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Effect of a Mixture of Probiotics (*Pediococcus acidilactici*, *Lactobacillus acidophilus*, and *Bacillus subtilis*) on the Physiological Status and Fish Health of the Nile Tilapia (*Oreochromis niloticus*)

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ABSTRACT

The present research evaluated the value of adding 3, 5, and 7g of a probiotic mixture of *Pediococcus acidilactici*, *Lactobacillus acidophilus*, and *Bacillus subtilis*/kg fish feed on the physiological status and fish health of the Nile tilapia (*Oreochromis niloticus*). A total of 120 Nile tilapia fish were divided into four equal groups fed on pellets containing different ratios of the probiotic mixture for three months. After the experimental period, the whole-body compositions (fats, protein, and ash content) of tilapia were affected significantly in all groups ($P \leq 0.05$). The immunological parameters, nitroblue tetrazolium (NBT) and lysozyme activities significantly increased in all treated groups. The results showed significant hematological parameters [Hematocrit (PCV), hemoglobin (Hb), erythrocyte counts (RBC), Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC)] and significant biochemical blood parameters [glucose, albumin, protein, globulin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT)] measured in serum after the experimental period. The number of *Vibrio* sp. isolated from the internal organs of experimental fish (muscles and liver) every month for three months was lower significantly in all treated groups than in the control. The relative level of protection (RLP) among the three treated groups after infection-challenge with *Vibrio harveyi* (0.1ml of culture suspension of pathogen containing 10^8 bacteria ml^{-1}) was higher than in the control, and the highest record was in the fourth group. The present research results support the use of a mix of *Pediococcus acidilactici*, *Lactobacillus acidophilus*, and *Bacillus subtilis* at a rate of 7g/ kg standard fish diets supplementation as immunostimulants in common fish diets.

INTRODUCTION

The aquaculture industry is enlarged quickly to equal the growing protein needs of people everywhere **(Gobi *et al.*, 2016)**. According to **Behera *et al.* (2018)**, tilapia is one of the world's most widely used commercially farmed fish, with global production now at

6.882 million metric tons and projected to reach 7.3 million metric tons by 2030. Accordingly, nearly 179 million tons, with a total sale value of 401 billion dollars **(FAO, 2020)**. As a result, approximately 179 million tons of fish were produced worldwide, with a total market value of 401 billion dollars **(FAO, 2020)**. Furthermore, profitable aquaculture operations depend on maximizing feed quality and efficiency to provide sufficient nutrition and growth **(Akter *et al.*, 2021)**. Fish feed costs may be decreased by using probiotics to enhance feed consumption and growth **(El-Saadony *et al.*, 2021)**.

Drug-resistant bacteria are created due to the extensive use of antibiotics and they can subsequently infect humans by the food cycle **(Wanja *et al.*, 2020)**. As a result, probiotics – microorganisms or their byproducts – are increasingly being used to enhance the host health. Probiotics aid in the development and reproduction of aquatic animals, prevent illness, increase immunity, facilitate digestion, promote growth, and act as a replacement for antibiotics **(El-Saadony *et al.*, 2021; Mondal *et al.*, 2022)**. By generating extracellular enzymes like lipase, protease, and carbohydrates, probiotics can promote the growth of aquatic animals **(Leonel Ochoa-Solano & Olmos-Soto, 2006)**. According to **Skjermo and Vadstein (1999)**, probiotic feed supplements can be given to fish through dietary supplements or water circulation.

Bacillus triggered fish's humoral and cell-mediated immune responses **(Xue *et al.*, 2020; Foysal *et al.*, 2021)**. *Bacillus* produces a variety of hydrolytic enzymes, including cellulases, proteases, and 1,3-glucanases, to aid in fish digestion **(Kuebutornye *et al.*, 2020)**.

Lactic acid bacteria (LAB) are used as probiotics in aquaculture and include *Lactobacillus* acidophilus and the Gram-positive *Bacillus subtilis* group. According to **Elshaghabee *et al.* (2017)**, *Lactobacillus* has primarily been studied as probiotics for animal feed and pharmaceuticals.

Recent studies have shown that the LAB and *Pediococcus acidilactici* can enhance enterocyte endocytic activity, improve intestinal microvilli morphology, and enhance weight gain, survival, and hemolymph antioxidant status in shrimp **(Castex *et al.* 2008, 2009)**. Nevertheless, the mechanisms behind these advantages are little known. When combined with lactic and acetic acid, a variety of bacteriocins (pediocins) from *P. acidilactici* strains have antagonistic effects on both Gram -ve and Gram +ve bacteria, most *Vibrio* spp. **(Castex *et al.*, 2008)**.

It is expected that the probiotic bacteria addition to fish diet will improve both health and performance. This research purpose was to examine how the hematological parameters, immunity, and microbiota of tilapia were influenced by using a mixture of three probiotics (*Pediococcus acidilactici*, *Lactobacillus acidophilus*, and *Bacillus subtilis*) as food additives.

MATERIALS AND METHODS

The experiment duration was 90 days and was performed at the Central Laboratory for Aquaculture Research, Abbassa, Sharkia, Egypt.

1. The experimental design

Fingerlings of Nile tilapia fish (*Oreochromis niloticus*) averaging about 54g were chosen for our research. The fish were put in twelve glass aquaria (70 × 40 × 60cm) supplied with aerated tap water. Fish were divided into four groups; each was supplied into three aquaria holding 10 fish. Commercial pellets (30% crude protein) were introduced to the groups in the food of 3% of their body weight at a daily rate. The 1st group (T1) was a control fed on a basal non-treated diet within each dietary level. The 2nd group (T2) was fed a diet supplemented with 3g mixture of *Pediococcus acidilactici, Lactobacillus acidophilus*, and *Bacillus subtilis*/kg standard fish diet. The 3rd set (T3) was fed a diet supplemented with 5g/kg feed of the same probiotics mixture. Also, the 4th group (T4) was fed a diet supplemented with a mixture with a ratio of 7g/ kg feed. The experimental nutrition to fish in all sets was three times a day and lasted for 12 weeks in total.

2. Experimental management

The fingerlings of the Nile tilapia were brought from the hatchery of Central Laboratory for Aquaculture Research , Abbassa, Sharkia. The water aquaria were provided with aerated, dechlorinated tap water. Fish feces and residuals were removed by siphoning using a plastic tube. About 50% of aquarium water was replaced by dechlorinated water daily. All water aquaria were provided with an air pump to provide fish with oxygen.

3. Measurements

3.1. Body composition and chemical analysis

After the experiment ended, five fish were taken from each treatment for chemical analysis of body composition, which was estimated according to **NRC (2011)**, as 5.64, 9.44, and 4.11 Kcal/g for protein, lipid, and nitrogen free extract (NFE), respectively.

*NEF = 100 - [H2O + (N×6.25) + Ash + CF or (NDF + ADF) Fiber + EE]

*NEF represented the most soluble carbohydrates, which are mainly starch, pectin, and hemicellulose.

3.2. Hematology and immunology

We withdrew blood from five fish per tank after the experimental period. Samples were withdrawn from the fish caudal vein using a 1.0 ml syringe. Erythrocyte counts (RBC), hematocrit (determined as % packed cell volume), hemoglobin (Hb), and lysozyme activity were determined by standard methods **(Svobodova *et al.*, 1991, 2001; Merrifield *et al.*, 2010)**. Blood parameters were estimated according to **Houston (1990)**,

as mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), and Mean corpuscular volume (MCV) were also calculated with standard formulas, MCHC (%) = 100. Hb / PCV, MCH (pg) =10. Hb / RBCs, and MCV (fl) = 10. PCV / RBCs.

The glucose concentration was detected using Boehringer Mannheim kits, as described by **Trinder (1969)**. The total protein was measured by the colorimetric method according to **Henry (1974)**, and albumin & globulin were detected according to **Busher (1990)**.

Aspartate and alanine aminotransferase (AST) and (ALT) activities were measured colorimetrically through the method of **Reitman and Frankel (1957)**.

Respiratory burst activity by measuring nitroblue tetrazolium activity (NBT):

The reduction of NBT (NBT reduction kit; Sigma-Aldrich) according to the manufacturer's instructions measured neutrophil respiratory burst by **Siwicki *et al*. (1985)** at 610nm.

- Lysozyme activity

An electric colorimeter with a turbidity measurement attachment was used to measure the lysozyme activity. The lysozyme content was determined based on the calibration curve and the extinction measured **(Schaperclaus *et al.*, 1992)**.

- Naturally infected tilapia

A total number of 30 clinically and the grossly diseased Nile tilapia (*Oreochromis niloticus*) were obtained from different fish farms in Abassa, Abo-Hammad, Sharkia, Egypt, and transferred alive or recently dead in an ice box to the Lab. The diseased fish were exposed to clinical and postmortem examinations, as **Plump and Bowser (1983)** described.

A- Clinical examination

It was done using the method described by **Plump and Browser (1983)** to describe abnormalities in the behavior of the tested fish.

B- Postmortem examination

For the body cavity examination, the fish were opened as described by **Plump and Browser (1983)**.

- Characterization and identification of the pathogenic bacterial isolates

Following 24-48 hours of growth at 30°C, cells cultured on selective media were exposed to Gram staining and cell configurations. Pigments, texture, and other cultural traits of the colony were found. The bacterial isolates were stained and subjected to biochemical examinations, such as indole, methyl red, citrate utilization, catalase, oxidase, urease, sucrose, maltose, and mannitol, then were determined through Bergey's Manual of Determinative Bacteriology **(Holt *et al.*, 1986, 1994)** and a set of identification keys.

-Microbiological analysis of feeding experiment

. Enumeration and isolation of *Vibrio* species

The bacteria were isolated in the feeding experiment under complete aseptic conditions from the internal and external organs (liver and muscles). The isolation was performed before and after the feeding experiment (every month for three months). *Vibrio* species were identified by using thiosulphate citrate bile salt sucrose agar medium (T.C.B.S) according to **Elston (1989)**.

Challenge test

After three months of feeding, tilapia were injected intraperitoneally with 0.1ml of virulent *Vibrio harveyi* (10^8 CFU/fish), which was centrifuged and resuspended in 0.85% NaCl. The control set was injected with 0.85% NaCl. Infected fish were observed daily for seven (7) days after inoculation, and mortalities were recorded **(Nadirah *et al.*, 2020)**.

Statistical analysis

It was done by using the one-way analysis of variance (ANOVA). It was performed according to **Murray (1975)** with SPSS statistical software. Data were exposed for test of homogeneity of variances and Duncan post-Hoc test. Data were considered significantly different when $P \leq 0.05$.

RESULTS

The whole-body composition (dry matter, fat, protein, and ash content) of experimental tilapia fish was affected significantly in all probiotic groups ($P \leq 0.05$) compared with the control (Table 1).

Table 1. Body composition (%) of the Nile tilapia fish after the experiment

Treatment	*Moisture*	*Crude protein*	*Ether extract*	*Ash*
T1	78.56 ± 0.14^{a}	68.03 ± 0.08^{a}	22.46 ± 0.23^{d}	14.38 ± 0.06^{a}
T2	76.84 ± 0.15^{c}	67.18 ± 0.08^{b}	26.58 ± 0.06^{a}	12.93 ± 0.1^{c}
T3	77.76 ± 0.12^{b}	67.94 ± 0.07^{a}	24.58 ± 0.15^{b}	13.1 ± 0.1^{c}
T4	78.22 ± 0.36^{ab}	67.35 ± 0.04^{b}	23.36 ± 0.19^{c}	14.06 ± 0.11^{b}
P. value	**0.003**	**0.001**	**0.001**	**0.001**

Means having different letters in the same column for the same parameter are significant ($P \leq 0.05$).

After the end of the experimental period, the following hematological parameters were measured and shown in Table (2): RBCs, Hb, HCT, MCV, MCH, and MCHC. The RBCs had significance which ranged between $1.35\pm0.02^{c} \times 10^{6}$/cmm in the control group

(T1) and 1.77±0.01^{a} × 10^{6}/cmm in treatment 4 (T4). Hb concentrations ranged significantly between 2.31±0.04^{c} and 4.32±0.07^{a} g/dl in T1 and T4, respectively. PCV was significantly increased to 30.29±0.24^{a}% in T4, while it was 19.35±0.29^{d}% in the control group (T1). MCV showed its highest value (170.79±0.39^{a} fl) in T4, while the lowest value (125.85±0.7^{c} 6fl) was recorded in T2. MCH showed its lowest value (17.04±0.09^{c} pg) in T1, while the highest value (24.4±0.24^{a} pg) was recorded in T4. MCHC values ranged between 11.92±0.06^{c} and 18.14±0.29^{a}g/ dl in T1 and T2, respectively.

Table 2. Hematological parameters and blood parameters at the end of the experiment

	RBCs ***(x 10^{6}/cmm)***	***Hb*** ***(g/dl)***	***PCV*** ***(%)***	***MCV*** ***(fl)***	***MCH*** ***(pg)***	***MCHC*** ***(g/dl)***
T1	1.35±0.02^{c}	2.31±0.04^{c}	19.35±0.29^{d}	143.01±0.03^{b}	17.04±0.09^{c}	11.92±0.06^{c}
T2	1.65±0.03^{b}	3.78±0.14^{b}	20.81±0.44^{c}	125.85±0.7^{c}	22.83±0.49^{b}	18.14±0.29^{a}
T3	1.72±0.03^{ab}	4.21±0.13^{a}	28.94±0.35^{b}	168.62±1.37^{a}	24.51±0.28^{a}	14.54±0.28^{b}
T4	1.77±0.01^{a}	4.32±0.07^{a}	30.29±0.24^{a}	170.79±0.39^{a}	24.4±0.24^{a}	14.28±0.12^{b}
P.v	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

Means having different letters in the same column for the same parameter are significant ($P \leq 0.05$).

The glucose, albumin, protein, globulin, AST (GOT), and ALT (GPT) were measured in serum after the period of experiment (Table 3). The results showed that the biochemical blood parameters measured were significantly changed. The glucose level showed the highest value (33.64±0.35^{a} mg/dl) in T4, while its lowest value (22.97±0.58^{c} mg/dl) was recorded in the control group (T1). Serum protein ranged from 3.59±0.12^{b} to 4.86±0.44^{a} g/dl in T1 and T4, respectively. Albumin values ranged from 0.86±0.08^{b} to 1.25±0.04^{a} g/dl in T1 and T4, respectively. The highest globulin value (3.61±0.40^{a}g/ dl) was observed in T4, while its lowest values (2.73±0.04^{b} and 2.74±0.05^{b} g/dl) were recorded in T1 and T2, respectively. AST showed its highest value (82.14±0.54^{d} U/I) in the control group (T1) and showed its lowest value (26.99±0.89^{a} U/I) in T4. ALT highest value (34.63±0.37^{a} U/I) was observed in the control group (T1), and its lowest value (12.6±0.49^{c} U/I) in T4.

Table 3. Serum biochemical analysis at the end of the experiment

	Glucose (mg/dl)	***Protein (g/dl)***	***Albumin (g/dl)***	***Globulin (g/dl)***	***AST (U/I)***	***ALT (U/I)***
T1	22.97±0.58^{c}	3.59±0.12^{b}	0.86±0.08^{b}	2.73±0.04^{b}	82.14±0.54^{d}	34.63±0.37^{a}
T2	23.55±0.36^{c}	3.88±0.12^{b}	1.13±0.07^{a}	2.74±0.05^{b}	50.96±0.98^{c}	34.66±0.31^{a}
T3	25.49±0.23^{b}	4.19±0.09^{ab}	1.19±0.03^{a}	3.00±0.06^{ab}	45.16±0.5^{b}	16.34±0.1^{b}
T4	33.64±0.35^{a}	4.86±0.44^{a}	1.25±0.04^{a}	3.61±0.40^{a}	26.99±0.89^{a}	12.6±0.49^{c}
P	0.0001	0.027	0.006	0.054	0.0001	0.0001

Means having different letters in the same column for the same parameter are significant ($P \leq 0.05$).

The result in Table (4) illustrated that the primary record of NBT was 1.78±0.01mg/ 0.1ml blood at start point and increased significantly at the first month of the experiment to be 1.97±0.02^{a} and 1.98±0.04^{a}mg/ 0.1ml blood for T2 and T3, respectively, and slightly increased in T4 to be 1.89±0.05^{ab}mg/ 0.1ml blood in comparison with T1 (1.81±0.02^{b} mg/0.1ml).

In the third month of the experiment, the NBT assay was significantly increased in all sets that contained a diet provided with a mixture of *Pediococcus acidilactici*, *Lactobacillus acidophilus* and *Bacillus subtilis* to 2.23±0.07^{a} and 2.3±0.03^{a}mg/ 0.1ml, blood for T2 and T3, respectively, and in T4 to be 2.02±0.07^{b}mg/ 0.1ml in comparison with T1 (1.87±0.03^{b}mg/ 0.1ml).

A significant difference was recorded between the NBT results in all treatments during the three months of the experiment.

Table 4. Effect of diet containing a mix of *Pediococcus acidilactici*, *Lactobacillus acidophilus* and *Bacillus subtilis* on a respiratory burst by using (NBT) mg/ml in *O. niloticus* during 3 months of feeding experiment

Treatment	**Zero time**	**First month**	**Second month**	**Third month**
T1	1.78±0.01	1.81±0.02^{b}	1.79±0.04^{c}	1.87±0.03^{b}
T2	1.78±0.02	1.97±0.02^{a}	2.1±0.04^{b}	2.23±0.07^{a}
T3	1.78±0.02	1.98±0.04^{a}	2.24±0.03^{a}	2.3±0.03^{a}
T4	1.78±0.01	1.89±0.05^{ab}	2.22±0.03^{a}	2.02±0.07^{b}
P v	0.988	0.027	0.0001	0.002

Means having different letters in the same column for the same parameter are significant ($P< 0.05$).

- Lysozyme activity

Lysozyme is a humoral innate defense parameter. The serum lysozyme activity was measured and shown in Table (5). The result showed that the lysozyme primary value was 1.75±0.03µg/ ml serum at start point and increased significantly at the first month of the experiment to be 1.89 ± 0.05^{bc} and 2.013 ± 0.05^{b}µg/ ml serum for T_2 and T_3, respectively, and in T_4 to be 2.33 ± 0.08^{a}µg/ ml serum in comparison with T_1 (1.79 ± 0.03^{c} µg/ml).

At the third month of the feeding experiment, serum lysozyme activity was significantly increased in all sets that had a diet supplemented with a mixture of *Pediococcus acidilactici, Lactobacillus acidophilus,* and *Bacillus subtilis* to be 2.4 ± 0.09^{c} and 2.82 ± 0.07^{b}µg/ ml serum for T_2 and T_3, respectively, and in T_4 to be 3.05 ± 0.04^{a}µg/ ml serum in comparison with T_1 (1.82 ± 0.05^{d} µg/ml).

There was a significant difference in lysozyme results (during 3 months) of the feeding experiment in all treatments.

Table 5. Serum lysozyme activity (µg/ml) in *O. niloticus* due to feeding by a diet supplemented with a mixture of *Pediococcus acidilactici, Lactobacillus acidophilus* and *Bacillus subtilis* during 3 months of feeding experiment

Treatment	Zero time	First month	Second month	Third month
T1	1.75±0.03	1.79 ± 0.03^{c}	1.93 ± 0.04^{b}	1.82 ± 0.05^{d}
T2	1.76±0.04	1.89 ± 0.05^{bc}	2.02 ± 0.06^{b}	2.4 ± 0.09^{c}
T3	1.79±0.02	2.013 ± 0.05^{b}	2.1 ± 0.06^{b}	2.82 ± 0.07^{b}
T4	1.79±0.01	2.33 ± 0.08^{a}	2.77 ± 0.08^{a}	3.05 ± 0.04^{a}
P v	0.318	0.0001	0.0001	0.0001

Means having different letters in the same column for the same parameter are significant ($P\leq$ 0.05).

- Clinical examination of naturally infected *Oreochromis niloticus*

Diseased fish showed decreased feed intake, imbalance, dullness, and swimming near the surface, in addition to respiratory distress, gasping, and engulfing the atmospheric air. The affected fish showed large irregular hemorrhage on all external body surfaces, petechial hemorrhage in skin, the base of fins, mouth, gill cover and eyes. The skin lost its pigmentation, loss of scales, abdomen and other areas. Fin, tail rot, prolapsed and congestion of the vent were also noticed in some cases, as in Fig. (1).

- Post-mortem examination of naturally infected *Oreochromis niloticus*

Post-mortem showed that the liver was yellowish-white in color with necrotic areas, hemorrhages in all internal organs, and ascetic fluid, which was yellowish in color and watery in consistency. Excessive mucous in gill pouches, as in Fig. (2).

Fig. 1. Nile-tilapia naturally infected with *Vibrio* spp. showing loss of scales, hemorrhage on the fins and fins rot

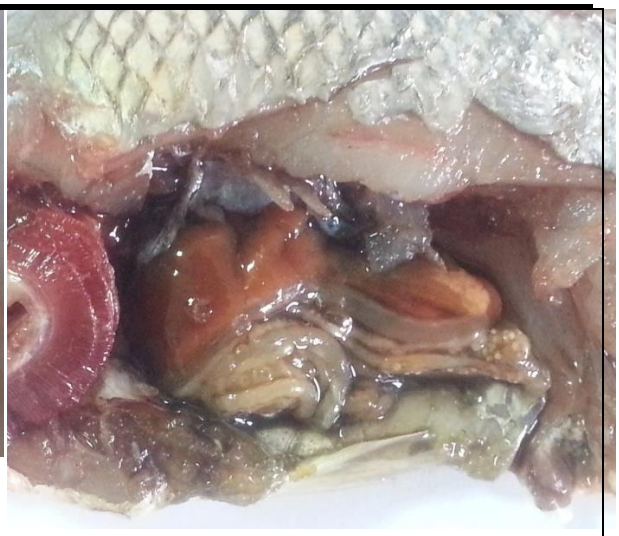

Fig. 2. Nile-tilapia naturally infected with *Vibrio* spp. showing necrosis on the liver and empty intestine

- Microbiological analysis of bacteria isolated from naturally infected tilapia

Bacteriological examination of collected fish samples was done from the initial organs (Liver and muscles).

A- Characterization and identification of bacteria isolated from examined *O. niloticus*

Isolate colonies are Gram-negative. On tryptic soy agar media (TSA), the colonies were large and circular with entire edges. They did not grow on Rimler shotts media (R.S), while on MacConkey agar, pale pin-headed colonies and T.C.B.S agar produced yellow color colonies. The isolates were curved, comma-shaped bacilli, motile; colonies were large and circular with entire edges (0.3-1.3mm) in diameter. They reacted positively to methyl red, Voges Proskauer, and citrate, produced Indole and fermented glucose, mannitol, rhamnose, and maltose, and were negative to lactose. They were positive for oxidase and catalase and produced nitrite. So it was known as *Vibrio* spp.

b- Effect of diet supplemented with the mixture of *Pediococcus acidilactici, Lactobacillus acidophilus,* and *Bacillus subtilis* on the count of *Vibrio* species in the liver and muscles of tested fish

Data in Table (6) indicate that, in the liver, the initial count of *Vibrio* species of tested fish liver was 21×10±1.15CFU/g at the start of the feeding experiment and decreased significantly at the first month of the experiment to be 14×10±0.58, 16×10±1.15, and 11×10±0.87CFU/g in T2, T3, and T4, respectively, if compared with T_1 (20×10±1.73CFU/g). In the third month of the feeding experiment, the count of *Vibrio* species of tested fish liver was completely inhibited for T2, T3, and T4. In muscles, the

count of *Vibrio* species of tested fish muscles was free at all treated (T2, T3, and T_4) and untreated fish at all feeding period (3 months). These indicated that *Vibrio* species was slightly more prevalent with T3 than T2 and T4 after feeding for 3 months. A significant difference was detected between *Vibrio* species counts in all treatments in the liver and muscles after 3 months of the experiment.

Table 6. Effect of diet supplemented with the mixture of *Pediococcus acidilactici*, *Lactobacillus acidophilus* and *Bacillus subtilis* on count of *Vibrio* species (CFU/g) in *O. niloticus* liver and muscles after 3 months of feeding experiment

ORGAN	**Liver**				**Muscles**			
Feeding time (month)	**T 1**	**T 2**	**T 3**	**T 4**	**T 1**	**T 2**	**T 3**	**T 4**
Zero time	b*$\mathbf{21\times10}$ ±1.15 c	b*$\mathbf{21\times10}$ ±0.58 d	c*$\mathbf{33\times10}$ ±1.73 c	b*$\mathbf{17\times10}$ ±1.15 c	**0** a	**0** a	**0** a	**0 a**
First month	c*$\mathbf{20\times10}$ ±1.73 c	ab*$\mathbf{14\times10}$ ±0.58 c	bc*$\mathbf{16\times10}$ ±1.15 b	a*$\mathbf{11\times10}$ ±0.87 b	**0** a	**0** a	**0** a	**0 a**
Second month	c*$\mathbf{11\times10}$ ±0.58 b	b*$\mathbf{7\times10}$ ±1.15 b	a*$\mathbf{2\times10}$ ±0.29 a	a*$\mathbf{1\times10}$ ±0.29 a	**0** a	**0** a	**0** a	**0 a**
Third month	**0 a**	**0 a**	**0 a**	**0 a**	**0 a**	**0 a**	**0 a**	**0 a**

Means with different letters within column differ significantly ($P \leq 0.05$).
Significances according to one-way ANOVA. Letters with * comparing within rows.

- Challenge test

Table (7) showed that the highest mortality percent after infection with pathogenic *Vibrio harveyi* was in T1 (80%) than in other treatments that fed a diet supplemented with 3g/kg, 5g/kg, and 7g/kg mixture of *Pediococcus acidilactici*, *Lactobacillus acidophilus*, *and Bacillus subtilis*, the mortality percents were (20 and 10) in T2 and T3, respectively, and there wasn't any mortality in T4. The highest RLP against *Vibrio harveyi* was in T4 (100%) and decreased in the other treatments to (0, 83, and 88%) for groups T1, T2 and T3 respectively. RLP was measured by the following equation: RLP = 1- [percentage of treated mortality/ percentage of control mortality] ×100.

Table 7. The mortality percent and relative level of protection in treated *O. niloticus* after infection-challenge with *Vibrio harveyi*

Vibrio harveyi	*Mortality %*	*RLP*
T1	80	0
T2	20	83
T3	10	88
T4	0	100

DISCUSSION

The aquaculture industry currently uses a wide variety of probiotics, including *Lactobacillus, Pediococcus, Bacillus, Bifidobacterium, Carnobacterium* spp., and *Streptococcus*, as well as *Flavobacterium, Cytophaga, Nitrobacter, Enterococcus, Pseudomonas, Aeromonas, Vibrio* spp., *Alteromonas, Nitrosomonas,* yeast (*Saccharomyces, Debaryomyces*), and others **(Irianto & Austin, 2002; Burr *et al.*, 2005; Sahu *et al.*, 2008)**.

The probiotics boost the immune system, improve feed digestion and increase survival **(Huerta-Rábago *et al.*, 2019)**. Major studies looking into how probiotics affect aquaculture have made use of dietary supplements **(Jahangiri & Esteban, 2018)**. Probiotics have been developed in laboratories, but there are now commercial products on the market. Isolates of *Pediococcus acidilactici* are among the essential bacterial species present in the majority of commercial products **(Salvador *et al.*, 2012)**. Numerous elements, including fish age, probiotic source, dose, water temperature, and salinity, can impact how effective probiotics are **(Jahangiri & Esteban, 2018)**.

Fish health status can be determined by tracking blood biochemical markers **(Adorian *et al.*, 2019)**. The study's probiotic-supplemented diet raised the hemoglobin content and red blood cell count. These study results imply that the use of *P. acidilactici* in *Oreochromis* sp. was both safe and effective in enhancing body condition. After feeding *Bacillus* to rainbow trout and tilapia, several researchers found no appreciable variations in RBC counts for control **(Capkin *et al.*, 2009)**.

Blood parameters such as Hb, Ht, RBCs, MCV, MCH, and MCHC in the probiotic sets of the current study were higher than in control set. The present findings are agreed with those of **Merrifield *et al.* (2010)**, who found that fish fed on diets supplemented with *Pediococcus acidilactici* had significantly higher blood values. The current results, however, contradict those of **Standen *et al.* (2013)**, who reported that

there was no significance in variations in tilapia hematological indices after feeding them *Pediococcus acidilactici*.

The fish erythrocytes volume indicates the health condition of the fish and determines any abnormalities resulting from the immunostimulants utilization. If the fish don't eat or suffer from infections, the hematocrit will be reduced **(Blaxhall, 1972)**.

The results of the protein and glucose profiles showed a notable rise in probiotic groups, supporting the hematological markers and growth parameters. These findings are in line with those of **Eissa *et al.* (2022)**, they found that supplying the feeds of seabass with *P. acidilactici* considerably raised glucose, albumin, globulin, and total protein levels in comparison to diets that were not supplemented. The current results verified that the protein pattern backed up the addition of *P. acidilactici's* growth-promoting effects. It can be concluded that adding 0.3g *P. acidilactici* to the rearing water of Nile tilapia yields the highest results because when *P. acidilactici* was added, the values of albumin, globulin and total protein were higher significantly and increase with increasing the levels of *P. acidilactici*. Hepatic enzymes express the liver physiological condition, and enzyme activity monitoring is any biochemical indicator that may be used to show how dietary additives affect fish health and metabolic activity **(Fadl *et al.*, 2020).** Since probiotics may significantly lower AST, ALT, and ALP enzymes, the findings of **Kurdomanov *et al.* (2019)** contradict the current investigation. However, our results contradict those of **Banerjee and Ray (2017)**, who made it clear that the probiotics had no discernible impact on the AST, ALT, or ALP enzymes. **Ghiasi *et al*. (2018)** found that a species of *P. acidilactici* fed whales had higher measurements of AST and ALT. The kind of probiotic and fish species used may be the cause of these disputes.

Likewise, **Abd E**l-**Rhman *et al.* (2009)** showed that the probiotics addition to the tilapia diet had a substantial impact on hematological markers. Conversely, *O. niloticus* which were fed a diet contains *Pediococcus acidilactici* **(Ferguson *et al.*, 2010)** and *Bacillus subtilis* **(Soltan & El-Laithy, 2008)** exhibited some variance. The hemoglobin and hematocrit contents of the control and other experimental fish groups fed a diet enriched with probiotics did not differ significantly.

C. gariepinus fingerlings that fed on a diet provided with *Lactobacillus acidophilus*, its concentrations of Hb, % hematocrit, ESR, RBC, WBC, PCV, MCH, MCHC, MCV, total serum protein, serum glucose, Cl, and cholesterol increased significantly than the control **(Al-Dohail *et al.*, 2009)**. This finding likely supports the idea that the fish groups that fed diets that contain probiotics were healthier than the controls, most likely as a result of lower levels of blood plasma cortisol, as assumed by **Rollo *et al.* (2006)** in the sea bream (*S. aurata*).

Total proteins, a blood protein fraction made up of albumin and globulin, were commonly used as indicators of the immunological and health profiles of aquatic animals. Concerning the elevation of the albumin and total protein in the treated groups, was due

to the *Bacillus subtilis* immuno-modulatory effect on the liver which activated the hepatocytes anabolic capacity producing blood proteins **(Marzouk *et al.*, 2008)**. According to **Abdelnour *et al.* (2023)**, its rise signifies a better immune system. Our findings are consistent with the results of **Magnadottir (2010)**.

According to the current findings, *B. subtilis* may boost the Nile tilapia immunological characteristics. These findings support the hypothesis made by **Kamgar and Ghane (2014)** that *B. subtilis* can boost rainbow trout immunological markers.

Compared to other diets, the control group's ALT and AST values were noticeably higher. This was agreed with the results of **Adorian *et al.* (2019)**, who found that fish feed on a diet contains 1×10^6 CFU g^{-1} *Bacillus* had lower parameters of liver enzymes (AST, ALT, and ALP) than control.

Our results were similar to **Mohamed *et al.* (2024)**, they found that using of commercial probiotics (*Bacillus toyonensis* and *Lactobacillus plantarum*) greatly enhanced the analytical parameters of the Nile tilapia's blood and plasma. AST and ALT levels were dramatically reduced in fish raised in water treated with probiotics, demonstrating a synergistic impact.

The activity of respiratory burst, particularly that of neutrophils and monocytes, is determined by the NBT test. The NBT test revealed noticeably higher levels in every examined group currently receiving probiotics to the control group. The set that received a combination of two probiotic bacteria exhibited greater values than the other groups, according to **Blaxhall (1972)**, which was supported by these data. This implies that the probiotics might improve immune responses that are not targeted. The treatment, which includes LAB, raises complement, lysozyme, and phagocyte activity **(Panigrahi *et al.*, 2004)**.

The Nitrobluetetrazolium assay (NBT) was utilized to detect phagocytic activity which acts as an immune response indicator, especially neutrophils and monocytes **(Jabs *et al.*, 1980)**. After activation of fish phagocytes, they were able to produce superoxide anion (O2-) and its reactive derivatives (hydrogen peroxide and hydroxyl radicals) during the consumption of intense oxygen, this process called the respiratory burst (Secombes, 1996). These species of reactive oxygen were toxic for pathogenic bacteria in fish. So, it was obvious that increased respiratory burst activity can be related to increased bacterial pathogen killing activity of phagocytes **(Sharp & Secombes, 1993)**.

The water containing *P. acidilactici* increased the lysozyme activity much higher in all treatment sets than in control. Our findings are in agreement with **Li *et al.* (2021)**, who verified that lysozyme has an important role in the immune system and can be triggered to fight off harmful bacterial infections. They said that the probiotics immunostimulant capacity in boosting the immune system of the fish was noticed by notable elevation of lysozyme activity of fish groups fed with diet has these microorganisms. Additionally, **Ye *et al.* (2011)** found that the Japanese founder's innate

immune response was enhanced by the addition of *Bacillus clausii* and either one or both fructose and mannan oligosaccharide supplementation. The probiotics addition to Nile tilapia rearing water enhanced the immune responses of fish as lysozyme and demonstrated the probiotics' capacity to stimulate the lytic activity of fish versus Gram-ve also, Gram+ve pathogens **(Kord *et al.*, 2022)**.

The lysozyme had an antibacterial effect by breaking the peptidoglycan in bacterial cell wall; predominantly Gram+ve bacteria; thereby make stimulating of bacterial phagocytosis by phagocytic cells **(Ellis, 1990)**. An increase in the lysozyme activity concentration in fish may be caused by infection or invasion by foreign materials **(Siwicki *et al.*, 1998)**.

The bacterial cell walls have peptidoglycan that have glycosidic linkages between N-acetylglucosamine and N-acetylmuramic acid which hydrolyzed by the cationic enzyme lysozyme. Some Gram +ve bacteria and even some Gram -ve bacteria can be lysed by lysozyme when combined with complement **(Paulsen *et al.*, 2001)**. The lysozyme activity significantly increased in fish meal provided with *Pediococcus acidilactici*, according to this study. Similarly, the Nile tilapia meal that has probiotics showed a substantial elevation in lysozyme activity **(Wang *et al.*, 2008)**. Generally, the clinically diseased fish showed decreased feed intake, loss of balance, roughness, and respiratory distress. The affected fish showed rapid opercula movement with nervous manifestation and loss of sensation just before death. Large irregular hemorrhage on all external body surfaces, petechial hemorrhage on skin, fins, mouth, gill cover, and eyes. Excessive mucus secretion, erratic scales, and skin erosion. Exophthalmia, corneal opacity, fin, abdomen swelling, tail rot, prolapsed, and congestion of the vent were also noticed. Red mouth and pale gills were observed in some cases of diseased fish.

According to reports, the primary bacterial infections in a number of marine fish species were *V. salmonicida, V. anguillarum* and *V. vulnificus* **(Austin & Austin, 1999)**. Major method of infection in fish was the bacterium entering the tissue of the host primarily through chemotactic activity, then deploying an iron-sequestering mechanism, which ultimately caused the fish to be harmed by extracellular products including hemolysin and proteases. Moribund seabream (*Sparu saurata*) obtained from a hatchery in Malta were found to frequently exhibit mucus secretion blood clots **(Haldar *et al.*, 2010)**. *V. harveyi* hemolysin activity may be the cause of the infection. Ascetic fluid, necrosis of intestine, liquified air bladder, and anemia are some of the usual signs of fish sickness brought on by pathogenic vibrio strains. Vibrios have also been seen to infect shrimp, and the hepatopancreas, gills, feed, and other organs could be the entry points. Vibrios penetrated the shrimp's epithelial cells and then colonized the host tissue **(Martin-Laurent *et al.*, 2001)**.

In this investigation, we found that *Bacillus subtilis* prevented *V. harveyi* from growing. According to earlier research, Bacillus's inhibitory effects may result from the pH of growing medium changing, the use of vital nutrients **(Yilmaz *et al.*, 2006)**. Furthermore, other researches have documented that *Bacillus* produces polypeptide antibiotics that are antagonistic to a variety of Gram- positive and Gram- negative bacteria **(Drablos *et al.*, 1999)**.

The current findings agreed with **Mohideen *et al.* (2010)**, who used probiotic bacterial strain *Bacillus* sp. that was obtained from fish intestines and antagonistic against *Vibrio Harvey*. Only one strain out of the nine isolates tested against *Vibrio Hervey* was able to limit the pathogen's growth more effectively than the others. According to **Jones and Hoffer (2002)**, *Bacillus sp.* produced hydrogen peroxides, lysozymes, siderophores, bacteriocins, and proteases that inhibited harmful microorganisms.

During the *V. alginolyticus* infection, probiotic therapy successfully decreased the mortality rate of the young shrimp. Thus, it makes sense to hypothesize that *L. acidophilus's* mode of action is predicated on the release of antibacterial peptides and competitive exclusion of the pathogen. According to **Uma *et al.* (1999)**, LAB considerably increases the survival rates of *P. indicus* larvae rearing water. According to **Ajitha *et al.* (2004)**, probiotic groups fed with *Lactobacillus* supplemented feed challenged with *V. alginolyticus* had higher survival rates for shrimp *P. indicus* (56–72%).

When assessing the effectiveness of probiotics, the antagonistic action against pathogens is crucial **(FAO/WHO, 2002)**. *Vibrio* and *Aeromonas* species, which are prevalent bacterial pathogens that cause diseases in aquatic animals, were antagonistic to the fermentation broths of *Lactobacillus plantarum* and *Pediococcu acidilactici* in many studies **(Uzun & Ogut, 2015; Wang *et al.*, 2020)**. By generating antimicrobial compounds and vying for adhesion sites and nutrients, some research suggested that LAB could reduce the amounts of bacterial pathogens **(Zhou *et al.*, 2012; Kumaree *et al.*, 2015; Soltani *et al.*, 2019)**.

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