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Original Article

Evaluation of probiotic adequacy, immunomodulatory effects and dosage application of *Bacillus coagulans* in formulated feeds for *Catla catla* (Hamilton 1822)

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Abstract: The present study was conducted to study the probiotic properties, antagonistic effect against pathogenic *Aeromonas hydrophila* of *Bacillus coagulans* isolated from intestine of healthy *Catla catla* Hamilton, 1822; and its optimum dosage for growth promotion and immunostimulation. The isolated *B. coagulans* from the gastrointestinal tract of *C. catla* was first assessed for its probiotic properties *viz*., antagonism towards pathogen and cell surface adhesion. A feeding trial of 90 days was conducted to optimize the inclusion level of *B. coagulans* in diets and *C. catla* fingerlings (avg. wt. 0.30±0.03g) were fed on feed supplemented with $1x10^3$ (diet D1), $2x10^3$ (diet D2), $3x10^3$ (diet D3) and $5x10^3$ (diet D4) *B. coagulans* CFU g^{-1} of feed in triplicate treatments. The growth and digestibility parameters, intestinal enzyme activities were significantly higher in group of fish fed on feed D3 ($3x10^3$ CFU g^{-1}) in comparison to other dietary treatments except for food conversion ratio which was significantly higher in control group. Significantly higher value of carcass protein level, lower excretion of metabolites (ammonia and phosphates), enhancement of non-specific immune response and increase of total Erythrocyte count (TEC) and total Leucocyte Count (TLC) were observed in fish fed with probiotics supplemented diets. The results obtained in the present study support the use of *B. coagulans* for better growth and proper nutrient utilization. The broken line analysis was carried out and polynomial fit curve further suggest that the optimum concentrations of *B. coagulans* as high as 3000 ($3x10^3$) CFU g^{-1} of feed is required for improving the overall physiological performance and enhancement of defense mechanisms in the fingerlings of *C. catla.*

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Introduction

Aquaculture is most promising, viable and fast-growing sector to provide nutritional security and its intensification is required to keep pace with surging need of animal protein. Intensification increases stress level in the animal as well as the environment. Disease outbreak is considered as most important constraint to its continued expansion. The application of antibiotics and chemotherapeutics to control diseases has led to serious problems such as the evolution of drug resistant pathogens, suppression of the aquatic animal's immune system, significant risk to human health and environmental hazards (Brogden et al., 2014; Allameh et al., 2015). An alternate approach to enhance disease resistance, immune responses and other health benefits is the administration of probiotics which play an important role in improving health of fish (Bandyopadhyay et al., 2015; Sivagami and Ronald, 2018). Merrifield et al. (2010) defined probiotics "as any microbial cell provided via the diet or rearing water that benefits the host fish, fish farmer or fish consumer, which is achieved, in part at least, by improving the microbial balance of the fish".

Many studies have reported that probiotic supplemented diets have a major impact on growth performance of fish (Gao et al., 2016; Bhatnagar and Lamba, 2017; Liu et al., 2018; Gobi et al., 2018; Sivagami and Ronald, 2018; Ullah et al., 2018). Probiotic are also known to improve intestinal enzymatic activities in fishes (Sivani et al., 2016; Makled et al., 2019). Zhang et al. (2018) reported that supplementation of *Lactobacillus delbrueckii* as

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probiotic enhanced growth performance and intestinal enzymatic activities as well as whole body composition of common carp. Selection of probiotics demands that is should be isolated from the gastrointestinal tract of host species intended to study (Patel et al., 2010; Makled et al., 2019) as commercially available probiotics are mainly from non-fish sources, which are believed to be unable to survive and/or remain viable at optimum concentrations in the fish intestine (Ghosh et al., 2008). Probiotics isolated from mature animals are well accepted in feeds of immature animals of same species (Gomez-Gil et al., 2000; Ghosh et al., 2003). *Bacillus* has been evaluated as probiotics in fish due to its antagonistic property, ability to enhance growth and immune response and is environment friendly to use (Shelby et al., 2006; Sumathi et al., 2014; Bhatnagar and Lamba, 2015, 2017; Bhatnagar and Saluja, 2019; Bhatnagar and Dhillon, 2019; Bhatnagar and Rathi, 2020). *Bacillus circulans* and *Bacillus* sp. have been isolated from the gut of *Catla catla* and *Cirrihinus mrigala* and their effect on growth, nutritional quality and immunity have been studied when incorporated in formulated diets (Bandyopadhyay and Patra, 2004). Studies were undertaken to isolate gut adherent potential probiotic bacterium to improve fish growth and digestibility in *C. catla* (Bhatnagar et al., 2012; Bhatnagar and Raparia, 2014; Bhatnagar and Saluja, 2019), and it was observed that significantly high growth performance can be achieved in the group of fishes fed on diet containing *B. coagulans*. In our earlier studies, three doses 200, 2000 and 20000 CFU per gram were used (Bhatnagar and Raparia, 2014), and it was found that optimum dose is somewhere near 2000 CFU g^{-1}, therefore to evaluate the proper dose four doses 1000, 2000, 3000 and 5000 CFU g^{-1} were used in the present study. However, it was felt that there is a need to assess the probiotic properties of this bacterium and its impact on growth performance, nutritional physiology and hematological parameters. Therefore, the present studies were conducted to evaluate the antagonistic properties of this probiotic species against *Aeromonas hydrophila* (major disease-causing agent in water), its influence on growth performance, blood parameters and immunity of *C. catla* and its optimum inclusion level in formulated diets.

Materials and Methods

Experimental Animals: Fingerlings of *C. catla* were obtained from "Sultan Fish Seed Farm" in Butana, Nilokheri, District Karnal (Haryana, India). Fingerlings were kept in glass aquarium of 30L capacity in laboratory conditions where temperature was maintained at 25±1°C. Fishes were acclimatized for 10 days prior to experiment start. Each aquarium was filled with de-chlorinated tap water and then stocked with 20 fingerlings with average body weight (BW) 0.30±0.03g. During the experiment, the water samples from all the aquaria were collected fortnightly and temperature, dissolved oxygen (DO), pH, electrical conductivity, calcium, chlorides and total alkalinity were measured following American Public Health Association (2017) to investigate the influence of supplemented feeds on quality of holding water. At the end of feeding trials, water samples from each tub were collected at two-hour intervals for the estimation of excretory levels of total ammonia ($N\text{-}NH4^{+}$) and reactive orthophosphate following APHA (2017), and calculated following Sumagaysay-Chavoso (2003).

Mass culture of *B. coagulans* and feed preparation: *Bacillus coagulans*, isolated from gut of *C. catla* (Bhatnagar et al., 2012) was used in the present studies. It was kept in nutrient agar slant at 4°C for further use. *Bacillus coagulans* was inoculated into conical flask (500 ml) containing nutrient broth and incubated at 30°C for 24 h in shaker incubator. The culture was centrifuged at 10000 rpm for 20 minutes at 4°C and supernatant was discarded, while the pellets were resuspended in phosphate buffer saline (PBS; pH 7.2). The suspension was similarly washed and recentrifuged four times and then quantified by spread plate technique (nutrient agar), incubated at 30°C for 24 h to determine the number of colonies forming units (CFU) (Bhatnagar and Raparia, 2014).

Table 1. Ingredient and Proximate composition (% dry weight basis) of experimental diets.

	Dietary treatments				
	DC (control)	**D1 (1000 CFU g^{-1})**	**D2 (2000 CFU g^{-1})**	**D3 (3000 CFU g^{-1})**	**D4 (5000 CFU g^{-1})**
Ingredient composition					
Groundnut oil cake	650.0	650.0	650.0	650.0	650.0
Rice bran	42.0	42.0	42.0	42.0	42.0
Processed soybean*	266.0	266.0	266.0	266.0	266.0
Wheat flour	32.0	32.0	32.0	32.0	32.0
Chromic oxide (Cr_2O_3)	10.0	10.0	10.0	10.0	10.0
Mineral mixture**	10.0	10.0	10.0	10.0	10.0
Probiotic bacterium (cells g^{-1}) *Bacillus coagulans*	0.0	1000	2000	3000	5000
*****Soybean was hydrothermally processed in an autoclave at 121°C (15 lbs for 15 minutes) to eliminate antinutrient factors (Garg *et al*., 2002). **Each kg has nutritional value: copper 312 mg, cobalt 35 mg, magnesium 2.114g, iron 979 mg, zinc 2 mg, iodine 15 mg, DL-methionine 1.920 g, L-lysine monohydrochloride 4.4 g, calcium 30%, phosphorous 8.25%.					
Proximate composition					
Crude protein (%)	39.80±1.36 ^{A}	38.24±0.98^{A}	39.51±0.86 ^{A}	39.86±0.79 ^{A}	39.35±0.79 ^{A}
Crude fat (%)	9.10±0.26 ^{B}	9.21±0.24 ^{B}	9.7±0.31 ^{A}	9.29±1.26 ^{B}	9.18±1.26 ^{B}
Crude fiber (%)	6.23±0.06 ^{A}	6.13±0.07 ^{A}	6.28±0.08 ^{A}	6.37±0.06 ^{A}	6.14±0.06 ^{A}
Total ash (%)	6.64±0.39 ^{A}	6.66±0.34 ^{A}	6.51±0.26 ^{A}	6.42±0.47 ^{A}	6.45±0.47 ^{A}
Moisture (%)	7.41±0.20 ^{A}	7.37±0.28 ^{A}	7.39±0.19 ^{A}	7.22±0.37 ^{A}	7.38±0.37 ^{A}
Nitrogen free extract (%)	30.82±1.42 ^{B}	32.38±1.49 ^{A}	31.84±1.11 ^{AB}	30.8±1.07 ^{AB}	31.5±1.07 ^{AB}
Gross energy (kJ g^{-1})	17.90±0.09 ^{B}	18.53±0.18 ^{A}	18.63±0.08 ^{A}	18.37±0.09 ^{A}	18.33±0.09 ^{A}
Feed phosphorus (%)	1.48±0.11 ^{C}	1.42±0.08 ^{C}	1.54±0.20 ^{B}	1.62±0.07 ^{A}	1.47±0.07 ^{C}

All values are Mean±S.E of mean. Means with different letters in the same row are significantly (P<0.05) different. (Duncan's Multiple Range test).

Feed was prepared by thoroughly mixing the ingredients (Table 1) followed by steaming for 20 minutes, cooled mixed thoroughly and then pellets were made by a hand pelletizer. Feed were air dried, probiotic were sprayed in respective concentrations and finally stored in vacuum sealed plastic containers at 4°C. In all the treatments, fishes were fed with respective diets daily at 4% BW in two instalments at 8:00 and 16:30 hours for 90 days. Growth parameters and enzymatic analysis was done using standard methods. Biochemical analysis of feed and fish carcass was carried out following AOAC (2019).

Antibiotic resistance study: *Bacillus coagulans* was examined for its inhibitory effects against the pathogenic *A. hydrophila* (IMTECH, Chandigarh) using Well diffusion assay (Lyon and Glatz, 1993). *Aeromonas hydrophila* was spread by a sterile swab, evenly, over the face of a sterile agar plate. A microbial suspension of each intestinal bacterial strain was applied in well at the centre of the agar plate (in a fashion such that the antimicrobial doesn't spread out from the centre) and incubated for 24 hours at 30º C to check the prevention of *Aeromonas* growth by the antibiotic activity. The strains that showed halos larger than 20 mm were considered positive.

Hydrophobicity assay: The cell surface hydrophobicity was evaluated according to the ability of the microorganisms to partition into hydrocarbon from phosphate buffer solution using the method of Savage (1992). Bacterial isolates were grown in nutrient broth (Merck, Germany) at 37°C for 24 h. After being centrifuged at 5000 rpm for 15 min, the pellets (bacterial precipitates) were washed twice with phosphate buffer solution and optical density (OD_{450}) of the bacteria at 450 nm adjusted to 0.5 A. About1 ml of bacterial suspension was added with 60 µl of a hydrocarbon *viz*., xylene (Fisher, England) and toluene (Merck, Germany), and vortexed for 1 min followed by determination of optical density of the water phase. Hydrophobicity was calculated according to the equation: [(OD_{450} before – OD_{450} after)/OD_{450} before] ×100 = % hydrophobicity.

Growth experiment

Experimental setup: The experiment was conducted

under laboratory conditions (25±1°C) in glass aquarium (30 L capacity) at Aqaculture Research Unit of Department of Zoology, Kurukshetra University, Kurukshetra. Each aquarium was filled with de-chlorinated tap water and then stocked with 20 fish fingerlings with average BW 0.3±0.03 g.

Five dietary treatments (DC, D1, D2, D3 and D4) were performed with three replicates of each treatment. In treatment 1 (DC), fishes were fed on artificial diet without probiotic bacteria (*i.e.*, control diet). Ingredient composition in g kg^{-1}: ground nut oil cake, 650; rice bran 42; hydrothermically processed soybean 266; wheat flour 32; mineral mixture 10. In treatment 2 (D1), fishes were fed on diet containing *B. coagulans* suspension in proportion 1000 ($1x10^3$) CFU g^{-1} of feed. In treatment 3 (D2), fishes were fed on artificial diet containing *B. coagulans* in proportion of 2000 ($2x10^3$) CFU g^{-1} of feed. In treatment 4 (D3), fishes were fed on artificial diet containing *B. coagulans* in proportion of 3000 ($3x10^3$) CFU g^{-1} of feed and in treatment 5 (D4), with proportion of 5000 ($5x10^3$) CFU g^{-1} of feed. All these diets were isocaloric and isoproteic with approximately 40% protein content. After spraying, the feed was air dried at room temperature and the bacterial concentration of feed ($CFUg^{-1}$) was calculated. Finally, the feed was stored in vacuumed plastic container at 4°C. All groups of fish were fed daily at 4% BW in 2 installments at 08:00 and 16:30 hours for 90 days. Growth parameters and enzymatic analysis was done using standard methods. Biochemical analysis of feed and fish carcass was carried out following AOAC (2019).

Blood parameters study: After the end of feeding trial, blood samples were collected from fingerlings of each dietary treatment for hematological diagnosis by using a heparinized syringe from caudal vein or heart by cardiac puncture (Lavanya et al., 2011). Blood samples of five fishes were pooled for analysis. EDTA (1 mg EDTA ml^{-1}) was used as anticoagulant in blood. Total Erythrocyte count (TEC) and total Leucocyte Count (TLC) were estimated by analyzing the blood samples from each treatment with the help of hemocytometer using a Neubauer's counting chamber.

Nonspecific immune response: Blood samples were heparinsed and immediately used for phagocytic assay (Park and Jeong, 1996).

Phagocytic assay: For phagocytic assay cells of freshly grown pathogenic bacteria *A. hydrophila* in 0.1 ml of PBS were added to 0.1 ml of blood sample (pooled samples of blood of five fishes mixed with EDTA as anticoagulant) of fishes of each treatment in sterile microplate. Blood was then incubated for 30 min at 25°C after thorough mixing in the well. The plate was removed and fifty ml of each suspension was transferred on glass slides to make smears. After air drying, smear was fixed in 95% ethanol, re-dried and stained with May Grunwald Giemsa. The phagocytic cells and phagocytosed bacteria were enumerated. Phagocytic ratio (PR) and phagocytic index (PI) were determined by enumerating 100 phagocytes per slide under a microscope. The average of three slides was calculated depending on the formula which is given below.

Phagocytic ratio (PR; i.e. percentage of cell with engulfed bacteria) = (No. of phagocytic cells with engulfed bacteria/No. of phagocytic cells) × 100.

Phagocytic index (PI; i.e. number of engulfed bacteria per cell) = No. of engulfed bacteria/No. of phagocytic cells.

Nitroblue tetrazolium (NBT) assay: The oxygen radical production by blood phagocytes during respiratory burst activity was measured through nitroblue tetrazolium (NBT) assay as described by Anderson and Siwicki (1995). Briefly, 0.1 ml of EDTA mixed blood from each treatment group was taken in Eppendorf to which 0.1 ml of 0.2% NBT solution was added. The mixture was incubated for 30 minutes at 25°C. From the suspension, 50 µl was taken, added to 1.0 ml N, N-dimethyl formamide in a glass tube and centrifuged at 3000g for 5 minutes. The optical density (OD) of the supernatant was measured at 540 nm in the spectrophotometer.

Challenge trial: After feeding for 90 days, 10 fishes from each treatment were challenged with *A. hydrohila* which has been cultured and maintained

Table 2. Growth performances and intestinal enzyme activities of *Catla catla* fed on Soybean based diets containing varying proportions of probiotic bacterium *Bacillus coagulans*.

Growth parameters	Dietary treatments				
	DC (Control)	D1 (1000CFUg^{-1})	D2 (2000CFUg^{-1})	D3 (3000CFUg^{-1})	D4 (5000CFUg^{-1})
Initial length (cm)	1.95±0.05^{A}	2.0±0.04^{A}	1.90±0.06^{A}	1.95±0.04^{A}	2.05±0.02^{A}
Initial weight (g)	0.32±0.02^{A}	0.29±0.01^{A}	0.31±0.02^{A}	0.32±0.03^{A}	0.31±0.02^{A}
Final weight (g)	1.40 ±0.03^{E}	1.87±0.04^{C}	2.08±0.05^{B}	2.30±0.03^{A}	1.69±0.02^{D}
Live weight gain (g)	1.09±0.02^{E}	1.57±0.04^{C}	1.77±0.04^{B}	1.98±0.03^{A}	1.4±0.02^{D}
Survival rate (%)	100^{A}	100^{A}	100 ^{A}	100 ^{A}	98.3±1.36^{A}
Growth (%) gain in BW	349.3±17.09^{E}	524.3±28.4^{C}	572.7±17.7^{B}	635.6±28.5^{A}	445.9±31.5^{D}
Growth/day (%) in BW	1.35±0.03^{C}	1.56±0.03^{B}	1.63±0.02^{AB}	1.77±0.02^{A}	1.5±0.04 ^{BC}
Specific growth rate (SGR) (%BW d^{-1})	0.72±0.02^{C}	0.85±0.02^{AB}	0.88±0.02$^{A\ B}$	0.99±0.01^{A}	0.81±.03^{B}
Feed conversion ratio (FCR)	2.6±0.09 ^{A}	1.96±0.04^{B}	1.74±0.02^{C}	1.56±0.03^{D}	1.99±0.04^{B}
Gross conversion efficiency (GCE)	0.41±0.02^{D}	0.53±0.01^{C}	0.65±0.01^{B}	0.80±0.02^{A}	0.59±0.01^{C}
Protein efficiency ratio (PER)	1.18±0.05^{E}	1.34±0.03^{C}	1.45±0.01^{B}	1.67±0.02^{A}	1.2±0.03^{D}
Apparent protein digestibility (APD) (%)	73.3±0.64^{E}	79.8±0.42^{C}	81.5±0.57^{B}	86.4±0.46^{A}	77.7±0.72^{D}

All values are Mean±S.E of mean. Means with different letters in the same row are significantly (P<0.05) different. (Duncan's Multiple Range test)

in the selective medium. Fishes from each replicate were immersed in a suspension of *A. hydrophila* ~ 10^5 CFU ml^{-1} followed by a second immersion ~10^7 CFU ml^{-1} after 7 days (Austin et al., 1995). Per cent survival was measured for 10 days based on observation that mortality reached its plateau after one week (Sahoo et al., 1998) and relative percentage survival was calculated by the following formula (Ellis, 1998) below:

RPS = 1- (Percent mortality in treated group/ Percent mortality in control group) × 100

Statistical analysis: Data were examined by one-way analysis of variance (ANOVA). When ANOVA identified differences among groups, a multiple comparison, Duncan's test was conducted to examine significant differences among treatments using Statistical Package for Social Science (SPSS-11.5) and significant differences were declared at P≤0.05. Data of experiments were further subjected to orthogonal polymonials (broken line regression analysis) for trend analysis (Zeitoum et al., 1976).

Results

Antagonistic activity of B. coagulans/antibiotic resistance assay: The size of zone of inhibition was found to be 19.0±1.6 and ranged between 15 to 21 mm (Fig. 1).

Hydrophobicity assay: The use of xylene, and toluene to evaluate the hydrophobic cell surface properties of the tested *B. coagulans* showed consistent positive results. The hydrophobicity of *B. coagulans* was 30.49±0.84% in xylene, and 22.79±3.96% in toluene.

Proximate composition: The average proximate composition of formulated diet revealed that the diets were isonitrogenous. Values of moisture, crude protein, crude fat, total ash, crude fiber and NFE are shown in Table 1 as % dry weight basis.

Fish growth, survival, digestibility and nutrient retention: The growth responses of test fish fed on experimental diets (DC to D4) are shown in Table 2. Fish satisfactorily accepted the experimental diet from the beginning of the experiment and maintained normal behavior throughout the experimental period. Survival rate (%) was high in all dietary treatments and slight mortality occurred only during the initial days of experiment. Statistical analysis revealed that the growth of fish in terms of weight gain (g), growth per cent gain (%) in body weight, growth per day (%) in BW and specific growth rate (SGR) were significantly (P<0.05) higher in treatment D3 in comparison to dietary treatments DC, D1, D2 and D4. Also, significantly (P<0.05) higher values of digestibility parameters *viz.,* apparent protein digestibility (APD), gross conversion efficiency

Table 3. Proximate carcass composition of *Catla catla* fed on Soybean based diets containing varying proportions of probiotics bacterium *Bacillus coagulans*.

Proximate composition	Initial value	Dietary treatments				
		DC (control)	D1 (1000 CFUg-1)	D2 (2000 CFUg-1)	D3 (3000 CFUg-1)	D4 (5000 CFUg-1)
Moisture (%)	73.07±0.36	70.65±0.51^{A}	69.5±0.34^{AB}	68.62±0.32^{B}	66.10±0.34^{C}	68.5±0.34^{B}
Crude protein (%)	8.90±0.06	11.93±0.21^{D}	14.41±0.07^{C}	16.34±0.19^{B}	17.04±0.21^{A}	13.99±0.07^{C}
Crude fat (%)	2.2±0.04	5.77±0.07^{A}	3.97±.06 ^{BC}	4.45±0.15^{B}	3.75±0.06^{C}	4.07±0.07^{BC}
Total ash (%)	3.6±0.06	4.27±0.18^{A}	4.15±0.10^{A}	3.95±0.09^{B}	3.93±0.04^{B}	4.29±0.10^{A}
Nitrogen free extract (%)	12.2±0.40	7.4±0.15^{C}	8.60±0.54^{B}	8.51±0.45^{B}	8.82±0.20^{A}	8.40±0.36^{B}
Gross energy (kJ/g)	5.06±.06	6.36±0.08^{D}	6.3±0.05^{D}	6.74±0.04^{B}	7.07±0.05^{A}	6.5±0.05^{C}
Phosphorus (%)	0.53±0.02	0.59±0.03^{D}	0.71±0.02^{AB}	0.67±0.03^{C}	0.69±0.03^{B}	0.73±0.02^{A}

All values are Mean±S.E of mean. Means with different letters in the same row are significantly (P<0.05) different. (Duncan's Multiple Range test).

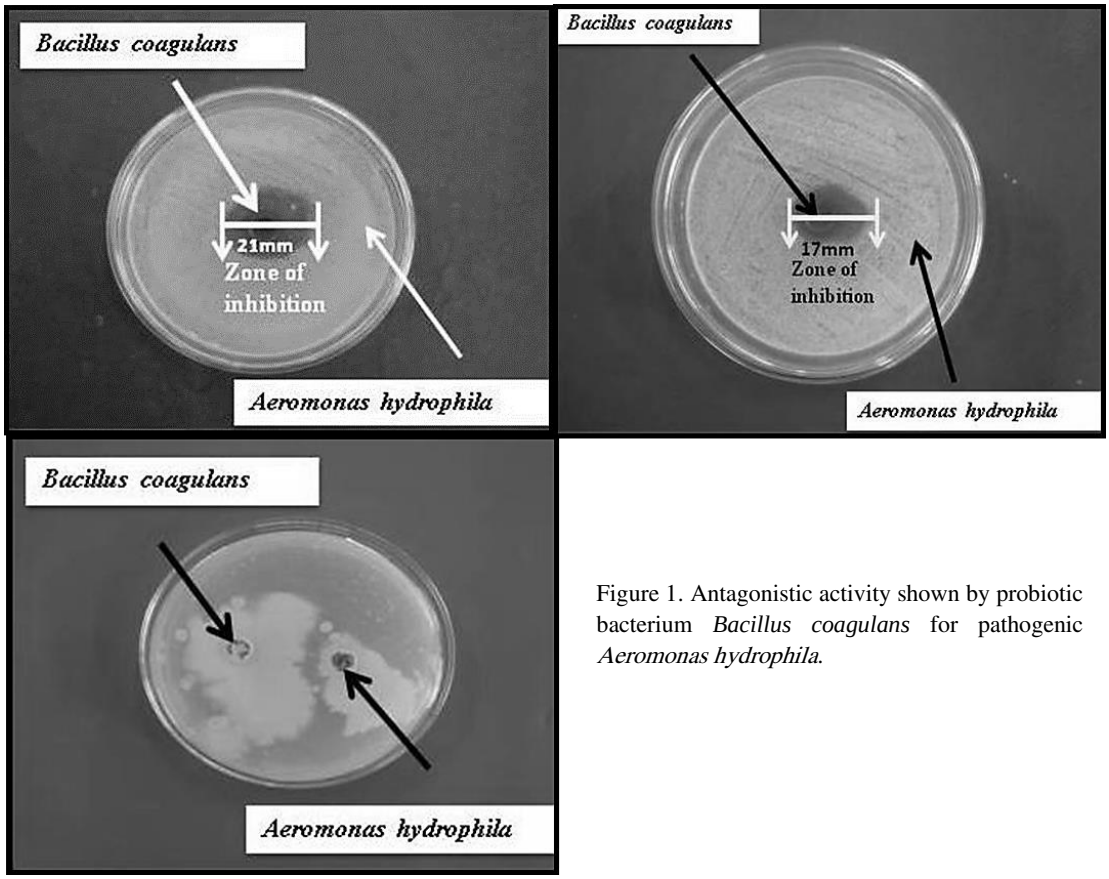

Figure 1. Antagonistic activity shown by probiotic bacterium *Bacillus coagulans* for pathogenic *Aeromonas hydrophila*.

(GCE) and protein efficiency ratio (PER) and significantly (P<0.05) lower FCR (1.56±0.03) were observed in the dietary treatment D3. The highest FCR was found in the control group (2.6±0.09) (Table 2). The data on weight gain revealed that initially up to 15 days not much variations were observed in the weight gain of group of fishes fed on varying dietary treatments. However, growth rate increased significantly (P<0.05) in the fishes fed on diet D3 after 30 till 90 days (Fig. 2). Diet containing *B. coagulans* at 3000 CFU g^{-1} of diet depicted 81.65% increase in weight gain in comparison to control diet (Fig. 3). Data of experiments were further subjected to orthogonal polymonials (broken line regression analysis) for trend analysis, also showed a clear dose dependent trend line curve. Polynomial curve fitting to the data of weight gain in the fingerlings of *C. catla* is shown in Figure 4.

Intestinal digestive enzyme activities: Intestinal digestive enzyme activities for protease, amylase and

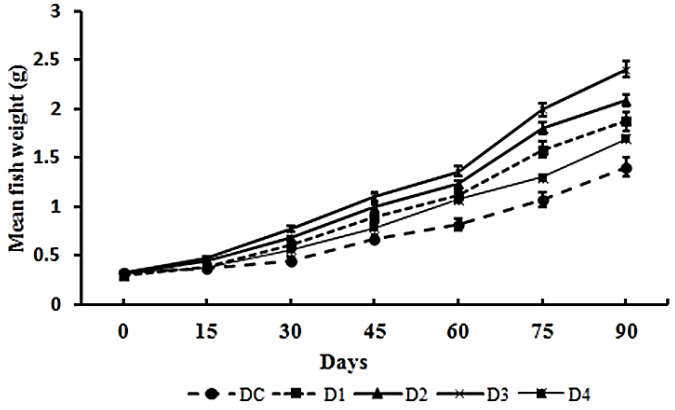

Figure 2. Increase in mean fish weight (g) ±S.E of mean of *Catla catla* fingerlings fed on diets supplemented with varying proportions of probiotics *Bacillus coagulans* (DC=control, D1=1000 cells g^{-1}, D2=2000 cells g^{-1}, D3=3000 cells g^{-1} and D4=5000 cells g^{-1} of diet) from day 15 to 90.

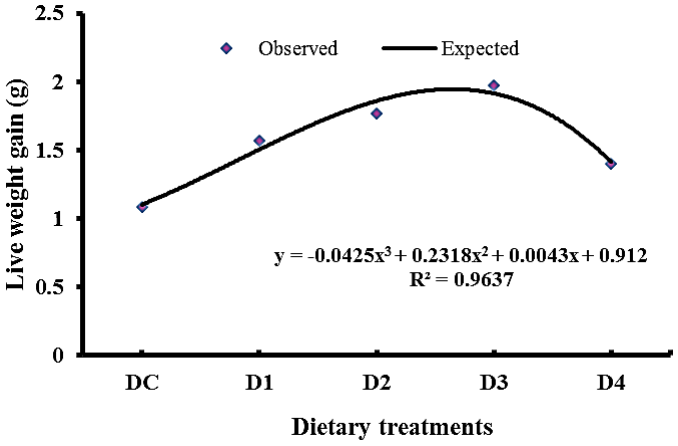

Figure 3. Polynomial fit curve using broken line analysis to show effect of *Bacillus coagulans* supplementation (DC=control, D1=1000 cells g^{-1}, D2=2000 cells g^{-1}, D3=3000 cells g^{-1} and D4=5000 cells g^{-1} of diet) fitting to the data of weight gain in the fingerlings of *Catla catla.*

cellulase were determined. It was found that specific activity of digestive enzymes was significantly (P<0.05) higher in all the dietary treatments in comparison to control group. The values showed an increasing trend from treatment DC to D3. Thereafter, with further increase in the inclusion level of probiotic bacteria (Diet-D4), containing *B. coagulans* in proportion of 5000 CFU g^{-1} of feed, the values decreased (Fig. 5).

Fish carcass composition: Initial and final carcass composition with respect to proximate nutrients of test fish on the basis of feeding trial is shown in Table 3. Crude protein (%) and gross energy (kJg^{-1}) were found

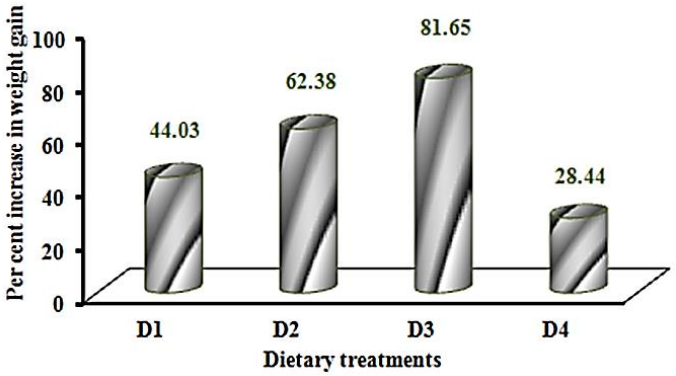

Figure 4. Per cent increase in growth of Catla catla fed on varying dietary treatments containing varying proportion of *Bacillus coagulans* (DC=control, D1=1000 cells g^{-1}, D2=2000 cells g^{-1}, D3=3000 cells g^{-1} and D4=5000 cells g^{-1} of diet).

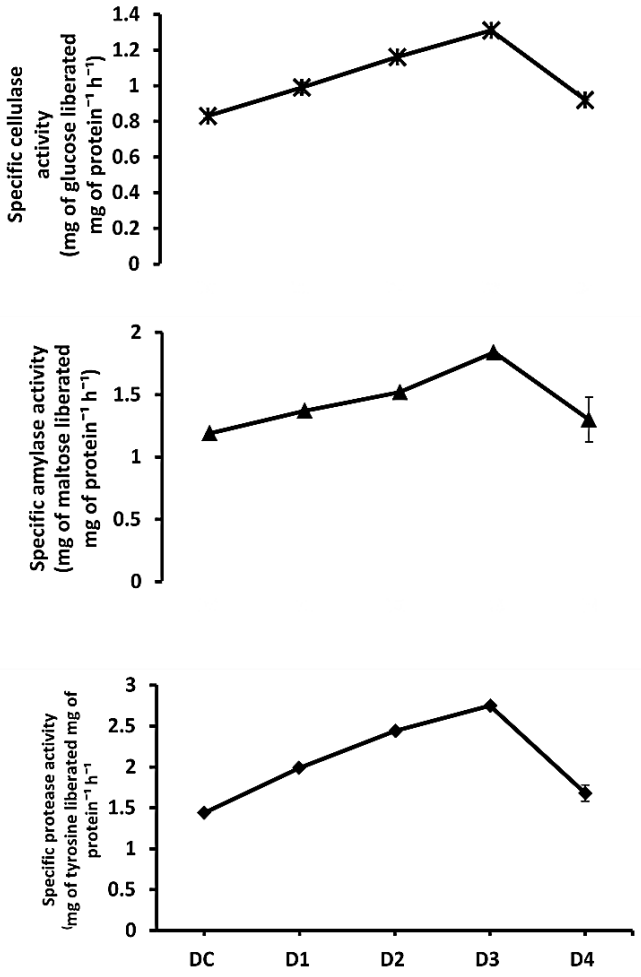

Figure 5. Intestinal Enzyme activities of *Catla catla* fed on varying dietary treatments containing varying proportion of *Bacillus coagulans* (DC=control, D1=1000 cells g^{-1}, D2=2000 cells g^{-1}, D3=3000 cells g^{-1} and D4=5000 cells g^{-1} of diet).

to be significantly (P<0.05) higher in the carcass of fish fed on diet D3. Moisture (%) and crude fat (%)

Table 4. Effect of fish fed on soybean-based diets with different proportion of probiotic bacterium *Bacillus coagulans* supplementation on water quality characteristics.

Physico-chemical parameters	Dietary treatments				
	DC (control)	D1 (1000 CFUg^{-1})	D2 (2000 CFUg^{-1})	D3 (3000 CFUg^{-1})	D4 (5000 CFUg^{-1})
Dissolved oxygen (DO) mgL^{-1}	6.4±0.07^{A}	6.1±0.02^{C}	6.4 ±0.08^{A}	6.3±0.10^{A}	6.1±0.10^{A}
pH	7.80±0.01^{A}	7.79±0.02^{A}	7.82±0.01^{A}	7.84±0.01^{A}	7.78±0.01^{A}
Conductivity (µ mho cm^{-1})	624.66±3.32^{B}	629±3.42^{B}	687.33±3.61^{A}	685.83±2.68^{A}	644.83±2.68^{AB}
Alkalinity(carbonates)	21.33±0.48^{B}	22.61±0.79^{AB}	24.76±0.33^{A}	24.80±0.58^{A}	24.2±0.58^{B}
Alkalinity(bicarbonates)	128.63±3.87^{C}	144.54±4.17^{AB}	149±5.33^{A}	143.5±4.20^{B}	144.5±4.20 ^{AB}
Chloride (mg L^{-1})	24.36±0.76^{A}	20.87±0.57^{B}	25.3±1.05^{A}	24.98±0.87^{A}	25.08±0.87^{A}
Calcium (mgL^{-1})	25.17±1.08^{AB}	24.19±0.96^{B}	22.73±0.53^{C}	26.41±0.98^{A}	19.41±1.92^{D}
Total dissolved solids	575.5±16.77^{A}	539.51±8.53^{B}	458.33±14.53^{E}	479.30±19.27^{D}	486.30±18.27^{C}
Total NH_3-Nexcretion (mg Kg^{-1}	1890.2±32.74^{A}	1287.31±19.4^{C}	752.8±16.36^{D}	619.3±13.4^{E}	1326.3±19.9^{B}
Total O-PO_4 production (mg Kg^{-1}	766.02±11.3^{A}	472.62±7.55^{C}	335.16±8.07^{D}	278.55±13.1^{E}	473.15±13.6^{B}

Means with different letters in the same row are significantly (P<0.05) different. (Duncan's Multiple Range test).

Table 5. Hematological Values of *Catla catla* fed on Soybean based diets containing varying proportions of probiotics bacterium *Bacillus coagulans*.

Treatments	Haematological parameters			
	RBC (10^6mm^3)		WBC (10^3mm^3)	
	Pre-Challenge	Post Challenge	Pre challenge	Post Challenge
DC (Control)	1.33±0.03^{E}	1.01±0.04 ^{D}	20.7±0.82 ^{E}	22.58±0.97^{D}
D1 (1000 CFUg^{-1})	1.64±0.06^{C}	1.51±0.03^{C}	32.3±1.2^{C}	36.52±1.4^{C}
D2 (2000 CFUg^{-1})	1.96±0.04^{B}	1.84±0.06^{B}	39.3±1.6^{B}	43.67±1.9^{B}
D3 (3000 CFUg^{-1})	2.4±0.08^{A}	2.15±0.03^{A}	50.5±2.1^{A}	53.58±2.5^{A}
D4 (5000 CFUg^{-1})	1.44±0.04^{D}	1.08±0.02^{D}	25.3±0.7^{D}	34.76±1.8^{C}

All values are Mean±S.E of mean. Means with different letters in the same column are significantly (P<0.05) different. (Duncan's Multiple Range test).

was found to be significantly (P<0.05) higher in dietary treatment DC. Nitrogen free extract (NFE) was found to be higher in diet D3. However, no significant (P<0.05) variations were observed in total ash (%) of carcass of fishes fed on different diets.

Effect of experimental diets on water quality characteristics: The data on water quality characteristics pertaining to five dietary treatments is presented in Table 4. In general, significant low values in total ammonia excretion and reactive phosphate production (mg Kg^{-1} BW d^{-1}) were recorded in fish fed on diet D3 supplemented with 3000 CFU g^{-1} of feed.

Haematological parameters: The RBC was significantly higher (P<0.05) in fishes fed on diet D3 (2.4±0.08) than in the control treatment DC (1.33±.03). In the present study, significant increase (P<0.05) in WBC count was observed in fishes of treatment D3 (50.5 ± 2.1) when compared to control treatment DC (20.7± 0.82). Among the post-challenge groups, DC showed significantly (P<0.05) lower RBC than the others. The post-challenge data showed increase in leukocyte count irrespective of the *B. coagulans* inclusion signify a possible increased infection and inflammatory response mediated by leukocyte against bacteria (Table 5; Fig. 6).

Phagocytic responses: Phagocytic ratios and phagocytic indices in the fish fed with varying proportion of *B. coagulans* were significantly (P<.05) higher than in control fish during the assay period. The highest values of phagocytic ratio (79.01±1.72) and phagocytic index (2.61±0.05) were observed in dietary treatment D3 and the lowest in fish fed on the control diet (59.58±1.19 and 1.75±.02, respectively). (Table 6; Fig. 7).

Table 6. Effect of *Bacillus coagulans* supplementation on phagocytic ratio and phagocytic index of *Catla catla*.

Fish group	Peripheral blood monocytes				
	Total no. of phagocytes	No. of ingesting phagocytes	Bacteria Cells within phagocytes	Phagocytic ratio (%)	Phagocytic index
DC (Control)	69.33±2.18	41.33±1.76	72.67±3.18	$59.58±1.19^{E}$	$1.75±0.02^{D}$
D1 (1000 CFUg^{-1})	67.6±2.02	46.4±2.4	95±2.30	$68.39±1.74^{C}$	$2.06±0.07^{C}$
D2 (2000 CFUg^{-1})	79.7±2.12	58.3±1.91	135.8±6.17	$73.17±1.09^{B}$	$2.31±0.03^{B}$
D3 (3000 CFUg^{-1})	84.2±2.41	66.3±1.83	173.6±3.67	$79.01±1.72^{A}$	$2.61±0.05^{A}$
D4 (5000 CFUg^{-1})	69.4±0.98	44±1.52	86±2.88	$63.5±2.41^{D}$	$1.95±0.04^{C}$

All values are Mean±S.E of mean. Means with different letters in the same column are significantly (P<0.05) different. (Duncan's Multiple Range test).

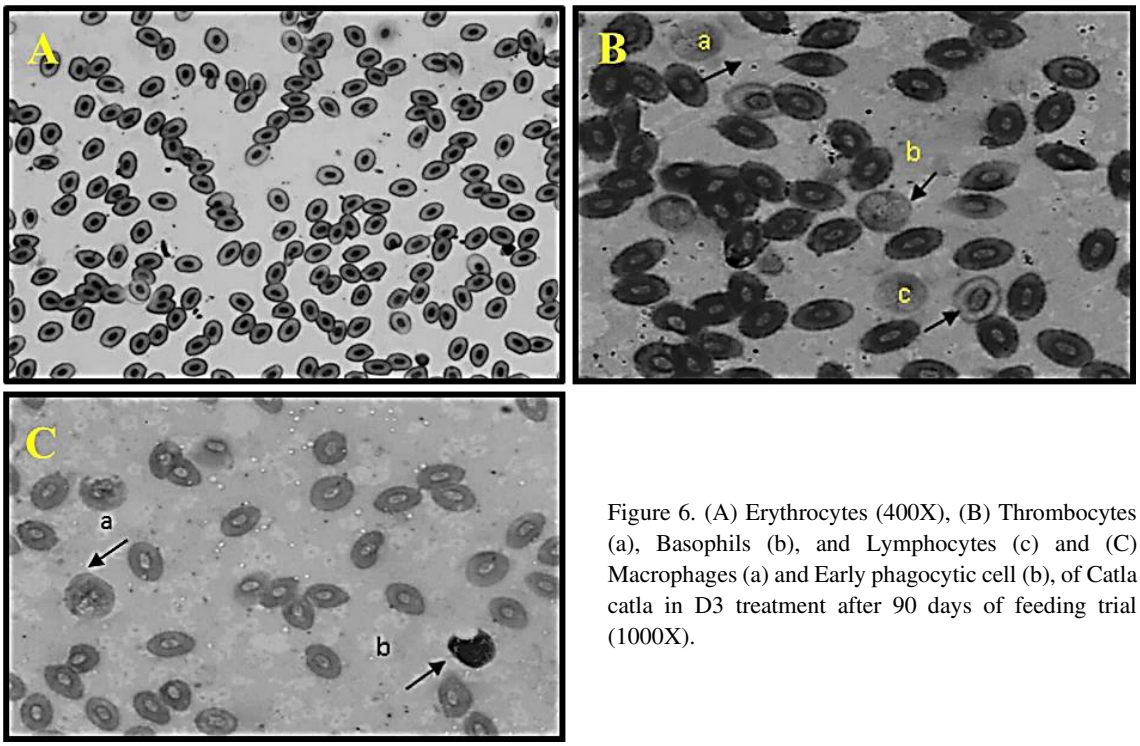

Figure 6. (A) Erythrocytes (400X), (B) Thrombocytes (a), Basophils (b), and Lymphocytes (c) and (C) Macrophages (a) and Early phagocytic cell (b), of Catla catla in D3 treatment after 90 days of feeding trial (1000X).

NBT assay: Respiratory burst activity of phagocytes was measured by reduction of Nitro Blue Tetrazolium (NBT) by intracellular superoxide radicals produced by leukocytes. The production of superoxide radicals was significantly influenced by the probiotic diets. Maximum increase in the NBT reduction value was observed in treatment D3 (Fig. 8).

Survival rate with challenge test: After challenge with *A. hydrophila*, the first mortality was recorded after 24 h. Mortality was recorded up to 10 days after challenge. Significantly (P<0.05) higher mortality (73.3%) was recorded in fishes of control group. The data on relative per cent survival is presented in the form of survivorship curve (Fig. 9). Treatment D2 and D3 fed groups showed significantly (P<0.05) higher relative percent survival, 86.36 and 90.9% respectively.

Clinical signs observed after challenge: The fish were sluggish and gradually lost their equilibrium 24-48 h after challenge with *A. hydrophila.* The clinical signs were characterized by hyperemic condition on the ventral side of the body, a visibly swollen abdomen and a slightly protruding reddish vent. The eyes of the infected fish were opaque and during the terminal stages the animals were seen floating dorsal side down at the water surface. The abdomen was distended due to accumulation of fluid in the peritoneal cavity. These changes were not evident in D3 group. Mortality

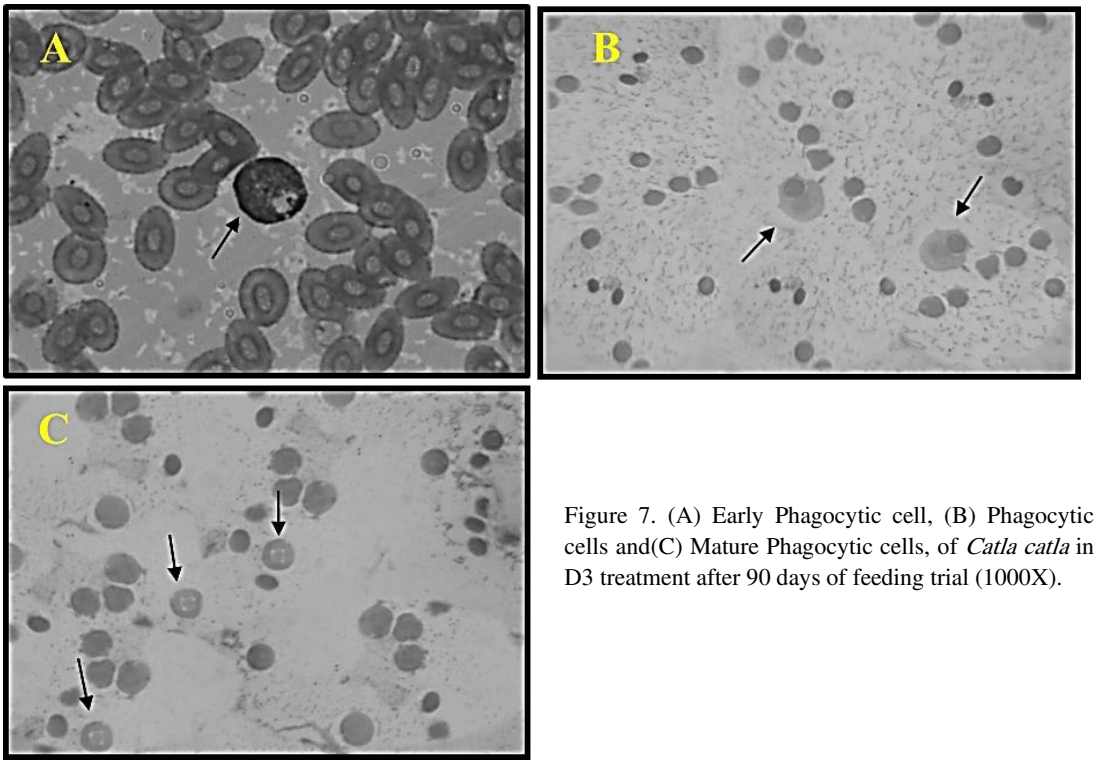

Figure 7. (A) Early Phagocytic cell, (B) Phagocytic cells and(C) Mature Phagocytic cells, of *Catla catla* in D3 treatment after 90 days of feeding trial (1000X).

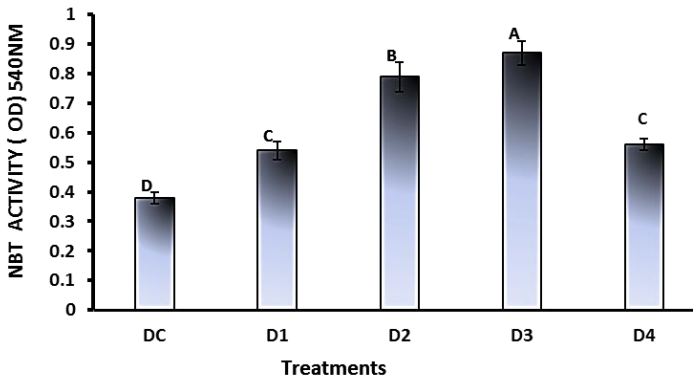

Figure 8. Nitro Blue Tetrazolium (NBT) activity of *Catla catla* fed on diet containing various proportions of probiotic bacterium *Bacillus coagulans*.

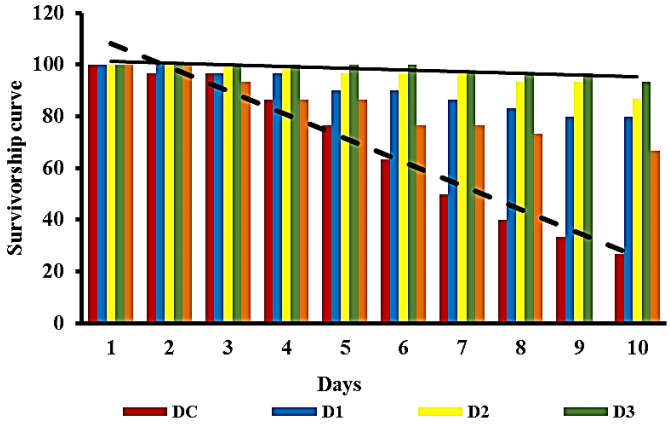

Figure 9. Survivorship curve of *Catla catla* in different dietary treatments containing varying proportion of *Bacillus coagulans* (DC=control, D1=1000 cells g^{-1}, D2=2000 cells g^{-1}, D3=3000 cells g^{-1} and D4=5000 cells g^{-1} of diet) challenged with *Aeromonas hydrophila*.

percentage was as low as 10.0±5.7 and 6.6±3.3 in treatment D2 and D3, respectively.

Discussions

In the present study, the novel strain of *B.coagulans* isolated from the gut of *C. catla* (Bhatnagar et al., 2012) was tested for antagonistic effect on the growth of common indicator fish pathogen *A. hydrophila* by the appearance of clear inhibition zone by well diffusion assay. The results revealed a clear zone of inhibition ranging from 15 to 21 mm with a mean value of 19.0±0.9 mm (Plate-6) clearly, indicating that this strain of *B. coagulans* can limit the growth of fish pathogen *A. hydrophila* by producing antimicrobials. Urdaci and Pinchuk (2004), Bhatnagar and Lamba (2015) and Bhatnagar and Dhillon (2019) have also reported that *Bacillus* species could produce a large number of antimicrobials.

The potential of probiotics is further inferred through the ability to adhere and to colonize in the intestinal tract. The hydrophobicity of this strain of *B. coagulans* was 30.49±0.84% in xylene and

22.79±3.96% in toluene, clearly revealing that this strain can colonize the gut of *C. catla* and has properties of successful probiotic. Mahdhi et al. (2011) have also advocated the ability of *B. subtilus* and *B. coagulans* to adhere to the intestinal surface and reported hydrophobicity with toluene 30.3±9.40 and 31.3±3.70 and with xylene 32.2±5.60 and 36.10, respectively. Bhatnagar and Lamba (2015) and Bhatnagar and Dhillon (2019) have also characterized properties of probiotics on the basis of hydrophobicity. These findings of the present study suggested that the isolated *B. coagulans* has potential to be the probiotic bacterium for *C. catla*.

In the present study, attempt has also been made to evaluate the optimum dose of probiotic supplementation in the formulated feed for *C. catla*. The optimum probiotic level which resulted in highest growth in *C. catla* fingerlings in terms of live weight gain (g), growth per cent gain, SGR (specific growth rate) and nutrient retention (PER, GCE and APD) was found to be around 3000 CFU g^{-1} of feed (treatment D3). The polynomial fit curve (broken line regression analysis) of weight gain also represented the optimum dose at dietary treatment D3 (*B. coagulans* @ 3000 CFU g^{-1}) with high R^2 values (0.9637, $y=-0.0425x^3 + 0.2318x^2+0.0043x+0.912$). FCR (feed conversion ratio) values decreased with each increase in the dietary probiotic content upto 3000 CFU g^{-1} of feed. Thereafter, further increase in dietary probiotic level resulted in increase in FCR and growth depression. The findings of the present study showed similarity with the study of Sivani et al. (2016) in which inclusion rate of probiotic bacterium increased after a certain level, a decrease in growth performance was observed. Although, all the feeds were isonitrogenous but the concentration of probiotics in dietary treatment D3 might have been helpful for proper nutrient utilization. High carcass crude protein and lesser nitrogen and phosphate excretion were also observed in dietary treatment D3 which can be attributed to proper probiotic concentration, whereas lesser carcass protein and greater nitrogen and phosphate excretion were observed in dietary treatment D4 which could have been due to the overall low feed utilization level.

The high APD (apparent protein digestibility) values for the diet containing *B. coagulans* at 3000 CFU g^{-1} of diet may be attributed to high dietary utilization. Ghosh et al. (2003) using *B. circulans* as probiotic in *Labeo rohita* fingerlings; Rengpipat et al. (1998) using *Bacillus* sp. as probiotics in *Paneus monodon*, Bhatnagar and Lamba (2015) using *B. cereus* in *C. mrigala*, Bhatnagar and Dhillon (2019) using *Aneurinibacillus aneurinilyticus* for *L. calbasu* also reported high values of APD values at doses coinciding with high growth performance.

The enhanced enzyme activity level in the gut because of extracellular enzyme production by *B. coagulans* might have helped in increasing the food absorption and thus resulted in high growth in treatment D3. Rani et al. (2004), Bhatnagar and Khandelwal (2009) and Makled et al. (2019) have reported extracellular enzyme production in significant amounts because of presence of suitable gut adherent enzyme producing microflora. The specific enzyme activities were also found highest in treatment D3 and lowest in control DC which may be due to better dietary protein utilization or due to colonization of probiotics bacteria and its exogenous enzyme production. When probiotics supplementation exceeds the optimum level, no further improvement in growth performance and nutritive physiology of the fish was observed, rather these parameters decreased. This might be due to the fact that probiotics bacteria incorporated in the feed might have competed amongst themselves and their colonization was not proper, resulting in the decline in exogenous/ extracellular enzyme production and thus low digestibility, low growth and high feed conversion ratio. These findings could be attributed to the specific feature of probiotic bacterium which stimulate the digestive system of host to increase the intestinal enzymatic activities (Eslamloo et al., 2012; Bhatnagar and Saluja, 2019) and inhibition of other harmful flora along fish gut (Makled et al., 2019) thus resulting in better growth performance of fish.

In aquaculture, water quality deteriorates mainly

due to accumulation of metabolic wastes such as ammonia and orthophosphate excretion in the holding water. *Bacillus* sp. reduces the quantity of ammonia and nitrite in the water as it degrades the organic matter and facilitates nutrients recycling (Skjermo and Vadstein, 1999; Sanders et al., 2003). The findings of Raparia and Bhatnagar (2016) and Bhatnagar and Lamba (2017) showed that dietary supplementation of *B. coagulans* and *B. cereus,* respectively, lowered the excretion of total ammonia ($N\text{-}NH^4$) and orthophosphate ($o\text{-}PO_4$), respectively. Similarly, in the present study, *B. coagulans* supplementation at 3000 CFU g^{-1} improved the water quality parameters and also reduced pathogenic bacteria load to significant levels.

RBC and WBC increased in yellowtail infected with *N. kampachi* (Ikeda et al., 1976). The result of the present experiment also revealed an increase in TLC and TEC counts in groups D2 and D3 compared to the control (DC). This indicated the heightened immune response in the fish fed on feed containing *B. coagulans*, probably due to its immunostimulatory effect. Similar findings were reported by Bandyopadhyay et al. (2015), Makled et al. (2017) and Bhatnagar and Dhillon (2019) where hematological parameters i.e. TLC and TEC count showed enhancement when fish were fed on probiotic supplemented diet.

It has been shown that *Bacillus* strains supplementation in diet could increase disease resistance in fish through the stimulation of cellular immune function, such as phagocyitc activity (Merrifield et al., 2009). Phagocytosis is responsible for early activation of the inflammatory response and is mediated by phagocytic cells such as neutrophils, monocytes and macrophages in fish (Kwak et al., 2003). Significant increases of phagocytic activity (PA) and phagocytic index (PI) was recorded in *E. coioides* fed *B. pumilus* or *B. clausii* containing diets for 60 days compared with those fed the control diet (Sun et al., 2010). Sumathi et al. (2014) reported that diets with *B. megaterium* and *Pontibacter* inclusion induced highest phagocytic ratio and phagocytic index in *L. rohita*. In present study also, significant increase in PA and PI were found in treatment D3 compared with those fed on control diet. In line with our finding, Bandyopadhyay and Patra (2004) found that isolated bacterium *B. circulans* PB7 could significantly improve the phagocytic ratio and phagocytic index of *C. catla* (Ham.). Bhatnagar and Dhillon (2019) in *L. calbasu* and Bhatnagar and Saluja (2019) in *C. catla* have also reported high PI and PA with high growth performance.

Zhou et al. (2010) confirmed the isolated probiotics *B. coagulans* 16 from the gut of *Oreochromis niloticus* enhances the immune and health status, thereby improving growth performance which supports the results of present studies for *C. catla*. However, they used culture of probiotics as water additives where as in present study probiotic bacterium was used as dietary supplement.

In *L. rohita* fed with feed containing *B. subtilis*, the survival rate after challenge with *A. hydrophila* was significantly higher in the treatment group compared to the control. Administration of yeast glucan enhances the survival of carp infected with *A. hydrophila* (Selvaraj et al., 2005). The per cent mortality during challenge trial with *A. hydrophila* was low in the groups fed with probiotic bacterial strain *B. coagulans* @ 3000 CFU g^{-1}. The survivorship plot indicated that there is a significant difference between the survivorship curves in each treatment; similar plot has been reported by Bhatnagar and Lamba (2017) and Bhatnagar and Dhillon (2019) in their studies on *C. mrigala* and *L. calbasu*, respectively. The high rates of establishment of bacterium in the gastro-intestinal tract of fish treated with *B. coagulans* have suppressed the *A. hydrophila* infection, which ultimately resulted in the higher survival in treatment D2 and D3 in present investigation.

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