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Immune response of saline tilapia (*Oreochromis niloticus*) to feed enriched with probiotic *Bacillus amyloliquefaciens* at high salinity

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Abstract. Tilapia is a widely favoured fish due to its substantial meat content and appealing taste. The incorporation of probiotics in tilapia farming can enhance the fish's immunity against diseases. Additionally, probiotics can increase the total protein content of the feed and maintain the balance of digestive microbes. This study aimed to investigate the immune response of saline tilapia fed a diet enriched with the probiotic *B. amyloliquefaciens* at a salinity level of 10 ppt. The research focused on various blood parameters in tilapia, including total erythrocytes, leukocytes, and hematocrit levels, as well as the food conversion ratio (FCR) and specific growth rate (SGR). The rearing method involved maintaining approximately 100 g of tilapia fry for 45 days, with four treatments and three replications. The treatments included a control without *B. amyloliquefaciens* and additions of the probiotic at concentrations of 10^3, 10^5, 10^6, and 10^7 CFU/mL. The study results indicated no statistically significant differences (P>0.05) in FCR values (1.16–1.24) and SGR (2.03–2.24% BW/day). However, the addition of *B. amyloliquefaciens* at a concentration of 10^7 CFU/mL led to an increase in erythrocyte count and hematocrit levels during the study period, with no observed effect on leukocyte count in saline tilapia reared at high salinity.

1 Introduction

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Tilapia (*Oreochromis niloticus*) is one of the most extensively cultivated fish species in Indonesia [1, 2]. In response to the growing demand for tilapia, farmers are exploring ways to enhance the health and growth of this fish in environments that may not be optimal, such as high-salinity conditions [3, 4]. Probiotics in fish are defined as living microorganisms that provide health benefits to their hosts, representing a promising solution [5, 6].

Excessive salinity levels can induce osmotic stress in fish, disrupting their internal homeostasis [7, 8]. This stress compromises the immune system, making fish more susceptible to diseases [9]. Research has shown that tilapia in high-salinity environments exhibit reduced activity of immune cells, such as lymphocytes and macrophages, which are essential for defense mechanisms.

Probiotics have been shown to enhance the immune response in fish through various mechanisms. They improve the health of the digestive tract, facilitating better nutrient absorption and overall health [10]. Additionally, probiotics contribute to the formation of beneficial microbiota in the intestines, which can inhibit pathogen growth [11]. Numerous studies have also demonstrated that probiotics can stimulate antibody production and enhance immune cell activity [12]. Probiotics offer an environmentally friendly alternative to antibiotics in aquaculture [13]. Erma's study [14] found that tilapia fed with probiotics under high salinity conditions had elevated levels of immunoglobulin M (IgM), a key antibody in the immune response. Moreover, fish fed with probiotics showed increased activity of T cells and B cells, which are vital for infection defense [15, 16].

Several studies have demonstrated that the administration of probiotics consisting of *Lactobacillus plantarum* to tilapia fish reared in high-salinity environments can result in an enhancement of immunoglobulin levels and lysozyme enzyme activity [17-19]. This evidence indicates that probiotics can enhance the immune system of fish, thereby increasing their resilience to environmental stressors, such as high salinity. This article will examine the impact of probiotic feed on the immune response of tilapia fish inhabiting high-salinity environments.

2 Materials and Methods

2.1 Preparation of fish

Prior to the commencement of the study, the tilapia seeds were acclimatised to a salinity of 15 ppt for a period of two weeks. The study employed a total of 12 containers, each with a capacity of 50 L and equipped with an aeration system. The stocking density of tilapia used was 20 fish with a length ranging from 7 to 9 cm, as reported by Rahmi et al. [20]. The fish were maintained in the containers for a period of 40 days, with feeding occurring three times a day (07.00, 12.00, 17.00) at a satiation level. Each container was filled with 20 liters of water at a salinity of 15 ppt and aerated to increase the dissolved oxygen levels within the maintenance medium.

2.2 Preparation of feed

The feed utilized in this research was formulated from a standard base and enhanced with *Bacillus amyloliquefaciens*, which was cultured in nutrient broth (NB). Initially, bacterial isolates were added to NB and incubated with agitation for 24 hours [21]. The bacterial suspension was then centrifuged at 6000 rpm for 10 minutes, after which the supernatant was discarded, and the bacterial pellet was resuspended in a saline solution. Subsequently, 1% of the feed was sprayed with this bacterial suspension [22, 23], air-dried, and then used.

2.3 Experimental Design

The experimental design employed was a completely randomized design (CRD) consisting of four treatments, each replicated three times. The treatments involved the addition of *Bacillus amyloliquefaciens* at concentrations of 10^3 CFU/mL (A), 10^5 CFU/mL (B), 10^7 CFU/mL (C), and 10^9 CFU/mL (D).

2.4 Fish performance indices

2.4.1 Blood profile

The total erythrocyte count was determined using the method described by Blaxhall and Daisley [24]. Blood samples, up to 0.2 mL, were collected using a red Sahli pipette marked to 0.5, followed by the addition of Hayem's solution up to the 101mark. Blood from one to two fish was drawn using a 1 mL syringe. The blood in the pipette was then mixed thoroughly for 3-5 minutes, with 2-3 drops discarded to remove any unmixed blood. The sample was then placed onto a haemacytometer covered with a cover glass. The haemacytometer was examined under a microscope, and erythrocytes were counted in five visible sample boxes. The total erythrocyte count was calculated using the following formula:

$$\text{Total erythrocytes} = \text{number of erythrocytes counted} \times 10^4 \ \frac{\text{cells}^3}{\text{mm}} \tag{1}$$

The leukocyte count was conducted using the protocol outlined by Blaxhall and Daisley [24]. Blood samples mixed with EDTA in a vacutainer were drawn up to the 0.5 mark using a pipette, which was then cleaned with a tissue. The Turk solution was subsequently aspirated into the same pipette until it reached the 11 marks. The mixture was thoroughly homogenized for approximately three minutes to ensure complete dissolution. Before introducing the solution into the counting chamber, two or three drops were discarded. The solution was allowed to settle for one minute, after which leukocytes were counted using a microscope with 10× or 40× objective lenses. The leukocyte count was calculated using the following formula:

$$\text{Total leukocyte} = \text{number of erythrocytes counted} \times 10^4 \ \frac{\text{cells}^3}{\text{mm}} \tag{2}$$

Hematocrit levels were determined using the method described by Anderson and Siwicki [25]. Blood was drawn to fill ¾ of the microhematocrit tube. The end of the tube containing the blood was sealed with crytoceal by dipping it approximately 1 mm deep to form a plug. The microhematocrit tube was then centrifuged at 5,000 rpm for 5 minutes. The length of the sedimented blood (a) and the total blood volume in the tube (b) were measured using a ruler. Hematocrit levels represent the proportion of blood cells (solids or sediment) in the blood fluid. The hematocrit value can be calculated using equation 3:

$$\text{Haematocrit} = (a/b) \times 100\% \quad (3)$$

2.4.2 Growth performance and feed utilization

At the beginningand the end of the feeding trial, fish were weighed. The following fish performance indices were calculated:

$$\text{Survival}(\%) = \frac{\text{total of fish alive}}{\text{total of fish used}} \times 100 \quad (4)$$

$$\text{Weight Gain (gr)} = \text{final weight} - \text{initial weight [26]} \quad (5)$$

$$\text{Specific Growth Rate (SGR)} = 100 \times \left[\frac{\text{Ln}(\sum \text{weight at the end of the study}) - \text{Ln}(\sum \text{weight at the beginning study})}{\text{rearing period (in days)}}\right] \text{[27]} \quad (6)$$

$$\text{Food Convertion Rate (FCR)} = \frac{\sum \text{weight at the end of the study} - \sum \text{weight at the beginning study}}{\text{Amount of feed consumed during the study period}} \text{[28]} \quad (7)$$

3 Results

3.1 Blood Profile

The blood profile of saline tilapia fish in each treatment for each week of maintenance, as indicated by total erythrocytes (ranging from 9×10^5 to 3.3×10^6 cells/mm³) (Fig. 1), total leukocytes (1,050×10³ to 1,600×10³ cells /mm³) (Fig. 2) and haemotocrit percentage (12.50 to 29.50%) (Fig. 3)

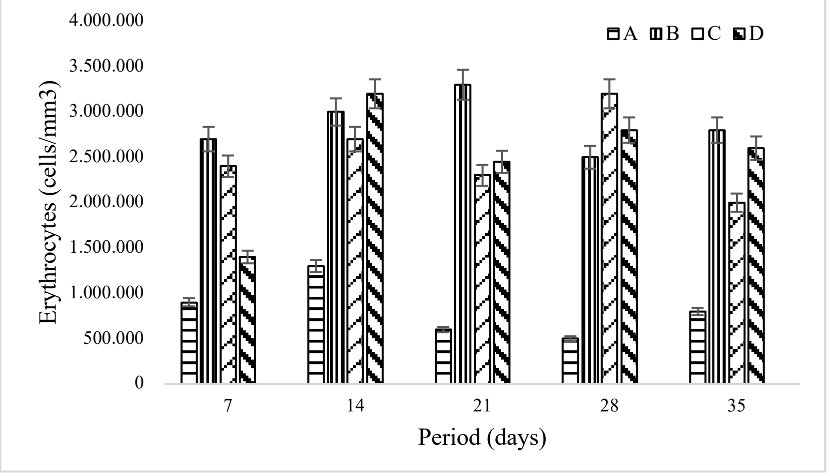

Fig. 1. Total of Erythrocytes observed over 35-day period in *Oreochromis niloticus*

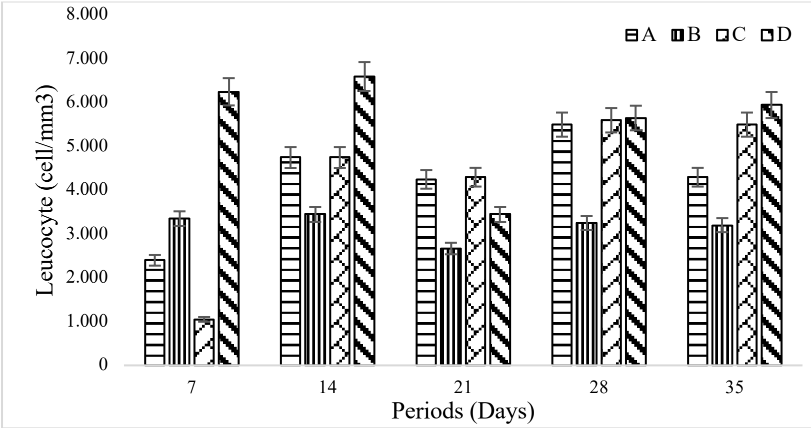

Fig. 2. Total of Leukocytes observed over 35-day period in *Oreochromis niloticus*

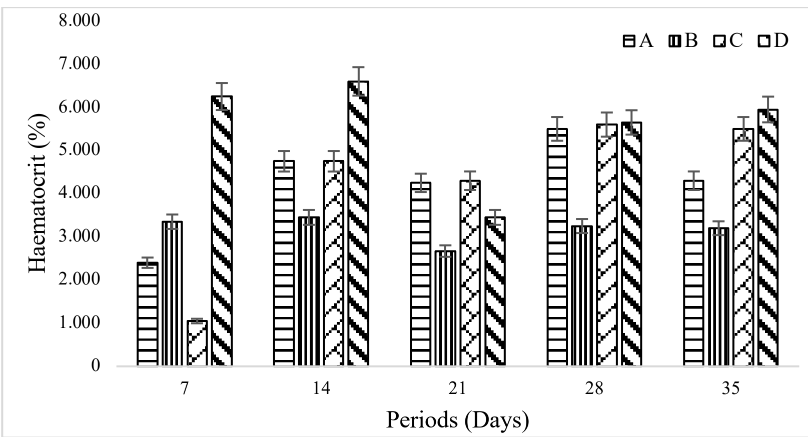

Fig. 3. Percentage of Haematocrit observed over 35-day period in *Oreochromis niloticus*

3.2 Tilapia Growth and Feed Utilization Analysis

The survival rate of saline-treated tilapia ranged from 83.33% to 86.67%, with no significant differences observed (P>0.05). The feed conversion ratio (FCR) varied between 0.91 and 1.15 (Table 2). In contrast, the addition of *B. amyloliquefaciens* at a concentration of 10^5 CFU/mL (B) resulted in a significant increase (P>0.05) in body weight (BW), which ranged from 2.24 to 3.63 grams, and in specific growth rate (SGR), which ranged from 0.61% to 1.32% per day (Table 1).

Table 1. Survival, Weight Gain (WG), Specific Growth Rate (SGR), and Food Convertion Rate during observation in *Oreochromis niloticus*

Treatments	Survival (%)	WG (gr)	SGR(%/hari)	FCR
A	80.00±3.75^a	2.24±1.67^a	0.61±0.001^a	0.91±0.02^a
B	86.67±2.46^a	3.63±1.03^b	1.32±0.000^b	1.05±0.13^a
C	85.00±4.03^a	2.80±2.05^a	0.80±0.001^a	1.15±1.29^a

D	83.33±2.76[a]	2.93±1.33[a]	1.01±0.001[a]	1.13±0.32[a]

Note: Different superscripts in the same parameter indicate significantly different treatment effects (Tukey's test; P<0.05). Mean ± standar deviasi (SD), n=3

4 Discussion

The results of the study showed that there was a greater increase in the number of erythrocytes in the diet supplemented with *B. amyloliquefaciens* 10^5 CFU/mL than in the other treatments (Fig. 1). The number of erythrocytes in this treatment increased significantly from day 7 to day 21, with the number of erythrocytes being 1.7×10^6 cells/mm^3 on day 7 and 3.3×10^6 cells/mm^3 on day 21. The erythrocyte count in this study was classified as normal condition. This is in line with the opinion of [28]; [29] that the normal range of total erythrocytes in tilapia is 2×10^4-3×10^6 cells/mm^3. Changes in temperature and salinity also affect the number of erythrocytes in tilapia [30]. Changes in temperature and salinity can increase the number of erythrocytes if given continuously. This is because as temperature and salinity increase, the activity of oxygen uptake by erythrocytes increases and the fish's body will compensate for the changes in oxygen deprivation by increasing the number of erythrocytes.

The results of the study showed that the treatment with the addition of *B. amyloliquefaciens* probiotics for the treatment of 10^5CFU/mL in saline tilapia feed did not show an increase in the number of leukocytes when compared to the treatment of 10^3 CFU/mL, 10^7 CFU/mL, and 10^9 CFU/mL. It can be observed that the addition of *B. amyloliquefaciens* probiotics at a concentration of 10^5 CFU/mL resulted in a non-significant increase in total leukocytes for each treatment over the course of the study period. This means that the addition of *B. amyloliquefaciens* probiotics can make the total leukocyte level from the beginning of the maintenance to the end of the maintenance of saline tilapia.

Leukocytes are integral to the immune response. In this study, leukocytes demonstrated no significant effect, despite being the most active component of the body's defense system, circulating in various forms within the bloodstream. Their primary function involves the destruction of infectious and toxic agents through phagocytosis and antibody formation [31]. The susceptibility of tilapia to disease at low temperatures can be attributed to fluctuations in leukocyte counts, either below or above normal levels. A reduced metabolic rate in fish leads to diminished immunity, increasing their vulnerability to diseases [32]. Elevated water temperatures enhance the immune response in fish. Variations in water quality during the growth period of tilapia, coupled with inadequate nutritional intake, can also result in decreased leukocyte counts, thereby compromising the immune system and increasing disease susceptibility [33, 34]. Additionally, increased temperatures can influence leukocyte counts in tilapia, leading to an elevation as part of the immune response to microorganisms.

The hematocrit levels observed during the study indicated that the treatment involved the addition of *B. amyloliquefaciens* probiotics at a concentration of 10^5 CFU/mL exhibited a range of 15.45-33.70%. This value indicates the optimal result after 35 days of study. The parameter that affects the measurement of red blood cell volume is the hematocrit. Haematocrit is defined as the percentage of red blood cell volume in the blood. Normal hematocrit values in tilapia range from 20 to 30%, with some marine fish exhibiting values of approximately 42% [35]. The hematocrit levels in the treatment based on the lowest value will gradually increase as the fish begin to adapt to the new environment and begin to obtain nutritional supplies from the food they eat. This is emphasized by [36], who states that hematocrit levels vary according to several factors, including nutritional intake, age, gender, body size and spawning period.

This suggests that the treatment, which includes the addition of probiotic *B. amyloliquefaciens* at a concentration of 10^5 colony-forming units (CFU) per milliliter, plays a pivotal role in enhancing the blood profile and overall resilience of tilapia. This is because it can boost the immune system response in tilapia through alterations in blood parameters, including total erythrocytes, total leukocytes, and haematocrit percentage, as observed during the course of the study. Furthermore, red blood cells are involved in the fish's innate and acquired immune response mechanisms, which contribute to the fish's body defense process. Erythrocytes can react to infections or other abnormalities within the fish's body [37, 38]. The hematocrit value in artificial feed supplemented with probiotics indicates that the synbiotic feed composition is optimal for the growth of tilapia, as it can elevate hemoglobin levels in the blood to attain normal values [23].

The survival rate of the saline tilapia (*O. niloticus*) was monitored throughout the duration of the study. A number of fish died in each treatment group. The variance analysis revealed that the inclusion of probiotics at varying doses in each treatment has no significant impact on the survival rate of saline tilapia (*O. niloticus*) ($P < 0.05$). However, Tukey's multiple comparison test indicated that the administration of different doses within the treatment had a significantly effect on the survival rate of saline tilapia.

The treatment involving the addition of probiotics, specifically *B. amyloliquefaciens* at a concentration of 10^5 CFU/mL, resulted in the highest body weight among saline tilapia. This outcome is hypothesized to be due to the probiotics meeting the necessary standards for effective feed digestion in saline tilapia. Previous studies have shown that incorporating probiotics into fish feed can increase body weight. This effect is attributed to the bacteria in probiotics, which improve digestion efficiency and nutrient absorption, thereby facilitating growth in both weight and length. Probiotics in feed can also enhance the nutritional profile, providing a source of easily digestible protein, biomass, and energy [39, 40, 41]. Additionally, probiotics can support production by promoting the growth and survival of cultured organisms [42].

The specific growth rate (SGR) was determined during the study, with the highest average treatment resulting from the addition of *B. amyloliquefaciens* probiotics at a density of 10^5 CFU/mL, exhibiting a rate of 1.32% per day. The analysis of variance (ANOVA) demonstrating that the administration of *B. amyloliquefaciens* probiotics had a statistically significant impact ($P < 0.05$) on the growth of saline tilapia (*O. niloticus*). The results of the Tukey post-hoc test demonstrated that there was a statistically significant difference between the treatment group that received the addition of *B. amyloliquefaciens* probiotics at a concentration of 10^5 CFU/mL and the treatment group that received the addition of *B. amyloliquefaciens* probiotics at other concentrations. The survival rate in saline tilapia demonstrated that the incorporation of *B. amyloliquefaciens* probiotics into commercial feed resulted in a superior outcome compared to the use of feed lacking the probiotic supplementation. It is hypothesised that the addition of probiotics enhances immunity and improves the survival of fish. The study demonstrated that the number of leukocytes in tilapia receiving probiotics in their feed increased. These findings suggest that the administration of prebiotics may increase fish resistance to infection and stress, which could contribute to improved fish survival. A high number of leukocytes indicates a stronger immune response to various disorders. In a similar vein, [23] posited that the use of probiotics can increase the survival rate and resistance of fish to pathogen infections. This is corroborated by the findings of [43], who observed that treatment without the addition of probiotics resulted in a lower growth rate value compared to the treatment with the addition of probiotics.

The present study considers water quality parameters as one of the variables under observation. It is important to note that the conditions of normal water quality cannot be considered in isolation from the management of cultivation. Stable water quality conditions in several parameters, including temperature, pH, ammonia and salinity, have been demonstrated to correlate with optimal growth rates [51].

Table 2. Water quality parameters during period of study

Paramaters	Range of value	Optimum value	References
Temperature (°C)	28-30	25-30	[45, 46]
Salinity (ppt)	10-10,5	20	[47]
pH	7.10-8.00	6.5 – 8.5	[48, 49]
Ammonia (ppm)	0.0558-0.0776	0,1	[50]

In this study, water quality measurements were conducted over a 40-day period, encompassing temperature, pH, and salinity. Ammonia levels remained below 0.1 ppm throughout the maintenance phase, indicating a satisfactory status. These findings demonstrate that the incorporation of probiotic feed is an effective method for maintaining water quality at a consistent and optimal level, making it a suitable approach for the cultivation of saline tilapia.

5 Conclusion

The research results clearly show that adding probiotic *B. amyloliquefaciens* to saline tilapia feed at a concentration of 10^5 CFU/mL optimises the total erythrocyte value, total leukocyte and haematocrit percentage. This is a significant finding that affects other treatments, including body weight and specific growth rate. The survival rate and feed conversion ratio of saline tilapia (O. *niloticus*) had no significant effect on saline tilapia during maintenance.

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