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Comparative study on the Therapeutic effects of probiotics mixture on oxidative stress Biomarkers in healthy and fungal-infected Rohu (*Labeo rohita*)

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Aquaculture production is the major source of food requirements to combat with ever growing food security issues and for this reason improving aquaculture production quantitatively along with quality is essential. Probiotics play a vital role in this scenario in different countries of the world. This study aimed to elucidate the effects of blended probiotics on healthy and diseased fish. This experimental trial was conducted in Fisheries Research Center Chashma, Mianwali for 74 days. Total 180 samples were used and grouped according to their average body size. Two different concentrations of probiotics as 2% and 4% were given to all treatment groups. Antioxidant enzymes activity was analyzed in both normal and fungal infected groups. Catalase activity increased significantly from T0 to T2 in both normal (53.7±2.98 to 75.9±2.56) and infected fish (81.1±1.66 to 92.4±5.95). Malondialdehyde (MDA) level showed a progressive rise, with values ranging from 518.7±4.57 to 534±8.12 in normal and 681±9.64 to 704±8.60 in infected fish. Peroxidase activity was evaluated from 698±5.33 (T0) to 728±18.3 (T2) in normal fish and from 1521±7.20 to 1604±31.6 in diseased fish. Superoxide dismutase (SOD) level was also improved ranging from 73.2±1.75 to 83.4±1.65 in normal and 91.3±1.16 to 99.6±2.12 in infected fish. These findings suggested that probiotics supplementation enhances antioxidant defense mechanisms particularly in fungus-infected fish. These results suggested that commercial blended probiotics work more efficiently and effects positively on antioxidant enzymes activity as it offers the promising applications of preventive as well as therapeutic measures in aquaculture.

Keywords: feed additives, probiotics, antioxidant enzymes, fungal diseases, aquaculture, fish nutrition

INTRODUCTION

Probiotics and other immuno-stimulants are used in aqua feed and are thought to be beneficial practices for improving the general health of aquatic animals as well as reducing the use of antibiotics and artificial medications, which are already prohibited in aquaculture. This is a result of several concerns with environmental contamination, human health issues, and the emergence of antibiotic-resistant bacteria. Numerous studies have already documented the benefits of probiotic on the welfare and health of various fish and prawns. On the other hand, it has been suggested that in treated animals, a multi strains probiotics might offer more advantages than single-strain probiotics due to the synergistic interaction of the probiotic strains (Mohammadi *et al.* 2021). The better and safe choice for feed additives is the probiotics that are emerging alternatives as prophylactics with extra benefit as enhancing immune response and toxin binding ability (Sarwar et al. 2019). Probiotics that are live microbes have the ability to balance the host good microbes and improve the antibody development that is why being suggested as adjuvants having potential over vaccines (Mohsin et al. 2021). Fish's nonspecific immune system primarily defenses against a variety of potential invading invaders, including bacteria, fungus, viruses, and parasites, through the actions of the lysozyme (Chiu et al. 2010). Probiotic-supplemented meals, either individually or in combination, has considerably higher serum enzymes activity of the fish (Harikrishnan *et al.* 2010). Lactobacillus spp., plays a number of beneficial biological functions mainly as immunity enhancement and pathogen resistant. In addition, it can survive in challenging environment of gastro-intestine and balanced the intestinal microbes (Yu et al. 2015).

In addition to different disease outbreaks, attack of parasites also causing massive demolition in aquaculture and it is also the reason of harmful effects on public health due to its consumption by the food chain. (Habib *et al.* 2019). Like other diseases, fungal borne infections are common and a serious problem in aquatic organisms, especially under conditions like poor water quality, physical injury or stress. The most prevalent pathogen from the fungus in freshwater are the species of the genera *Achlya*, *Branchiomyces* and *Saprolegnia* and are known to cause saprolegniasis, a particular disease characterized by cotton like mycelia growth on the infected organism's skin, gills or on the eggs. These infections often occur as secondary invaders following the parasitic, bacterial or viral infections and can affect the integumentary barrier of the infected fish that leads to systematic infections, osmotic imbalance and mortality. Fungal outbreaks are more common in hatcheries and during handling or transport where minor injuries or stress can predispose fish to opportunistic fungus colonization (Faruk and Anka, 2006). These fungal borne diseases are most common in brood stock and can occur at other life stages of eggs and fish as well. It causes low productivity in aquaculture and increases the mortality rate sometimes as high as 80-100% in egg at incubation period (Iqbal et al. 2012). The bio-therapeutic alternatives has been considered as probiotics to antibiotics with supportive effects to the host in maintaining the health, improving immune response, increasing antioxidant functions and restraining the pathogenic damages (Rajput and Li, 2012).

The main reason for poor growth and increased mortality rates in intense larvae culture is considered to opportunistic pathogenic micro-biota. Therefore, to maintain the sustainable larvae production an alternative to antibiotics therapeutic approach is necessary in environment friendly operation at fish hatcheries. For this reason, effective probiotics can be investigated in the hatcheries to combat fatal pathogenic challenges (Talpur et al. 2012). According to World Health Organization, probiotics as live microorganisms can provide various health benefits when introduced to the host's body (WHO, 2006; Abu-Akkada and Awad, 2015). It is recognized that probiotics can suppress the growth of harmful pathogens within the body by helping to maintain the healthy intestinal balance and are associated with reducing the allergies and inflammation in susceptible animals (Li et al. 2019).

Water-dwelling active fungal agents infect fish populations particularly at juvenile stage and adult individuals, causing eggs and larvae to decompose. Typically seen as a secondary infection in fish populations, fungal infections develop themselves in lesions resulting from mechanical harm caused by initial agents such as bacteria, viruses, and parasites, hence altering the disease's diagnosis. The most prevalent and commercially significant fungal disease is saprolegniasis. Winter fungus or winter kill syndrome are other names for this disease. The most prevalent Saprolegnia that cause saprolegniasis are *S. parasitica* and *S. invaderis* (Ozcan and Arserim, 2022). Stress and unfavorable environmental factors cause the immune system to be suppressed. Fungi start to establish as a result of infections caused by bacteria and viruses when there is an excess of mucus production (Khoo, 2000). According to Kutama et al. (2013), it harms fish's epidermal tissues and can spread throughout the body, beginning with the head and fins. In aquaculture, these white patches fungal infections are problematic particularly during spawning season and post handling stress. Their eggs are also highly susceptible, as this saprolegnia colonize the body surface that impairs gas exchange and spreads rapidly to others (Barde et al. 2020). Therefore, this experiment was conducted to evaluate the impact of dietary blended probiotics on the activity of key antioxidant enzymes in *Labeo rohita*, in order to determine whether probiotics supplementation enhances the fish antioxidant defense system and reduces oxidative stress.

MATERIALS AND METHODS

Experimental Design, Feed Formulation and Feeding

The experimental trial of 8 weeks was conducted in the Fisheries Research Center Chashma, Mianwali, Pakistan, in glass aquariums having capacity of 500 liter with 300-liter water filled, from October 15 to December 30, 2023 including acclimatization period. Total 180 samples of Rohu fish with average weight of 150 ± 5.01 g of both normal fish without the obvious signs and symptoms of diseases and diseased fish with obvious morphological disease symptoms were used in this experimental trial. After collection, specimens were acclimatized for 14 days. After this period, samples were divided into 6 groups each with 30 samples.

To formulate the experimental diets, various feed ingredients including fish meal, soybean oil, rice polish, wheat bran and corn flour were dried and then ground into uniform particle size. Proximate composition having crude protein contents (30%), ash contents (8%), crude fat contents (6%), moisture contents (9%) and crude fiber contents (5%) of all these ingredients were analyzed following the standard procedures outlined by AOAC (2006) and Saini et al. (2014) for all groups and the final preparations are presented in Table 1. Further the basal diet was mixed with 2 different concentrations (2% and 4%) of commercially available probiotics designed as a mixture that equally contains 5 different strains of *Bacillus* spp. including *B. pumilus*, *B. amyloliquefaciens*, *B. licheniformis*, *B. megaterium* and *B. subtilis* for all treatment groups.

Table 1: Components of experimental diet with 2 different concentrations of probiotics for all the treatments

Component	Treatment 0	Treatment 1	Treatment 2
Total feed weight (g)	100	100	100
Crude protein (%)	30.0	30.0	30.0
Probiotics (%)	0	2	4
Main ingredients (g)			
Fish meal	20.0	20.0	20.0
Soybean	20.0	20.0	20.0
Rice polish	35.0	35.0	35.0
Corn gluten	23.0	23.0	23.0
Additives (g)			
vitamins	1	1	1
Molasses	1	1	1

Table 2: Fish body parts or organs affected by fungal species

Fish body parts/organ	Fungal species	Fungal colonies
Head	*Aspergillus spp*., *Penicillium spp*.	a & b (1)
Eyes	*Aspergillus spp*.	a (2)
Caudal fin	*Penicillium spp*., *Alternaria spp*.	a & b (2)
Scales	*Penicillium spp*., *Aspergillus spp.*	a (2) & b (4)
Gills	Alternaria spp.	b (1)

Note: fungal colony a & b (2) means, two colonies of two different species identified and both were processed; colony a (2) & b (4) means, two different species in which one has two and second has four colonies identified and processed.

Fish feed was given manually two times a day. All the aquariums under the use for this experiment were equipped with the thermostat to maintain the require temperature (23 to 28˚C) of the sample fish and automated aerators for good supply of oxygen as 6 to 7 mg/L throughout the study period. Uneaten food material as a waste were daily siphoned out from aquaria for regulating the feed.
Almost 20-30 % water was exchanged with fresh water every two to three days and 100% water were replaced once a week to maintain the environment suitable for the good survival of fish.
In the present study, diseased fish were examined for pathological symptoms that were mainly consistent with fungal infections. The observed disease samples were have white and black spots on their various body parts specially on head, eyes, scales and caudal fin. These fungal spots are most common in aquaculture mostly in fish populations in winters and can significantly impact fish health, its survival and market value. These fungi are opportunistic in nature and infect the fish that have less immunity due to stress, poor water quality, injuries and/or co-infections. White fungal infections in aquaculture are most commonly *Aspergillus spp*. The infected fish represent white to grayish cotton like patches mostly on the skin, fins, gills or eyes as listed in Table 2. These infections occur at the site of physical damage and progress to the inner tissue layers if not treated on time. These infections impair respirations and osmoregulatory functions and in severe cases leads to death.

Hydro-chemical Analysis
With a portable meter (HQ30D, Hach Lange), various water parameters which includes temperature, pH, dissolved oxygen (mg/L), and electrical conductivity (S/cm) were measured and maintained on daily basis.

Extraction of Antioxidant Enzymes
Enzymes were extracted from liver, kidney and lungs samples taken from all the treatment and control groups. By following the procedure used earlier by Aziz et al. (2021) and Kausar et al. (2025) as samples (0.5g) were mixed with 2 mL of ice-cold phosphate buffer (extraction solvent, pH 7.0, 50 mM; disodium phosphate heptahydrate ($Na_2HPO_4 \cdot 7H_2O$) and KH_2PO_4). Samples were homogenized and then centrifuged (10,000 rpm for 15 min, and 4°C) and the supernatant was recovered. The procedure was performed twice for each sample. The resulting extracts were used to analyze activities of the different enzymes such as;

Superoxide Dismutase (SOD)
Samples were estimated for SOD activity assayed according to the established method (Du et al. 2006). A 15 W fluorescent bulb and an aluminum foil-lined were utilized for carrying out the photo-induced reaction. By applying a spectrophotometer, the solution's absorbance at 560 nm was determined (Hitachi U-2800, Tokyo, Japan).

Catalase (CAT)
Catalase activity in samples were assayed by a method described by (Lewis et al. 2005). This assay solution comprised of 50 mM buffer of phosphate having 7 pH, 59 mM of hydrogen peroxide and 0.1 ml of enzymes extraction, and level of CAT activity was measured by measuring the absorbance change in the reaction solution at 240 nm continuously after 20 seconds.

Peroxidase (POD)
POD activity was assessed following the methodology outlined by (Lewis et al. 2005). This assay solution included distilled water, buffer of phosphate as 200 mM with 7 pH, 200 mM of guaiacol, hydrogen peroxide 400 mM and 15 µl samples for the determination of peroxidase activity. After the sample has been added, the reaction was begin. Every 20 seconds, the reaction solution's change in absorbance at 470 nm was measured.

Table 3 (a): Mean values of antioxidant enzymes in normal fish samples subjected to different treatments over study period

Treatment	Catalase (U/mg protein)	MDA (nmol/g tissue)	Peroxidase (U/mg protein)	SOD (U/mg protein)	TAC (mmol/L)	TOS (µmol H_2O_2 Eq/L)
T0	53.7 ± 2.98	518 ± 4.57	698 ± 5.33	73.2 ± 1.75	2.11 ± 0.105	22279 ± 16.3
T1	60.0 ± 1.49	522 ± 5.13	715 ± 15.9	78.9 ± 2.60	2.80 ± 0.249	22329 ± 42.2
T2	75.9 ± 2.56	534 ± 8.12	728 ± 18.3	83.4 ± 1.65	3.02 ± 0.271	22385 ± 51.7

Table 3 (b): Mean values of antioxidant enzymes in diseased fish samples subjected to different treatments over study period

Treatment	Catalase (U/mg)	MDA (nmol/g)	Peroxidase (U/mg)	SOD (U/mg)	TAC (mmol Trolox/L)	TOS (µmol H_2O_2 Eq./L)
T0	81.1 ± 1.66	681 ± 9.64	1521 ± 7.20	91.3 ± 1.16	2.35 ± 0.18	23794 ± 47.6
T1	85.1 ± 5.72	688 ± 5.93	1580 ± 22.6	96.2 ± 2.30	2.75 ± 0.17	24181 ± 186.0
T2	92.4 ± 5.95	704 ± 8.60	1604 ± 31.6	99.6 ± 2.12	3.02 ± 0.33	25316 ± 117.0

Malondialdehyde (MDA)

Using the approach of (Rehman et al. 2021) the level of lipid per oxidation in the samples was determined by the amount of malondialdehyde that is product of the lipid per oxidation. Samples were homogenized in 0.1% of tricarboxylic acid. At 14,462 rpm for 5 minutes, the homogenate was centrifuged. 0.05% TBA and 20% TCA were added to 1M portion of the supernatant. The combination was heated at 95°C for 30 minutes, followed by a fast cooling in the ice bath. The absorbance for supernatant at 532 nm was measured after centrifuging at 14,462 g for 10 min and the results for nonspecific absorption at 600 nm was subtracted. The extinction coefficient, which is 155 mM per cm, was used to compute the MDA contents.

Total Antioxidant Capacity (TAC)

The ABTS assay is based on the reduction of radical cat-ion that is 2, 2-azino-bis 3-ethylbenzothiazoline-6-sulfonate which has bluish green color, into its original form that is colorless by antioxidants. According to the antioxidant concentration, antioxidants decolorize ABTS (Nenadis et al. 2007). The extract of sample, reagent 1 (a combination of the sodium phosphate, glacial acetic acid, hydrogen peroxide and ABTS) and reagent 2 (a blend of sodium acetate solution and glacial acetic acid having pH 5.8) all these three samples and reagents were included in the test mixture. After mixing the contents in tubes, they were let to stand for 6 minutes. At 660 nm, absorbance was measured. A calibration curve was created using ascorbic acid. The milli-molar ascorbic acid equivalent to L-1 unit was used to express the TAC values.

Total Oxidant Status (TOS)

This activity was assessed using the method of (Erel, 2005) which involves measuring ferric ion using xylenol orange and oxidizing ferrous ions into ferric ions using oxidants which are present within the sample. Reagent R1, Reagent R2, and sample extract were all included in the test mixture. After 5 minutes, a spectrophotometer was used to detect the absorbance at 560 nm. The results were given in M water vapor equivalent/L.

Data analysis:

The obtained data were computed and analyzed by using one way analysis of variance (ANOVA) and Post-Hoc Tukey's honestly significant difference test (HSD) was applied to check out the differences among group means at α = 5%. In addition to these analysis, Principal Component Analysis (PCA) was also conducted to explore patterns, relationships and clustering among variables, providing a comprehensive multivariate overview of the dataset.

RESULTS AND DISCUSSION

During the research trial, *Labeo rohita* in both normal and diseased conditions were cultured under controlled conditions with distinct feeding strategies. These strategies included Treatment 1, where fish were provided with a diet containing 2% probiotics; Treatment 2, with 4% probiotic inclusion and a control group as T0 was maintained for both health statuses without any probiotic supplementation. The trial was conducted for duration of 74 days including 14 days of acclimatization period. In diseased samples obvious black and white patches of fungal infections were noted at various spots of the body as described in figures 1, 2 and 3 which showed the infections on head, eyes, caudal fin and scales.

Figure 1: Comparison of fish samples showing black fungal infectious patches on scales (left) and after probiotic supplements (right). The diseased specimen exhibiting dark necrotic patches on scales while treated fish shows better pigmentation.

Moreover, the rate of white fungal patches were high in samples and was covering more surface area of infected fish. The caudal fins in various samples were under rotting condition on sampling. These infections on various body parts may indicates serious pathological conditions if the fungal hyphae grow extensively on head covering eyes that can leads to blindness and penetration to brain. At this level it's impossible to treat these conditions and fishes die eventually. The fin infection is less pathogenic as compared to others as fishes survive but if it remains untreated for long time it can leads to its complete damage that will hinders in swimming. Upon completion of 74-days period, data were collected and analyzed to access antioxidant enzyme activity. The recorded data were then subjected to statistical analysis to determine any significant difference among the groups.

Figure 2: Comparison of fish samples showing white fungal infectious patches on head and eyes (left) and post treatment with probiotics (right). The diseased specimen displaying white mycelial patches around eyes whereas treated fish shows clear eyes with absence of fungal mass.

Three genera named *Aspergillus spp*., *Penicillium spp*., and *Alternaria spp*. were seen in the diseased samples that were affecting most of their body parts. *Aspergillus spp*. was more abundantly present than others as high as 59.4%, *Penicillium spp*. as 23.1% and *Alternaria spp*. as 17.5%.

A comparative analysis of antioxidant enzyme activities and oxidative stress indicators in treatment fish that were subjected to different concentrations of probiotics revealed distinct biochemical profiles across groups as represented in Figure 4 (a and b); (c and d). Superoxide dismutase (SOD), peroxidase, catalase and total antioxidant capacity (TAC) levels significantly increased with treatment intensity. In normal fish group, catalase activity increased markedly from T0 (53.7±2.98 U/mg) to T2 (75.9±2.56 U/mg) that exhibiting significantly higher values than T0 and T1 ($p < 0.001$).

Figure 3: Comparison of fish suffering from fin rot due to fungus attack (left) and after probiotic based recovery (right). The diseased fish shows discolored and eroded fins in contrast to the treated fish which displays normal fin and its edges.

A similar trend were observed for SOD (T0: 73.2±1.75 U/mg; T2: 83.4±1.65 U/mg) and peroxidase (T0: 698±5.33 U/mg; T2: 728±18.3 U/mg) indicating a strong up regulation of enzymatic defense under T2. TAC also increased significantly (T0: 2.11±0.105 mmol/L; T2: 3.02±0.271 mmol/L) reflecting the overall enhanced antioxidant capacity. Tukey's HSD post-hoc analysis confirmed T2 as statistically superior treatment for all these antioxidant markers. Malodialdehyde (MDA), a marker of lipid peroxidation, showed a slight but statistically significant increase under T2 (534±8.12 nmol/g) as compared to T0 (518±4.57 nmol/g) indicating the elevated oxidative damage despite enhanced enzymatic defenses. Similarly total oxidant status (TOS) increased (T0: 22279±16.3 µmol H_2O_2 Eq/L to T2: 22385±51.7 µmol H_2O_2 Eq/L) suggesting a rise in systematic oxidative burden with high treatment dose. Remarkably, T0 and T1were not significantly different in MDA, pointing the comparable protective effects against lipid peroxidation. In diseased fish group, treatment T2 also resulted in pronounced improvements in antioxidant parameters. Catalase and SOD activities were highest in the T2 group (92.4±5.95 U/mg and 99.6±2.12 U/mg, respectively), indicating enhanced antioxidant defense. Similarly, total antioxidant capacity (TAC) as (3.02±0.33 mmol Trolox/L) and peroxidase activity (1604±31.6 U/mg) were also significantly elevated in T2, while markers of oxidative damage such as malondialdehyde (MDA) as (T2: 704±8.60 nmol/g; T1: 688±5.93 and T0: 681±9.64 nmol/g), and total oxidant status (TOS) as (T2: 25316±117.0 µmol H_2O_2 Eq./L; T1: 24181±186.0 µmol H_2O_2 Eq./L and T0: 23794±47.6 µmol H_2O_2 Eq./L) were lowest in the T0 group as suggesting that T2 boosts antioxidant activity in both normal as well as diseased fish groups as mentioned in Table 3 (a and b). The ANOVA test followed by Tukey's HSD confirmed significant differences ($p < 0.001$) across treatments for all biochemical parameters for both groups. T2 was consistently associated with the highest and T0 with the lowest antioxidant enzyme activities. The Principal component Analysis (PCA) as shown in figure 5 (a and b) revealed strong correlation among peroxidase, MDA and TOS along Dim1 (74.4%) forming one functional axis (Fig. 5).

By lessening the harmful effects of free radicals on the cellular constituents of fish, antioxidant enzymes contribute to the defense mechanisms needed to shield fish from oxidative injury (Hoseinifar et al. 2021; Chowdhury and Saikia, 2020). Furthermore, MDA serves as a marker for fish lipid peroxidation (LPO). Thus, probiotic-based diets may enhance the antioxidant activity of the supplemented *P. hypophthalmus* fingerlings, as suggested by the considerable increase in hepatic CAT and SOD enzyme activities and the drop in hepatic MDA concentrations observed in the probiotic-supplemented groups (Martinez-Alvarez *et al.* 2005). The findings of our study aligned with those presented by Wang et al. (2017), reported that hepatic CAT, SOD, and total capacity for antioxidants in common carps were considerably boosted by a probiotic cocktail of Bacillus species. Similar results were seen in rainbow trout, where dietary multispecies probiotics dramatically raised glutathione-S-transferase (GST) enzyme levels and lowered MDA concentrations (Giannenas *et al.* 2015). *O. niloticus* fed meals with *B. licheniformis* showed increased SOD enzyme activity in their serum and mucous (Gobi *et al.* 2018). Additionally, feeding native *Bacillus* species caused higher levels of SOD and CAT enzymes in the skin mucosa and gastrointestinal specimens of Nile tilapia

(Kuebutornye et al. 2020).

The antioxidant enzymes catalase (CAT) and SOD shield the host against oxidative stress. Reactive O_2 is dismutase into H_2O_2 by SOD, whereas H_2O_2 is broken down by CAT (Li et al. 2015).

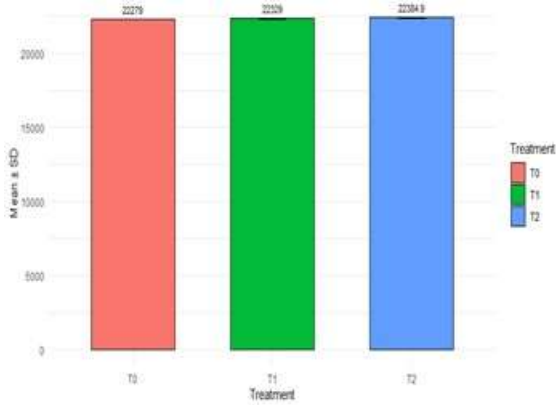


Figure 4 (a)

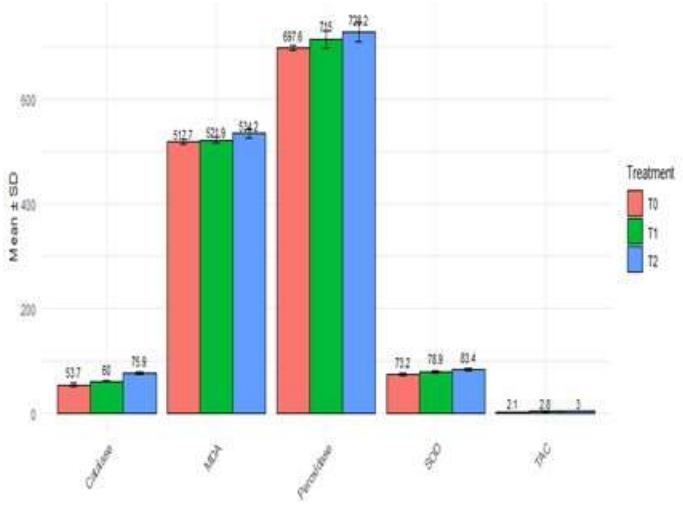


Figure 4 (b)

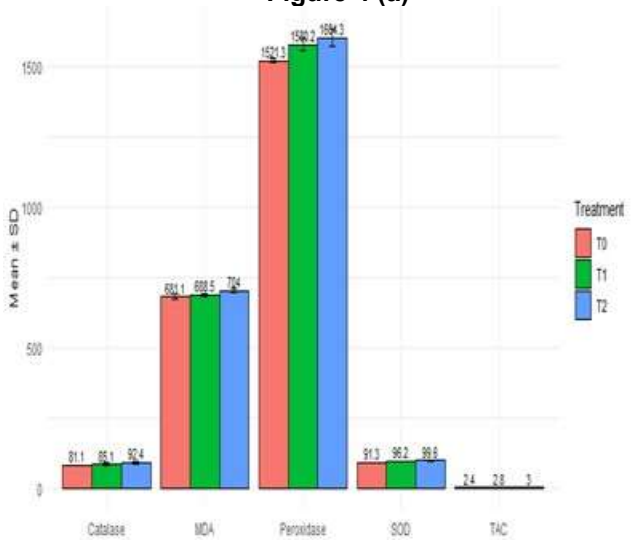


Figure 4 (c)

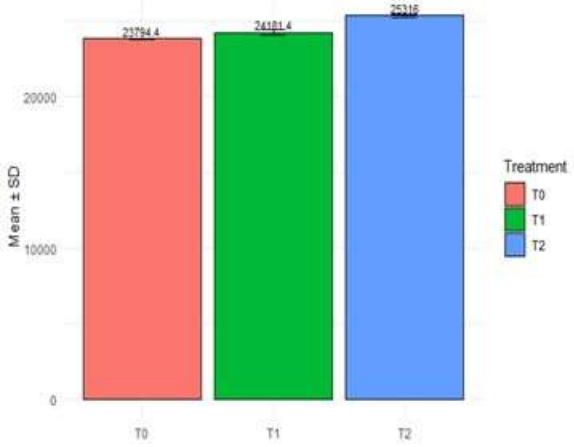
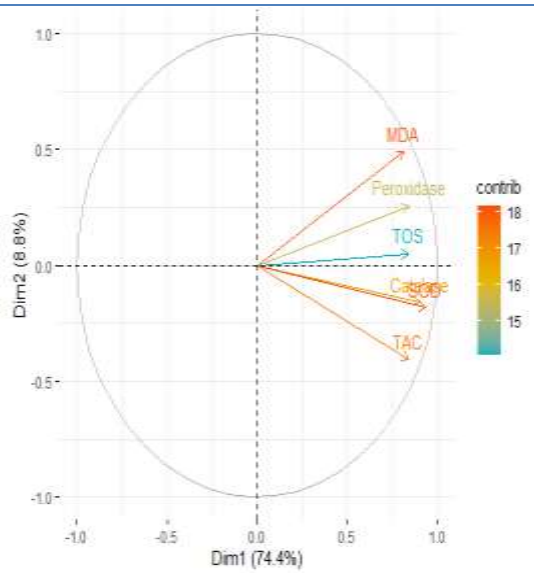
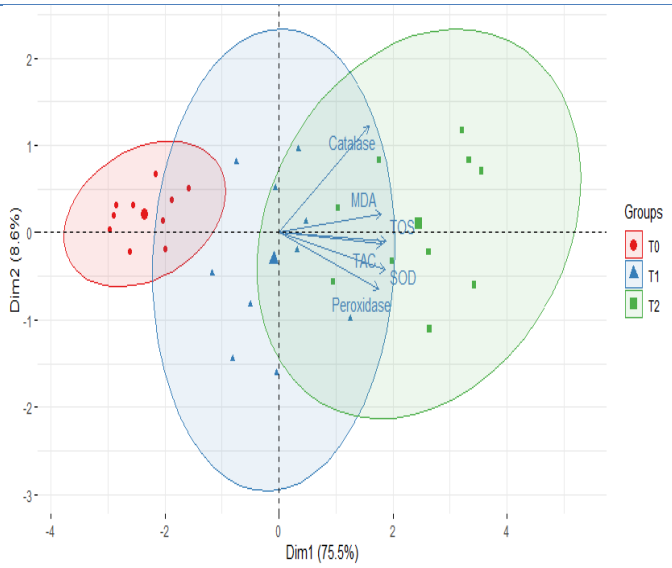


Figure 4 (d)

Figure 4 (a, b): and 4 (c, d): Compares the biochemical response of normal and diseased fish samples respectively, subjected to various treatments. The figure highlights differences in key biochemical parameters of both health status.





5 (a) **5 (b)**

Figure 5 (a) and 5 (b): presents the PCA biplot comparing antioxidant enzymes activity between normal and diseased fish samples. The plots illustrate the variation and clustering patterns, revealing distinctions in antioxidant responses associated with health status.

The other findings in tilapia serum demonstrated increased SOD and CAT activity in each of the treated groups in comparison to the control group. The use of probiotic, especially at concentrations of 7 or 10 gkg-1, may enhance the antioxidant properties of tilapia, as indicated by improved CAT and SOD activities (Wang *et al.* 2017).

The focus of well-being of aquatic creatures kept in captivity has shifted from curative treatments to preventive ones. Probiotics have been investigated for their effectiveness against fish diseases through inhibitory tests and their capacity to create extracellular enzymes as an environment friendly substitute for antibiotics. Numerous investigations have examined the separation of *Bacillus* species from Rohu, with potential applications as a probiotic option (Choudhary *et al.* 2021; Dutta and Ghosh, 2021). This study employed a prospective combination of probiotic candidates on common freshwater fish cultivator, Rohu. The fish were observed to respond favorably to the probiotics both as a preventative measure and as a therapeutic alternative to antibiotics against common fish fungal diseases (Dien *et al.* 2022). This study examined the probiotic's inhibitory potential against fish infections caused by fungi, which is a reason for concern in aquaculture since they can result in high rates of morbidity and mortality.

When provided dietary probiotics, Nile tilapia showed a considerably higher rate of survival towards *A. hydrophila* as compared to the control. Probiotic diets including *B. subtilis* and *L. lactics* have been shown in earlier research to increase the diseases immunity of Nile tilapia over bacteria (Aly *et al.* 2008). Probiotics may have strengthened Nile tilapia's non-specific immunity, which in turn increased their resistance to *A. hydrophila* infection. Additionally, these findings supported earlier research on the improved resistance to disease brought on by probiotic supplements (Raida *et al.* 2003). The most significant advantage of probiotics is their ability to modulate the immune system, which enhances the host's defenses against infections. These findings imply that immuno-stimulants, like probiotics, can improve the host's health by inducing immune responses, changing the makeup of the intestinal microbiota, and producing compounds that inhibit the activity of harmful agents (Balcazar *et al.* 2007). The current findings suggest that the combination of five probiotics improved the fish antioxidant defense system and immunity to infections. These micro diets, which depend on the culture of five bacterial strains, have the potential to be a viable, safe, and environmentally friendly alternative to modern control measures in aquaculture. However, more research is needed to determine the effects of specific probiotics on immune function and gut modulation.

CONCLUSIONS

This comparative findings suggested that the blended probiotics exert significant therapeutic effects in alleviating oxidative stress in normal and fungal infected fish. By enhancing antioxidant defense mechanisms and regulating the oxidative biomarkers, these probiotics contribute in improving health and resilience. These results highlights the potential of probiotics as an effective and eco-friendly strategy for promoting fish health and sustainable aquaculture practices. Further research is recommended to optimize probiotics formulations and dosages to obtained maximum benefits for various fish species.

Supplementary materials
Not applicable.

Author contributions
Conceptualization and methodology, B.R. and K.R.; software, H.H. and Y.M.; validation, K.R.; formal analysis, B.T. and H.H.; investigation, B.R. and Y.M.; resources, B.R.; data curation, K.R; writing-original draft preparation, B.R.; writing-review and editing, B.R., B.Z. and R. S.E.T.; visualization, N.Z. and B.T.; supervision, K.R.; project administration, B.R.; funding acquisition, B.R. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement
The study was approved by the Bioethical Committee of the Office of Research, Innovation and Commercialization (ORIC) University of Agriculture Faisalabad, Pakistan.

Informed Consent Statement
Not applicable.

Data Availability Statement
All of the data is included in the article/Supplementary Material.

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Conflict of interest
The authors declared that present study was performed in absence of any conflict of interest.

REFERENCES

Abu-Akkada SS and Awad AM, 2015. Protective effects of probiotics and prebiotics on *Eimeria tenella*-infected broiler chickens. Pak Vet J 35: 446-450.

Aly SM, Abdel-Galil GA, Abdel-Aziz GA and Mohamed MF, 2008. Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of Tilapia nilotica (*Oreochromis niloticus*) to challenge infections. Fish Shellfish Immunol 25: 128-136.

AOAC. 2006. Official Method of Analysis. 18th Edt. Washington, D.C: Association of Official Analytical Chemist.

Aziz S, Abdullah S, Anwar H, Latif F and Mustfa W, 2021. Effect of Engineered Nickel Oxide Nanoparticles on Antioxidant Enzymes in Freshwater Fish, *Labeo rohita*. Pak Vet J 41: 424-428.

Balcazar JL, Blas ID, Ruiz-Zarzuela I, Vendrel D, Girones O and Muzquiz JL, 2007. Enhancement of the immune response and protection induced by probiotic lactic acid bacteria against furunculosis in rainbow trout (*Oncorhynchus mykiss*). Immunol Med Microbiol 51: 185-193.

Barde RD, Deshpande M, Nagthane N, Darak O and Baig MMV, 2020. A review of Saprolegnia infection in freshwater fishes and control of the saprolegniosis. Sustain Humanosphere 16: 702-711.

Chiu CH, Cheng CH, Gua WR, Guu YK and Cheng W. 2010. Dietary administration of the probiotic *Saccharomyces cerevisiae* P13 enhanced the growth, innate immune responses, and disease resistance of the grouper, *Epinephelus coioides*. Fish Shellfish Immunol 29: 1053-1059.

Choudhary P, Das R, Saurabh S, Pradhan S, Swain P, Mishra SS and Das BK, 2021. Evaluation of the effect of mono or multi-microbe probiotics on growth, serum enzymes and non-specific immune

parameters in rohu *Labeo rohita* (Hamilton, 1822) fingerlings. Indian J Fish 1: 82-92.

Chowdhury S and Saikia SK, 2020. Oxidative stress in fish: a review. J Sci R es 12: 145-160.

Dien LT, Ngo TP, Nguyen TV, Kayansamruaj P, Salin KR, Mohan CV, Rodkhum C and Dong HT. 2022. Non-antibiotic approaches to combat motile Aeromonas infections in aquaculture: current state of knowledge and future perspectives. Rev Aquacult 15: 333-366.

Du J, Daniels DH, Asbury C, Venkataraman S, Liu J, Spitz DR and Cullen JJ, 2006. Mitochondrial production of reactive oxygen species mediates dicumarol-induced cytotoxicity in cancer cells. J Biol Chem 281: 37416-37426.

Dutta D and Ghosh K, 2021. Improvement of growth, nutrient utilization and haemato-immunological parameters in rohu, *Labeo rohita* (Hamilton) using *Bacillus tequilensis* (KF623287) through diets or as water additive. Aquac Nutr 27: 29-47.

Erel O, 2005. A new automated colorimetric method for measuring total oxidant status. Clin Biochem 38: 1103-1111.

Faruk MAR, and Anka IZ, 2017. An overview of diseases in fish hatcheries and nurseries. Fundam Appl Agric *2*: 311-316.

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

Gobi N, Vaseeharan B, Chen JC, Rekha R, Vijayakumar S, Anjugam M and Iswarya A, 2018. Dietary supplementation of probiotic *Bacillus licheniformis* Dahb1 improves growth performance, mucus and serum immune parameters, antioxidant enzyme activity as well as resistance against *Aeromonas hydrophila* in tilapia *Oreochromis mossambicus*. Fish Shellfish Immunol 74: 501-508.

Habib SS, Naz S, Sadeeq A, Iqbal MZ, Ali M, Hanif R, Malik MA, Khan N, Gul K and Mushtaq A, 2019. Prevalence of ectoparasites in carp fingerlings of Chashma Lake in District Mianwali Punjab Province, Pakistan. Int J Biosci 14: 468-474.

Harikrishnan R, Balasundaram C and Heo MS, 2010. *Lactobacillus sakei* BK19 enriched diet enhances the immunity status and disease resistance to streptococcosis infection in kelp grouper, *Epinephelus bruneus*. Fish Shellfish Immunol 29: 1037-1043.

Hoseinifar SH, Yousefi S, Van-Doan H, Ashouri G, Gioacchini G, Maradonna F and Carnevali O, 2021. Oxidative stress and antioxidant defense in fish: the implications of probiotic, prebiotic, and synbiotics. Rev Fish Sci Aquac 29: 198-217.

Iqbal Z, Sheikh U and Mughal R, 2012. Fungal infections in some economically important freshwater fishes. Pak Vet J 32: 422-426.

Kausar R, Hameed A, Jabbar J, Rehan S, Iqbal T, Hameed A and Usman M, 2025. Antioxidant and Oxidant Profiles in Thigh and Breast Meat of Pakistani Domestic Chicken Breeds. Pak Vet J 45: 174-183.

Khoo L, 2000. Fungal diseases in fish. J Exot Pet Med 9: 102-111.

Kuebutornye FKA, Wang Z, Lu Y, Abarike ED, Sakyi ME, Li Y, Xie CX and Hlordzi V, 2020. Effects of three host-associated Bacillus species on mucosal immunity and gut health of Nile tilapia, *Oreochromis niloticus* and its resistance against *Aeromonas hydrophila* infection. Fish Shellfish Immunol 97: 83-95.

Kutama AS, Umar S, Fagwalawa LD, Ahmed MK and Bilkisu AI, 2013. Detection of fish saprolegniasis in thomas dam and challawa commercial fish pond. Global Advanced Res J Agric Sci 2: 315-321.

Lewis A, Du J, Liu J, Ritchie JM, Oberley LW and Cullen JJ, 2005. Metastatic progression of pancreatic cancer: changes in antioxidant enzymes and cell growth. Clin Exp Metastasis 22: 523-532.

Li A, Wang Y, Suolang S, Mehmood K, Jiang X, Zhang L, Li Z, Waqas M, Iqbal M, Basang W and Li J, 2019. Isolation and identification of potential bacillus probiotics from free ranging yaks of Tibetan Plateau, China. Pak Vet J 39: 377-382.

Li J, Xu Y, Jin L and Li X, 2015. Effects of a probiotic mixture (*Bacillus subtilis* YB-1 and *Bacillus cereus* YB-2) on disease resistance and non-specific immunity of sea cucumber, *Apostichopus japonicus* (Selenka). Aquac Res 46: 3008-3019.

Martinez-Alvarez RM, Morales AE and Sanz A, 2005. Antioxidant defenses in fish: biotic and abiotic factors. Rev Fish Biol Fisheries 15: 75-88.

Mohammadi G, Rafiee G, Tavabe KR, Abdel-Latif HMR and Dawood MAO, 2021. The enrichment of diet with beneficial bacteria (single- or multi- strain) in biofloc system enhanced the water quality, growth performance, immune responses, and disease resistance of Nile tilapia (*Oreochromis niloticus*). Aquaculture 539: 1-16.

Mohsin M, Li L, Aleem MT, Habib YJ, Shehata AI, Afzal MZ, Abbas RZ, Abbas A and Guangwen Y, 2021. Immunogenicity and Protective Efficacy of Probiotics with EtIMP1C against *Eimeria tenella* Challenge. Pak Vet J 41: 274-278.

Nenadis N, Lazaridouand O and Tsimidou MZ, 2007. Use of reference compounds in antioxidant activity assessment. J Agric Food Chem 14: 5452-5460.

Ozcan F and Arserim NB, 2022. Fungal diseases in fish. BSJ Agri 5: 48-52.

Raida MK, Larsen JL, Nielsen ME and Buchmann K, 2003. Enhanced resistance of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against *Yersinia ruckeri* challenge following oral administration of

Bacillus subtilis and *B. licheniformis* (BioPlus2B). J Fish Dis 26: 495-498.

Rajput IR and Li WF, 2012. Potential role of probiotics in mechanism of intestinal immunity. Pak Vet J 32: 303-308.

Rehman T, Naz S, Hussain R, Chatha AMM, Ahmad F, Yamin A, Akram R, Naz N and Shaheen A, 2021. Exposure to heavy metals causes histopathological changes and alters antioxidant enzymes in fresh water fish (*Oreochromis niloticus*). Asian J Agric Biol 1: 1-11.

Saini VP, Ojha ML, Gupta MC, Nair P, Sharma A and Luhar V, 2014. Effect of dietary probiotic on growth performance and disease resistance in *Labeo rohita* (Ham.) fingerlings. Int J Fish Aquat Stud 1: 7-11.

Sarwar N, Mehmood A, Sheraz A and Noman M, 2019. Protective Effect of Probiotics in Combination with Vaccination on Antibody Response, Biochemical and Hematological Indices in Broilers. Pak Vet J 39: (3).

Talpur AD, Memon AJ, Khan MI, Ikhwanuddin M, Daniel MMD and Abol-Munafi AB, 2012. Control of *Vibrio harveyi* infection in blue swimming crab, *Portunus pelagicus* larvae by the gut isolated lactic acid bacteria under challenge bioassay. Pak Vet J 32: 408-411.

Wang L, Ge C, Wang J, Dai J, Zhang P and Li Y, 2017. Effects of different combinations of Bacillus on immunity and antioxidant activities in common carp. Aquac Int 25: 2091-2099.

Yu H, Yu J, Zhu Y, Li Y and Tang L, 2015. Comparison of Improved Effect of Antibacterial and Antiviral Activity of Four Probiotic Lactobacillus Expressing Porcine Lactoferrin in Mice. Pak Vet J 35: 274-278.