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## Research Article

# Effects of dietary supplementation of probiotics, yeast and digestive enzymes on the antioxidant status and hematological analysis of grass carp (*Ctenopharyngodon idella*)

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## ABSTRACT

Probiotics is widely used in aquaculture. Aquaculture is the controlled cultivation of aquatic organisms in a range of freshwater and marine environments. Present study examined the impact of probiotics (*Bacillus Subtilis*, *Lactobacillus plantarum*), yeast (*Saccharomyces cerevisiae*) and digestive enzymes (Amylase, Protease, Lipase & Pancreatin) on the antioxidant status and hematological analysis of grass carp reared in glass aquaria at the Aquaculture and Fisheries Laboratory, Department of Zoology, Wildlife and Fisheries, PMAS-Arid Agriculture University Rawalpindi, Pakistan. A total of 90 fingerlings of grass carp were reared in the laboratory, having the same environmental conditions. The fish were fed in different groups viz; Control group (C) were fed with commercially available supplementary fish diet while group one (T1) and group two (T2) were fed with Supplementary feed with addition of 2g/kg and 4g/kg consortium of probiotics, yeast and digestive enzymes respectively. The growth parameters were checked on a fortnightly basis to adjust the quantity of feed given. At the end of the experiment (90 days), random sampling was done from each group to analyze antioxidant status and hematological profile. The Results show that supplementary diets with addition of probiotics, yeast and digestive enzymes enhanced the antioxidant status in group 1 (50.092 µg/mL); Hematological parameters i.e., Red Blood Cells (RBC), were observed significantly higher in T2 group ( $3.10 \times 10^5/\mu\text{L}$ ) than T1 and Control group, while WBC; white blood cells ( $9.94 \times 10^4/\mu\text{L}$ ) and Hemoglobin (Hb) were significantly higher in T1 group (8.30 g/dL) than T2 and Control group ( $P < 0.05$ ). The results of the present study suggest the addition of consortium of probiotics, yeast and digestive enzymes in fish feed can improve the health status of grass carp. Such component used as feed additives are significant in achieving a sustainable and efficient fish production.

**Keywords:** Probiotics; digestive enzymes; antioxidant status; hematological analysis; grass carp.



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## INTRODUCTION

Aquaculture refers to the rearing of aquatic animals, including fish, crustaceans, and water devilling plants, in various water environments (Mohsin et al., 2017). Probiotics have been extensively used in aquaculture for many years to maintain the growth of species. Probiotics are known for helping control diseases and keeping the water clean (Dima et al., 2022). With the continuous growth of the global population, the demand for food, mainly protein-rich sources have significantly increased (Feng et al., 2024). Fish is a vibrant fount of protein, minerals, vitamins, and healthy fats, playing a key role in tackling global malnutrition and dietary issues (Yang et al., 2019)

Pakistan has introduced several exotic fish species including carps, tilapia and trout into its fresh water system for purposes like sport fishing, yield enhancement and biological control. These species include grass carp, bighead carp, silver carp, common carp, goldfish, and tilapia and trout species (Khan et al., 2011). The Grass carp (*Ctenopharyngodon idella*) is the ultimate significant cultured freshwater fish, accounting for over 10% of all aquaculture production worldwide (FAO, 2018). Due to the increasing human population and the depletion of wild fish stocks, controlled environment aquaculture techniques increase the production efficacy of fish, although they face problems like overcrowding, resource competition, and deteriorating water quality. As a result, fish experience biological tension, oxidative damage, and weakened resistance, making them more susceptible to disease (Yu et al., 2025). Probiotics are live microbial supplements that enter the host's digestive tract and strengthen the gut microbiome, increasing the host's ability to grow and tolerance to illness (Gil et al 2000; Dawood et al 2018). Microbial supplements such as *Bacillus subtilis*, *Lactobacillus plantarum*, *Saccharomyces cerevisiae* and *Lactobacillus acidophilus* are used as dietary supplements to increase the oxidative defense capacity, immunity and hematology of grass carp (Bucio et al., 2005). *B. subtilis* is frequently used as supplementary feed. Vitamins and amino acids are two of its nutritive metabolites, enhance intestinal microbiota, improve animal growth, and control immunity (Shao et al. 2020). *Bacillus subtilis* has demonstrated the ability to improve antioxidant defense, immune function, and digestive enzyme activity in a range of cultured species, such as laying hens, yellow croaker, white shrimp, and tilapia. Furthermore, *B. subtilis* is easy to produce and store, with no reported harmful or toxic effects on aquaculture species (Shao et al., 2021). The immunological parameters and antioxidant capacity of fish are improved by the use of yeast as dietary supplements (Li et al., 2012). Gastrointestinal enzymes such as lysine, amylase, and protease are added to the food of grass carp to improve the growth characteristics and antioxidant capacity. The concentration of superoxide dismutase and malondialdehyde rises in intestine as the grass carp consume lysine (Huang et al., 2021). Oxidative damage arises when the production of reactive oxidants surpasses the organism's antioxidant defense system. Antioxidants enzymes and vitamins protect against this damage. In aquatic species, antioxidant levels often increase in response to oxidative stress, serving as biomarkers of environmental impact (Lenartova et al., 1997). Oxidative stress cause diseases such as hypertension, aging process, diabetes, cancer, and the Alzheimer's disease (Moreno et al., 2014). Oxidative stress occurs due to the overexpression of reactive species which formed regularly during the metabolism. The antioxidant enzymes such as catalase, superoxide dismutase prevent the body from oxidative damage (Tang et al., 2019). The intestinal failure, intestinal inflammation, and even intestinal tissue cell death are occurred by the excessive oxidative stress (Bhattacharyya et al., 2014). The antioxidant system called defense system work against the oxidative stress, and is crucial for preserving the gut and intestinal functionality (Circu & Aw, 2011). Probiotics like *Bacillus subtilis* relief grass carp, boost their immunity, and protect their liver from oxidative damage brought on by bacterial infections like *Aeromonas hydrophila* (Guo et al., 2022). Hematological parameters are the indicator of fish health that examine the physiological and chemical changes in the body of fish. Total white blood cells, hemoglobin concentration, erythrocytes, are determined by analysis of hematological parameters. Temperature, sex, age, nutrition, size and maturity are the factors that affect the hematological parameters (Ejraei et al., 2015). The purpose of this study is to evaluate the antioxidant status and hematological analysis of grass carp by using the consortium of probiotics, yeast and digestive enzymes as dietary supplement in fish feed.

## MATERIALS AND METHODS

### Fingerlings Collection and Study Site

The fingerlings of grass carp were obtained from the Fisheries complex Rawal Town Islamabad and the experimental trial was set up at the Aquaculture and Fisheries Laboratory, Department of Zoology, Wildlife and Fisheries, Faculty of Sciences, PMAS-Arid Agriculture University Rawalpindi.

### Stocking and Acclimatization

The samples were properly tag and transported to Aquaculture and Fisheries Laboratory at Department of Zoology, Wildlife and Fisheries, PMAS-AAUR in polythene bag filled with oxygen and water. The fish were stocked in 6 glass aquaria having size of [1×1×1.5 ft3 (length × width × height)], filled with equal amount of water and acclimatized for one week. All the tanks were equipped with aerators to provide fresh oxygen. The fish were fed with commercially available floating pellets, 2mm in size, containing 30% crude protein.

### Study Plan

Fingerlings of grass carp with an initial mean body weight  $2.40 \pm 0.219$  grams were stocked in six glass aquaria at a density of 10 fingerlings per aquarium. The supplementary diet was given to the grass carp with different concentration

on daily basis. The fish were fed in three different groups. C = control group was fed with supplementary diet without addition of probiotics and group one (T1) and group (T2) were fed with Supplementary feed with addition of 2g/kg and 4g/kg consortium of probiotics, yeast and digestive enzymes respectively at the rate of 5% body weight of the fingerlings in each aquaria. The experiment was carried out on grass carp fingerlings for 90 days. Leftover food and feces were pumped out on daily basis, and water was exchanged with fresh water every 2–3 days to ensure proper water quality.

### **Growth Experiment**

Growth performance was measured in terms of survival, total length and weight gain rate. The weight of fish was measured on fortnightly basis with the help of weighing balance. Feeding rate was controlled based on fish body weight during the entire trial. The growth variables were evaluated by using the given formula:

Weight gain (WG (g)) = Final body weight (FBW (g)) – Initial body weight (IBW (g))

### **Sampling and Analysis**

At the end of the experiment the meat and blood sample of grass carp (5 to 7) was collected from each group (C, T1 and T2) for the analysis of antioxidant status and hematological parameters respectively.

#### **Antioxidant Status**

The antioxidant status was determined through 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay that prevent the body from antioxidant damage. The DPPH free radical scavenging method of antioxidant determination was purposed by Brand's Williams in 1995. The freshly prepared 0.001g of DPPH was added to the 5mL absolute ethanol. Incubate the mixture at room temperature in the dark for 30 minutes. Fresh fish tissue sample was used in the DPPH assay. Fish tissue samples were homogenized in 3mL Phosphate-buffered saline (PBS) or Tris-HCl buffer (pH 7.4). The homogenate is extracted at 12,000g for 15-20 minutes at 4°C to separate the supernatant containing soluble proteins and antioxidants in fish samples. The supernatant was used as the main stock for the assay and serial dilutions were performed. This allows the reaction between the DPPH and the antioxidant present in fish extract. The spectrophotometer (Spectro 23, Labomed, Inc., USA) was used to check the absorbance of subsequent mixture at 517 nm against blank and control.

#### **Hematological Analysis**

Hematology of fish was examined including RBCs, WBCs count and Hb. Fish samples that were randomly chosen from each of the treatments group was anesthetize with clove oil. For hematological analysis blood sample of grass carp was collected from the puncture heart through sterilized 2cc syringe and stored in Ethylenediaminetetraacetic acid (EDTA) containing vials for transportation. The blood sample was diluted with dilution solutions, which contain formalin, sodium citrate, purified water and cresyl brilliant blue. The sample was analyzed through Neubauer Hemocytometer (Improved Neubauer, Paul Marienfeld GmbH & Co. KG, Germany). The Giemsa staining technique was used for analysis of WBCs. The hemoglobin was examined using the cyanmethemoglobin procedure, which employs Drabkin's reagent.

#### **RBCs Counting**

RBCs count was determined using a Neubauer hemocytometer following standard manual microscopy protocols. Whole blood was collected in an Eppendorf tube and diluted 1:100 with an isotonic diluent to prevent hemolysis and ensure accurate cell separation. The diluted sample was thoroughly mixed to achieve a homogenous suspension and subsequently loaded into the hemocytometer chamber via capillary action using a micropipette. The chamber was allowed to stand for a few minutes to permit cell settling. Microscopic examination was conducted at 400× magnification, and erythrocytes were enumerated within the five small central squares of the central grid, as per the established counting method.

#### **WBCs Counting**

The manual counting method is used for WBCs like RBCs. A microscope is used to count the WBCs within the in large boxes of defined grid area. WBC counts in blood samples provide information on hematological illnesses, infections, and inflammatory reactions.

#### **Heamoglobin Measurements**

Tetrameric hemoglobin makes up the red blood cells of fish. The amount of hemoglobin in your blood is determined by the hemoglobin test. The spectrophotometric method of cyanmethemoglobin is utilized to analyze the concentration of hemoglobin. The bloody substance was mixed with Drabkin reagent, causing hemoglobin to change into stable methemoglobin. A spectrophotometer was then adjusted at a wavelength of 540 nm, and the findings were compared to the reference standard.

## Statistical Analysis

Data collected on antioxidant status and hematological analysis was subjected to statistical analysis. All the data was evaluated using one-way analysis of variance (ANOVA) with Duncan's multiple range test (DMRT) to figure out the significant differences between the treated groups by SPSS software at 5 % level of significance ( $p < 0.05$ ). Obtained data was expressed through mean  $\pm$  standard deviation within the treated groups.

## RESULTS AND DISCUSSION

### Growth Outcomes

During the experiment the growth/weight of fingerlings were noted on fortnightly basis to determine the effects of supplementary diets on grass carp. The probiotic, yeast and digestive enzymes supplemented feed groups showed improved average weight gain as compared to control feed (Table 1).

Table 1. Average growth performance of grass carp by dietary supplementation of probiotics, yeast and digestive enzymes.

Growth Parameters	Control	Treatment1	Treatment 2
IBW(g)	2.40 $\pm$ 0.219	2.42 $\pm$ 0.184	2.42 $\pm$ 0.287
FBW(g)	3.25 $\pm$ 0.349	3.44 $\pm$ 0.643	3.63 $\pm$ 1.064
WG(g)	0.85 $\pm$ 0.412 <sup>c</sup>	1.02 $\pm$ 0.669 <sup>b</sup>	1.21 $\pm$ 1.102 <sup>a</sup>

Means  $\pm$ Standard deviation with different superscript is notably different ( $P < 0.05$ ). IBW, average initial body weight; FBW, average final body weight; WG, weight gain. C=control (supplementary feed without addition of probiotics, yeast and digestive enzymes), T1 was fed with Supplementary feed with addition of 2g probiotics, yeast and digestive enzymes) and T2 was fed with Supplementary feed with addition of 4g probiotics, yeast and digestive enzymes).

Table 2. Evaluation of antioxidant status of grass carp by dietary supplementation of probiotics, yeast and digestive enzymes.

Treatments	Antioxidant Status ( $\mu\text{g/mL}$ )
Control	45.831 $\pm$ 2.789 <sup>a</sup>
Treatment 1	50.092 $\pm$ 1.446 <sup>b</sup>
Treatment 2	40.948 $\pm$ 3.261 <sup>c</sup>

Means standard error with different superscripts is notably different ( $P < 0.05$ ).C= control group (supplementary diet without addition of probiotics, yeast and digestive enzymes), T1 was fed with Supplementary feed with addition of 2g probiotics, yeast and digestive enzymes) and T2 was fed with Supplementary feed with addition of 4g probiotics, yeast and digestive enzymes).

Table 3. Evaluation of hematological analysis of grass carp (mean standard errors) by dietary supplementation of probiotics yeast and digestive enzymes.

Parameters	Control	Treatment1	Treatment2
RBC $\times 10^5$ (Cell/m <sup>3</sup> )	1.70 $\pm$ 0.25 <sup>b</sup>	1.66 $\pm$ 0.45 <sup>c</sup>	3.10 $\pm$ 0.20 <sup>a</sup>
WBC $\times 10^4$ ( $\mu\text{l}$ )	3.85 $\pm$ 2.03 <sup>b</sup>	9.94 $\pm$ 0.65 <sup>a</sup>	0.45 $\pm$ 0.12 <sup>c</sup>
HGB(g/dL )	7.99 $\pm$ 0.12 <sup>a</sup>	8.30 $\pm$ 0.15 <sup>b</sup>	-3.70 $\pm$ 1.15 <sup>c</sup>

RBC, red blood cells; WBC, white blood cells and HGB, heamoglobin. Means with different superscripts a, b, and c in the same row are significantly different ( $P < 0.05$ ).

### Antioxidant Status

After the completion of the experiment the antioxidant status of grass carp was determined. The antioxidant status was significantly higher in group one (T1) than group two (T2) and control. The fish fed with 2g/kg consortium of probiotics, yeast and digestive enzymes in feed revealed higher antioxidant status as compared to control and group twoT2 (Table 2).

### Hematological Parameters

The hematological parameters were determined at the end of the experimental trial and the results were given in table 3. The highest RBCs were noted in group two (T2) and lowest in group one (T1) and Control. The highest WBCs were

noted in T1 and Control and lowest in T2 group. The hemoglobin highest noted in T1 followed by control and T2. The blood parameters were significantly higher in probiotics fed groups then control group.

Probiotics is widely used in aquaculture and it reduced the use of antibiotics, boost immunity, regulate gut microorganisms, resistance, and improve sustainability by using strains that have a wide tolerance and synergistic effectiveness (Abbas et al., 2025). The nutritional supplementation of *Bacillus sp.*, *Paracoccus marcusii*, and *Lactobacillus* strains improved the growth response and immunity of aquatic animals (Xue et al., 2020). *B. subtilis* and *L. plantarum* are now the most widely used microbial supplements in aquaculture due to their many benefits (El-Saadony et al., 2021). Nutritional supplementation of *B. Subtilis* increase the immunity of grass carp by enlightening the water standard (Laloo et al. 2007; Borges et al. 2008; Wang et al. 2008). The effects of dietary supplementation of probiotics (*Bacillus Subtilis*, *Lactobacillus plantarum*), yeast (*Saccharomyces cerevisiae*) and digestive enzymes (Amylase, Protease, Lipase & Pancreatin) on grass carp was studied in the present research experiment. In the present study the fish nurtured with dietary supplements with addition of probiotics, yeast and digestive enzymes shows improved growth performance, antioxidant status and hematological parameters. Excess ROS can cause oxidative stress, which is damaging to the host's cells, and that indicates per oxidative tissue damage and is a key sign of oxidative tension (Dragun et al., 2017).

*B. subtilis* improved the antioxidant capacity and reduce tissue damage in the intestine of grass carp and also increasing the levels of alkaline phosphatase (AKP) and catalase (CAT) in the blood (Tang et al., 2019). *B. subtilis* supplementation has been shown to improve the digestive enzymes of grass carp (Wu et al., 2012), *Litopenaeus vannamei* (Zokaeifar et al., 2012), and tilapia (Liu et al., 2017a). The yeast cells contain vitamins, that have significantly positive effects on antioxidant activity and defense response of aquatic organisms (Zhang et al., 2019). *B. licheniformis* Dahb1 at concentrations of  $10^5$  and  $10^7$ CFU g<sup>-1</sup> boosted growth, strengthened defense responses, and increased antioxidant enzyme activity in the tilapia (*O. mossambicus*) (Gobi et al., 2018). The highest antioxidant status was recorded in T1 that was fed with supplementary diet with addition of 2g/kg probiotics, yeast and digestive enzymes and lowest was recorded in T2 that was fed with 4g/kg supplementary feed with addition of probiotics, yeast and digestive enzymes in feed.

The dietary supplementation of probiotics improved the level of RBCs count, total leucocytes, Hb concentration, MCH and MCHC in common cap fingerlings. The biochemical and hematological parameters are the indicators of the health of fish (Satheesh kumar et al. 2012). Mohammadian et al., (2020) examined the effects of a probiotic called *Lactobacillus bulgaricus*, with a concentration of 108 CFU per gram, on blood health and immune system role in Rainbow Trout (*Oncorhynchus mykiss*). The results indicated that fish fed with probiotic experienced improvements in their blood parameters compared to control group. The yeast probiotics such as *S. cerevisiae* significantly improved the growth and blood profile in rohu (*Labeorohita*) (Jahan et al., 2021).

The hematology of grass carp in present study were noted different among experimental groups. In the current study probiotics, yeast and digestive enzymes supplementary diet shows improvement in blood variables, the result showed highest level of RBCs in T2, WBCs in T1 and hemoglobin in T1. Use of 2g/kg consortium of probiotics, yeast and digestive enzymes in basal diet could be better option for improving fish growth and production of culture system. Moreover, further study is recommended to explore the effect of commercial probiotic on antioxidant status and hematology at some other concentration to obtain better results.

## CONCLUSION

The present study indicates that dietary supplementation with the consortium of probiotics (*Bacillus subtilis*, *Lactobacillus plantarum*), yeast (*Saccharomyces cerevisiae*), and digestive enzymes (amylase, protease, lipase, and pancreatin) positively enhances the growth response, antioxidant status, and hematological profile of grass carp (*Ctenopharyngodon idella*).

## AUTHOR'S CONTRIBUTION

T Naz conducted experiment. M Z Anjum supervised the experiment. M Irfan and M Q Khan provided statistical assistance. S Urooj, V Arshad, H Qamar, T Ijaz and B Mushtaq are contributed in data collection.

## FUNDING

No funding was available for this project.

## AVAILABILITY OF DATA AND MATERIAL

The data supporting the results of this study is available from the corresponding author, upon reasonable request.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

N/A

## CONSENT FOR PUBLICATION

I, the undersigned, consent to the publication of my identifiable information.

## CONFLICT OF INTERESTS

The authors declare there is no conflict of interest.

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