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BENEFICIAL EFFECTS OF DIETARY PROBIOTIC MIXTURE ON THE HEMATO-BIOCHEMICAL PARAMETERS OF NILE TILAPIA

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Abstract

Probiotics are useful bacteria in promoting the development, health status and survival of aquatic animals. This current experiment was focused to check the hemato-biochemical parameters of Nile Tilapia (*Oreochromis niloticus*) by using probiotic mixture. Fish fingerlings were collected and then acclimatized for 2 weeks in 70 L glass aquaria. The fish feed was prepared by mixing different probiotics which include *Bacillus subtilis*, *B. megaterium*, *B. pumilus*, *B. licheniformis* and *Enterococcus faecalis* in the basal diet and the trial was carried out for 90 days. Four treatments were designed for trial, Control T_0 (without probiotic), T_1 (2 g probiotic), T_2 (4 g) and T_3 (6 g). Each aquarium contained equal fish with three replicates and fed according to 5% of their body weight twice a day. Physico-chemical parameters of water were monitored. After each month in the trial, the hemato-biochemical parameters were measured. The value of RBCs, Hb, Hct and PLT increased in T_2 with 4 g probiotic instead of other treatment groups. WBCs were increased in T_3 group. While in MCV, MCH and MCHC a significant decrease was observed. In biochemical parameters which include Total Protein, Albumin and Globulin, a significant increase in T_3 was observed. The current investigation showed that probiotics improve the hematological and biochemical status of Nile Tilapia.

Keywords: Nutritional Supplements, Feed additives, Blood profile, Fish Health.

INTRODUCTION

The aquaculture industry plays a critical role in human nutrition and fulfilling future demand for aquatic food items, accounting for 50% of world fish consumption. The substantial rise in human population has prompted the aquaculture sector to step up its

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efforts to fulfill rising fish demand in a sustainable manner (Boyd et al., 2020). Global fish consumption per capita has nearly doubled over the last half-century and is currently comparable to poultry in edible weight (Edwards et al., 2019). Over the last 25 years, aquaculture productivity has grown faster than most other food commodities, and the sector has grown into a global enterprise (Garlock et al., 2022; Falcon et al*.,* 2022). In 2020, aquaculture accounted for 56% of fish available for direct human consumption worldwide (FAO, 2022).

Intensive and semi-intensive aquaculture activities have been recognized as the most promising sectors for maintaining food security in society. In recent years, medicinal plants, probiotics, synbiotics and prebiotics have sparked widespread attention as prospective feed additives for improving fish disease resistance, growth performance and immunity as well as an alternative to antibiotics and chemotherapeutics (Giri et al., 2014).

Modernization and intensification of all sectors of animal husbandry are dependent on the rational organization, volume, and quality of feed production (Allameh et al., 2017). Nowadays, additives are enhanced with artificial fish feed. Feed additives are eatable materials that are very subtly added to fish diets to improve the feed, which in return boost the growth performance and lower fish death rates (Dada, 2015). Nowadays, functional feed additives are used in place of chemotherapeutics and antibiotics (Yousefi et al*.,* 2018). These additives enhance fish physiological functions, immunological response, growth and health performance as compared to regular additives. Phytochemicals, mycotoxin binders, organic acids, immunological boosters, yeast products, enzymes, prebiotics and probiotics are a few examples of functional feed additives (Alemayehu et al*.,* 2018).

Probiotic feed additives are increasingly being utilized in complex feeds because they benefit the animal health by enhancing immunity, increasing survival, reducing feed conversion, and increasing weight growth (Mazanko et al., 2022). Probiotics are live or dead components of the microorganisms that have positive impact on the host and the environment. Probiotics respond differently depending on the situation (Venugopal et al*.,* 2016).

Probiotics are useful bacteria for the host's nutrition. Probiotics have been proposed to work in a variety of ways, which include (i) competitive exclusion through the production of inhibitory substances, (ii) competition for chemicals, nutrients, and energy, (iii) competition for adhesion sites, (vi) hematological parameter, (v) involvement in digestion, (vi) role in macro and micronutrients, and (vii) lower virulence (Noor et al*.,* 2020).

Compared to a single-strain application, the use of probiotics as multi-strain or multi-species dietary supplements has been demonstrated to provide greater advantages, since a mixture of probiotics may allow for the incorporation of diverse modes of probiotic activity in one go. Lactic acid bacteria (LAB), such as *Enterococcus faecium*, are commonly used as probiotics in fish farming and may be detected in the intestines of healthy fish. Supplementinon with *E. faecium* also increases final weight and blood

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phagocytes. Combining *Lactobacillus* and *Bacillus* strains boosts the immunological responses. The combined use of *L. acidophilus* and *B. subtilis* on Nile Tilapia (*O. niloticus*) shows minimal advantage over solo usage in terms of immune parameters and survival. (Aly et al., 2008).

Hematology is an essential biomarker for determining fish health and a species capacity to adapt to its surroundings. Hemato-biochemical parameter framework is crucial serving as a major bio-indicator and is important due to the fact that it permits efficient fish health monitoring (Adeyemo, 2007). WBC, RBC, Hct, Hb, platelet count, MCH, MCV and MCHC levels belong to the hematological parameters of fish that depend on the species, egg and sperm maturation and age (Gabriel et al*.,* 2011). Hematological parameters are essential diagnostic tools for assessing and tracking metabolic and morphological changes in fish (Fazio, 2019). Increased or decreased blood parameters are thought to be a sign of an unwell condition, environmental stress, or tissue damage. Similarly to aquatic creatures, hematologic data have been utilized to assess fish health and monitor the effects (Javed & Usmani 2015).

Serum biochemistry refers to the chemical examination of serum (a component of blood other than plasma). The serum contains a variety of components such as enzymes, protein, hormones, and lipids. Testing these compounds provides information on the body tissues and organs, as well as the animal's metabolic status. Serum biochemical markers (glucose, total protein, etc.) are utilized to distinguish between the animal's good physiological condition and anomalies caused by sickness in the organism (Kataria et al., 2010). Blood contains two forms of protein: globulin and albumin, which can be measured separately or combined in a single test tube termed total protein. This test examines the quantity of complete protein in the sample. Albumin levels indicate dehydration and the function of the kidneys, liver, and digestive tract. The level of globulin indicates the generation of antibodies and inflammation. A high concentration of globulin promotes immune-mediated illness, infectious disorders, and cancer. The muscles and heart require glucose, a persistent and rapid energy source. Total serum protein indicated the nutritional status of the animal as well as fish health (Shahsavani et al., 2010).

Nile Tilapia (*O. niloticus*) is an omnivore fish that is indigenous to Africa and a member of the Cichlidae family. Nile Tilapia have a brown or grey appearance and typically have deeply compressed, lateral bodies with some stripes or bands on their body especially the tail (Villamil et al*.,* 2014). Tilapia is a very economically valuable fish species that is widely cultivated across the world. Due to its rapid development, productivity and resistance to both biotic and abiotic stressors, *O. niloticus* has recently acquired the moniker "chicken fish". It is the most lucrative fish in warm water aquaculture because it grows quickly in water compared to other fish species (Abdel-Daim et al*.,* 2020). The aims of this research were to analyze the effect of probiotic mixture on hemato-biochemical parameters of fish Nile Tilapia.

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MATERIALS AND METHODS

This study was carried out at Fisheries Farm, University of Agriculture, Faisalabad. Following 2 weeks of acclimatization, Nile tilapia, *O. niloticus*, (n = 60) were randomly stocked into three separate glass tanks of 70 L each and belonging to the same farm. Each tank (T_0, T_1, T_2 and T_3) were subdivided into three equal replicates, 5 fish each. Throughout the phase of acclimatization, fish were fed on a standard diet according to 5% of its body weight. Constant oxygen was supplied to test media maintained with the help of a capillary system. All the parameters like Total Hardness, pH, temperature of water etc. in the aquarium were maintained to maximize the benefits of probiotics.

Preparation of Feed

Commercial probiotics of Big Fish Aquatic Probiotic was added at ratio 2, 4 and 6 grams, by using different probiotics which include *B. subtilis, B. megaterium, B. pumilus, B. licheniformis and E. faecalis.* Then basal diet and water was poured gradually into the mixture to make a stiff paste and make their pellets and were air dried overnight. Placing the probiotic-enriched fish diet mixture in a clean, dry container or resealable plastic bag and sealing them tightly prevent the moisture and contaminants from entering and for daily feeding.

Diet Composition of Fish

Before the meal was composed and formulated, dietary components from the nearby fish feed market were collected and their chemical composition was evaluated by (AOAC, 1995). These feed ingredients were crushed and sieved until they reached the desired particle size before being added to the diet (Table 2.1).

Before preparing the diet, nutritional components were bought from the local fish feed market and their chemical composition was tested by (AOAC, 1995). Before being included into the diet table, these feed components were crushed and sieved to achieve the desired particle size.

Blood sampling and serum separation

During the trial, blood samples were collected after each month. Blood samples were taken from the caudal blood vessels (v. caudalis) using a sterile syringe. Each sample was divided into two parts: The first part was transferred into a 2ml sterile test tube with EDTA for hematological assay, and the second part was kept in a 2ml plain Eppendorf tube for serum separation. Blood was left to clot at 4°C for 60 min. After that, tubes were centrifuged at 1008g using an Eppendorf centrifuge for 10 min for serum separation. The serum was collected in Eppendorf tubes and stored at -40°C until analyses.

Hematological analysis

The following blood parameters were measured Neubauer hemocytometer to calculate white and red blood cells count, the method (Blaxhall & Daisley, 1973). The standard

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microhematocrit of Snieszko were used to calculate hematocrit (Hct). The blood indices which include MCV, MCH, and MCHC were computed by using different formulas.

MCV (fl) = Hct x 10/ number of RBC

MCH (pg) = Hb x 10/ number of RBC

MCHC (g per dL) = Hb x 100/ RBC

Biochemical analysis

Serum total protein was determined colorimetrically using commercial kits (TP0100, Sigma-Aldrich, USA). Serum albumin was measured using bromocresol green binding method (Doumas et al., 1971). Serum globulin was calculated by subtracting albumin values from total protein. Albumin/globulin (A/G) ratio was calculated by dividing albumin values by globulin ones.

Table 2.1: Fish feed preparation with different ingredients for the control group and at 2%, 4% and 6% probiotic addition

Ingredients	Control	T_1	T_2	T_3
Fish Meal	48	36	36	36
Soybean Meal	-	12	12	12
Wheat Flour	13	13	12	12
Wheat Bran	18	16	16	15
Rice Bran	15	13.5	12.5	13.5
Soy Oil	-	4	4	4
Fish Oil	4	-	-	-
Vit. And Min.	-	2	2	2
Lysine	2	0.5	0.5	0.5
Methionine	-	1	1	1
Probiotics		2	4	6
Total	100g	100g	100g	100g

Statistical Evaluation

After performing, the data was statistically estimated by analysis of variance (ANOVA) under CRD and Tuckey's test by using the software SPSS 16.0. Correlation coefficients among all variables was used to find out the relationships among different variables. Data were presented in three replicates as mean ± standard error (SE).

RESULTS

Hematological parameters

This study examined the variations in fish hemato-biochemical parameters by feeding Nile Tilapia with a conventional commercial diet along with varying dosages of a probiotic mixture. Table 3.1 represents the effect of mixture of probiotics at different ratios and it indicated that during the study trial.

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As compared to control and treated groups, the value of red blood cells increased up to 4.12±0.023 and it was found to be maximum in T_2 with 4g probiotic. With increasing the ratio of mixture of probiotics, it was observed that the white blood cells revealed different variation in their values and their value was maximum in T_3 with ratio of 6g probiotics.

Highest mean hemoglobin value was recorded in T_2 (6.1078±0.05) with 4g probiotic compared to T_3 (5.69±0.06) and T_1 (2.3122±0.02). The highest variation of hematocrit was observed in T_2 (31.4667±0.35) followed by T_3 (28.7223±0.68) and T_1 (25.1886±0.24). In Fig. 3.1 the graph of hematological parameters were measured on monthly basis in detail to describe the value of parameters.

The MCV and MCH showed varying variations in their values as the ratio of probiotic mixture increased; their value decreased. Maximum decrease in MCV value occurred in T_2 up to 102.38±0.02 and for MCH maximum decrease was up to 32.847±0.04 in T_2. During the probiotic exposure, the value of MCHC decreased and maximum decrease occurred up to 28.4047±0.04 in T_3. The value of platelets increased and it was maximum in T_2.

Biochemical parameters

Biochemical parameters also revealed different variations during probiotic mixture exposure. During the study period, it was found that total protein increased and was maximum up to 7.1689±0.02 in T_3.

During the probiotic exposure, the value of albumin and globulin increased, and the maximum increase in value was up to 4.0544±0.03 and 4.0367±0.01 respectively in T_3. Fig. 3.2 indicates that by increasing the concentration of probiotic the value of serum biochemical parameters increased and it was found to be maximum in T_3 with 6g probiotic mixture.

Table 3.1: Hematological and biochemical parameters of Nile Tilapia fed with Bacillus strains mixture probiotic

Parameters	Control	T_1 (2g probiotic)	T_2 (4g Probiotic)	T_3 (6g Probiotic)
RBCs (× 10^6 /µL)	1.764467±0.01	2.3122±0.02	4.12±0.023	3.5999±0.016
WBCs (×10^3 /µL)	30.3756 ±0.1	32.4033±0.06	31.5953±0.05	34.1056±0.08
Hb (g/dL)	4.3566±0.01	2.3122±0.02	6.1078±0.05	5.69±0.06
MCV (fL)	106.19±0.03	105.79±0.05	102.38±0.02	104.08±0.04
MCH (pg)	39.668±0.11	37.4603±0.08	32.847±0.04	34.849±0.04
MCHC (g/dL)	31.4037±0.03	30.148±0.02	29.4037±0.03	28.4047±0.04
Hct (%)	28.7223±0.68	25.1886±0.24	31.4667±0.35	28.7223±0.68
Plt (×10^6 /µL)	2.5867±0.07	2.8622±0.06	3.9233±0.08	3.1722±0.12
TLP (g/dL)	4.7667±0.04	5.0633±0.03	6.4111±0.03	7.1689±0.02
Alb (g/dL)	1.65±0.01	2.2367±0.02	3.3678±0.02	4.0544±0.03
Glb (g/dL)	2.2444±0.008	2.7933±0.05	3.3389±0.02	4.0367±0.01

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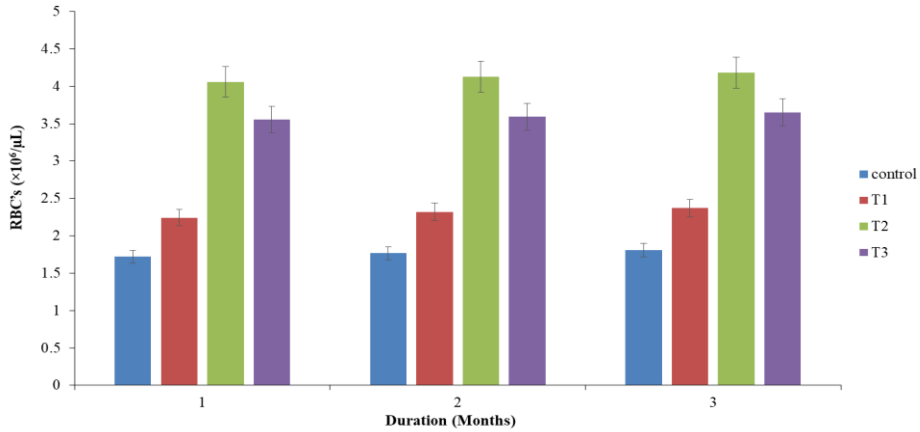
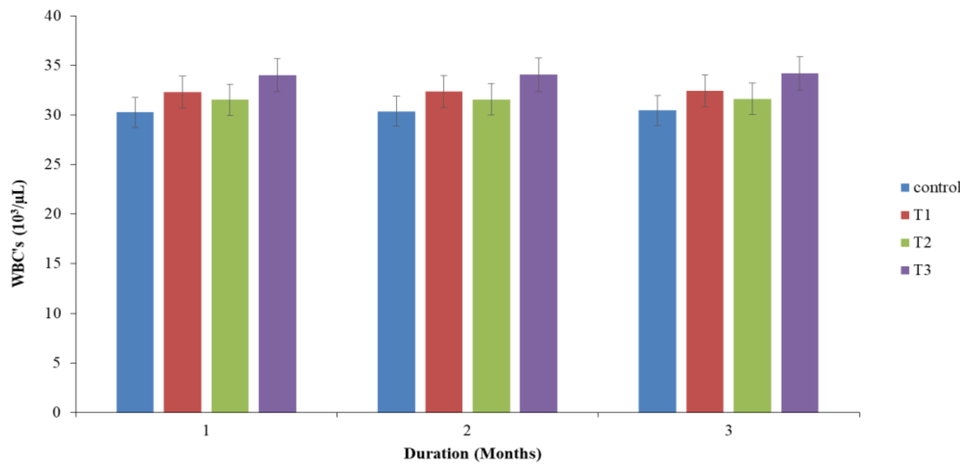
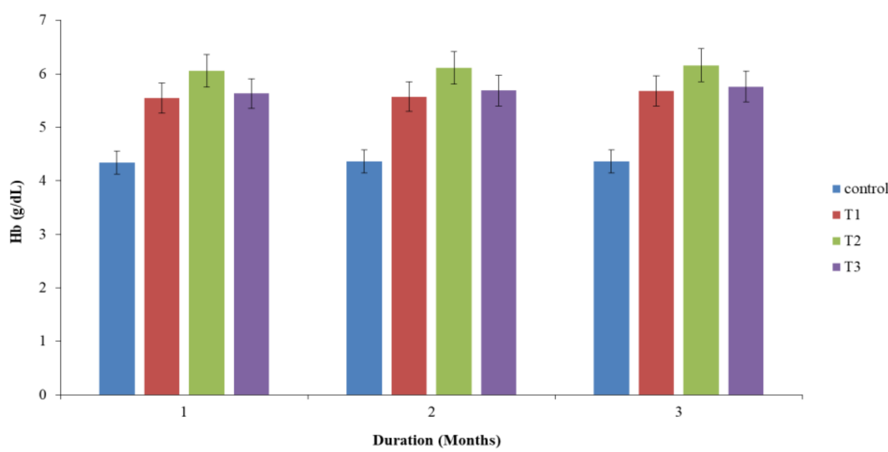


(a) Graphical representation of RBC's



(b) Graphical representation of WBC's



(c) Graphical representation of Hb

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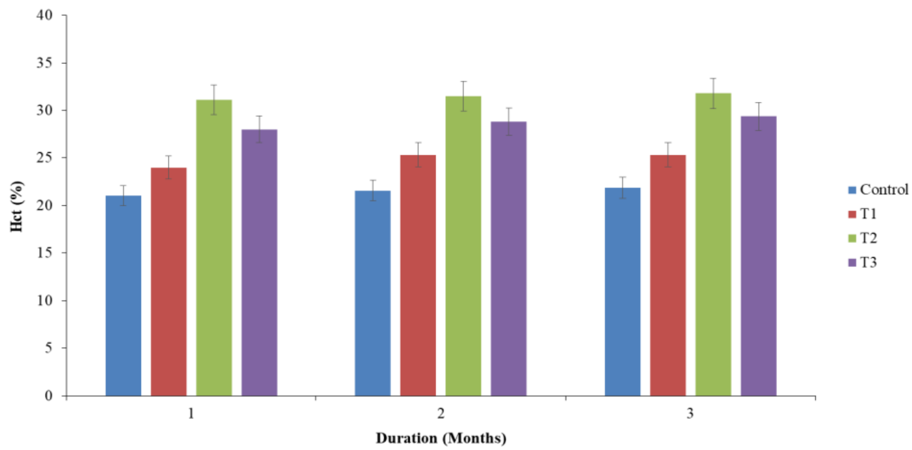

(d) Graphical representation of Hct

Figure 3.1: Graphical representation of Hematological Parameters of Fish Nile Tilapia (a) Red Blood Cells (b) White Blood Cells (c) Hemoglobin (d) Hematocrit between control and treated groups after feed with (2g, 4g and 6g probiotic mixture)

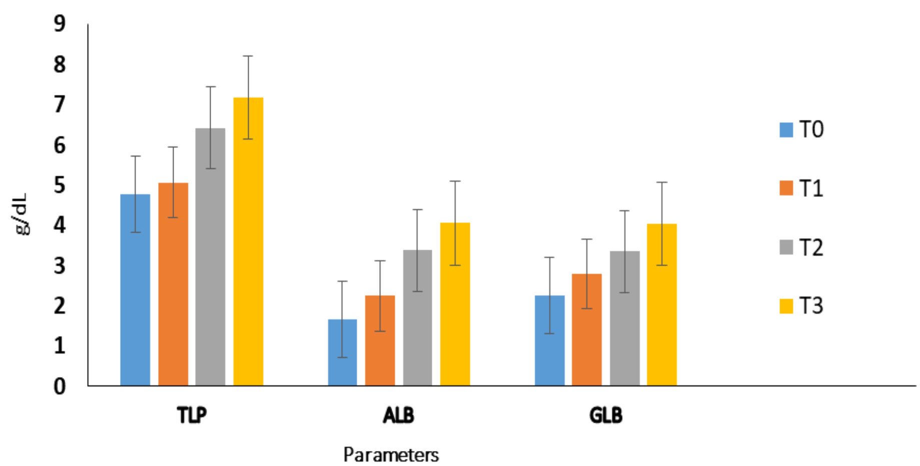

Fig 3.2: Serum Total Protein, albumin and globulin of Nile Tilapia after feeding with (basal diet, 2g, 4g and 6g of probiotic mixture)

DISCUSSION

The objective of the present research work was to evaluate the effect of mixture of dietary probiotics on the Hemato-biochemical parameter of fish Nile Tilapia (*O. niloticus*). Hematology is an essential biomarker for determining fish health and a species capacity to adapt to its surroundings environment (Adeyemo, 2007). Hematological measures are commonly used to determine the potential changes in fish health status following feeding

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with functional feed additives under cultural circumstances (Hassan et al., 2018). During the present research, the overall improvements in hematological characteristics were reported by feeding probiotics might indicate a role of probiotics in stimulating certain immune and stress responses of fish.

By giving probiotics, the recent study demonstrated a positive effect on hematological parameters. The present study revealed that Nile Tilapia exposed to mixture of probiotic exhibited a significant increase in red blood cells (RBCs), white blood cells (WBCs), hemoglobin (Hb), hematocrit (Hct) and platelets (PLT) while revealed a significant decrease in mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC). Similar result were reported by Irianto & Austin (2002) that probiotic usage was related to higher RBC and leucocyte counts in fish.

The use of commercial probiotics in this study considerably enhanced the blood and serum biochemical parameters of Nile Tilapia. Similar results were also reported by Eissa et al. (2024) showed increased RBCs, Hb and MCHC in Red Tilapia groups fed on *B. subtilis*, suggesting that *Bacillus* stimulated fish immune and anti-stress responses. In a similar study, increase in Hct and Hb values were exposed in Nile Tilapia fed with *B. amyloliquefaciens* as were monocytes in *Labeo rohita* fed on *B. subtilis*. Previous research on the supplementation of multi strain probiotics (MSP) in fish diets also demonstrated increased levels of Hb, RBCs, MCH, and WBCs, which is similar to our work (Tabassum et al. 2021; Yaqub *et al*. 2021).

In addition, other studies have shown that the application of MSP containing *L. sporogenes*, *L. acidophilus*, *B. subtilis*, *B. licheniformis*, and *Saccharomyces cerevisiae* in *Cirrhinus mrigala* and *L. acidophilus* and *B. subtilis* in *O. niloticus* can raise WBCs, which is also consistent with the results of the current research (Aly et al. 2008; Sharma et al. 2010). Additionally, the elevated WBC numbers could be a sign of enhanced innate immunity (Rajikkannu et al. 2015).

In recent study, significant increase in serum analysis was detected (albumin, lysozyme, globulin and total protein) between all treatments. Mohapatra et al. (2014) found that fish fed on probiotic diet had a substantially higher serum albumin to globulin ratio (A/G ratio) ($P<0.01$) and increased with higher water temperature. Eissa et al. (2023) reported similar result in protein content, albumin, and globulin levels in Nile Tilapia after feeding a *Pediococcus acidilactic* supplemented meal. A recent study found comparable findings in Beluga fish *(Huso huso*) treated with *P. acidilactic* and Rainbow Trout (*Oncorhynchus mykiss*) supplemented with lactic acid bacteria supplements. Furthermore, increased levels of globulin, albumin and serum protein in fish were assumed to indicate a strong innate immune response. Eissa et al. (2024) investigated that feeding Red Tilapia on diets enriched with *B. subtilis*, showed elevated total protein, albumin, and globulin values along with significant decreases in glucose. The increased blood proteins may be linked to effective feed utilization and high availability of digested proteins and amino acids in fish intestines.

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CONCLUSION

The present research was conducted to evaluate the effect of dietary probiotics mixture on the Hemato-biochemical parameter of fish Nile Tilapia. The results of present research showed that probiotics have a highly positive impact on the hemato-biochemical parameters of fish. After feed with 2g, 4g and 6g of probiotic mixture, considering the overall performance and it was concluded that 4g of dietary probiotic mixture was highly beneficial for the hematological parameters and 6g of probiotic mixture was highly beneficial for biochemical parameters of fish Nile Tilapia. These findings highlight the potential of dietary probiotics as a useful supplement in aquaculture operations aiming at improving Nile Tilapia health and performance. To optimize these advantages in aquaculture settings, more research should be done on the ideal dosage and long-term impacts.

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