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Probiotics Supplementation for Improving Growth Performance, Nutrient Digestibility and Hematology of *Catla catla* Fingerlings Fed Sunflower Meal-Based Diet

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DR conducted the study and wrote the manuscript. SMH acquired funds, administered and supervised the project. MH, MZHA and EN curated data, and reviewed and edited the manuscript.

Key words
***Catla catla*, Probiotics, Growth performance, Hematological indices, Nutrient digestibility**

ABSTRACT

The use of probiotics as feed additives has been extensively explored and serves a protective function against gut pathogenic microorganisms. The purpose of this study was to examine the effects of supplementing *Catla catla* fingerlings fed a diet including sunflower meal (SFM) with probiotics (Protexin®) on growth performance, nutritional digestibility, and hematological indicators. Six test diets and one control diet (0 g/kg) were formulated before the beginning of the experiment, with varying amounts of probiotics (0, 0.5, 1, 1.5, 2, 2.5 and 3 g/kg) added to the basal diet. Fish growth performance, nutritional bioavailability, and hematological indices all improved significantly ($p<0.05$) after being supplemented with probiotics. Fish fed a diet supplemented with probiotics at a rate of 2.5 g/kg had substantial improvement in body weight (20.29 g), body wt.% (272.78 %), feed conversion ratio (1.24) and specific growth rate (1.46). At a supplementation dosage of 2.5 g/kg, the maximum apparent digestibility coefficients (ADC) were observed for gross energy (73.54 %), crude protein (74.57 %), and crude fat (77.53 %). The total quantity of RBCs, WBCs, platelets, hemoglobin (Hb), and hematocrit (Ht) all changed significantly when fish were given a probiotic dosage of 2.5 g/kg. Incorporating probiotics at a dose of 2.5 g/kg resulted in the best growth performance, highest nutritional digestibility, and best hematological indicators in the fingerlings. Finally, the optimal dosage of probiotics for supplementation was shown to be 2.5 g/kg.

INTRODUCTION

Since aquaculture is the most practical source of nutritional protein, it is growing at an incredibly fast pace (FAO, 2018). The production of affordable fish feed that complies with all dietary requirements is essential for the success of this sector (Ahmad *et al*., 2021). Also, it is performing a major role in food safety and poverty control (Fiedler *et al*., 2016). The Food and Agriculture Organization estimated that the world's aquaculture output reached 87.5 million tons of animal production in the year 2020. It is anticipated that the output of aquatic foods would rise by an additional 15 percent by the year 2030. Whereas, aquaculture's production of finfish was predicted to account for 76% in 2020 (FAO, 2022). 8.7% of the world's carp production is composed of Indian major carps (IMCs) (FAO, 2014). One member of IMC that like to feed on the surface is *Catla catla*. Its distinctive flavor is a key source of its high price and popularity. Its diet contains both phytoplankton and zooplankton and resides in south Asian freshwaters.

Aquatic animals may benefit from a diet that includes fish meal. Fish meal is very nutritious, with the highest concentration of minerals, fats, vitamins, and high-quality protein as compare to any protein food source (Cho and Kim, 2011). However, unsustainable supply, increasing

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demand and high prices of fishmeal make it mandatory to search for alternative protein sources (Lim *et al*., 2011). Alternative to this fish meal, plant by products are being used singly or in combination to formulate cost effective feed (Enterria *et al*., 2011).

Sunflower meal (SFM) may prove a promising alternative to fishmeal as protein source because of its local availability and lower cost. Moreover, it is free of toxic and growth-reducing factors (Rehman *et al*., 2014). As a result of anti-nutritional factors (ANFs), scientists in the aquaculture industry are investigating the efficacy of feed by adding supplements like probiotics.

Probiotics are defined as non-digestible feed supplements (Gibson, 2004) that benefit the host by enzymatic contribution to enhance the feed quality, growth promoting factors and improve the immune response (Harikrishnan *et al*., 2010). These probiotics play important part in the survival of fish (Villamli *et al*., 2003) as well as increases growth rate by improving feed absorption. The gut pathogenic bacteria may be combated with the help of dietary probiotics.

The key purpose of this investigation was to examine the impacts of probiotic supplementation on the growth performance, nutritional digestibility, and hematology of *C. catla* fingerlings fed diets based on sunflower meal.

MATERIALS AND METHODS

The trial was carried out in the Fish Nutrition Laboratory, Department of Zoology, Government College University, Faisalabad.

Fish feeding experiment setup

Fingerlings of *C. catla* were taken from the Government Fish Seed Hatchery on Satyana Road in Faisalabad. Fingerlings were immersed in a 0.5% saline solution for a minute or two to kill any ecto-parasites and avoid fungal diseases. These were acclimated for two weeks in V-shaped water containers up to 70 L before the experiment. Throughout that time, they received basal feed once every day.

Formulation of feed

The SFM and other feed materials were obtained from an industrial feed mill. Prior to the formation of diets of experimental trial, all of the feed components were chemically tested (Table I) using standard procedures (AOAC, 1995). An inert marker called chromic oxide (Cr_2O_3) was included in all of the diets at a 1% concentration. An electric mixer was used to combine all the contents of ingredients. Water and fish oil were subsequently added to make suitable dough and then passed through feed pelletizer to form feed pellets (Lovell, 1989). Seven experimental diets were formulated by addition of graded levels (0, 0.5, 1, 1.5, 2, 2.5 and 3 g/kg) of probiotics (Protexin® CFU/g = 2×10^9; A multi-strains Probiotics Animal Nutrition, Probiotics International Ltd, England) (Table II). These diets were all dried in a cool, shaded spot and then stored in an oven at 4 degrees Celsius.

Feeding session and sample gathering

Experimental diets were fed to the fish at the rate of 4% of their live wet weight twice (8:00 am and 4:00 pm) a day. After one hour of the feeding session, the uneaten feed was collected and oven dried at 60°C for the purpose of feed intake analysis. Careful feces collection was performed to lower the risk of nutrient runoff into the water supply and were passed through the drying process in an oven at 65 degrees Celsius.

Growth assessments

For growth evaluation, fifteen fingerlings of *C. catla* which were stocked in each replicated water tank, were weighed at the start and at the end of the growth trial. Standard formulas were used in order to determine fish growth characteristics such as weight gain (g), weight gain %, feed conversion ratio (FCR), and specific growth rate (SGR) (NRC, 1993).

Table I. Chemical composition (%) of feed ingredients (Dry matter basis).

Ingredients	Dry matter (%)	Gross energy (Kcal/g)	Crude protein (%)	Crude fat (%)	Crude fiber (%)	Ash (%)	Total carbo-hydrate (%)
Fish meal	92.49	3.79	45.27	8.58	1.63	21.51	23.01
Wheat flour	91.54	2.74	9.89	2.23	2.81	3. 25	81.82
Corn gluten (60%)	91.99	4.19	59.12	5.96	2.36	2.22	30.34
Rice polish	93.89	4.21	13.54	10.25	3.89	6.89	65.43
SFM*	93.34	3.25	39.17	5.52	2.21	8.36	44.74

*SFM, Sunflower seed meal.

Table II. Composition (%) of SFM based diets supplemented with probiotics.

Ingredients	Test diet-1 (Control)	Test diet-2	Test diet-3	Test diet-4	Test diet-5	Test diet-6	Test diet-7
Probiotics (g/Kg) Protexin*	0	0.5	1	1.5	2	2.5	3
SFM	50	50	50	50	50	50	50
Fish meal	14.5	14.5	14.5	14.5	14.5	14.5	14.5
Wheat flour	12	11.5	11	10.5	10	9.5	9
Rice polish	12	12	12	12	12	12	12
Fish oil	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Vitamin premix	1	1	1	1	1	1	1
Mineral mixture	1	1	1	1	1	1	1
Ascorbic acid	1	1	1	1	1	1	1
Chromic oxide	1	1	1	1	1	1	1

Probiotics were used at the expense of wheat flour. *Protexin consists of *Lactobacillus plantarum, L. bulgaricus, L. acidophillus, L. mamophilus, Bifidobacterium bifidum, Streptococcus faecium, Aspergillus oryzae* and *Candida printolopesi* (CFU/g=2×109).

Weight gain % = Final weight – Initial weight/ Initial weight ×100

SGR = Final weight – Initial weight/ No. of experiment days ×100

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

Nutrient digestibility assessment

In order to determine the apparent digestibility coefficient (ADC %) of crude protein (CP), crude fat (CF) and gross energy (GE) of feed and stored fecal samples, AOAC's protocols were followed (AOAC, 1995). The oven-drying technique was used at 105 degrees Celsius for 12 h to determine the water content of fish excrement and feed. Kjeldahl apparatus was used to estimate CP. CF was determined by extraction with petroleum ether for 12 h using soxhlet system. GE of samples was approximated by oxygen bomb calorimeter.

The apparent digestibility coefficient (ADC %) of crude protein, crude fat and gross energy of experimental diets were determined by the formula described in NRC (1993).

ADC (%) = 100 – 100 × (Percent marker in diet × Percent nutrient in feces/ Percent marker in diet × Percent nutrient in feces)

Hematological assessment

Clove oil (Sigma-Aldrich Co. LLC) at a concentration of 60 mg/L was used to anesthetize the fingerlings in each tank. Since clove oil is insoluble in water, it was dissolved in ethanol instead. The blood was extracted from the caudal vein of dead fish using a heparin-induced syringe, and the samples were sent to the Molcare Lab at the Department of Biochemistry at the University of Agriculture in Faisalabad, Pakistan, to be analyzed for hematological parameters. A micro hematocrit centrifuge (Hematokrit 24 Hettich) was used to measure the hematocrit (Ht) of blood by centrifuging blood-filled heparin-induced capillary tubes for 5 min at 12000 rpm (Brown, 1980). Red blood cells (RBCs), platelets (PLT) and white blood cells (WBCs) were counted using a hemocytometer equipped with a neubauer counting chamber (Blaxhall and Daisley, 1973). Hemoglobin (Hb) was measured by Wedemeyer and Yastuke (1977) method. To estimate mean corpuscular hemoglobin concentration (MCHC), mean cell volume (MCV) and mean corpuscular hemoglobin (MCH), following equations were used:

MCV = PCV/RBC × 10

MCH = Hb/ RBC × 10

MCHC = Hb / PCV × 100

Statistical assessment

Finally, a one-way analysis of variance was performed on data pertaining to growth efficiency, ADC% of crude fat, crude protein, gross energy, and hematological parameters (Steel *et al*., 1996). Tukey's honest significant difference test was performed to evaluate the statistical significance of differences in treatments, and a value of $p<0.05$ was regarded to indicate statistical significance (Snedecor and Cochran, 1991). CoStat (version 6.303, PMB 320, Monterey, CA 93940 USA) (computer software) was used for statistical analysis. Graphs were generated using version 23 of IBM's statistical programme, SPSS.

RESULTS

Growth performance

Table III showed the impact of probiotic supplementation on the growth of *C. catla* fingerlings fed an SFM-based diet. Fingerlings given probiotic-supplemented SFM-based diets showed statistically

Table III. Growth performance of *C. catla* fingerlings fed graded levels of probiotics supplemented SFM based diets.

Diets probiotics (g/kg)	Survival (%)	Initial weight (g)	Final weight (g)	Feed intake (g)	Weight gain (g)	Weight gain %	FCR	SGR
Test diet I (0)	97.78	7.45	23.74	0.31	16.29	218.84	1.73	1.29
Test diet II (0.5)	97.78	7.47	24.16	0.32	16.69	223.60	1.73	1.30
Test diet III (1)	95.56	7.46	25.66	0.31	18.20	244.17	1.51	1.37
Test diet IV (1.5)	100.00	7.45	26.44	0.30	18.99	254.98	1.44	1.41
Test diet V (2)	97.78	7.46	26.97	0.30	19.51	261.60	1.40	1.43
Test diet VI (2.5)	100.00	7.44	27.73	0.28	20.29	272.78	1.24	1.46
Test diet VII (3)	100.00	7.46	25.52	0.31	18.06	242.05	1.52	1.37

Data are means of 3 replicates.
For details of test diets, see Table II.

significant changes ($p<0.05$) in terms of survival rate (%), weight increase (g), weight gain (%), FCR, and SGR (Table III). Importantly, most of the growth parameters of *C. catla* started to increase with increase in probiotic supplementation level up to 2.5 g/kg. The best growth parameters were noticed at 2.5 g/kg supplementation of probiotics which were 20.29 g, 272.78%, 1.24 and 1.46 for WG, WG %, FCR and SGR, respectively. However, poor values of growth parameters were found in control group.

Quadratic regression analysis demonstrated that 2.09, 2.23 and 2.13 g/kg levels of probiotics supplementation are optimum levels for weight gain (g), SGR and FCR, respectively in *C. catla* fingerlings (Fig. 1).

Table IV. Compositions (%) of SFM based diets supplemented with probiotics.

Test diet (Probiotics level g/Kg)	Crude protein (%)	Crude fat (%)	Gross energy (Kcal/g)
Test diet I (0)	31.86	6.90	3.96
Test diet II (0.5)	31.87	6.91	3.94
Test diet III (1)	31.85	6.91	3.93
Test diet IV (1.5)	31.86	6.89	3.94
Test diet V (2)	31.87	6.90	3.95
Test diet VI (2.5)	31.84	6.92	3.95
Test diet VII (3)	31.84	6.90	3.94

Data are means of 3 replicates.
For details of test diets, see Table II.

Nutrient digestibility

Tables IV and V showed the calculated values of crude fat, crude protein, and gross energy in the test diets and in the feces of fish given probiotics supplemented SFM meal-based diets, respectively. These results indicated that fish fed SFM based diets supplemented with probiotics released least amounts of gross energy, crude fat and crude protein in feces at 2.5 g/kg and followed by 2 g/kg probiotics level. The highest ADC% of CF (78%), CP (75%) and GE (74%) were noticed at 2.5 g/kg probiotics supplementation level which varies significantly from other treatments (Table VI).

Table V. Compositions (%) of feces of *C. catla* fingerlings fed SFM based diets supplemented with probiotics Analyzed.

Test diet (Probiotics level g/Kg)	Crude protein (%)	Crude fat (%)	Gross energy (Kcal/g)
Test diet I (0)	16.45	3.80	2.09
Test diet II (0.5)	14.74	3.65	1.91
Test diet III (1)	13.09	3.22	1.78
Test diet IV (1.5)	11.40	3.35	1.50
Test diet V (2)	10.97	2.44	1.32
Test diet VI (2.5)	8.65	1.66	1.12
Test diet VII (3)	12.69	2.51	1.46

Data are means of 3 replicates.
For details of test diets, see Table II.

Table VI. Apparent nutrient digestibility (%) of *C. catla* fingerlings fed SFM based probiotics supplemented test diets.

Test diet (Probiotics level g/Kg)	Crude protein (%)	Crude fat (%)	Gross energy (Kcal/g)
Test diet I (0)	51.59	48.37	50.42
Test diet II (1.5)	56.67	50.58	54.69
Test diet III (1)	61.46	56.34	57.62
Test diet IV (1.5)	66.59	54.57	64.47
Test diet V (2)	67.71	66.77	68.54
Test diet VI (2.5)	74.57	77.53	73.54
Test diet VII (3)	62.53	65.74	65.20

Data are means of 3 replicates.
For details of test diets, see Table II.

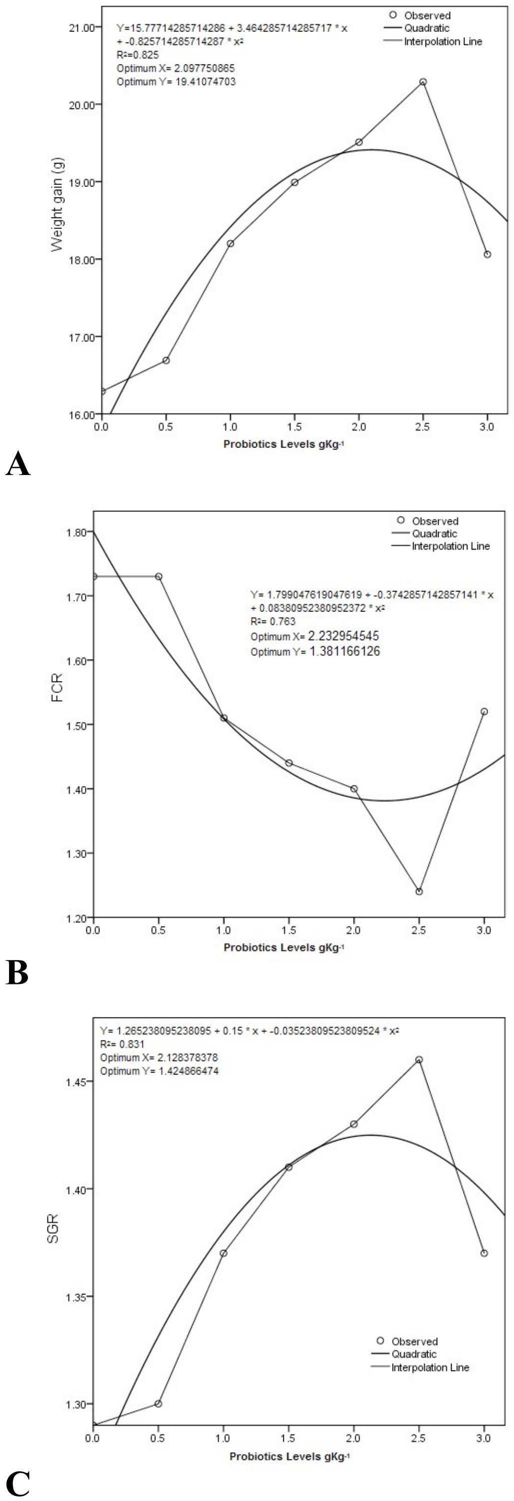

Fig. 1. The relationship between growth performance factors A, weight gain (g), optimum at 2.09; B, SGR (%) optimum at 2.23, C, FCR (%) optimum at 2.13 of *C. catla* fed SFM based diet.

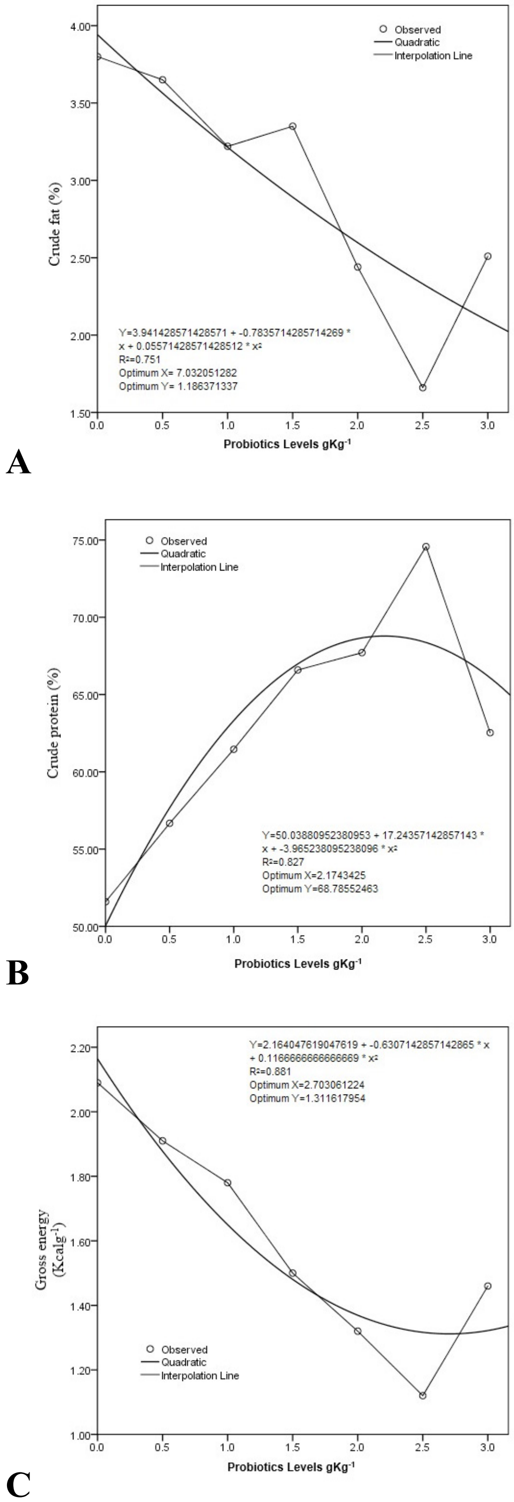

Fig. 2. The relationship between nutrients digestibility. A, crude fat (%) optimum at 0.751; B, crude protein (%) optimum at 2.17 and C, gross energy (Kcal/g) optimum at 2.18 of *C. catla* fed SFM based diets.

Table VII. Hematology of *C. catla* fingerlings fed different graded levels of probiotics (Protexin) supplemented SFM based diets

Test diets (Probiotics level g/Kg)	RBC (10^6mm^{-3})	WBC (10^3mm^{-3})	PLT	Hb (g/100ml)	PCV (%)	MCHC (%)	MCH (pg)
Test diet I (0)	1.40	6.11	54.34	5.97	22.36	27.97	34.89
Test diet II (0.5)	1.64	6.91	56.10	6.05	20.84	26.39	40.07
Test diet III (1)	2.01	7.44	58.43	6.93	23.26	31.97	47.16
Test diet IV (1.5)	2.51	8.08	61.59	7.56	25.02	35.62	60.67
Test diet V (2)	2.97	8.31	66.09	8.11	24.49	35.08	63.46
Test diet VI (2.5)	3.19	8.65	67.25	8.79	22.60	34.33	58.96
Test diet VII (3)	2.46	7.39	62.27	6.19	20.00	30.98	50.01

Data are means of 3 replicates.
For details of test diets, see Table II.

Quadratic regression analysis showed that 0.75, 2.18 and 2.17 g/kg levels of probiotic supplementation are optimum dietary levels for crude fat, gross energy and crude protein in *C. catla* fingerlings fed SFM meal-based experimental diets (Fig. 2).

Hematology

Effect of probiotic supplementation on the hematology of *C. catla* fingerlings fed SFM meal-based diet is given in Table VII. Maximum improvement in RBCs (3.19×10^6 mm^{-3}), WBCs (8.65×10^3 mm^{-3}), PLT (67.25), Hb (8.79 g/100ml) and Ht (34.34%) values were observed in 2.5 g/kg supplementation level of probiotic following 2 g/kg probiotic level. On the contrary, lower values of RBCs (1.40×10^6mm^{-3}), WBCs (6.11×10^3 mm^{-3}), PLT (54.34) and Hb (5.97 g/100ml) were found in control group. Moreover, maximum PCV (25%) and MCHC (35.62%) values were noticed when fish were fed with 1.5 g/kg dosage of probiotic level. While, maximum values of MCH (63.46pg) were estimated at 2 g/kg dosage of probiotics supplementation.

Quadratic regression analysis showed that 2.45, 2.00, 2.59, 1.88, 1.44 and 2.36 g/kg levels of probiotic supplementation are optimum dietary levels for RBCs, WBCs, PLT, Hb, PCV and MCV for *C. catla* fingerlings fed SFM based experimental diets (Fig. 3).

DISCUSSION

In aquaculture, dietary uses of probiotics have been extensively explored and proved to be efficient at enhancing the growth performance of aquatic animals. Probiotics raise feed value, the digestive enzyme contribution, block pathogenic bacteria, and boost immunological response in terms of promoting growth. In the current investigation, weight gain (g), weight gain %, FCR and SGR in case of growth were significantly enhanced in *C. catla* fingerlings when fed with probiotics supplemented SFM diet. These results also showed that growth performance linearly increase with increase in probiotics supplementation level up to 2.5 g/kg. This shows that 2.5 g/kg probiotic level is the ideal range of supplementation for *C. catla* fingerlings fed SFM based diet. Furthermore, a non-significant increase in survival rate (%) was also recorded in the present study. According to Hussain *et al*. (2021), the administration of 2 g/kg probiotics in a diet based on corn gluten meal is ideal for enhancing the growth performance of *Cyprinus carpio* fingerlings. Another recent study demonstrated that the ultimate FBW (28.2 g), WG (19.1 g), WG % (209.8 g) and SGR (1.88%/ day) of European sea bass given the feed supplemented with probiotics (3 g/kg) were considerably greater than that of fish fed with control diet (Eissa *et al*., 2022). This is because probiotics replace harmful bacteria and lower the pH of the digestive tract, creating an environment favorable for digestive enzymes and hence, boosting growth parameters.

Generally, the application of probiotics in fish diets results in more nutrient digestibility. The present research results demonstrated that supplementing the test diets with probiotics significantly improved protein digestibility in *C. catla* fingerlings fed SFM-based diet. Furthermore, SFM based diets supplemented with probiotics released least amounts of gross energy, crude fat and crude protein in feces at 2.5g/kg probiotics level. Less excretion of nutrients in feces shows that the probiotics have the ability to improve the absorption of nutrients present in diet. In a recent study, it was conducted that Siberian sturgeon had improved feed utilization, nutritional digestibility, and health condition when they were fed a diet supplemented with probiotics (Shekarabi *et al*., 2022). Moreover, *Channa striata* fingerlings fed the probiotic known as *Lactobacillus acidophilus* had been shown to boost both the digestion of nutrients and the performance of growth (Munir *et al*., 2016). The use of probiotic bacteria in aquaculture has evolved as a strategy for lowering the prevalence of

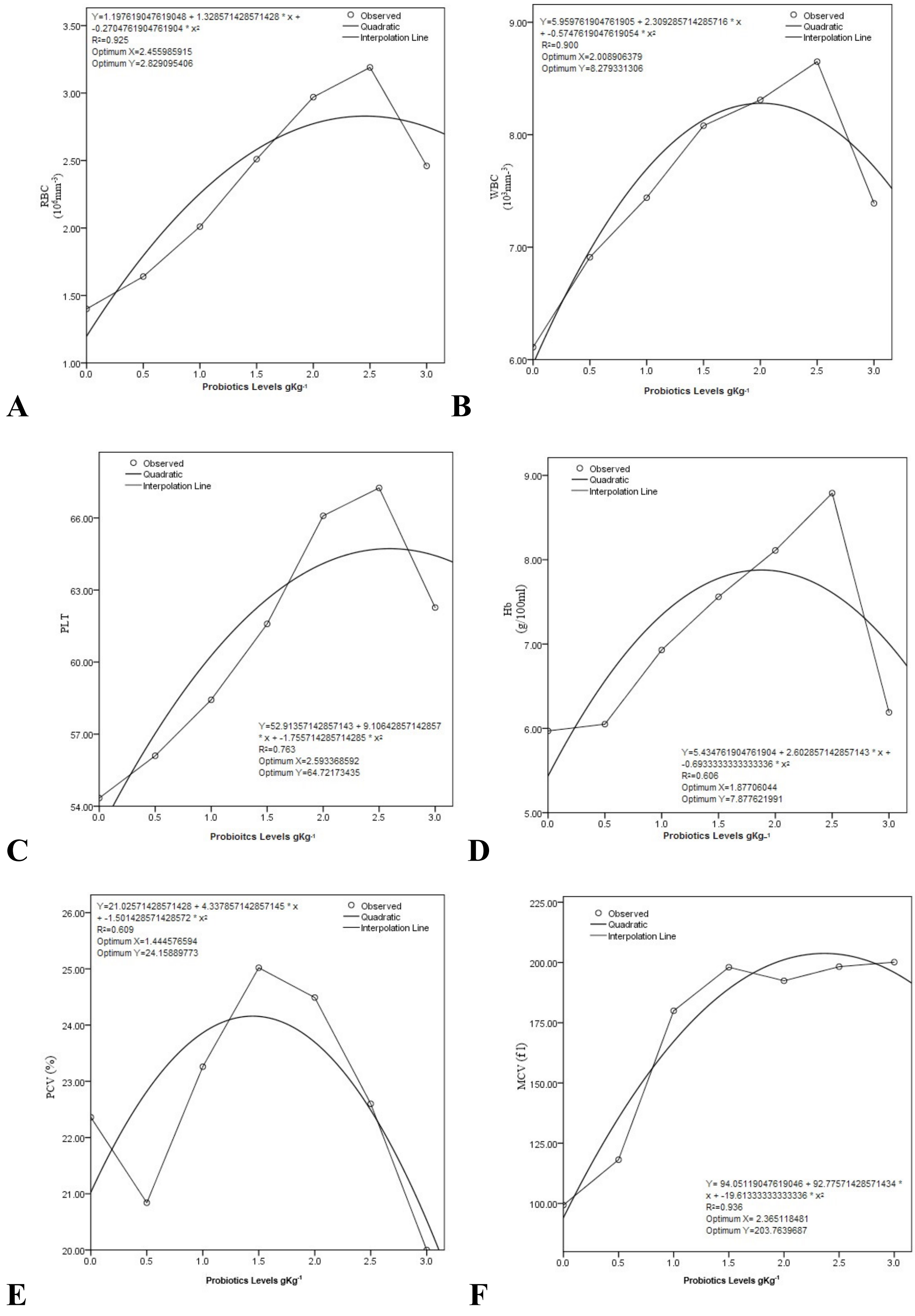

Fig. 3. The relationship between hematological parameters A, RBCs ($10^6 mm^{-3}$) optimum at 2.45; B, WBCs ($10^3 mm^{-3}$) optimum at 2.00; C, PLT optimum at 2.59; D, Hb (g/100ml) optimum at 1.88; E, PCV (%) optimum at 1.44 and F, MCV (pg) 2.36 of *C. catla* fed SFM based diets.

diseases, increasing nutrient uptake, improving villi height in small intestine and bettering ecological conditions. In the current study, *C. catla* fingerlings showed highest RBCs, WBCs and platelets when fed with probiotics supplemented SFM based diet. *C. catla* fingerlings showed highest RBCs, WBCs and platelets when supplied with 2.5 g/kg probiotics supplemented SFM meal-based diet. Hussain *et al*. (2021) found that supplementing *C. carpio* fingerlings' diets with 2g/kg of probiotics improved their hematological indices. Research done by Eissa *et al*. (2022) showed that a dose of 3 grams of commercial probiotics enhanced hematological parameters in sea bass (*Dicentrarchus labrax*) (Eissa *et al*., 2022). Consistent with our findings, Rajikkannu *et al*. (2015) found that a probiotics level of 107 CFU/g in a diet based on soybean meal significantly improved the RBCs of *L. rohita* and *C. carpio*. In contrast to our research, Ferguson *et al*. (2010) reported non-significant changes in Hb and Ht contents in *Oreochromis niloticus* supplemented with *B. subtilis* and Biogen® respectively. It is now possible to draw the conclusion that a number of different species, feeding sites, and environmental conditions have a part in contributing to the differences in results.

CONCLUSION

In conclusion, probiotics are valuable bacteria that aid the host organism and help defend it from other potentially hazardous bacteria. The addition of probiotics to the diet of *C. catla* fingerlings that were given a diet consisting of sunflower meal-based diet resulted in substantial improvements in the growth performance, nutritional digestibility, and hematology of the fish. When compared to different levels of supplementation, the administration of probiotics at a dosage of 2.5 g/kg was shown to be the most efficient and optimal.

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IRB approval

The experiment was carried out in-line with the IRB guidlines of Government College University, Faisalabad.

Ethical statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Statement of conflict of interest

The authors have declared no conflict of interest.

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