; T1 - *B. subtilis*AN11; T2 - *C. bifermentans* CPS222; T3 -, *B. subtilis*AN11 and *C. bifermentans* CPSS2

lysozyme is an important defense molecule which takes part in non-specific immunity of host (animals) that disrupts bacterial cell walls by shattering glycosidic connections in the peptidoglycan layers of bacteria. Further, it acts specifically on the cell wall of Gram positive bacteria and on the inner peptidoglycan layer of Gram negative bacteria after complement and other enzymes have disrupted the outer cell wall (Yano, 1996; Saurabh and Sahoo, 2008). Hence, the present finding suggest that multi-species probiotic might stimulate leucocytes to produce increased levels of lysozymes and lead to activation of phagocytic cells and complement system to protect the host against wide range of pathogenic bacteria.

Myeloperoxidase activity

The total myeloperoxidase (MPO) content of serum increased significantly ($p<0.05$) in all the treatment groups than that of control group at both the time points (Fig. 1c). It was significantly higher ($p<0.05$) in T3 followed by T1 and T2. In general, the release of myeloperoxidase enzyme mostly by the azurophilic granules of neutrophils during oxidative respiratory burst activity, as evaluated by the serum peroxidase level, plays a key role in antimicrobial activity. During a respiratory burst, hydrogen peroxide is used to make hypochlorous acid (Dalmo *et al*., 1997). The reduction in the activity may be the indication of contaminants or stress (Anderson and Siwicki, 1994). Significant increase in myeloperoxidase activity was found in *C. carpio* fed *B. coagulans*, *B. licheniformis* and *P. polymyxa* supplemented diets after 80 days of probiotics feeding (Gupta *et al*., 2014). On the contrary, myeloperoxidase activity was not affected in Senegalese sole, *Soles senegalensis* fed mono and multi-species probiotics (Batista *et al*., 2015).

Serum bactericidal activity (SBA)

The serum bactericidal activity, in all the probiotic treated groups against *A. hydrophila* was significantly higher than in the untreated control group (Table 5). However, the group which was given a mixture of *B. subtilis* AN11 and *C. bifermentans* CPSS2 showed higher bactericidal activity ($p<0.05$) than the other probiotic treated groups. Similarly, Aly *et al*. (2008) observed significant increase in bactericidal activity of

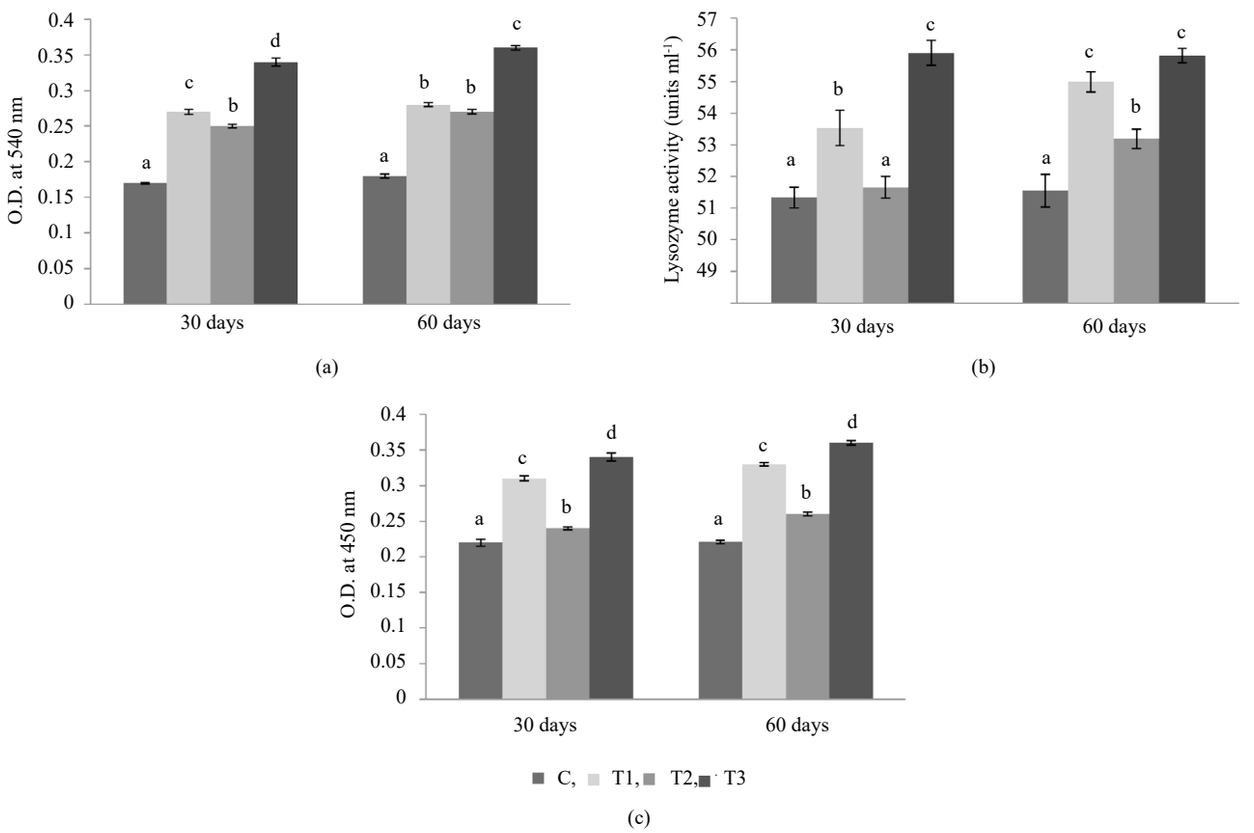

Fig. 1: (a) Respiratory burst activity, (b) Serum lysozyme activity and (c) Myeloperoxidase activity of *L. rohita* fingerlings treated with mono or multi-microbe probiotics. Values are expressed as mean±SE (n = 15). Treatments bearing different superscripts differ significantly ($p<0.05$). C - control; T1 - *B. subtilis*AN11; T2 - *C. bifermentans* CPS222; T3 - *B. subtilis* AN11 and *C. bifermentans* CPSS2

Table 5. Serum bactericidal activity of *L. rohita* fingerlings against pathogenic bacteria *A. hydrophila* after feeding probiotics for 30 and 60 days

Treatment	30 days (No. of bacterial colonies)	60 days (No. of bacterial colonies)
C	468.80±4.74[d]	482.8±2.06[d]
T1	271.00±2.88[b]	279.8±2.42[b]
T2	328.40±4.63[c]	335.60±4.58[c]
T3	253.80±2.06[a]	251.80±3.00[a]

Different superscripts in the same column indicate significance difference ($p<0.05$) amongst different treatments. Values are expressed as mean±SE (n = 15). C - Control; T1 - *B. subtilis*AN11; T2 - *C. bifermentans* CPS222; T3 - *B. subtilis*AN11 and *C. bifermentans* CPSS2

tilapia fed with *B. subtilis* and *L. acidophilus* after one month of feeding trial. The administration of *B. subtilis* in feed of *L. rohita* and *C. catla* had resulted in increase in serum bactericidal activity (Kumar *et al*., 2008; Kumar *et al*., 2015). The higher bactericidal activity observed might be due to increased secretion of lysosomal enzymes.

In conclusion, the present study has revealed that dietary administration of *B. subtilis* AN11 and *C. bifermentans* CPSS2 in combination, significantly ($p<0.05$) improved growth, enzymatic activity and non-specific immunity in *L. rohita* fingerlings after 60-days of feeding trial. Hence, further studies may be conducted in outdoor facilities to investigate the overall health impact including physiological health parameters in *L. rohita* to ascertain the effect of application of such probiotic bacteria in aquaculture systems.

Acknowledgements

All the authors are grateful to the Director, ICAR-CIFA, Bhubaneswar, for providing facilities including wet laboratory to carry out the present investigation. All the authors are also thankful to ICAR, New Delhi, for providing financial support in the form of Institutional project (Pr. No. I-88-B).

References

Aly, S. M., Ahmed, Y. A., Ghareeb, A. A. and Mohamed, M. F. 2008. Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of *Tilapia nilotica* (*Oreochromis niloticus*) to challenge infections. *Fish Shellfish Immunol.,* 25: 128-136. http://dx.doi: 10.1016/j.fsi.2008.03.013.

Anderson, D. P., Moritomo, T. and de Grooth, R. 1992. Neutrophil, glass-adherent, nitroblue tetrazolium assay gives early indication of immunization effectiveness in rainbow trout. *J. Vet. Immunol. Immunopathol*., 30: 419-429. https://doi.org/10.1016/0165-2427(92)90110-C.

Anderson, D. P. and Siwicki, A. K. 1994. Duration of protection against *Aeromonas salmonicida* in brook trout immunostimulated with glucan or chitosan by injection or immersion. *Progressive Fish Culturist*, 56: 258-261.

Bandyopadhyay, P. and Mohapatra, P. K. 2009. Effect of a probiotic bacterium *Bacillus circulans* PB7 in the formulated diets: on growth, nutritional quality and immunity of *Catla catla* (Ham.). *J. Fish Physiol. Biochem.*, 35: 467-478. https://doi.org/10.1007/s106 95-008-9272-8.

Banerjee, G. and Ray, A. K. 2017. The advancement of probiotics research and its application in fish farming industries. *Vet. Sci. Res. J.,* 115: 66-77. https://doi: 10.1016/j.rvsc.2017.01.016.

Baruah, A., Das, R., Kumar, S., Roy, P., Sahu, A., Panda, S. P., Mishra, S. S. and Das, B. K. 2016. A novel and effective C*lostridium bifermentans* CPSS2 against selected fish pathogenic bacteria. *Int. J. Adv. Res.,* 4: 2140-2150. https://doi: 10.21474/IJAR01/1700.

Batista, S., Ramos, M. A., Cunha, S., Barros, R., Cristovao, B., Rema, P., Pires, M. A., Valente, L. M. and Ozorio R. O. 2015. Immune responses and gut morphology of Senegalese sole (*Solea senegalensis*, Kaup 1858) fed monospecies and multispecies probiotics. *Aquac. Nutr.,* 21: 625-634. https://doi.org/10.1111/anu.12191.

Beck, B. R., Kim, D., Jeon, J., Lee, S. M., Kim, H. K., Kim, O. J., Lee, J. I., Suh, B. S., Do, H. K., Lee, K. H. and Holzapfel, W. H. 2015. The effects of combined dietary probiotics *Lactococcus lactis* BFE920 and *Lactobacillus plantarum* FGL0001 on innate immunity and disease resistance in olive flounder (*Paralichthys olivaceus*). *Fish Shellfish Immunol.*, 42: 177-183. https://doi: 10.1016/j.fsi.2014.10.035.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.*, 72: 248-254. https://doi.org/10.1016/0003-2697(76)90527-3.

Cha, J. H., Rahimnejad, S., Yang, S. Y., Kim, K. W. and Lee, K. J. 2013. Evaluations of *Bacillus* spp. as dietary additives on growth performance, innate immunity and disease resistance of olive flounder (*Paralichthys olivaceus*) against *Streptococcus iniae* and as water additives. *Aquaculture,* 402: 50-57. https://doi.org/10.1016/j.aquaculture.2013.03.030.

Chen, Y. J., Min, B. J., Cho, J. H., Kwon, O. S., Son, K. S., Kim, H. J. and Kim, I. H. 2006. Effects of dietary *Bacillus*-based probiotic on growth performance, nutrients digestibility, blood characteristics and fecal noxious gas content in finishing pigs. *Asian-Australas J. Anim. Sci.,* 19: 587-592. https://doi.org/10.5713/ajas.2006.587.

Choi, J. Y., Shinde, P. L., Ingale, S. L., Kim, J. S., Kim, Y. W., Kim, K. H., Kwon, I. K. and Chae, B. J. 2011. Evaluation of multi-microbe probiotics prepared by submerged liquid or solid substrate fermentation and antibiotics in weaning pigs. *Livest. Sci.,* 138: 144-151. https://doi.org/10.1016/j.livsci.2010.12.015.

Dalmo, R. A., Ingebrigtsen, K. and Bogwald, J. 1997. Non-specific defence mechanisms in fish, with particular reference to the reticuloendothelial system (RES). *J. Fish. Dis.*, 20: 241-273. https://doi.org/10.1046/j.1365-2761.1997.00302.x.

Das, A., Nakhro, K., Chowdhury, S. and Kamilya, D. 2013. Effects of potential probiotic *Bacillus amyloliquifaciens* FPTB16 on systemic and cutaneous mucosal immune responses and disease resistance of catla (*Catla catla*). *Fish Shellfish Immunol.,* 35: 1547-1553. https://doi.org/10.1016/j.fsi.2013.08.022.

Das, B. K., Neha Nidhi, R. G., Roy, P., Muduli, A. K., Swain, P., Mishra, S. S. and Jayasankar, P. 2014. Antagonistic activity of cellular components of *Bacillus subtilis* AN11 against bacterial pathogens. *Int. J. Curr .Microbiol. App. Sci.,* 3: 795-809.

Dawood, M. A., Koshio, S., Ishikawa, M. and Yokoyama, S. 2015. Interaction effects of dietary supplementation of heat-killed *Lactobacillus plantarum* and β-glucan on growth performance, digestibility and immune response of juvenile red sea bream, *Pagrus major*. *Fish Shellfish Immunol.*, 45: 33-42. https://doi.org/10.1016/j.fsi.2015.01.033.

De, B. C., Meena, D. K, Behera, B. K., Das, P., Mohapatra, P. D. and Sharma, A. P. 2014. Probiotics in fish and shellfish culture: immunomodulatory and ecophysiological responses. *Fish Physiol. Biochem.*, 40: 921-971. DOI 10.1007/s10695-013-9897-0.

Doumas, B. T., Watson, W. A. and Biggs, H. G. 1971. Albumin standards and the measurement of serum albumin with bromocresol green. *Clinica Chimica Acta*, 31: 87-96. https://doi.org/10.1016/0009-8981(71)90365-2.

Duncan, D. B. 1955. Multiple range and multiple F tests. *Biometrics,* 11: 1-42. https://doi.10.2307/3001478.

Ellis, A. E. 1990. Lysozyme assays. In: Stolen, J. S., Fletcher, T. C., Anderson, D. P., Roberson, B. S. and Van Muiswinkel, W. B. (Eds.), *Techniques in fish immunology*, SOS Publications, Fair Haven, USA, p. 101-103.

FAO/WHO, 2001. Health and nutritional properties of probiotics in food including powder milk with liver lactic acid bacteria. *Report of a Joint FAO/WHO Expert Consultation*, Cordoba, Argentina. Food and Agriculture Organisation of the United Nations, Rome, Italy and World Health Organisation, Geneva, Switzerland, 34 pp. http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf?ua=1.

Fast, M. D., Sims, D. E., Burka, J. F., Mustafa, A. and Ross, N. W. 2002. Skin morphology and humoral non-specific defence parameters of mucus and plasma in rainbow trout, coho and Atlantic salmon. *Comp. Biochem. Physiol.*, *Part A*, 132: 645-657. https://doi.org/10.1016/S1095-6433(02)00109-5.

Fridovich, I. 1995. Superoxide radical and superoxide dismutases. *Ann. Rev. Biochem.*, 64: 97-112.

Giannenas, I., Karamaligas, I., Margaroni, M., Pappas, I., Mayer, E., Encarnaca, P. and Karagouni, E. 2015. Effect of dietary incorporation of a multi-strain probiotic on growth performance and health status in rainbow trout (*Oncorhynchus mykiss*). *Fish Physiol. Biochem.*, 41: 119-128. https://doi. 10.1007/s10695-014-0010-0.

Giannenas, I., Papadopoulos, E., Tsalie, E., Triantafillou, E. L., Henikl, S., Teichmann, K. and Tontis, D. 2012. Assessment of dietary supplementation with probiotics on performance, intestinal morphology and microflora of chickens infected with *Eimeria tenella*. *Vet. Parasitol.*, 188: 31-40. https://doi.org/10.1016/j.vetpar.2012.02.017.

Giri, S. S., Sukumaran, V., Sen, S. S. and Jena, P. K. 2014. Effects of dietary supplementation of potential probiotic *Bacillus subtilis* VSG 1 singularly or in combination with *Lactobacillus plantarum* VSG 3 or/and *Pseudomonas aeruginosa* VSG 2 on the growth, immunity and disease resistance of *Labeo rohita*. *Aquac. Nutr.,* 20: 163-171. https://doi.org/10.1111/anu.12062.

Guardiola, F. A., Cuesta, A., Arizcun, M., Meseguer, J. and Esteban, M. A. 2014. Comparative skin mucus and serum humoral defence mechanisms in the teleost gilthead seabream (*Sparus aurata*). *Fish Shellfish Immunol.,* 36: 545-551. https://doi.org/10.1016/j.fsi.2014.01.001.

Gupta, A., Gupta, P. and Dhawan, A. 2014. Dietary supplementation of probiotics affects growth, immune response and disease resistance of *Cyprinus carpio* fry. *Fish Shellfish Immunol.*, 41: 113-119. https://doi.org/10.1016/j.fsi.2014.08.023.

Hai, N. V. 2015. Research findings from the use of probiotics in tilapia aquaculture: A review. *Fish Shellfish Immunol.*, 45: 592-597. https://doi.org/10.1016/j.fsi.2015.05.026.

Irianto, A. and Austin, B. 2002. Probiotics in aquaculture. *J. Fish Dis.*, 25: 633-642. https://doi.org/10.1046/j.1365-2761.2002.00422.x.

Kaneko, J. J. 1989. *Clinical biochemistry of domestic animals.* 4th edn. Academic Press Inc., New York, USA.

Kumar, R., Mukherjee, S. C., Ranjan, R. and Nayak, S. K. 2008. Enhanced innate immune parameters in *Labeo rohita* (Ham.) following oral administration of *Bacillus subtilis*. *Fish Shellfish Immunol.*, 24: 168-172. https://doi.org/10.1016/j.fsi.2007.10.008.

Kumar, R., Mukherjee, S. C., Ranjan, R., Vani, T., Brahmachari, R. K. and Nayak, S. K. 2015. Effect of dietary supplementation of *Bacillus subtilis* on haematological and immunological parameters of *Catla catla* (Hamilton). *Aquac. Int.*, 23: 1275-1292. https://doi.10.1007/s10499-015-9883-x.

Lan, R. X., Lee, S. I. and Kim, I. H. 2016. Effects of multistrain probiotics on growth performance, nutrient digestibility, blood profiles, faecal microbial shedding, faecal score and noxious gas emission in weaning pigs. *J. Anim. Physiol. Nutr.*, 100: 1130-1138. https://doi.org/10.1111/jpn.12501.

Lara-Flores, M., Olvera-Novoa, M. A., Guzman-Mendez, B. E. and Lopez-Madrid, W. 2003. Use of the bacteria *Streptococcus faecium* and *Lactobacillus acidophilus* and the yeast *Saccharomyces cerevisiae* as growth promoters in Nile tilapia (*Oreochromis niloticus*). *Aquaculture,* 216: 193-201. https://doi.org/10.1016/S0044-8486(02)00277-6.

Leja, K., Myszka, K. and Czaczyk, K. 2013. The ability of *Clostridium bifermentans* strains to lactic acid biosynthesis in various environmental conditions. *Springer Plus,* 2:44. https://doi.org/10.1186/2193-1801-2-44.

Lin, H. L., Shiu, Y. L., Chiu, C. S., Huang, S. L. and Liu, C. H. 2017. Screening probiotic candidates for a mixture of probiotics to enhance the growth performance, immunity and disease resistance of Asian seabass, *Lates calcarifer* (Bloch), against *Aeromonas hydrophila*. *Fish Shellfish Immunol.,* 60: 474-482. https://doi.org/10.1016/j.fsi.2016.11.026.

Marikovsky, M., Ziv, V., Nevo, N., Harris-Cerruti, C. and Mahler, O. 2003. Cu/Zn superoxide dismutase plays important role in immune response. *J. Immunol.,* 170(6): 2993-3001. DOI: https://doi.org/10.4049/jimmunol. 170.6. 2993.

Markowiak, P. and Slizewska, K. 2018. The role of probiotics, prebiotics and synbiotics in animal nutrition. *Gut Pathogens*, 10: 21. https://doi.org/10.1186/s13099-018-0250-0.

Mehrim, A. I. 2009. Effect of dietary supplementation of Biogen (Commercial probiotic) on mono-sex Nile tilapia *Oreochromis niloticus* under different stocking densities. *J. Fish. Aquat. Sci.*, 4: 261-173.

Merrifield, D. L., Bradley, G., Baker, R. T. and Davies, S. J. 2010. Probiotic applications for rainbow trout (*Oncorhynchus mykiss* Walbaum) II. Effects on growth performance, feed utilisation, intestinal microbiota and related health criteria post-antibiotic treatment. *Aquac. Nutr.*, 16: 496-503. https://doi.org/10.1111/j.1365-2095.2009.00688.

Min, B. J., Kwon, O. S., Son, K. S., Cho, J. H., Lee, W. B., Kim, J. H., Park B. C. and Kim, I. H. 2004. The effect of *Bacillus* and active yeast complex supplementation on the performance, fecal *Bacillus* counts and ammonia nitrogen concentrations in weaned pigs. *J. Anim. Sci.*, 82(Suppl. 1): 26. https://doi.org/10.1016/j.livsci.2008.04.018.

Mohapatra, S., Chakraborty, T., Prusty, A. K., Pani Prasad, K. and Mohanta, K. N. 2014. Beneficial effects of dietary probiotics mixture on hemato-immunology and cell apoptosis of *Labeo rohita* fingerlings reared at higher water temperatures. *PloS One,* 9(6): e100929. doi:10.1371/journal.pone.0100929.

Nayak, S. K., Swain, P. and Mukherjee, S. C. 2007. Effect of dietary supplementation of probiotic and vitamin C on the immune response of Indian major carp, *Labeo rohita* (Ham.). *Fish Shellfish Immunol.*, 23: 892-896. https://doi.org/10.1016/j.fsi.2007.02.008.

Newaj-Fyzul, A., Al-Harbi, A. H. and Austin, B. 2014. Developments in the use of probiotics for disease control in aquaculture. *Aquaculture,* 431: 1-11. https://doi.org/10.1016/j.aquaculture.2013.08.026.

Nordeste, R., Tessema, A., Sharma, S., Kovac, Z., Wang, C., Morales, R. and Griffiths, M. W. 2017. Molecules produced by probiotics prevent enteric colibacillosis in pigs. *BMC Vet. Res.,* 13: 335. https://doi.org/10.1186/s12917-017-1246-6.

Park, Y., Moniruzzaman, M., Lee, S., Hong, J., Won, S., Lee, J. M., Yun, H., Kim, K. W., Ko, D. and Bai, S. C. 2016. Comparison of the effects of dietary single and multi-probiotics on growth, non-specific immune responses and disease resistance in starry flounder, *Platichthys stellatus*. *Fish Shellfish Immunol.,* 59: 351-357. https://doi.org/10.1016/j.fsi.2016.11.006.

Quade, M. J. and Roth, J. A. 1997. A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. *Vet. Immunol. Immunopathol.*, 58: 239-248. https://doi.org/10.1016/S0165-2427(97)00048-2.

Ramesh, D., Vinothkanna, A., Rai, A. K. and Vignesh, V. S. 2015. Isolation of potential probiotic *Bacillus* spp. and assessment of their subcellular components to induce immune responses in *Labeo rohita* against *Aeromonas hydrophila*. *Fish Shellfish Immunol.*, 45(2): 268-276. https://doi.org/10.1016/j.fsi.2015.04.018.

Rao, Y. V., Das, B. K., Jyotyrmayee, P. and Chakrabarti, R. 2006. Effect of *Achyranthes aspera* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. *Fish Shellfish Immunol.*, 20: 263-273. https://doi.org/10.1016/j.fsi.2005.04.006.

Saurabh, S., Choudhary, A. K. and Sushma, G. S. 2005. Concept of probiotics in aquaculture. *Fishing Chimes,* 25(4): 23-26.

Saurabh, S. and Sahoo, P. K. 2008. Lysozyme: An important defence molecule of fish innate immune system. *Aquac. Res.*, 39: 223-239. https://doi.org/10.1111/j.1365-2109.2007.01883.x.

Seal, B. S., Lillehoj, H. S., Donovan, D. M. and Gay, C. G. 2013. Alternatives to antibiotics: a symposium on the challenges and solutions for animal production. *Anim. Health Res. Rev.*, 14(1): 78-87. https://doi.org/10.1017/S1466252313000030.

Sharifuzzaman, S. M., Abbass, A., Tinsley, J. W. and Austin, B. 2011. Subcellular components of probiotics *Kocuria*SM1 and *Rhodococcus* SM2 induce protective immunity in rainbow trout (*Oncorhynchus mykiss*, Walbaum) against *Vibrio anguillarum*. *Fish Shellfish Immunol.*, 30: 347-353. https://doi.org/10.1016/j.fsi.2010.11.005.

Sheikhzadeh, N., Heidarieh, M., Pashaki, A. K., Nofouzi, K., Farshbafi, M. A. and Akbari, M. 2012. Hilyses®,

fermented *Saccharomyces cerevisiae*, enhances the growth performance and skin non-specific immune parameters in rainbow trout (*Oncorhynchus mykiss*). *Fish Shellfish Immunol.*, 32: 1083-1087. https://doi.org/10.1016/j.fsi.2012.03.003.

Soetan, K. O., Aiyelaagbe, O. O. and Olaiya, C. O. 2010. A review of the biochemical, biotechnological and other applications of enzymes. *Afr. J. Biotechnol.*, 9: 382-393.

Soto, J. O. 2017. Bacillus probiotic enzymes: External auxiliary apparatus to avoid digestive deficiencies, water pollution, diseases and economic problems in marine cultivated animals. In: *Advances in food and nutrition research, vol 80*. Elsevier Inc., p 15-35. https://doi.org/10.1016/bs.afnr.2016.11.001.

Standen, B. T., Peggs, D. L., Rawling, M. D., Foey, A., Davies, S. J., Santos, G. A. and Merrifield, D. L. 2016. Dietary administration of a commercial mixed-species probiotic improves growth performance and modulates the intestinal immunity of tilapia, *Oreochromis niloticus*. *Fish Shellfish Immunol.*, 49: 427-435. https://doi.org/10.1016/j.fsi.2015.11.037.

Stefanaki, C., Bacopoulou, F. and Michos, A. 2018. The impact of probiotics' administration on glycemic control, body composition, gut microbiome, mitochondria and other hormonal signals in adolescents with prediabetes-A randomised, controlled trial study protocol. *Contemp. Clin. Trials Commun.,* 11: 55-62. https://doi.org/10.1016/j.conctc.2018.06.002.

Thy, H. T., Tri, N. N., Quy, O. M, Fotedar, R., Kannika, K., Unajak, S. and Areechon, N. 2017. Effects of the dietary supplementation of mixed probiotic spores of *Bacillus amyloliquefaciens* 54A and *Bacillus pumilus* 47B on growth, innate immunity and stress responses of striped catfish (*Pangasianodon hypophthalmus*). *Fish Shellfish Immunol.*, 60: 391-399. https://doi.org/10.1016/j.fsi.2016.11.016.

Tseng, D. Y., Ho, P. L., Huang, S. Y., Cheng, S. C., Shiu, Y. L., Chiu, C. S. and Liu, C. H. 2009. Enhancement of immunity and disease resistance in the white shrimp, *Litopenaeus vannamei*, by the probiotic, *Bacillus subtilis* E20. *Fish Shellfish Immunol.*, 26: 339-344. https://doi.org/10.1016/j.fsi.2008.12.003.

Wang, Y. 2011. Use of probiotics *Bacillus coagulans*, *Rhodopseudomonas palustris* and *Lactobacillus acidophilus* as growth promoters in grass carp (*Ctenopharyngodon idella*) fingerlings. *Aquac. Nutr.*, 17: 372-378. https://doi.org/10.1111/j.1365-2095.2010. 00771. x.

Wiegertjes, G. F., Voorthuis, P., Groeneveld, A., Van, Muiswinkel, W. B., Stet, R. J., Bongers A. B. and Doulabi, B. Z. 1996. Characterisation of isogenic carp (*Cyprinus carpio* L.) lines with a genetically determined high or low antibody production. *Anim. Genet.,* 27: 313-319. https://doi.org/10.1111/j.1365-2052.1996.tb00975.x.

Xia, Y., Lu, M., Chen, G., Cao, J., Gao, F., Wang, M., Liu, Z., Zhang, D., Zhu, H. and Yi, M. 2018. Effects of dietary *Lactobacillus rhamnosus* JCM1136 and *Lactococcus lactis* subsp. *lactis* JCM5805 on the growth, intestinal microbiota, morphology, immune response and disease resistance of juvenile Nile tilapia, *Oreochromis niloticus*. *Fish Shellfish Immunol.*, 76: 368-379. https://doi.org/10.1016/j.fsi.2018.03.020.

Yano, T. 1996. The nonspecific immune system: humoral defense. In: Hoar, W. S., Randall, D. J., Iwama, G. and Nakanishi, T. (Eds.), *The fish immune system: Organism, pathogen and environment,* Academic Press, Massachusetts, USA, p. 105-157.

Zhou, X., Tian, Z., Wang, Y. and Li, W. 2010. Effect of treatment with probiotics as water additives on tilapia (*Oreochromis niloticus*) growth performance and immune response. *Fish Physiol. Biochem.,* 36: 501-509. DOI 10.1007/s10695-009-9320-z.

Date of Receipt : 31.07.2019
Date of Acceptance : 25.11.2021