

# Evaluation of potential applications of dietary probiotic (*Bacillus licheniformis* SB3086): Effect on growth, digestive enzyme activity, hematological, biochemical, and immune response of Tilapia (*Oreochromis mossambicus*)

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## Abstract

The present study was designed to evaluate the dietary effect of dietary supplementation of *Bacillus licheniformis* on growth, hematological and immune parameters of *Oreochromis mossambicus* fingerlings. A feeding trial of 8 weeks was carried out, in which fish were fed at a rate of 2% body weight with four experimental diets, including a control diet and three supplemented diets such as,  $10^5$ ,  $10^7$  and  $10^9$  CFUg<sup>-1</sup> respectively. At the termination of feeding experiment, growth related parameters, hematological and immune response of fish were evaluated. Feed conversion ratio (FCR) was significantly decreased, inferring high efficiency in utilizing feed. In similar groups, hematological parameters such as total erythrocyte count, total leukocyte, total serum protein, blood glucose level and hemoglobin count were significantly increased ( $P < 0.05$ ), with higher probiotic concentration. Similarly, digestive enzyme activity of protease, lipase and amylase was also higher in fish fed with increased concentration of probiotic. Diet groups having  $10^9$  CFUg<sup>-1</sup> of probiotic was observed with elevated immune response, as levels of lysozyme activity, respiratory burst activity and phagocytic activity were significantly higher ( $P < 0.05$ ) in this group as compared to control group. In conclusion, supplementation of diet with *Bacillus licheniformis* can enhance growth performance, and health status of *O. mossambicus*.

## Introduction

Now-a- days, Aquaculture is one of the most expeditious meat production sectors and is the major source of availability of proteins to the society (Kamran et al., 2020). It is envisioned as a potential resource to address food and nutrition security in the future (Halwart, 2020). Fish farming accounts for 46.8 % of total fish production in the world (FAO, 2020). Human population is growing at an exponential rate and annual fish growth rate has downturned in recent years which is worrisome. (Blanchard et al., 2012; Afridi et al., 2019). The declining global fish populations necessitates that

aquaculturists strengthen their aquaculture practices to meet the increasing proteins demand (Ahmad et al., 2020; Naylor et al., 2021).

On a global basis, sustainable aquaculture with high rate of production is crucial for the survival of this sector (Bossier and ekasari, 2017). However, limitations of natural resources and impact on the environment are the challenges in the development of sustainable aquaculture industry (Carvalho et al., 2020; Bridson et al., 2020). In the recent few decades, there has been extensive influx of antibiotics in the aquatic bodies in pursuit of disease control. This situation has resulted in numerous environmental complications, such as drug

resistance and bioaccumulation in aquatic life forms. There is a dire need to address this issue on urgent basis at not only in aquaculture industry but also in public health sector (Kim et al., 2017; Dawood et al., 2018; Adel and Dawood, 2021). Recently, use of probiotics has emerged as a reliable alternative which can mitigate the use of excessive antibiotics in aquaculture (Olmos et al., 2020; Won et al., 2020; Waiyemitra et al., 2020; Seethalakshmi, 2021). In aquaculture, probiotics have been used for a long time but in the last few years they have become an integral part of the growth promoting supplements and immune modulating additives. In fish aquaculture, probiotics administration supplemented with feed can inhibit growth of pathogens, modulate immunity, and enhance growth rate (Amir et al., 2019; Kuebutornye et al., 2020; Tachibana et al., 2021; Moustafa et al., 2021; El-Saadony et al., 2021; Liu et al., 2021; Hassaan et al., 2021). For example, *Bacillus subtilis*, *Bacillus* sp. KUAQ1 and *Bacillus* sp. KUAQ2 are reported to be potential probiotics known for their effect on growth enhancement and immune stimulation of *Tilapia* spp. fish (Sookchaiyaporn et al., 2020; Tachibana et al., 2021). These probiotics can alter the innate immune response; however, little is known about their effect on mucosal immunity in fish (Sheikhzadeh et al., 2012, Sutthi and Doan, 2020; Wang et al., 2020). Some probiotics, such as *Bacillus* spp., have also been known to enhance the antioxidant potential of various fish associated enzymes (Gobi et al., 2016).

Among the potential candidates of probiotics, gram-positive spore-producing bacteria belonging to the genus *Bacillus* spp. can be instrumental in fish aquaculture systems. Due to their resistant spores, they can withstand harsh environmental conditions. Also, they can produce important enzymes, such as lipase, protease, and amylase and are now emerging as one of the most widely used probiotics in aquaculture industry (Wang et al., 2019; Kuebutornye et al., 2020). *Bacillus licheniformis* is one of the promising candidates in this context (Mohammad et al., 2017; El-Sadoony et al., 2021) which can be isolated from soil, hot springs and from the feathers of ground dwelling birds, such as sparrows (Vigneshwaran et al., 2013). It is a gram positive, mesophilic bacterium which can survive at high temperatures; the optimal temperature for its growth is around 50°C (Kalishwaralal et al., 2009). It can produce antibacterial peptides in the intestinal tract of fish leading to better health and enhanced growth of fish (Jin et al., 2021). *Bacillus licheniformis* is also helpful as it is involved in the immunity boost in fish and tends to adhere to the gastrointestinal tract of fish and produces bacteriocins, thus reducing the the risk of infection there (Dawood and koshio, 2016). For instance, supplementary use of *B. licheniformis* at  $1 \times 10^5$  CFU/g and  $1 \times 10^6$  CFU/g use rates improved the growth, immune and antioxidant status of grass carp (Qin et al., 2020). Also, *B. licheniformis* BCR 4-3, with supplementation rate of  $3 \times 10^6$  CFU/L, has been reported to have a boosting effect on growth and

survival of *Litopenaeus vannamei*. The same effect has been reported on sea cucumber, with optimal probiotic concentration of  $10^7$  CFU/g (Garcia-medel et al., 2020; Lu et al., 2021).

On the other hand, omnivorous fish, such as *Tilapia* spp., are gaining rapid popularity particularly in Asian countries, such as Pakistan. Availability of *Tilapia* spp. in brackish and sea water resource in another reason why *Tilapia* spp. aquaculture trend is at the rise (Iqbal et al., 2012; Yaqub et al., 2019). To make this business viable, it is pertinent to prevent this fish from disease and avoid later economic losses (Zhang et al., 2013; Acar et al., 2015). Traditionally, various antimicrobials, parasiticides and feed additives are extensively used in the fish aquaculture (Naiel et al., 2020). However, there is a need to substitute this trend with probiotics to attain eco-friendly and more economic fish aquaculture system (Monica and Jayaraj, 2021). The current study was designed to analyze the effect of *B. licheniformis* on growth performance, digestive enzymes activity, hematological, biochemical, and immune parameters of *Tilapia* Mozambique (*Oreochromis mossambicus*).

## Materials and Methods

### Experimental Fish

Mozambique *Tilapia* (*Oreochromis mossambicus*) fingerlings were obtained from Manawan Fish hatchery, Lahore, Pakistan. After receiving the fish at the experimental location, the fish were acclimatized in concrete tanks (120L capacity) for 2 weeks under continuous flow of water. The temperature in the water tanks was maintained at  $27 \pm 2.0^\circ\text{C}$ , pH  $7.2 \pm 0.5$  and an average flow rate of  $500 \text{ L h}^{-1}$ . During this period, the fish were provided 35% crude protein diet (2% of their body weight) and the fish health was monitored regularly by observing their skin and gill color, any external injuries, and symptoms of disease. Before the start of the experiment, fish were selected for the trial on the following basis: (a) No change in their skin and gills color (b) Skin with no sign of infection or bruise (c) Normal movement in flowing water (Strzyżewska et al., 2016, Strzyżewska et al., 2017; Basirun et al., 2019).

### Probiotic Culture

The probiotic strain of *B. licheniformis* was isolated from soil and its molecular characterization was made by 16S rDNA gene sequencing (NCBI GenBank accession no KT443923.1). This bacterium has been reported as probiotic in previous studies. In the current experiment, stock culture of the probiotic was used which had been previously maintained in sterile glycerol at  $-20^\circ\text{C}$  in the laboratory. For further experimental use, fresh cultures were prepared on agar plates. Then a single colony was inoculated in nutrient broth and was incubated overnight in shaker incubator. By preparing serial dilutions, colony forming units of the bacterium in each

dilution were calculated by taking optical density (OD) at 600nm. The culture was centrifuged at 10,000g for 20 minutes and pellets were resuspended in PBS for further use. Three concentrations of probiotic viz.  $10^5$ ,  $10^7$  and  $10^9$  cfu  $g^{-1}$  were used in experimental diets.

### Preparation of Experimental Diets

The prepared concentrations of probiotic bacterium were mixed with basal diet (Table 1). The diets were converted into pellets. then air dried for 48 hours and stored at  $-4^{\circ}C$  for further experimental use. Survival of probiotic bacterium in the diet was assessed during the trial by preparing saline dilutions of the diet and plating 0.1 ml of this dilution on nutrient agar plates at  $36^{\circ}C$  for 24 hours. The diets which retained the desirable colony count were used in the experiment.

### Feeding Schedule

After the acclimatization period, healthy fish were transferred into aquaria that were triplicated for each diet. The control diet was, D1, having no probiotic, and the others, D2, D3, and D4 contained the experimental diets with  $10^5$ ,  $10^7$ , and  $10^9$  cfu  $g^{-1}$ , respectively. Each aquarium contained 15 fish; temperature and air quality of water in each aquarium was maintained according to standard conditions described previously. The experimental fish were provided basal diet and experimental diets for 8 weeks, twice daily. Continuous water exchange and removal of feces was done on regular basis to ensure best water quality in each aquarium.

### Growth Measurement

Mean weight of fish in all aquaria was calculated before the feeding trial. After that, gross weight of equal number of fishes from each aquarium was calculated on weekly basis. At the end of trial, 5 fish from each aquarium (15 from each diet group) were randomly collected and their individual and mean body weight

was calculated. Thereafter, growth parameters including percent weight gain (WG%), specific growth rate (SGR%), feed conversion ratio (FCR) and survival rate (%SR) were calculated using the formulae already used by Zhou et al., (2013).

### Hematological Parameters

After 8 weeks, five fish from each aquarium (15 from each diet group) were randomly collected and were anesthetized by MS-222 and blood was collected from caudal vein by using heparinized syringe and was stored in K2 VACUTTE<sup>®</sup> EDTA tubes. Then, using the already described protocol of Ullah et al. (2018), the hematological parameters, such as RBCs, WBCs, HCT, HB, MCH, MCV, MCHC were determined.

### Intestinal Enzymes Activity

At the end of the experiment, three fish per aquarium (nine fish/group) were used for intestinal enzymes activity analysis. For this purpose, the fish were anesthetized with MS-22, aseptically dissected at low temperature and their gastrointestinal tracts were removed carefully and snap frozen in nitrogen liquid and kept at  $20^{\circ}C$  for determination of Protease, Lipase and Amylase activities by using respective standard method (Amir et al., 2019; Ahmad et al., 2020).

### Biochemical and Immunological Parameters

For biochemical and immunological parameters, the serum was collected from the blood of five fishes from each aquarium by using heparinized syringe in VACUTTE<sup>®</sup> EDTA tubes (red top). Blood was centrifuged for 300 second at 3000 rpm; and serum was separated in microtube and stored at  $4^{\circ}C$ . The method of Ullah et al. (2018) was adapted for the analysis of immunoglobulins, total serum protein, lysozyme, and amino transferase activity. Whereas, the respiratory burst and phagocytic activity was measured by using fresh heparinized blood (Devi et al., 2019).

**Table 1.** Formulation of feed and proximate composition of diets.

Ingredients	Control D1(Inclusion level 0g/100g)	D2 (Inclusion level $10^5$ g/100g)	D3(Inclusion level $10^7$ g/100g)	D4 (Inclusion level $10^9$ g/100g)
Fish meal	18	18	18	18
Soybean meal	15	15	15	15
Sunflower meal	15	15	15	15
Gluten 60	17	17	17	17
Wheat bran	17	17	17	17
Rice bran	15	15	15	15
Vit. Premix	1	1	1	1
Fish oil	1	1	1	1
Cfu $g^{-1}$	0.00	$10^5$	$10^7$	$10^9$
<b>Proximate Composition %age</b>				
Crude Protein	36.7			
Crude Lipid	9.1			
Ash	6.1			

### Peroxidase Activity

Peroxidase activity of serum was calculated by measuring its reduction capability towards hydrogen peroxide at 470nm (Civello et al., 1995).

The activity of peroxidase enzyme was measured using the expression given below:

$$\text{Activity} \left( \frac{\text{Units}}{\text{mL}} \right) = \frac{\Delta A/3}{26.6 \times 60/3000}$$

### Glucose Content

The blood sample was collected from fish and was centrifuged for 10 minutes at 10000g. The obtained serum was collected and incubated with glucose oxidase reagent for 30 minutes at 25°C. Blood glucose concentration was determined by using the following expression:

$$\text{Glucose concentration} = \frac{\text{Sample absorption (470nm)}}{\text{Standard absorption} \times \text{Concentration of standard}}$$

### Challenge Test

At the end of the experiment, 3 fish from each tank (9 from each diet group) were set for acclimatization for one week. All the groups were exposed to fresh culture of pathogenic bacteria, *Staphylococcus aureus*. Suspension of *S. aureus* were injected intraperitoneal to the fish at the concentration of  $2.24 \times 10^7$  CFU/g suspended in PBS (Das et al., 2011). The bacterial strain was isolated from infected *L. rohita* at Institute of Industrial Biotechnology, GC University, Lahore. Challenge test study was conducted for 15 days. During that period, mortality as well as disease symptoms were observed closely in each group.

### Statistical Analysis

One-way ANOVA, followed by LSD post-hoc test was performed to calculate significant difference between growth performance, hematological parameters, intestinal enzyme activity, biochemical and immunological parameters, etc. of different feeding

groups. All statistical tests were performed using SPSS version 2.0. Significant level was set at  $P < 0.05$  and the results were presented as mean  $\pm$  SD. For graphical presentation GraphPad prism software version 5 was used.

## Results

### Growth Performance

*Bacillus licheniformis*-supplemented diet showed significant positive effect on the growth of *Tilapia* (Table 2). ANOVA revealed significant difference in final body weight ( $n=3$ , ANOVA,  $F_{3,11}=96.4$ ,  $P < 0.001$ ), weight gain ( $n=3$ , ANOVA,  $F_{3,11}=64.9$ ,  $P < 0.001$ ), weight gain percent ( $n=3$ , ANOVA,  $F_{3,11}=6.15$ ,  $P < 0.001$ ), SGR ( $n=3$ , ANOVA,  $F_{3,11}=7.15$ ,  $P < 0.001$ ), FCR ( $n=3$ , ANOVA,  $F_{3,11}=109$ ,  $P < 0.001$ ) and Survival percentage ( $n=3$ , ANOVA,  $F_{3,11}=54$ ,  $P < 0.001$ ). The Post-hoc LSD test indicated high growth performance of probiotic-fed *Tilapia* as compared to the fish fed with control diet. Among probiotic-supplemented fed groups, the D4 ( $10^9$  cfu/g) group showed maximum growth response, survival rate and lowest FCR.

### Hematological Parameters

The probiotic, *B. licheniformis*-supplemented, fed groups showed significantly improved hematology parameters of *Tilapia* as compared to control (Table 3). ANOVA indicated significant differences in WBCs ( $n=3$ , ANOVA,  $F_{3,11}=48.6$ ,  $P < 0.001$ ; Figure 1), RBCs ( $n=3$ , ANOVA,  $F_{3,11}=38.2$ ,  $P < 0.001$ ; Figure 1), blood glucose level ( $n=3$ , ANOVA,  $F_{3,11}=37.0$ ,  $P < 0.001$ ; Figure 2), HCT % ( $n=3$ , ANOVA,  $F_{3,11}=47.1$ ,  $P < 0.001$  (Table 3), Hb ( $n=3$ , ANOVA,  $F_{3,11}=18.7$ ,  $P < 0.001$  (Table 3), MCH ( $n=3$ , ANOVA,  $F_{3,11}=40.8$ ,  $P < 0.001$ , (Table 3) and MCHC ( $n=3$ , ANOVA,  $F_{3,11}=61.6$ ,  $P < 0.001$  (Table 3). The Post-hoc LSD test indicated significantly varied hematological parameters of probiotic-fed *Tilapia* as compared to fish fed with control diet. Among groups fed upon probiotic-supplemented diet, the D4 group ( $10^9$  cfu/g) showed the highest concentration of HCT (%), Hb (g/dl), MCHC ( $\text{g dL}^{-1}$ ), WBCs, RBCs and lowest concentration of MCH (pg) and blood glucose level.

**Table 2.** Comparative effects of dietary supplementation with the probiotic, *Bacillus licheniformis*, and control diet on growth and survival of *Oreochromis mossambicus* ( $n=3$ ).

Test Diets	Diet 1	Diet 2	Diet 3	Diet 4	P - value
Treatments	Control	$10^5$ CFU/g	$10^7$ CFU/g	$10^9$ CFU/g	
Mean initial weight (g)	5.4 $\pm$ 1.1	5.3 $\pm$ 0.9	5.1 $\pm$ 1.4	5.3 $\pm$ 1.2	
Mean final weight (g)	34.1 $\pm$ 2.2 <sup>d</sup>	40.3 $\pm$ 2.39 <sup>c</sup>	43.4 $\pm$ 2.32 <sup>b</sup>	48.2 $\pm$ 3.9 <sup>a</sup>	0.001
Average weight gain (g)	28.7 $\pm$ 1.9 <sup>d</sup>	35.0 $\pm$ 2.11 <sup>c</sup>	38.3 $\pm$ 3.1 <sup>b</sup>	42.9 $\pm$ 4.05 <sup>a</sup>	0.001
% Weight gain	533.2 $\pm$ 66.4 <sup>b</sup>	667.8 $\pm$ 59.9 <sup>ab</sup>	757.3 $\pm$ 69.1 <sup>a</sup>	819.1 $\pm$ 70.4 <sup>a</sup>	0.001
SGR (%)	2.1 $\pm$ 0.3 <sup>b</sup>	2.3 $\pm$ 0.31 <sup>ab</sup>	2.4 $\pm$ 0.32 <sup>a</sup>	2.5 $\pm$ 0.23 <sup>a</sup>	0.001
FCR	1.4 $\pm$ 0.2 <sup>d</sup>	1.3 $\pm$ 0.27 <sup>c</sup>	0.9 $\pm$ 0.21 <sup>b</sup>	0.7 $\pm$ 0.1 <sup>a</sup>	0.001
Survival Rate (%)	79.3 $\pm$ 7.9 <sup>c</sup>	87.5 $\pm$ 7.88 <sup>b</sup>	90.1 $\pm$ 8.77 <sup>b</sup>	97.6 $\pm$ 9.12 <sup>a</sup>	0.001

One-way ANOVA followed by LSD Post-Hoc test shows a pairwise comparison between stocks. Variables in the same row with different superscripts are significantly different ( $P < 0.05$ ) statistically. D1 fish group was fed upon basal diet, while D2, D3 and D4 groups were fed upon diet that included different levels of the probiotic, *B. licheniformis*:  $10^5$  CFU/g,  $10^7$  CFU/g and  $10^9$  CFU/g, respectively.

**Intestinal Enzyme Activity**

Activity of protease, lipase, and amylase in the intestine of probiotic-fed groups was significantly higher as compared to the control group (Table 4). ANOVA revealed significant differences in protease activity ( $n=3$ , ANOVA,  $F_{3,11}=46.4$ ,  $P<0.001$ ) (Table 4), Lipase ( $n=3$ , ANOVA,  $F_{3,11}=54.7$ ,  $P<0.001$ ) (Table 4), and Amylase ( $n=3$ , ANOVA,  $F_{3,11}=33.6$ ,  $P<0.001$ ) (Table 4). Among the probiotic-fed groups, the D4 group ( $10^9$  cfu/g) showed highest activity of protease, lipase and amylase.

**Immunological and Biochemical Parameters**

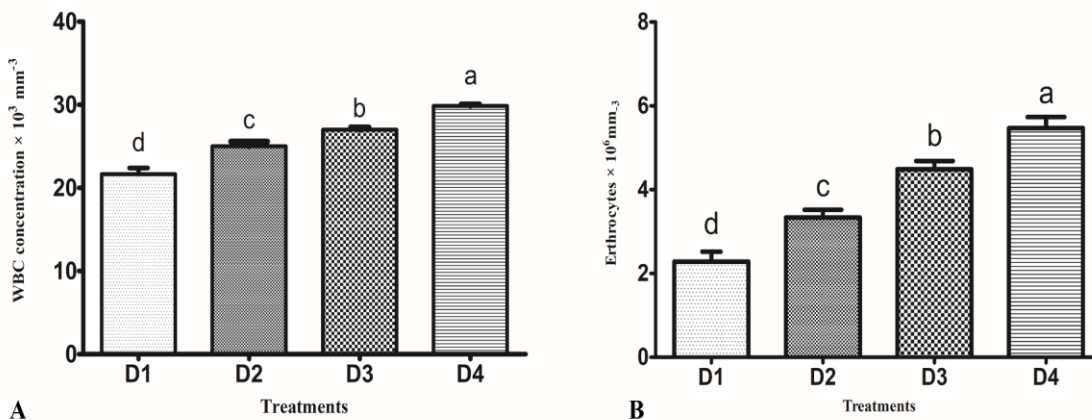
The probiotic-supplemented fed groups showed significantly improved results of immunological and biochemical parameters of *Tilapia* as compared to

control (Table 5). One-way ANOVA suggested significant variation of total serum protein ( $n=3$ , ANOVA,  $F_{3,11}=15$ ,  $P<0.001$ ) (Figure 2), total serum peroxidase activity ( $n=3$ , ANOVA,  $F_{3,11}=11.8$ ,  $P<0.001$ , (Figure 3), immunoglobulins ( $n=3$ , ANOVA,  $F_{3,11}=36.2$ ,  $P<0.001$ ) (Table 5) and lysozyme activity ( $n=3$ , ANOVA,  $F_{3,11}=17.1$ ,  $P<0.001$ ) (Table 5), respiratory burst ( $n=3$ , ANOVA,  $F_{3,11}=75.0$ ,  $P<0.001$ ) (Table 5), and phagocytic activity ( $n=3$ , ANOVA,  $F_{3,11}=54.0$ ,  $P<0.001$ ) (Table 5). The post-hoc LSD test indicated significant effect on biochemical and immunological parameters of probiotic-fed *Tilapia* as compared to the fish raised on the control diet. Amongst probiotic-supplemented fed groups, the D4 ( $10^9$  cfu/g) group showed highest immunoglobulins (mg/ml), lysozyme activity ( $\mu\text{g/ml}$ ), respiratory burst (% age) and phagocytic activity (% age).

**Table 3.** Comparative effects of dietary supplementation with the probiotic, *Bacillus licheniformis*, and control diet on hematological parameters of *Oreochromis mossambicus* ( $n=3$ ).

Hematological Parameters	Experimental Fish Groups				P - value
	D1	D2	D3	D4	
HCT (%)	26±1 <sup>d</sup>	28.7±1.5 <sup>c</sup>	33±1 <sup>b</sup>	37.7±1.5 <sup>a</sup>	0.001
Hb (g/dl)	6±0.8 <sup>c</sup>	7.8±0.4 <sup>b</sup>	8.6±0.1 <sup>ab</sup>	9.9±0.9 <sup>a</sup>	0.001
MCH (pg)	46.6±2.1 <sup>a</sup>	42.3±0.6 <sup>b</sup>	39±1 <sup>c</sup>	34.4±1.5 <sup>d</sup>	0.001
MCHC (g dL <sup>-1</sup> )	25.5±0.4 <sup>d</sup>	26.6±0.5 <sup>c</sup>	28.2±0.3 <sup>b</sup>	29.6±0.4 <sup>a</sup>	0.001

One-way ANOVA followed by LSD post-hoc test shows pairwise comparison between the groups. Variables with different superscripts in the same row are statistically significantly different ( $P<0.05$ ). D1 group was fed on basal diet while D2, D3 and D4 fish groups were fed with diet that included different levels of the probiotic, *Bacillus licheniformis*:  $10^5$  CFU/g,  $10^7$  CFU/g, and  $10^9$  CFU/g, respectively.



**Figure 1.** Effect of dietary *Bacillus licheniformis* supplementation on WBCs (A) and RBCs (B) of *Tilapia* fingerlings. Variables with different superscripts are significantly different statistically ( $p < 0.05$ ). D1 fish group was fed on basal diet while D2, D3 and D4 groups were fed upon diets that included different levels of the probiotic, *Bacillus licheniformis*:  $10^5$  CFU/g,  $10^7$  CFU/g, and  $10^9$  CFU/g, respectively.

**Table 4.** Comparative effects of dietary supplementation with the probiotic, *Bacillus licheniformis* and control diet on activity of intestinal enzymes of *Oreochromis mossambicus* ( $n = 3$ ).

Intestinal enzymes (U mg <sup>-1</sup> )	Experimental Fish Groups				P-value
	D1	D2	D3	D4	
Protease activity	9.7±0.5 <sup>c</sup>	11.9±0.7 <sup>b</sup>	13.0±0.4 <sup>b</sup>	15.5±0.7 <sup>a</sup>	0.001
Lipase activity	2.5±0.2 <sup>d</sup>	3.5±0.3 <sup>c</sup>	4.6±0.3 <sup>b</sup>	5.5±0.4 <sup>a</sup>	0.001
Amylase activity	1.5±0.3 <sup>d</sup>	2.2±0.2 <sup>c</sup>	2.7±0.2 <sup>b</sup>	3.5±0.4 <sup>a</sup>	0.001

One-way ANOVA followed by LSD post-hoc test shows pairwise comparison between the groups. Variables with different superscripts in same row are significantly different statistically ( $p < 0.05$ ). D1 fish group was fed on basal diet, while D2, D3 and D4 groups were fed on diet that included

**Challenge Test**

The probiotic-fed group showed significantly lower mortality and higher survival percentage as compared to the control group. ANOVA indicated significant differences among all the groups ( $n=3$ , ANOVA,  $F_{3,11}=18$ ,  $P<0.001$ ) (Figure 4). The post-hoc LSD test indicated significantly higher survival percentage in D4 ( $10^9$  cfu/g) group, followed by D3, D2 and D1 groups (Figure 4). Among probiotic-supplemented fed groups, the D4 ( $10^9$  cfu/g) group showed highest survival percentage, and lowest mortality.

**Discussion**

The use of probiotics in aquaculture as dietary supplements with a view of eco-friendly and sustainable approach to enhance fish growth performance and health is gaining support based on substantial evidence. Some *Bacillus* species are promising candidates for their use in aquaculture (Doan et al., 2020; Monica and Jayaraj, 2021).

**Growth Performance**

The findings of present study suggest that inclusion of *B. licheniformis* in diet of *O. mossambicus* enhances its growth rate and immune response. The present study results strengthen previously reported findings concerning *B. subtilis* and *B. licheniformis* when

incorporated in the fish feed resulting in higher growth, nutrient digestibility and higher protein efficiency ratio (Gobi et al., 2018; Romanova et al., 2020; Jin et al., 2021; Nayak, 2010). Also, some other previously performed studies suggest that probiotic supplemented diet can increase feed intake, production of vitamins, and can enhance the digestive activity of various enzymes (Gobi et al., 2018). Recently, inclusion of *B. licheniformis* in the diet of *Cyprinus carpio* and mrigal fry has been reported to result in better growth which report seems to strengthen the present study findings (Zhang et al., 2021).

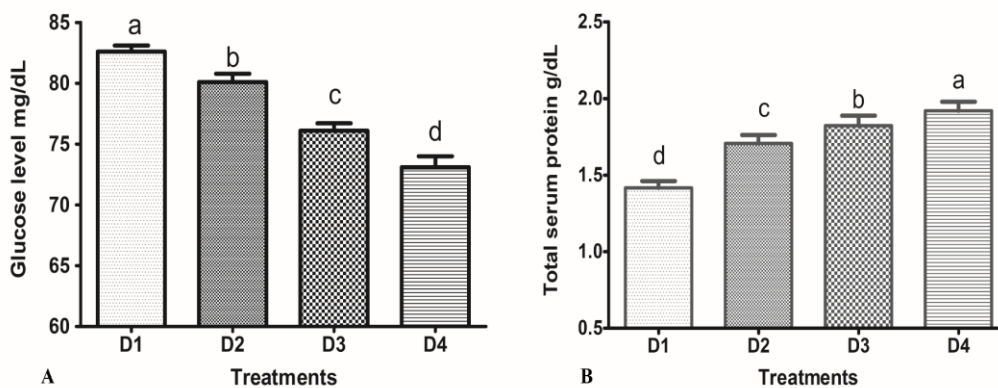
**Feed Conversion Ratio (FCR)**

Inclusion of probiotics (*Bacillus* spp.) has been observed to result in better optimized FCR in Nile Tilapia (*Oreochromis niloticus*), which observation is in compliance with the results of previously performed similar studies showing optimal concentration of  $10^8$  cfu/g for probiotic (Samson et al., 2020). Similarly, studies with grass carp (*Ctenopharyngodon idella*) have shown that the probiotic, *B. subtilis*-based diet resulted in improved FCR as compared to control group (Shah et al., 2021). A decreased value of FCR, is also a good sign that can be attributed to increase in the activity of digestive enzymes, intestinal health, and decrease in stress levels in culture environment. Also, improved FCR can be seen as *O. niloticus* gains maturity, owing to

**Table 5.** Comparative effects of dietary supplementation with the probiotic, *Bacillus licheniformis*, and the control diet on biochemical and immunological parameters of *Oreochromis mossambicus* ( $n = 3$ ).

Biochemical and Immunological Parameters	Experimental Fish Groups				P-value
	D1	D2	D3	D4	
Immunoglobulins (mg ml <sup>-1</sup> )	7.7±0.2 <sup>d</sup>	8.9±0.7 <sup>c</sup>	10.6±0.4 <sup>b</sup>	11.6±0.5 <sup>a</sup>	0.001
Lysozyme activity (µg ml <sup>-1</sup> )	7.8±0.4 <sup>c</sup>	9.2±0.6 <sup>b</sup>	10.3±0.4 <sup>ab</sup>	11.1±1.0 <sup>a</sup>	0.001
Respiratory burst (%age)	0.3±0.2 <sup>c</sup>	0.6±0.1 <sup>b</sup>	1.1±0.1 <sup>b</sup>	2.1±0.2 <sup>a</sup>	0.001
Phagocytic activity (%age)	1.5±0.4 <sup>d</sup>	2.3±0.1 <sup>c</sup>	2.8±0.1 <sup>b</sup>	3.7±0.3 <sup>a</sup>	0.001

One-way ANOVA followed by LSD post-hoc test shows pairwise comparison between the groups. Variables with different superscripts in the same row are significantly different ( $P<0.05$ ) statistically. D1 fish group was fed on basal diet, while D2, D3 and D4 fish groups were fed upon diet that included different levels of the probiotic, *Bacillus licheniformis*:  $10^5$  CFU/g,  $10^7$  CFU/g and  $10^9$  CFU/g, respectively.



**Figure 2.** Effect of dietary *Bacillus licheniformis* supplementation on blood glucose level (A) and total serum protein (B) of *Tilapia* fingerlings. Variables with different superscript are significantly ( $p < 0.05$ ) different statistically. D1 group was fed upon basal diet, while D2, D3 and D4 groups were fed upon diets including different levels of the probiotic, *Bacillus licheniformis*:  $10^5$  CFU/g,  $10^7$  CFU/g and  $10^9$  CFU/g, respectively.

improvement in its digestive system (Rodde et al., 2020). Thus, probiotics in diet can cause modifications gut flora of fish as well as simulate digestive activity beneficial for efficient utilization of biomolecules in feed (Amir et al., 2019).

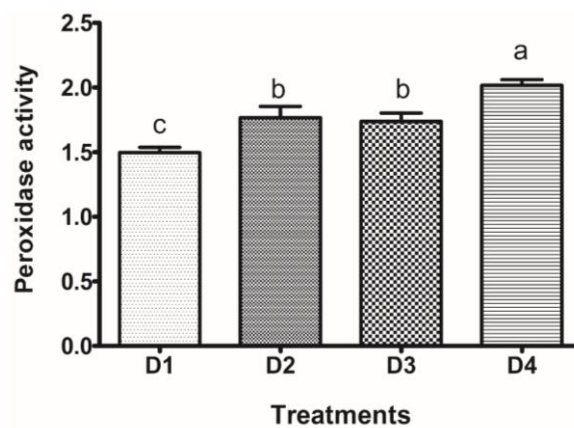
**Immune Modulation**

Modulation of immune response by use of probiotics is also a well-documented fact. Alteration of immune response takes place by adhesion and colony formation by probiotics in fish intestine. Probiotics and immune system interact through microbially- associated molecular patterns which involve peptidoglycan and lipoteichoic acid. This interaction takes place by various types of receptors, such as Tol-like receptors. Probiotic can enhance immune activity in fish by interacting with various types of immune cells (Gobi et al., 2018). Some previous studies have also suggested an increase in serum and mucus protein in *Labeo rohita* and *Catla catla* treated with different types of probiotics, such as *B. subtilis* and *B. amyloliquifaciens* (Mohapatra et al. 2012; Sutthi and Doan, 2020). Similarly, in the present study, the total serum protein concentration was observed to

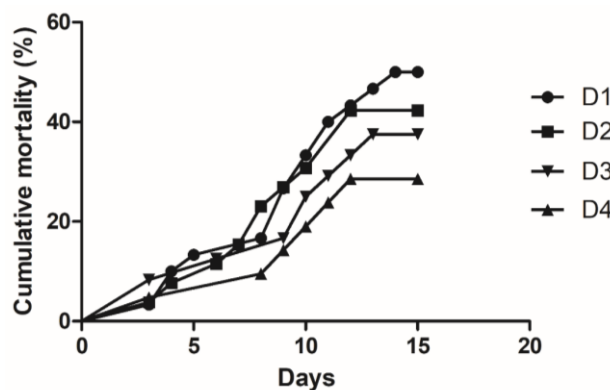
higher in *O. mossambicus* treated with the probiotic *B. licheniformis* Dahb1 diets. These proteins are known for their association with lysozymes, immunoglobulins and lectins which are important in the defensive mechanism against pathogens. The present study suggests that supplementation of *O. mossambicus* diet with *B. licheniformis*, can improve the concentration of serum-associated proteins, thereby resulting in increased serum proteins level.

**Hematological Parameters**

Administration of probiotics can increase white blood cells count in fish which is also associated with enhanced immunity. *Oreochromis niloticus* supplemented diet with *Bacillus pumilus* (Aly et al., 2008) and Nile *Tilapia* fed with *Lactobacillus plantarum* also showed significantly increased blood leucocytes. In the present study, higher number of leucocytes in diet with highest concentration of the probiotic were recorded, while in other probiotic based groups, there was no statistically significant difference among white blood cell counts.



**Figure 3.** Effect of dietary *Bacillus licheniformis* supplementation on total serum peroxidase activity of *Tilapia* fingerlings. Variables with different superscript are significantly ( $p < 0.05$ ) different statistically. D1 group was fed on basal diet, while D2, D3 and D4 fish groups were fed upon diet that included different levels of the probiotic, *Bacillus licheniformis*:  $10^5$  CFU/g,  $10^7$  CFU/g, and  $10^9$  CFU/g, respectively.



**Figure 4.** Cumulative percentage mortality of *Tilapia* fingerlings after challenged with *S. aureus*. (D1, fed on basal diet, while D2, D3 and D4, were fed upon diet including different levels of the probiotic, *Bacillus licheniformis*  $10^5$  CFU/g,  $10^7$  CFU/g and  $10^9$  CFU/g, respectively.)

Other hematological parameters, such as hemoglobin concentration (HB), mean hemoglobin concentration (MHC), mean corpuscular hemoglobin concentration (MCHC) and Hematocrit (HCT) have not been documented previously with reference to *B. licheniformis*. In the present study, however, a significant increase in levels of these hemoglobin parameters were noticed indicating higher carrying capacity of oxygen in blood. Sufficient availability of oxygen to the fish ensures fish good health. Shareef and Dabbagh (2009) evaluated hemoglobin concentration in chicks when treated with yeast. Dahiya et al. (2012) had reported the effect of probiotics on hemoglobin parameters in the Indian Magur (*Clarius Batrachus*). Similarly, Firouzabakhsh et al. (2011) had determined similar parameters using protexin as a probiotic and reported increased levels of hemoglobin in the fingerlings.

### Enzyme Activity

Lysozyme enzyme of fish's innate immune system works by prevention of biofilm formation and adherence of pathogens by stimulating a phagocytic activity. It disintegrates the polysaccharide cell wall of bacteria, and thus provides protection against pathogens. Nile *Tilapia* fed with increased probiotic concentration showed increase in lysozyme activity (Pirarat et al., 2011). *Catla catla* fed with probiotic *B. amyloliquifaciens* containing diet at concentration of  $10^8$  and  $10^9$  showed increased lysozyme activity (Das et al., 2013). In the present study, activity of lysozyme enzyme in the blood serum of *Tilapia* was significantly higher in fish treated with *B. licheniformis* containing diet. Further investigation to assess the lysozyme activity in mucus of fish fed with diets containing different probiotics is suggested.

The potential of *B. licheniformis* to increase the nutrient digestibility of aquatic organisms by stimulating enzymatic secretions, such as amylase, protease, cellulase has been documented in various aquatic organisms. Nejad et al. (2006) had reported elevated levels of these enzymes in Indian White Shrimp (*Fenneropenaeus indicus*) by using *Bacillus* strains as probiotics. Similarly, Suzer et al. (2008) had reported increased activity of digestive enzymes in seabream when treated with *Lactobacillus* spp. as probiotic. Here, the increased levels of protease, amylase and lipase is the major reason for significant increase in growth rate. It seems that *B. licheniformis*, due to its high enzymatic potential, improves the absorption, digestion, and availability of given nutrients in an efficient way.

Effect of *B. licheniformis* to enhance phagocytic and respiratory burst activity of plasma in aquatic animals is less documented. In the present study, increased respiratory burst activity and phagocytic activity can cause enhanced bactericidal activity. Respiratory burst is associated with increased level of reactive oxygen species and free radicals which are

associated with higher toxic levels for invading microbes. Also, this process is carried out by phagocytic cells which recognize any pathogens that enter the blood. Meidong et al. (2021) reported the increased respiratory burst activity in catfish when treated with *Bacillus* spp. probiotics. Similarly, Farnendes et al. (2021) reported increased respiratory burst activity in shrimp, when treated with different *Bacillus* spp. In the present study, the experimental diets have high rate of respiratory burst activity as compared to control diet which indicates increased metabolism and oxygen consumption through probiotic (*B. licheniformis*) supplementation.

### Challenge Test

Use of probiotics in aquatic culture systems increases animal's resistance against potential pathogens; Gobi et al. (2016) reported challenge test with *Aeromonas hydrophila* in *Tilapia* using bacterial probiotic. Similarly, in the present study infection with *staphylococcus aureus* showed mortality in the control group, whereas probiotic-fed fish groups showed relatively lower mortality. Resistant to infection was due to modulated immune response, attained with *B. licheniformis* as a probiotic. With probiotic bacteria present in fish, pathogenic bacteria become weakened with less availability to nutrients as compared to probiotics. So, probiotic bacteria can compete with other invading microbes, making fish less susceptible to disease, and less prone to mortality.

The increase in growth rate can be attributed to increased digestive enzymatic activity, enhanced appetite, degradation of indigestible components as well as modulation of intestine morphology. There was an improvement in immune system which could be attributed to probiotic colony formation and adhesion to inner gut lining. Also, mucus and serum proteins levels were reported to be higher in fish with enhanced immune activity through probiotics.

This study describes the potential impacts of *B. licheniformis* on growth rate, survival rate, feed conversion ratio, hematological and immune parameters in *O. mossambicus*, when treated under controlled fish rearing conditions. The positive impact of the probiotic can be attributed to its vital role in enhancing digestibility and increasing enzymatic and cellular activities related to immune response and digestion. Based on these results, use of *B. licheniformis*-supplemented diet can be considered as effective, eco-friendly, and viable practice to increase fish production in aquaculture of *Tilapia* and other fish species.

### Conclusion

This study, in general, shows that *Bacillus licheniformis* is a potential probiotic for use in *tilapia* farming. When used with concentration of  $10^9$ CFU/g in basal diet, it showed significant improvement in growth



rate and feed conversion ratio. Also, with increasing concentration of probiotic in experimental diet, tilapia showed significant improvement in immunity. Hence, probiotic *B. licheniformis* can be used as alternative to other therapeutic and feed additives for sustainable aquaculture practices.

### Ethical Statement

The handling of experimental animals (Tilapia juveniles) was performed in accordance with the recommendations in the international guiding principles for biomedical research involving animals (ICLAS, 2012). All manipulation was performed under anesthesia, and all efforts were made to minimize animal stress.

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### Author Contribution

AY: Supervised the Project, approved the final version to be published.

MNA: Carried out experimental and laboratory work, Wrote the manuscript.

MK: Co supervised the Project, did statistical analysis, and contributed the final version of manuscript

IM: Conceived and planned the project, helped in experimental work.

### Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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