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Investigation of Potential Probiotic Bacterium (*Bacillus subtilis* CCI3) in the Formulated Diets on Immunity, Growth Performance and Nutritional Quality of *Cyprinus carpio*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

In the present investigation *Bacillus subtilis CCI3* was evaluated for use as a probiotic supplement in the feeds for the fingerlings of *Cyprinus carpio.* The outcome of supplement on the feed utilization efficiency, growth performance, and immune response was evaluated. *Cyprinus carpio* fingerlings

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(avg. wt. 27.75 ± 0.045 g) were fed feed supplement with 2 x 10^4 (feed BS1), 2 x 10^5 (feed BS2) and 2 x10^6 (feed BS3) *Bacillus subtilis* cells per 100g feed for 60 days @ 5 % of the body weight per day in two equal installments in triplicate treatments. The control diet (CC) was not supplemented with the *Bacillus subtilis*. All the feeds are isocaloric and isonitrogenous. Feed BS2 fed fishes showed better growth, significantly ($p \leq 0.05$) higher protein efficiency ratio (PER), highest RNA: DNA ratio and a lower feed conversion ratio (FCR) than the other experimental feeds. Feed BS2 fed fishes also showed highest carcass protein and lipid than the others. Intestinal protease and α – amylase activity was observed significantly higher ($p \leq 0.05$) in BS2 feed treated fishes and also significantly ($p \leq 0.05$) greatest GPT and GOT values were observed in feed BS2 fed fishes but lowest in control (C). Highest TSP, albumin, globulin was observed in BS2 treated fishes after 60 days feeding trial. But lowest glucose level was observed in the same treatment. TEC, TLC, Hct and Hb values were highest in BS2 treated fishes. After feeding trial the specific and non-specific immunity levels and disease resistance of fish were also studied. NBT, antibody titer and serum bacterial activity were highest in diet BS2 fed fishes. After feeding trial the fishes were challenged for 10 days by bath exposure to *Aeromonas sp.* (A2) [10^5 CFU ml^{-1}, 1hr. and after 7 days 10^7 CFU ml^{-1}, 1hr].

Keywords: Cyprinus carpio; Bacillus subtilis CCI3.

1. INTRODUCTION

Probiotic definitions differ to a great extent depending on the source but accepted definition was proposed by Fuller [1] as a live microbial feed supplement which beneficially affects the host animal by improving its microbial balance. “The demand for animal protein for human consumption is at present on the rise and is largely supplied with terrestrial farm animals. In terms of protein production aquaculture is an all-time more important option in animal” [2,3,4]. “This activity requires high-quality feeds with high protein content which should contain not only necessary nutrients but also complementary additives to keep organisms healthy and favor growth. However, considerable efforts are still being made to develop alternative or supplementary methods to improve fish health. Among such methods, the prophylactic use of probiotics and immune-stimulants has attracted particular interest” [5]. “Such approaches can be implemented at larval and early fry stages where vaccines cannot be used. At these stages’ mortality can be high due to abundance of opportunistic pathogens even where the initial infection pressure is low” [6].

Throughout the past 50 years, numerous trials were conducted with microorganisms known as probiotics in efforts to improve food species and human health and welfare. Fuller [1] were shown that “suitable probiotic application improves intestinal microbial balance, and hence improved food absorption further Cole and Fuller [7] recorded reduced pathogenic problems in the gastrointestinal tract”. Several probiotic species were used including *Saccharomyces* sp. [8], *Bacillus* sp. [9,10], and mixed cultures [11]. “By means of some trial, growth promotions were clearly verified in poultry and pigs compared with control groups. Those results were most promising and gave confidence that further improvements in probiotic applications were possible” [12]. “Probiont *Vibrio alginolyticus* applied to salmon could reduce diseases caused by *Aeromonas salmonisida, Vibrio ordali* and *Vibrio aguillarium”* was found by Austin et al. [13]. In the present investigation different feeds were made by incorporating a potential probiotic bacterium *Bacillus subtilis CCI3* for *Cyprinus carpio*. The main aims of this experiment were to study effects of the strain regarding nutritional quality, immune response, survival, growth performance and digestibility against fish pathogenic *Aeromonas sp.* after challenge trial of *Cyprinus carpio.*

2. MATERIALS AND METHODS

Healthy fingerlings of *Cyprinus carpio* (Common Carp) having an initial measurement of 27.75 ± 0.045g provided by Government Fisheries Farm, Hadapsar, Pune were acclimated for about 1-week prevailing laboratory condition of water temperature (25 – 29ºC) and pH(7.2 – 7.8).

2.1 Preparation of Experimental Feeds

The four prepared feeds (CC, BS1, BS2 and BS3) were formulated using locally available ingredients as shown in Table 1 Feed formulation

was done basically by "square-method" using determined values of protein content of the ingredients as shown in Table 2. "Percentage of each ingredient required was calculated precisely providing allowance for the premix. Dough was prepared and the feeds were pelleted separately with local made hand pelletizer for preparation of a kg feed". [4] The pellets were dried in a thermostatic oven (Kumar, Mumbai, India) at 37ºC to less than 10% moisture [14] and stored in airtight jars at room temperature. Proximate compositions of the four prepared feeds (CC, BS1, BS2 and BS3) are detailed in Table 3.

Table 1. Proximate composition of different components used in trial feeds for *Cyprinus carpio*

Ingredients	Soybean Meal	Potato Starch	Fish Meal
Moisture (%) *	1.85	1.75	8.2
Dry matter (%)	93.15	93.25	91.8
Crude Protein (%)	38.52	29.86	54.76
Crude Lipid (%)	18.56	4.53	1.13
Crude fiber (%)	4.4	11.12	2.48
Ash (%)	5.32	11.92	18.77
NFE (%)	21.35	35.18	9.66
Gross Energy (kcal g^{-1})	17.24	11.9	20.125

**Expressed as percentage of fresh weight, while crude protein, crude lipid, crude fiber, ash, nitrogen free extract and gross energy are expressed as percentage of dry matter. Each datum is mean of three different treatments*

Table 2. Formulation of Different trial feeds for *Cyprinus carpio*

Ingredients	Experimental feeds (%)			
	Diet 1 (Control)	Diet 2 (BS1)	Diet 3 (BS2)	Diet 4 (BS3)
Soybean Meal	60	60	60	60
Potato Starch	20	20	20	20
Fish Meal	10	10	10	10
Cod Liver Oil [a]	5	5	5	5
Mineral [b]	2.5	2.5	2.5	2 .5
Vitamin [c]	2.5	2.5	2.5	2.5
Probiotic [d] Concentration (cfu/100 g of feed)	**----**	2 $x10^4$	2 x 10^5	2 $x10^6$

Table 3. Proximate composition of different feeds (dry matter basis) for *Cyprinus carpio*

Ingredients	Diet 1 (CC)	Diet 2 (BS1)	Diet 3 (BS2)	Diet 4 (BS3)
Dry matter (%)	93.32	91.87	91.38	90.95
Crude Protein (%)	39.87	39.90	39.92	39.95
Crude Lipid (%)	7.61	7.63	7.63	7.15
Crude fiber (%)	5.9	5.86	5.88	5.85
Ash (%)	1.44	1.52	1.56	1.64
NFE *	33.50	31.96	31.39	31.36
Gross Energy (kcal g^{-1})	18.73	18.75	18.84	18.89
P/E ratio mg CP **/ kJ energy	21.29	21.28	21.18	21.14
Ca/P ratio	2.42	2.48	2.50	2.56

** Nitrogen free extract ** CP: Crude Protein*
Each datum is mean of three different treatments

"The probiotic bacterium *Bacillus subtilis* PB4(*CCI3*) isolated from the intestine of *C. carpio* were grown for 48 hrs. at 30°C with Nutrient agar media (Hi-media, India). The bacterial culture was centrifuged at 5000 rpm and the pellet was resuspended in sterile saline water" [4]. The experimental diets were prepared by absorbing suspension of the probiotic bacteria. The prepared feeds were spread in the sterile trays and the absorption was achieved by spraying the suspended probiotic bacteria in 2 x 10^4 (BS1), 2 x10^5 (BS2) and 2x10^6 (BS3) *Bacillus subtilis CCI3* cells per 100g feed. "After spraying, the feed was air dried in a vent hood at the room temperature overnight and the moisture content and the bacterial concentration in the feeds (CFU 100 g^{-1}) was calculated. The bacterial concentration was calculated 1.72 x 10^4, 1.87 x 10^5 and 1.76 x10^6 CFU / 100 g of feed BS1, BS2 and BS3 respectively. The control feed (CC) was not supplemented with the *Bacillus subtilisCCI3* finally the feed was stored in vacuumed heavy-duty plastic containers at 4ºC" [15]. Routine checking of the bacterial concentration in the feeds didn't show any marked variation.

2.2 Studies on Growth and Dietary Performances

The experimental setup consisted of 15 rectangular aquariums (triplicates of each treatments) capacity of 20 liter with continues aeration. Each aquarium was stocked with seven fishes. Water quality (temperature, pH, dissolved oxygen, total alkalinity, total NH_3) was monitored at weakly intervals following the methods provide in APHA – AWWA – WPCF (1998). Fishes were fed twice daily at 8.00 and 11.00 hr. at 5% body weight [16] in two equal installments. The net weight was recorded every 15 days with an electronic balance and feed quality was readjusted after every weighing period of 15 days. For evaluating the dietary performances, the nutritional indices like live weight gain (LWG), average daily growth (ADG), feed conversion ratio (FCR), specific growth rate (SGR) and protein efficiency ratio (PER) were used.

2.3 Proximate Analysis

Proximate analyses of ingredients, feeds, fecal matter and body carcass were determined following the methods provided in AOAC (1990). Moisture content was determined by drying the samples in hot air oven (Kumar, Mumbai, India) at 110ºC for 24 hr. Crude protein content (Total Kjeldahl Nitrogen X 1.25) were estimated by micro-kjeldahl method. Crude lipid contents were determined by the soxhlet extraction method using petroleum ether (boiling point: 40 – 60ºC) in the electro –thermal Soxhlet apparatus. After extraction of lipid, the defattend samples were used for the estimation of crude fiber following Patra [17]. Ash content was estimated by incinerating samples in a muffle furnace at 500 ± 50ºC for 1hr.

"One hour after feeding, the left-over feed was initially siphoned out and equal amount of water replenished. For fecal matter analyses, pooled fecal matter was collected into Petri dishes from the bottom of the aquarium at every two hours by the help of pipette" [18]. The collected material was stored at – 20º C [19]. The collected material was dried in an oven (Kumar, Mumbai, India) at 55º C, ground and preserved in airtight containers.

2.4 Biochemical Analysis

DNA (Deoxy-ribo Nucleic acid) and RNA (Ribo-Nucleic acid) contents in the 200 mg liver (hepatopancreas) tissues were estimated as per the scheme given by Munro and fleck [20]. The activity of the digestive enzymes, protease and α-amylase in the intestine of fishes were estimated according to the method Bernfeld [21] and modified by Snell and Snell [22]. The GOT and GPT activity in the liver were determined following the method Bernfeld [21], while ACP activity was determined the method of Bramley [23] and ALP activity by Rosauki [24].

2.5 Study of Blood Parameters

"Blood samples were collected by heparinized syringe from caudal vein for hematology. EDTA (Ethylene Diamine Tetra acetic acid) was used as anticoagulant. 1.0mg EDTA ml^{-1} of blood or 1 drop of 1.0% solution 5 ml^{-1} of blood was used for hematology. Hematological parameters were estimated according to the method" of Wintrobe [25]. "MCV, MCH, MCHC were calculated by using standard formulae" [26]. Blood samples were collected in the laboratory for serological diagnosis by syringe from caudal vein and heart. Determination of the total serum protein (TSP) and albumin were estimated following the method of Kulow [27]. "The globulin content is the difference between the total protein and the albumins. The estimation of glucose was carried out by glucose oxidase method" [28].

2.6 Determination of Immunity Levels

"At the end of the feeding trial i.e. on day 60, fishes of the experimental tanks of each group were labeled to collect serum samples and analyzed for agglutination titer using microtiter plates following" [29]. "Collected sera were stored at 20ºC until analysis. Blood was collected from the fish by using a 2 ml glass syringe rinsed with an anticoagulant (EDTA). Then the blood was transferred into the heparinized vial and mix properly. 0.1 ml of freshly prepared NBT solution was added to 0.1ml of the heparin mixed blood and 15 µl of stimulant solution in the incubating bottle. The bottles were incubated at 37ºC for 10 minutes and at 26ºC for another 10 minutes. 50 – 70µl of this blood was transferred onto a clean slide and makes a thick smear with a spreader slide. The slides were air dried and stain with Wright's stain. For staining with the Wright's stain, first blood the slide with 1ml of the staining solution for 30 seconds then 1 ml of distilled water was added and keep for another 30 seconds. The stain was then poured off and slide was dried. Then the slide was observed under oil immersion lens at 100 X. the positive cells, had the violet colored formazan granules in the cytoplasm. The percentage of the positive cells gave the idea about the non-specific immune status of the organism" [29].

2.7 Challenge Trial

After feeding for 60 days, the fishes in each treatment were challenged with *Aeromonas species* (A2), which had been cultured and maintained in the *Aeromonas* medium (TSA, Hi-Media). Fishes in all replicated immersed in a suspension of *Aeromonas* A2, ~ 10^5 CFU ml^{-1} according to Austin et al. [13].

2.8 Statistical Analysis

As all the analysis was carried out on pooled samples of a given lot, standard errors or standard deviation of means were calculated. However, for evaluating the dietary performances, nutritional indices, enzymatic activities and RNA: DNA ratio, different hematological, serological, immunological parameters and challenge trials; correlation and regression test was performed through SPSS packages. Significant differences between the means of the treatment were tested. Two-factor without replication Analysis of Variance (ANOVA) was applied to defect the significant differences in growth and survivability between the treatments

3. RESULTS AND DISCUSSION

The proximate composition of different ingredients used for preparing experimental feeds for *Cyprinus carpio* was presented in Table 1. The crude protein percentage of soyabean meal, potato starch and fish meal were 38.52, 29.86 and 54.76 respectively, whereas the crude lipid percentage was 18.56, 4.53 and 1.13 respectively. All the four experimental feeds (CC, BS1, BS2 and BS3) were almost isocaloric and isonitrogenous. The average crude protein percentage on dry matter basis was around 39.91 and the gross energy was around 18.80 kJ g-1 Table 3.

Table 4 A and Fig. 1 represents the growth of *Cyprinus carpio* in relation to various feeds from the table and the Fig. 1 it is indicated that significantly ($p \leq 0.05$) highest growth (45.54 ± 0.075 g) was obtained from feed BS2 fed fishes, whereas lowest growth (40.12 ± 0.0120 g) was observed in case of feed CC fed fishes. Significantly ($p \leq 0.05$) differences of growth between the treatments was further confirmed by two factors without replication ANOVA analysis where *F Crit* < F (Table 4B). Fish fed BS2 showed highest growth in terms of weight gain percent (132.74 ± 0.410 and least was found in feed CC (103.42 ± 0.230) (Table 5). Significantly ($p \leq 0.05$) highest SGR, PER and lowest FCR were observed in feed BS2 fed *Cyprinus carpio*, whereas lowest SGR,PER and highest FCR were observed in feed CC fed fishes (Table 5).

Table 6 represents the Initial and final carcass composition in *Cyprinus carpio* with relation to various trial feeds. The carcass composition of the fishes revealed an apparent increase in the final carcass protein and lipid ($p \leq 0.05$) over the initial protein and lipid. Highest carcass protein was observed in BS2 fed fishes (63.72 ± 0.050) and least in fish fed with CC. Among the treatment significantly ($p \leq 0.05$) highest crude lipid percentage (22.05 ± 0.009 %) was recorded in feed BS2 feed fishes, while lowest in control (CC). These results indicate that enhancement of carcass quality by probiotic supplemented (*Bacillus subtilis* CCI3) feeds may be due to enzymatic activity in the gut and thereby better nutrient utilization.

Proximate composition of fecal matter of *Cyprinus carpio* during 60 days feeding trial was presented in Table 7. Fecal matter proximate analysis revealed significantly ($p \leq 0.05$) least nitrogen excretion (12.16 ± 0.044 %) in fish fed BS2 whereas highest (17.52 ± 0.039 %)) in fish fed feed CC (Table 7). The crude lipid remained between 3.64 ± 0.007 % (feed BS2) and 3.98 ± 0.011 % (feed CC).

Different water quality parameters of different treatment tanks during the 60 days feeding trial of *Cyprinus carpio* were represented in Table 8. The relation of different water quality parameters did not follow any specific trend may be of controlled conditions and isocaloric feeds. The water quality during the study period remained in the following ranges: pH, 7.41 ± 0.119 to 7.56 ± 0.251; total alkalinity 112.37 ± 4.6 to 139.87 ± 4.2; DO, 4.44 ± 0.362 to 5.02 ±0.621; total ammonia, 0.548 ± 0.038 to 0.1312 ± 0.020 and average temperature 30.00 ± 1.255. It was reported by Ngan and Phu, (2011) that *Bacillus* spp. were associated with improvement of water quality, reduction of pathogenic *vibrios* in culture environment, enhancement of survival and growth rate, and the improved health status of juvenile *Penaeus monodon*.

Significantly ($p \leq 0.05$) greatest RNA: DNA ratio (1.48 ± 0.009) was registered in fish fed feed BS2 and least (1.37 ± 0.012) in fish fed feed CC treated fishes (Table 9, Fig. 2). It is also observed that RNA: DNA ratio of fishes increased in all the treatments over the initial RNA: DNA ratio. Intestinal Protease and alpha amylase activity were significantly ($p \leq 0.05$) highest in feed BS2 fed *Cyprinus carpio* (5.90 ± 0.05 and 0.32± 0.07) and lowest in feed CC fed fishes (3.60 ± 0.03) (Table 10). The activity of protease increased with increase of dietary protein [30], but in this experiment all the feeds were isonitrogenous and feed BS2 showed greater protease activity might be due to greater dietary protein utilization. Saigal et al. [31] reported that the activity of amylase is correlated with the carbohydrate content of the diet. In this study, although all the feed compositions were same, but feed BS2 fed fishes showed greater amylase activity, which greater carbohydrate utilization. This study also showed that amylase activity was greater in all the test feeds compared to initial, which was also indicated that this probiotic bacterial strain might be able to synthesized the carbohydrate content of the diet. The ACP activity in the liver was greatest ($p \leq 0.05$) in case of fish fed feed BS2 (2.65 ± 0.010) and least (2.05 ± 0.030) in case of fish fed feed CC (Table 10). Similarly, ALP activity in the liver was shown the similar trend (Table 10). Significantly ($p \leq 0.05$) highest GOT (0.053 ± 0.001) and GPT (0.088 ± 0.003) values were registered in feed BS2 fed fishes, whereas lowest (0.040 ± 0.001) GOT values was recorded in feed CC fed fishes and GPT value (0.041 ± 0.003) in feed CC fed fishes. The highest VSI and HIS was found in feed BS2 (11.55± 0.04 and 2.65 ± 0.05) (Table 11).

"Hematological value of *Cyprinus carpio* after 60 days feeding trial was presented in Table 12. and hematological value in fishes after challenge trial were presented in Table 11. Significantly ($p \leq 0.05$) highest TEC, TLC, Hb and Hct were registered in fed BS2 treated fishes, while lowest in feed CC fed fishes. Blood is a patho-physiological reflector of the whole body and therefore, blood parameters are important in diagnosing the status of fish" [26], particularly when a bacterial strain is incorporated in feed. In this present study it was observed that all the blood parameters in all the treatments were similar to standard [17] and "BS2 fed fishes showed superior in compare to others, which was not only indicated the positive impact but also demonstrated a stable physiological reflection of the whole body" [26].

After 60 days feeding trial survivability of fishes were determined by challenge trial with pathogenic. Significantly ($p \leq 0.05$) highest survivability (93.33%) was observed in feed BS2 fed fishes and least in feed CC treated fishes (11.66%) (Table 15A, Fig. 3). From ANOVA analysis it was found that the survivability between the treatment and days were significantly ($p \leq 0.05$) different (Table 15B).

After challenge trial significantly ($p \leq 0.05$) decrease in the value of glucose and albumin globulin ratio of feed BS2 was found.

"The observation draws attention to an essential inference that the probiotic concentration, which was used in this feed, might be helpful for optimum dietary utilization. Thus, the bacilli are thought to antagonize potential pathogens in the aquatic environments. This is curious because it is generally accepted that laboratory cultures do not survive well when re-introduced into the natural environment; the cells being often outcompeted / antagonized by the natural

microflora" [32]. "Nevertheless, a direct benefit to the use of the bacilli was the reduction in the use of chemicals in the aquatic environment and in enhanced growth of famed species" [33].

Table 4A. Growth (g) of *Cyprinus carpio* in relative to various feeds

Feeds	Average Weight (g)				
	Initial	**15 days**	**30 days**	**45 days**	**60 days**
CC	27.75 ± 0.045	30.85 ± 0.018	34.69 ± 0.054	37.04 ± 0.084	40.45 ± 0.097[a]
BS1	27.78± 0.060	30.76 ± 0.075	35.98 ± 0.088	38.12 ± 0.096	42.86 ± 0.0110[b]
BS2	27.73± 0.045	31.45 ± 0.053	35.15 ± 0.068	39.68 ± 0.072	45.54 ± 0.075[c]
BS3	27.78± 0.030	30.74 ± 0.075	33.88 ± 0.082	31.01 ± 0.109	40.12 ± 0.120[d]

Results are mean of ten separate determinations (Mean + SE)
Figures having Different letter (superscripted) in the same column are significantly different ($p \leq 0.05$)

Table 4B. two factor without replication ANOVA analysis of Growth of *Cyprinus carpio*

Source of variation	SS	df	MS	F	P-Value	F crit
Probiotic concentration	13.55988	3	4.51996	4.298321	0.028145	3.490295
Days	528.2073	4	132.0518	125.5766	1.11E-09	3.259167

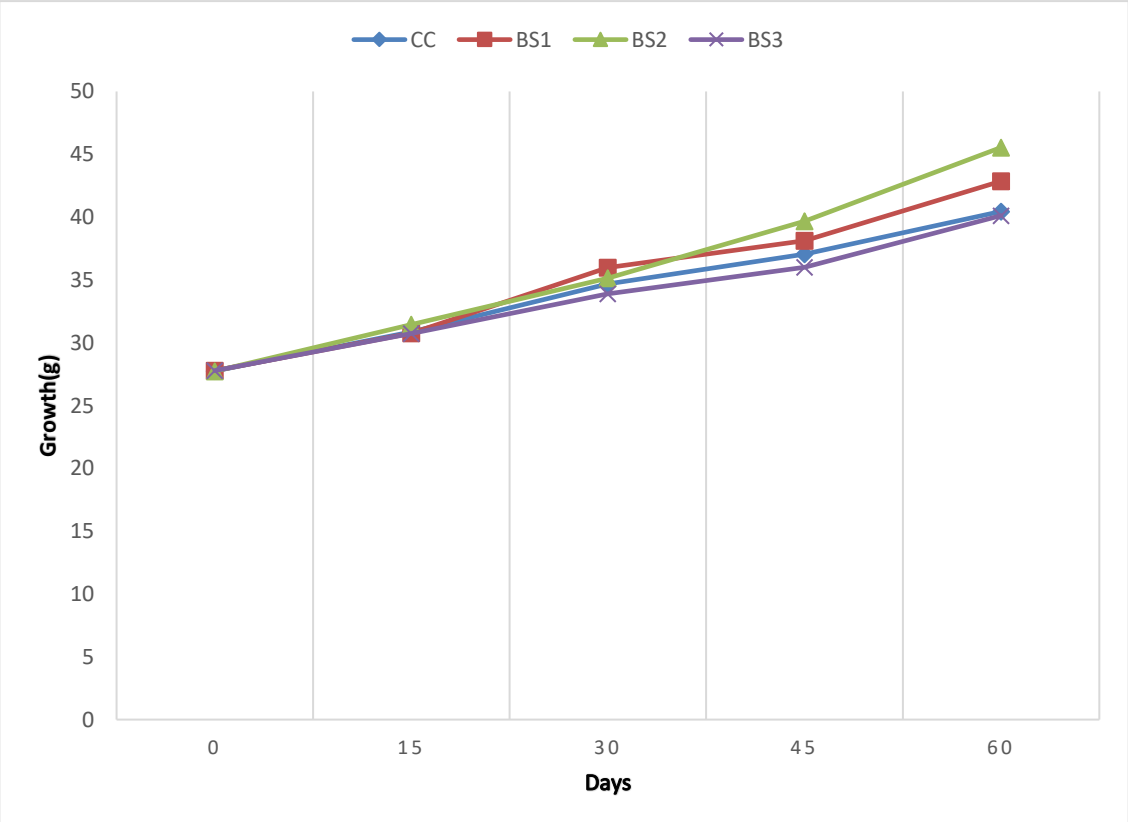

Fig. 1. Growth (g) of *Cyprinus carpio* in relation to various feeds

Table 5. Initial and final body weight, Live weight gain, Average daily growth, FCR, SGR and PER of *Cyprinus carpio* in relation to various trial feeds

Feeds	Fish Weight (g)		Live weight gain (g)	Weight gain %	FCR	SGR	PER
	Initial	Final					
CC	27.75 ± 0.025	51.45 ± 0.027	28.7 ± 0.006[a]	103.42 ± 0.230[a]	2.90 ± 0.004[a]	1.18 ± 0.002[a]	0.8647 ± 0.007[a]
BS1	27.78± 0.030	58.86 ±0.033	31.08 ± 0.012[b]	111.879 ± 0.421[b]	2.681 ± 0.011[b]	1.25 ± 0.005[b]	0.9346 ± 0.008[b]
BS2	27.73±0.032	64.54 ±0.028	31.81 ± 0.010[c]	132.74 ± 0.410[c]	2.259 ± 0.005[c]	1.41 ± 0.003[c]	1.1084 ± 0.006[c]
BS3	27.78±0.027	57.12 ±0.025	29.34 ± 0.007[d]	105.61 ± 0.236[d]	2.84 ± 0.004[d]	1.20 ± 0.006[d]	0.8812 ± 0.007[d]

Results are mean of ten separate determinations (Mean + SE)
Figures having Different letter (superscripted) in the same column are significantly different ($p \leq 0.05$)

Table 6. Initial and final carcass composition of *Cyprinus carpio* in 60 days experimental trials

Carcass composition (%)	Experimental feeds				
	Initial	CC	BS1	BS2	BS3
Moisture (%)	74.16 ± 0.288[a]	74.24 ± 0.312 [a]	75.62 ±0.300 [a]	75.52± 0.0.217 [a]	75.21± 0.217 [a]
Crude Protein (%)	59.12± 0.050 [a]	60.33± 0.044[b]	62.42 ± 0.057 [b]	63.78± 0.050 [b]	63.25± 0.050 [b]
Crude Lipid (%)	15.28±0.010 [a]	10.12± 0.012[a]	21.14 ± 0.011 [b]	22.05± 0.009 [b]	20.45 ±0.020 [b]
Ash (%)	13.22±0.010 [a]	12.14± 0.016 [b]	12.58 ± 0.007 [b]	12.87± 0.010 [b]	12.64± 0.009[b]

Results are mean of ten separate determinations (Mean + SE)
Figures having Different letter (superscripted) in the same row are significantly different ($p \leq 0.05$)

Table 7. Proximate composition of faecal matter for *Cyprinus carpio* in 60 days experimental trial

Composition (% dry matter)	Experimental Feeds			
	CC	BS1	BS2	BS3
Crude Protein (%)	17.52± 0.039[a]	14.35±0.045[b]	12.16±0.044[c]	13.48±0.020 [c]
Crude Lipid (%)	3.98±0.011[a]	3.69±0.010[b]	3.64±0.007[b]	3.87±0.012 [c]
Ash (%)	9.42±0.011[a]	1.31±0.005b	5.19±0.008 [a]	1.31±0.010 [a]

Results are mean of ten separate determinations (Mean + SE)
Figures having Different letter (superscripted) in the same row are significantly different ($p \leq 0.05$)

Table 8. Various water quality parameters of different treatment tanks during the 60 days feeding trials of *Cyprinus carpio*

Parameters	Experimental tanks			
	CC	**BS1**	**BS2**	**BS3**
Temperature (°C)	30.0 ± 1.225	30.0 ± 1.225	30.0 ± 1.225	30.0 ± 1.225
pH	7.41 ± 0.119	7.56 ± 0.215	7.52 ± 0.212	7.53 ± 0.2.16
Total Alkalinity (ppm)	139.87 ± 4.2	127.92 ± 4.5	125.37 ± 5.2	112.37 ± 4.6
DO (ppm)	4.95 ± 0.438	4.62 ± 0.471	5.02 ± 0.621	4.44 ± 0.362
Total NH_3 excretion (mg/kg^{-1})	1312 ± 0.020	610.42 ± 0.03	548.38 ± 0.024	645.58 ± 0.028

Results are mean of five separate determinations (Mean + SE of mean)

Table 9. Muscle RNA/DNA ratio in *Cyprinus carpio* treated with different experimental feeds

Feeds		RNA/DNA ratio
Initial		1.16 ±0.006
Final	CC	1.37±0.012[a]
	BS1	1.45±0.001[b]
	BS2	1.48±0.009[c]
	BS3	1.41 ±0.007[d]

Results are mean of five separate determinations (Mean + SE of mean)
Figures having Different letter (superscripted) in the same column are significantly different ($p \leq 0.05$)

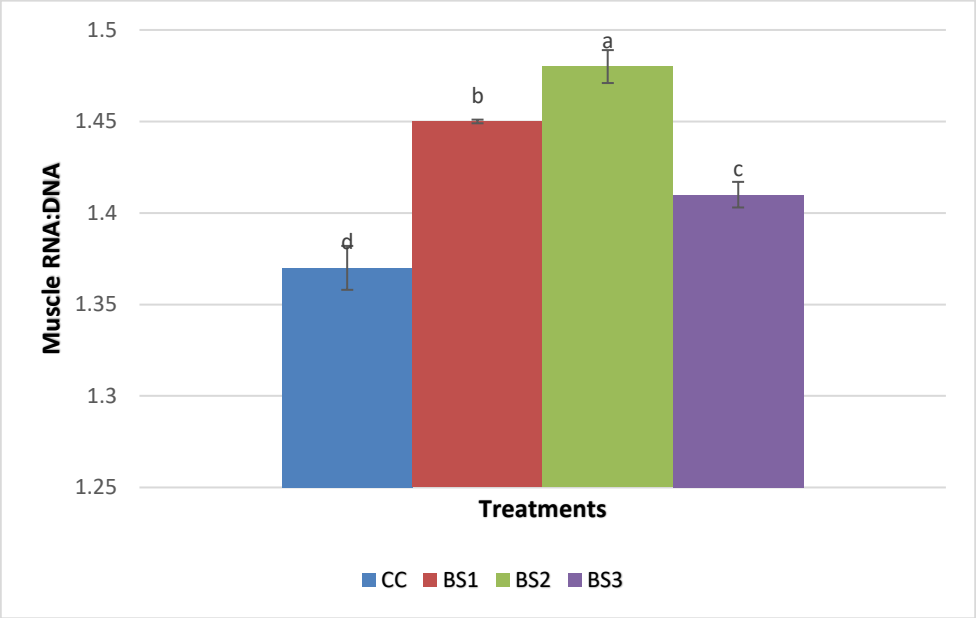

Fig. 2. Final Muscle RNA:DNA ratio in *Cyprinus carpio* in relation to various feeds

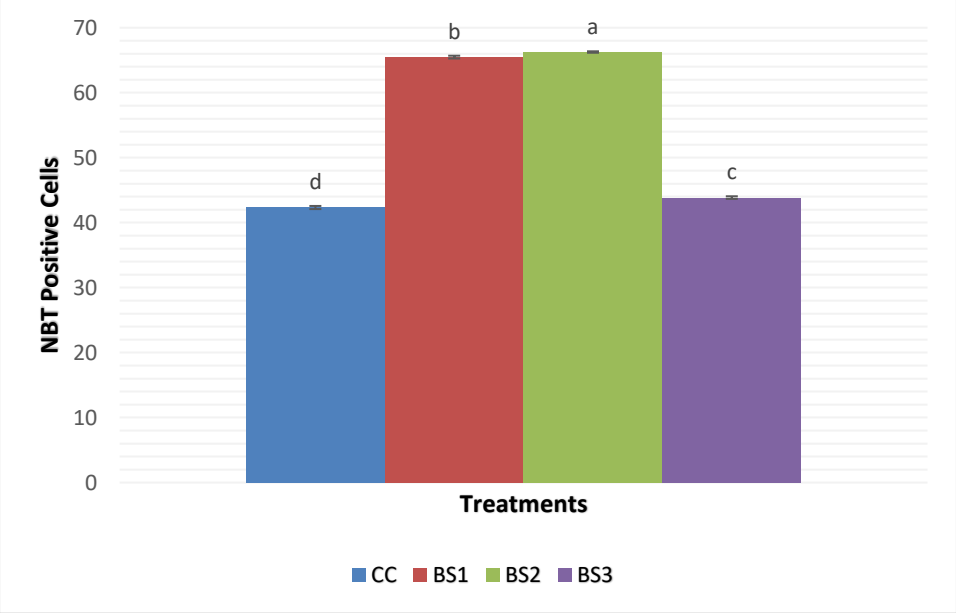

Fig. 3. NBT positive values of *Cyprinus carpio* in relation to various feeds

Table 10. Effect of different experimental feeds on muscle protein, muscle glycogen, enzymatic activities (amylase, Protease, lipase, ACP, ALP, GOT and GPT) under laboratory conditions (LD 12:12 at 28 ± 1°C) -60 days treatment

Parameters	Diets			
	CC	BS1	BS2	BS3
Muscle Protein (mg g^{-1})	108 .65 ±1.76^{c}	115.09±1.23^{b}	128.05±1.26^{b}	111.07±1.21^{b}
Muscle Glycogen (mg g^{-1})	1.71 ±0.03^{c}	1.65±0.02^{a}	1.42±0.03^{a}	1.44±0.02^{b}
Liver Glycogen (mg g^{-1})	2.44 ±0.03^{a}	2.22±0.02^{b}	2.18±0.03^{c}	2.40±0.035^{a}
Total amylase activity (mg g^{-1} h^{-1})	0.21±0.02^{e}	0.28±0.04^{c}	0.32±0.07^{d}	0.29±0.03^{a}
Specific amylase activity (mg g^{-1} h^{-1})	0.11±0.02^{b}	0.14±0.03^{a}	0.17±0.03^{b}	0.15±0.03^{b}
Total Protease activity (mg g^{-1} h^{-1})	3.60±0.03	3.98±0.05	5.90±0.05	5.10±0.05
Specific Protease activity (mg g^{-1} h^{-1})	1.35±0.04	1.85±0.03	2.60±0.05	2.28±0.04
Total Lipase activity (mg g^{-1} h^{-1})	0.08±0.03	0.17±0.02	0.25±0.01	0.20±0.02
Specific Lipase activity (mg g^{-1} h^{-1})	0.10±0.03	0.15±0.02	0.18±0.03	0.16±0.03
ACP	2.05±0.030	2.17±0.021	2.65±0.010	2.58±0.005
ALP	12.03±0.08	13.50±0.008	15.60±0.013	14.10±0.020
GOT	0.040±0.001	0.044±0.001	0.053±0.001	0.046±0.001
GPT	0.041±0.003	0.068±0.003	0.088±0.003	0.069±0.001

Results are mean of five separate determinations (Mean + SE of mean) Means bearing Different letter (superscripted) in the same row are significantly different ($p \leq 0.05$) mg of tyrosine liberated/mg of protein/minute, mg of maltase liberated/mg of protein/minute ,micromole fatty acid liberated/mg of protein/hour

ACP= Acid Phosphatase (EC 3.1.3.2)

ALP=Alkaline Phosphatse (EC 3.1.3.1)

GOT=Glutamate-oxaloacetate transaminase (E.C.2.1.1.1)

GPT= Glutamate-pyruvate transaminase (E.C.2.1.1.2)

Table 11. Effect of different experimental feeds on VSI and HSI under laboratory conditions (LD 12:12 at 28 ± 1°C) 60 days treatment

Parameters	Diets			
	CC	BS1	BS2	BS3
VSI[1]	1.90±0.30^{d}	9.85±0.03^{b}	11.55±0.04^{a}	10.07±0.02^{b}
HSI[2]	1.61 ±0.16^{d}	1.70±0.13^{c}	2.65±0.05^{a}	1.88±0.04^{b}

[1]Viscero-Somatic [2]Hepato-Somatic

Results are mean of five separate determinations (Mean + SE of mean)

Means bearing Different letter (superscripted) in the same row are significantly different ($p \leq 0.05$)

Table 12. Hematological value of *Cyprinus carpio* after feeding trial

Parameters	Treatments			
	CC	BS1	BS2	BS3
TEC (x 10^6 mm^3)	1.61 ±0.004[a]	1.86±0.006[b]	2.08±0.002[c]	1.52±0.003[d]
TLC (x 10^6 mm^3)	18.9 ±0.010 [a]	22.5±0.002[b]	21.4 ±0.008 [c]	20.1±0.009[d]
Hb (g%)	10.4±0.009	10.9±0.002 [b]	11.4±0.12 [c]	9.8 ±0.009 [d]
Hct (%)	28.4±0.016 [a]	30.75±0.020[b]	31.42±0.031[c]	28. 1± 0.022[d]
MCV (µm^3 $cell^{-1}$)	169.589±0.142 [a]	160.814±0.106[b]	148.612±0.187[c]	172.94± 0.121[d]
MCH (pg$cell^{-1}$)	41.49±0.036 [a]	47.022±0.048 [b]	47.38±0.034 [c]	51.87± 0.032 [d]
MCHC (g 100ml Hct^{-1})	29.911±0.011[a]	32.02±0.008[b]	34.99±0.012[c]	30.89± 0.09 [d]

Results are mean of five separate determinations (Mean + SE of mean)
Means bearing Different letter (superscripted) in the same row are significantly different ($p \leq 0.05$)

Table 13. Effect of different experimental feeds on NBT Positive values and Albumin: Globulin ratio of *Cyprinus carpio*

Parameters	Treatments			
	CC	BS1	BS2	BS3
NBT positive cells (%)	42.33± 0.241[a]	65.48± 0.213[b]	61.26± 0.112[c]	43.86± 0.202[d]
Albumin: Globulin ratio	1.76± 0.011[a]	1.66 ± 0.007[b]	1.58 ± 0.012[c]	1.69 ± 0.009[d]

Results are mean of five separate determinations (Mean + SE of mean)
Means bearing Different letter (superscripted) in the same column are significantly different ($p \leq 0.05$)

Table 14. Detection of Glucose level and antibody titer of *Cyprinus carpio*

Parameters	Treatments			
	CC	BS1	BS2	BS3
Glucose	74.2 ± 0.011[a]	74.5± 0.015[b]	75.8 ± 0.019[c]	70.45 ± 0.010[d]
Antibody titer	47.02± 2.040[a]	73.83 ± 2.010[b]	151.72 ± 3.240[c]	59.98± 2.440[d]

Results are mean of five separate determinations (Mean + SE of mean)
Means bearing Different letter (superscripted) in the same column are significantly different ($p \leq 0.05$)

Table 15A. Survivability of *Cyprinus carpio* in a challenge trial with *Aeromonas Species* for 10 days

Days	Treatments							
	CC		BS1		BS2		BS3	
	Ser No.	Sur%	Sur No.	Sur%	Ser No.	Sur%	Ser No.	Sur%
1	30	100	30	100	30	100	30	100
2	30	100	30	100	30	100	30	100
3	28	83.33	30	100	30	100	30	100
4	24	80	30	100	30	100	28	93.33
5	19	63.33	29	91.66	30	100	25	83.33
6	15	50	28	93.33	30	100	23	71.66
7	14	41.66	26	81.66	30	100	22	73.33
8	12	40	25	83.33	30	100	16	53.33
9	8	21.66	20	61.66	29	91.66	13	43.33
10	5	11.66	14	41.66	88	93.33	12	40

Means bearing Different letter (superscripted) in the same row are significantly different ($p \leq 0.05$)

Table 15B. Two factor without replication ANOVA analysis of Survivability of Cyprinus *carpio*

Source of variation	SS	df	MS	F	P-Value	F crit
Probiotic concentration	8240.773	9	915.6415	4.374732	0.000656	2.152607
Days	53390.79	4	13347.7	63.77234	7.68E-16	2.633532

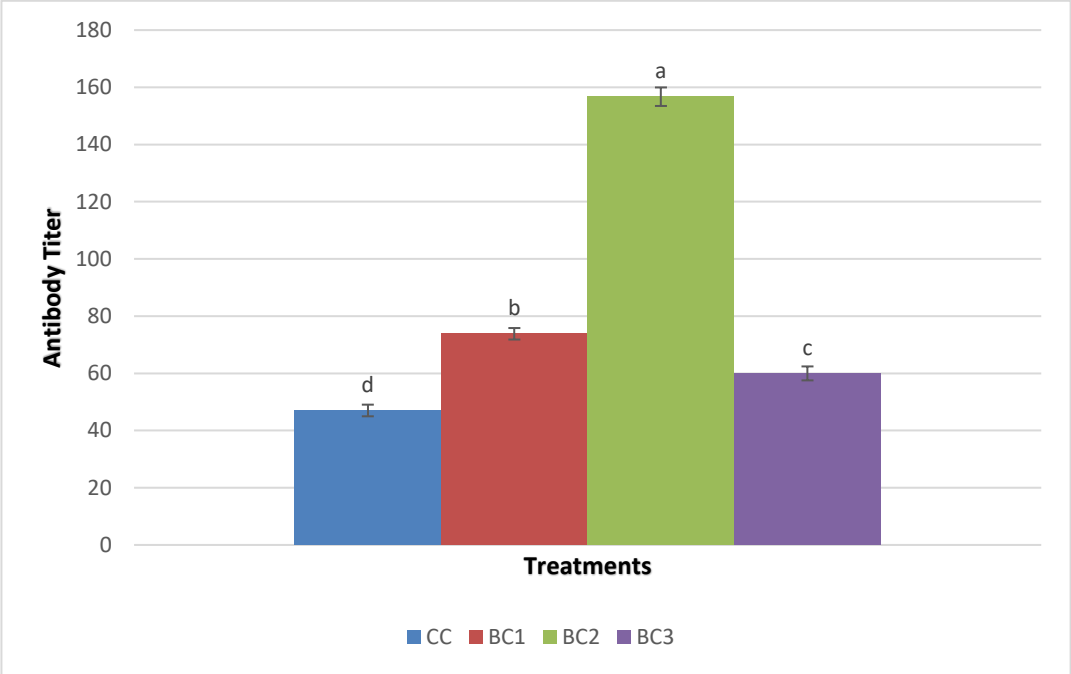

Fig. 4. Specific immune response (circulating antibody titer) from *Cyprinus carpio* in relation to various feeds

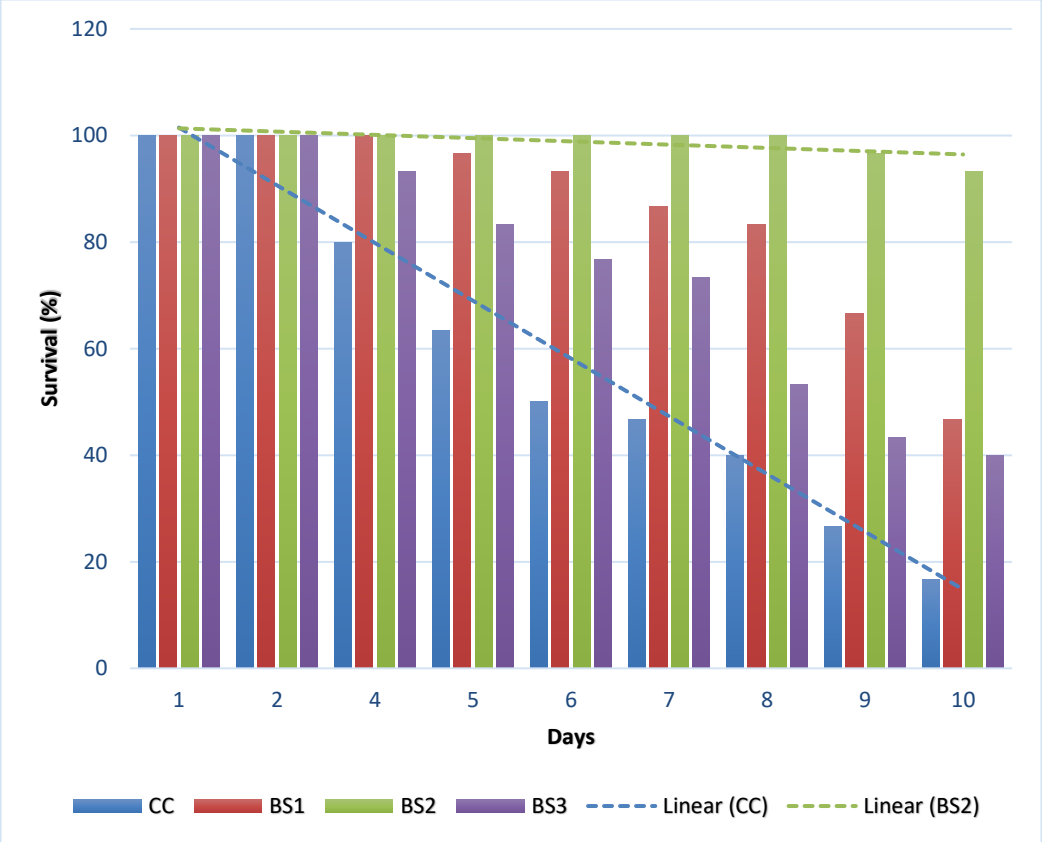

Fig. 5. Survivability of *Cyprinus carpio* in a challenge trial with *Aeromonas hydrophila* for 10 days

Table 16. Hematological value of *Cyprinus carpio* after challenge trial

Parameters	Treatments			
	CC	BS1	BS2	BS3
TEC (x 10^6 mm^3)	1.58 ±0.004[a]	2.02 ±0.004[b]	2.51±0.002[c]	1.84±0.002[d]
TLC (x 10^6 mm^3)	1.22 ±0.010 [a]	2.65 ±0.002 [b]	3.77 ±0.008[c]	2.15 ±0.009[d]
Hb (g%)	9.8 ±0.009 [a]	10.5 ±0.002 [b]	10.2 ±0.006 [c]	9.8 ±0.009 [d]
Hct (%)	28.51±0.014 [a]	30.12±0.018 [b]	34.12 ±0.021 [c]	28.21±0.022 [d]
MCV (µm^3 $cell^{-1}$)	169.26±0.116 [a]	134.24±0.121[b]	154.04± 0.147 [c]	174.31±0.125 [d]
MCH (pg$cell^{-1}$)	52.652 ±0.042 [a]	45.78 ± 0.018 [b]	41.58±0.042 [c]	42.98 ±0.012 [d]
MCHC (g 100ml Hct^{-1})	32.144 ±0.031[a]	30.925 ± 0.018[b]	21.095 ± 0.025 [c]	28.822±0.37[d]

Results are mean of five separate determinations (Mean + SE of mean)
Means bearing Different letter (superscripted) in the same row are significantly different ($p \leq 0.05$)

Table 17. Detection of Glucose level and Albumin: Globulin ratio of *Cyprinus carpio* after challenge trial

Parameters	Treatments			
	CC	BS1	BS2	BS3
Glucose (mg $100ml^{-1}$)	119.3 ± 0.022[a]	84.7± 0.035[b]	95.8 ± 0.015[c]	99.8 ± 0.014[d]
Albumin: Globulin ratio	2.16± 0.010[a]	1.56 ± 0.017[b]	1.41 ± 0.014[c]	1.78 ± 0.016[d]

Results are mean of five separate determinations (Mean + SE of mean)
Means bearing Different letter (superscripted) in the same column are significantly different ($p \leq 0.05$)

"The use of probiotics has been accompanied by a concomitant reduction in the levels of antimicrobial compounds (particularly antibiotics) used in aquaculture and in improved appetite and / or growth performance of the farmed species. In particular, it is important to determine whether or not probiotic actually tastes good or does it modify the feed thereby improving digestibility (and taste)" [4].

Kennedy et al. [34] used "*Bacillus* 48 to enhance the quality and viability of common snook, *Cetropomus undecimalis* (Bloch). These workers found that *Bacillus* improved the survival of larvae, increased food absorption by enhancing protease levels and gave better growth. Also, the probiotic decreased the number of suspected pathogenic bacteria in the gut". It is noteworthy that Chang and Liu [35] used "*Bacillus toyoi* and *Enterococcus faecium* SF 68 from commercial products to reduce Edwardsiellosis in European eel, *Anguilla anguilla* (L.)". An extracellular protease producing bacteria *Bacillus circulans* (Lr 1.1) was isolated by Ghosh et al. [36] from the gut of *Labeo rohita,* fingerlings, and used as supplement in the diets and the effect of supplement on growth performance and utilization efficiency of *L. rohita.* Similarly, it was reported by Parthasarathy and Ravi [37] that "plasma total protein of *Catla catla* decreased when the fish were fed with diets supplemented with *Lactobacillus plantarum* or a mixture of *Lactobacillus plantarum* and *Bacillus megaterium*. It is clear from these studies that the effect of supplemental probiotic on fish health, immune response and hematology may be species-specific, depending on probiotic type, dose and administration route". Osman et al. [38] observed "improvement in growth and immune status in cultured *Oreochromis niloticus* treated with probiotic bacteria *Micrococcus"* [39-43].

In the above investigations, although, all the feeds were iso-nitrogenous but the concentration of probiotics in BS2 feed might be helpful for proper nutrient utilization. Whole body carcass composition and lesser nitrogen egestion is attributable to proper probiotic concentration. RNA: DNA is known to provide dependable indication of growth trend. The various results find out from present investigation triggers for the utilization of probiotic (*Bacillus subtilis CCI3*) for optimal growth, appropriate use of nutrients, further it confirms that it work as important immunostimulant in *Cyprinus carpio. The* statistically significant data ($p \leq 0.05$) were observed in all the parameters of immunity, higher survival against the pathogenic *Aeromonas species* infection, thus indicating as a potent immunostimulant in *Cyprinus carpio.* The findings of this study suggest that the concentration of probiotic *Bacillus subtilis CCI3* applied in feed was able to increase the overall physiological performances and enhanced the defense mechanism in the fingerlings of *Cyprinus carpio*.

4. CONCLUSION

In the above investigations, although, all the feeds were iso-nitrogenous but the concentration of probiotics in BS2 feed might be helpful for proper nutrient utilization. Whole body carcass composition and lesser nitrogen egestion is attributable to proper probiotic concentration. RNA:

DNA is known to provide dependable indication of growth trend. The various results find out from present investigation triggers for the utilization of probiotic (Bacillus subtilis CCI3) for optimal growth, appropriate use of nutrients, further it confirms that it work as important immunostimulant in *Cyprinus carpio*. The statistically significant data (p 0.05) were observed in all the parameters of immunity, higher survival against the pathogenic Aeromonas species infection, thus indicating as a potent immunostimulant in *Cyprinus carpio*. The findings of this study suggest that the concentration of probiotic Bacillus subtilis CCI3 applied in feed was able to increase the overall physiological performances and enhanced the defense mechanism in the fingerlings of *Cyprinus carpio*.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies have been used to draft this article.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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