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Enhancing of growth performance, immunity and disease resistance in Nile tilapia, *Oreochromis niloticus*, by using dietary probiotics supplementation

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Abstract
This study evaluated the effect of probiotics on the growth performance, some immunological, biochemical and hematological parameters of Nile tilapia. Eight weeks feeding trials were conducted to examine the effect of three types of probiotics, multi-strain probiotics (6×10^7 cfu/g), *Bacillus subtilis* (1×10^{11} cfu/g) and *Saccharomyces cerevisiae* (2.6×10^{10} cfu/g) in commercial names Protexin, Biogen-S and Diamond V respectively. Nile tilapia (24±0.02 g) was fed probiotic-incorporated diets as well as control diet (free of probiotics) in triplicates. At 4th and 8th week of feeding trial, the growth performance of fish fed different probiotics was higher than those fed the control diet. No significant difference was observed among fish fed different probiotics for 4 or 8 weeks. Blood profile showed an increase in hemoglobin (Hb), red blood cell (RBCs), packed cell volume (PCV), total protein, and globulin while aspartate aminotransferase (AST), alanine aminotransferase (ALT) levels decreased in fish fed different types of probiotics. Probiotics supplementation improved fish innate immunity based on lysozyme and respiratory burst activities over the control diet evaluated at 4th and 8th week. Likewise, significant up-regulation of the expression of cytokine genes, interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF α) estimated at 8th week by using quantitative RT-PCR were significantly higher than the control. After experimental period (8 weeks) fish from each group were challenged by pathogenic *Aeromonas hydrophila* (4×10^8 cfu/ml) which exhibited better relative survival percentages than the controls. Dietary supplementation of probiotics improves growth performance, immune status and disease resistance in Nile tilapia. Also, the best results related to growth obtained from groups fed multi-strain probiotic.

Keywords: growth, challenge, immunity, real time RT-PCR, Probiotics, proinflammatory cytokine.

1. Introduction
Aquaculture is one of the fastest growing sectors of food production. Tilapia is a widespread freshwater cultured fish that can be reared under wide ranges of environmental conditions and can accept different protein sources in its diet (Welker and Lim, 2011) [68]. Outbreaks of viral, bacterial and fungal infections have caused significant economic losses in aquaculture worldwide. In addition, substantial broodstock mortality has been reported in farms due to poor environmental conditions, unbalanced nutrition, generation of toxins and genetic factors (Kautsky *et al*., 2000) [25]. In past decades, prevention and control of fish diseases were achieved by using chemical additives and veterinary drugs, especially antibiotics, even though such methods were later found to be responsible for generating significant risks to public health by promoting the selection, propagation and persistence of bacterial-resistant strains (FAO, 2016) [16].
The interest of consumers for safe, pharmaceutical-free products, and the need of a sustainable aquaculture have encouraged the scientific research community to use probiotics as an ecofriendly health strategy to counteract aquaculture diseases (Balcázar *et al*. 2006, Wang *et al*. 2008b) [8, 66].
Probiotics are live microorganisms which confer health benefits on host when administered via the feed or to the rearing water. The beneficial effects of probiotics, such as improvement of food utilization, modulation of intestinal microflora, enhancement of immune responses and

antagonism to pathogens, have been demonstrated (Balcázar *et al*., 2006; Irianto and Austin, 2002; Kesarcodi-Watson *et al*., 2008; Merrifield *et al*., 2010; Nayak, 2010; Wang *et al*., 2008b) [8, 22, 26, 35, 37, 66].

It has been shown that the modulation of immune response by probiotics comprised different responses in a variety of domestic animals and fish, including the induction of proinflammatory cytokines, the activation of natural killer cells, production of mucosal and systemic antibodies, increasing the phagocytic lysozyme and complement activities (Matsuzaki and Chin, 2000; Panigrahi *et al*., 2004; 2005) [33, 40].

Probiotic strains could inhibit the pathogenic bacteria by the production of inhibitory compounds as bacteriocins, siderophores, lysozymes, proteases, hydrogen peroxide, formation of ammonia and diacetyle (Verschuere *et al*. 2000) [63], competition for essential nutrients and adhesion sites (Vine *et al*., 2004) [65]. In addition, the cell walls components of probiotic bacteria, such as β-glucan, lipopolysaccharide and peptidoglycans were attributed to the immunostimulatory effects in fish (Rengpipat *et al*., 2000; Gullian *et al*., 2004) [48, 20]. Among the various benefits of probiotics, immunomodulatory activity is note-worthy in improving the overall health status of the fish. However, there is limited research available for immunomodulatory activity of probiotics, especially for the long-term use of probiotics in fish diets. Therefore, the current study was conducted to evaluate different commercial probiotics as feed supplements for Nile tilapia through estimation of their effects on growth performance, physiological, and immunological parameters. Also, fish challenge against pathogenic bacteria, *Aeromonas hydrophila* was evaluated.

2. Materials and Methods

2.1. Probiotics

Probiotics with the trade name Protexin, Biogen-S, and Diamond V were used in the present study. Protexin, is a multi-strain probiotic, contains Lactobacillus plantarum, Lactobacillus rhamnosus, Bifidobacterium bifidum, Enterococcus faecium, Candida pintolepesii, Aspergillus oryzae in isolated forms (Not less than 6×10^7 cfu/g) obtained from Australian Co., Ltd. Biogen-S contains Bacillus subtilis natto (Not less 1×10^{11} cfu/g) obtained from The Samu median co., Ltd. Diamond V contains Saccharomyces cerevisiae (2.6×10^{10} cfu/g) obtained from Cedar Rapids, lowa, USA.

2.2. Fish and experimental design

Apparently healthy monosex Nile tilapia (Oreochromus niloticus) was collected from the nursery ponds of the Central Laboratory for Aquaculture Research, Abbassa, Abo-Hammad, Sharqia, Egypt, and kept in indoor fiberglass tank for two weeks for adaptation.

Fish (24.01±0.02 g) were distributed in 12 glass aquaria (70×60×50 cm) distributed as 20 fish per aquarium. Fish were divided into four groups in three replicates. The first group (T1) fed with control diet (basal diet) with no probiotic that was formulated as shown in Table 1. The proximate analysis of the basal diet indicated 39.9% crude protein, 10.89% crude lipid and 3.68% fiber according to the AOAC (2000). In the experimental diets, the 2nd group (T2) fed with (basal diet + Protexin). The 3rd group (T3) fed with (basal diet + Biogen) and the 4th group (T4) fed with (basal diet + Diamond), where they were added at a rate of 1 g/kg diet. Probiotics were included to the basal diet through infusion then evenly coated with molasses according to method described by Noordiyana *et al*. (2015) [39]. The fish were tested diets fed three times daily at the rate 3% of live body weight for 8 weeks (Silva *et al*., 2015) [55]. The aquaria were supplied with dechlorinated tape water. The average of the temperature was 25°C ± 1°C and the oxygen was adjusted for continuous aeration by using electrical air pumping compressors (RINA, Italy). Two-third of the aquarium water was changed daily. The fish per each aquarium were weighted weekly from the beginning of the feeding experiment and the diets amount was adjusted according to the increase in the total body weight.

Table 1: Ingredients and chemical composition of the basal diet.

Ingredients	Percentage of die
Ground yellow corn Fish oil	26.5
Soya bean meal	22
Fish oil	5
Meat meal	29
Fish meal	25
Mineral and vitamin mixture (premix)	1.5
Calculated chemical analysis (%)	
Crude protein	39.9
Crude lipid	10.89
Crude fiber	3.68
Ash	9.11
Moisture	10.58

2.3. Growth performance and feed utilization parameters

The average weight-gain (AWG), specific growth rate (SGR) and feed conversion ratio (FCR) was calculated accordingly through the following equations:

WG% (g/fish) = (Average final weight (g) – Average initial weight (g))/ Average initial weight (g) × 100. (Jauncey and Ross, 1982)

SGR % = 100 [ln final body weight (g) – ln initial body weight (g)]/experimental period (day). (Siddiqui *et al*., 1988)

FCR (%) = Feed intake (g) / Body weight gain (g) × 100. (De Silva and Anderson, 1995) [10].

2.4. Hematological and biochemical parameters

Three blood samples were collected from caudal vein according to Feldman *et al*. (2000) [17] and were divided into three groups. The first sample group was taken at the 4th week of feeding experiment; the second group was taken at the 8th week while the third group was taken post experimental infection. Before sampling, fish were starved for 24 h. Fifteen fish were used from each treatment (T1 to T4) to obtain blood samples (five fish per aquarium).

For heamatological analysis, the blood samples were collected with anticoagulant 10% ethylenediaminetetraacetate (EDTA) to measure hematocrit (Ht), hemoglobin (Hb), red blood cells (RBCs) and white blood cells (WBCs). Ht was determined as described by Dacie and Lewis (1991) [9]. (Hb) was determined by using cyanmethemoglobin colorimetric method after centrifugation at 540 nm according to Stoskopf (1993) [58]. The Total leukocytic count (WBCs) and erythrocytic count (RBCs) was carried out by using hemocytometer and special diluting fluid (Natt-Herrick) as indicated by (Martins *et al*., 2004) [31]. Differential leukocytic count was performed according to Feldman *et al*. (2000) [17].

For biochemical analysis, the blood samples were allowed to clot overnight at 4°C and then centrifuged at 3000 rpm for 10 min. The non-hemolyzed serum was collected and stored at -

20°C until use. Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) activities were determined calorimetrically according to the method described by Reitman and Frankel (1957) [47]. Total protein (TP), albumin and globulin were levels assayed by the method of Grant *et al*. (1987) [19], Doumas *et al*. (1981) [12] and Doumas and Biggs (1972) [11] respectively. The serum glucose was determined according to (Trinder, 1969) [62].

2.5. Respiratory burst
Blood sample of 0.1 ml was placed into micro titer plate and equal amount of 0.2% nitro blue tetra-zolium (NBT) solution was added and incubated for 30 minutes at room temperature. 0.1 ml of NBT blood cell suspension was taken and added to a glass tube containing 1 ml N, N-dimethyl formamide and centrifuged for 5 min at 3000 rpm. The respiratory burst activity was read in spectrophotometer at 620 nm in 1 ml cuvettes (Siwicki *et al*., 1985) [56].

2.6. Lysozyme activity
The lysozyme activity was measured by using turbidity measurement according to Schäperclaus *et al*. (1992) [52]. A series of dilution was prepared by diluting the standard lysozyme from hen egg-white (Fluka, Switzerland) and mixed with Micrococcus lysodeikticus (ATCC NO. 1698 Sigma) suspension for establishing the calibration curve. Ten µl of standard solution or serum were added to 200 µl of Micrococcus suspension (35 mg of Micrococcus dry powder/95 ml of 1/15 M phosphate buffer + 0.5 ml of NaCl solution). The changes in the extinction were measured at 546 nm by measuring the extinction immediately after adding the solution which contained the lysozyme (start of the reaction) and after 20 min incubation of the preparation under investigation at 40 °C (end of the reaction). The lysozyme content was determined based on the calibration curve and the extinction measured.

2.7. Molecular analysis: RNA extraction and gene expression by quantitative RT (reverse transcriptase)-PCR
Total RNA was extracted from the head kidney tissue at the end of the feeding period using RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's protocol. The quality and RNA concentration were assessed spectrophotometry by nanodrop spectrophoto-meter at A260 and A280 according to Wilfinger *et al*. (1997) [70]. Approximately 1 mg of total RNA from each of the tissue samples was reverse transcribed to produce cDNA using a Quantitect® Reverse Transcription kit (Qiagen, Germany) where the manufacturer's instructions have been followed. Initially, PCR was performed using primers for β-actin, which served as reference (ref) gene for both the test sample and the control (calibrator) sample (Table 2). The expressions of interleukin-1 (IL-1β) and tumor necrosis factor alpha (TNF-α) were examined using the primers presented in Table 2 (Selim and Reda 2015) [53]. The PCR conditions were as follows: 94 °C for 5 minutes, 40 cycles of 94 °C, 55 °C and 72 °C for 10, 30 and 40 seconds respectively, and extension at 72 °C for 7 minutes. Generate a melt curve using the Applied Biosystem real-time PCR system software. The obtained data were analyzed according to the method described by Livak and Schmittgen (2001) [29] which is also known as the $2^{-\Delta\Delta CT}$ method.

Table 2: Primers used for the examination of the expressions of cytokine genes in the head kidney tissues of *Oreochromis niloticus*.

Gene	Primer sequence	Product size	Ta
βActin	F. TGGGGCAGTATGGCTTGTATG. R. CTCTGGCACCCTAATCACCTCT.	165 bp	60 °C
TNF- α	F. GCTGGAGGCCAATAAAATCA. R. CCTTCGTCAGTCTCCAGCTC.	339 bp	60 °C
IL1β	F. TGCTGAGCACAGAATTCCAG. R. GCTGTGGAGAAGAACCAAGC.	371 bp	60 °C

2.8. Challenge test
At the end of 8th week, pathogenic *A. hydrophila* (0.5 ml of 10^8 bacterial cells ml^{-1}) was injected I/P to 21 fish/treatment (Aly *et al*., 2008) [4]. Inoculated fish were observed daily for 14 days and their mortalities were recorded. The relative level of protection (RLP) among the challenged fish was determined according to Ruangroupan *et al*. (1986) [50].

RLP % = 100 – (treatment mortality %/control mortality %) × 100

2.9. Determination of antibody titer
0.2 ml of serum was mixed with 2.4 ml of physiological saline in an agglutination tube and a dilution series was established. 1 ml of antigen suspension having 4.8×10^9 *A. hydrophila* per ml was added to each dilution stage to result in dilution sequences 1:25, 1:50, 1:100 and so on. The agglutination samples were allowed to stay at room temperature. The first reading was recorded after 30 minutes and the second after 3 to 6 hours. The result was checked again after 24 hours. The agglutination titer was the least dilution stage at which a positive agglutination occurred (Schäperclaus *et al*. 1992) [52].

2.10. Statistical analysis
The data were subjected to homogeneity test. Statistical analysis was performed using the one way and two-way ANOVA according to Tamhane and Dunlop (2000) [60], to test the effect of probiotics treatment and the duration of feeding. Duncan test was used as a post-hoc test. Data were considered significantly different when $P < 0.05$. It was performed with SPSS statistical software version 11.0.

3. Result
3.1. Growth performance
Fish fed probiotics-enriched diets showed higher growth than those fed the control diet (Table 3). There was no significant difference (P <0.05) in growth performance among fish fed different probiotics. The experimental groups (T2, T3 and T4) were significantly different (P<0.05) in both average final weights and weight gains from the control (T1). The highest averages of final weight and weight gain were observed in the T2. Specific growth rate was not different (P>0.05) between the experimental groups T2, T3 and T4, but the differences between each of these groups versus the control were statistically significant (P<0.05). The T2 showed the highest SGR, which was significant (P<0.05) in comparison with the

control and also to the dietary- supplemented groups. The treated groups also showed significant difference (P<0.05) in reduction in feed conversion ratio (FCR) compared to the control.

Table 3: Effect of probiotics on growth performance of Nile tilapia, *O. niloticus* fed different probiotics for the first and the second month.

Duration of treatments										
Parameters			**First Month**					**Second Month**		
Treatment	**Initial body weight (g)**	**Final body weight (g)**	**Body gain %**	**SGR* (%g/d)**	**FCR****	**Initial body weight (g)**	**Final body weight (g)**	**Body gain %**	**SGR (%g/d)**	**FCR**
T1 (Control)	24.01±0.02	32.99±0.28^{b}	37.42±1.10^{b}	1.06±0.03^{b}	2.51±0.07^{b}	24.01±0.02	40.01±1.52^{b}	66.64±6.19^{b}	0.85±0.06^{b}	3.07±0.28^{b}
T2 (Protexin)	24.03±0.02	35.44±0.17^{a}	47.49±0.62^{a}	1.30±0.01^{a}	2.04±0.02^{a}	24.03±0.02	49.29±0.75^{a}	105.09±3.04^{a}	1.20±0.02^{a}	2.07±0.05^{a}
T3 (Biogen-S)	24.04±0.01	35.38±0.53^{a}	47.13± 2.2^{a}	1.29±0.05^{a}	2.06±0.08^{a}	24.04±0.01	47.45±2.49^{a}	97.35±10.3^{a}	1.13±0.09^{a}	2.25±0.19^{a}
T4 (Diamond-V)	24.01±0.01	34.52±0.09^{a}	43.77±0.39^{a}	1.21±0.01^{a}	2.19±0.02^{a}	24.01±0.01	47.48±1.88^{a}	97.73±7.79^{a}	1.13±0.06^{a}	2.18±0.16^{a}
One way ANOVA	P Value									
	0.491	0.002	0.002	0.002	0.001	0.491	0.025	0.025	0.018	0.019

SGR*. Specific growth rate, FCR**. Food conversion ratio. The values were given as means (±S.E.) of three replicates. Means values with different superscript letters within the same columns are differ significantly at (P ≤ 0.05). Means with same letters within column non-differ significantly, P ≤ 0.05 (a- c).

3.2. Hematological and biochemical parameters

All probiotics-supplemented groups were different from the control (P<0.05) in hemoglobin density, numbers of RBC sand hematocrit values within the two tested times of the study (Table 4). The highest hemoglobin density and hematocrit values were observed in T2 (Protexin supplemented group) at the 8th week. Otherwise, there was no significant difference (P < 0.05) in RBCs count among fish fed different probiotics. Neither duration of feeding nor its interaction with probiotics were of significant importance for all erythrogram parameters (P <0.05), except for Hb density where it was significantly affected by feeding period. There was no significant difference (P>0.05) in Mean Corpuscular hemoglobin concentration (MCHC) of treated fish compared to the control.

The results of leukocytes examination (Table 5) showed significant rise (P<0.05) in total leukocytic count as well as lymphocyte and monocyte percent in all groups compared to the control. Among the experimental groups, the T2 represented the highest count of lymphocytes and monocyte in comparison with the control at the 8th week. However, the percentages of neutrophils showed a marked decrease (P<0.05) in the experimental fingerlings compared to the control within the 4th and 8th week. On the contrary to erythrogram results, feeding period of probiotcs was of significant importance (P<0.05).

Table 6 showed a significant decrease (P< 0.05) in the serum levels of ALT, AST and glucose along the experimental periods were noticed in probiotics-supplemented groups in comparison with control (T1). Neither duration of probiotic feeding nor its interaction with probiotics had a significant effect on studied fish for transaminases. On the other hand, there was a significant (P<0.05) increase in globulin and total protein of treated groups than the control ones.

Table 4: Effect of probiotics on erythrogram of Nile tilapia fed different probiotics for the first and the second month.

Groups	Month	Hb (g%)	RBCs (106/µl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (%)
T1 (control)	1	3.05±0.09^{c}	1.07±0.02^{b}	12.60±0.26^{d}	117.97±0.84^{a}	28.33±0.65^{a}	24.03±0.7^{ab}
	2	3.11±0.05^{c}	1.15±0.07^{b}	13.23±0.38^{d}	115.91±4.02^{a}	27.26±1.39^{ab}	23.5± 0.46^{ab}
T2 (protexin)	1	3.82±0.16^{ab}	1.63±0.06^{a}	17.03±0.42^{ab}	104.42±1.78^{b}	23.54±1.95 ^{b}	22.5±1.49^{ab}
	2	4.20±0.17^{a}	1.6±0.07^{a}	17.13±0.54^{a}	110.7±4.62^{ab}	27.19±1.83^{ab}	25.05±0.1^{ab}
T3 (Biogen-S)	1	3.58±0.11^{b}	1.46±0.08^{a}	16.35±0.72^{abc}	108.47±1.88^{ab}	23.82±0.25^{b}	21.98±0.61^{b}
	2	3.99±0.19^{ab}	1.64±0.06^{a}	16.4±0.38^{abc}	104.71±2.8^{b}	24.44±1.23^{ab}	23.87±1.28^{ab}
T4 (Diamond-V)	1	3.60±0.06^{b}	1.46±0.06^{a}	15.22±0.4^{c}	101.34±5.7^{b}	23.96±1.05^{b}	23.67±0.28^{ab}
	2	3.98±0.22^{ab}	1.57±0.05^{a}	15.67±0.23^{bc}	100.15±2.63^{b}	25.39±0.7^{ab}	25.42±1.33^{a}
Two way ANOVA	P Value						
Prob.		0.0001	0.0001	0.0001	0.002	0.047	0.461
Month		0.008	0.081	0.377	0.941	0.211	0.059
Prob. × Month		0.582	0.438	0.926	0.485	0.337	0.449

The values were given as means (±S.E.) of three replicates. Means values with different superscript letters within the same columns are differ significantly at (P ≤ 0.05). Means with same letters within column non-differ significantly, P ≤ 0.05 (a- c).

Table 5: Effect of probiotics on leukogram of Nile tilapia fed different probiotics for the first and the second month.

Groups	Month	WBCs (× 103/ µl)	Neutrophil (%)	Lymphocyte (%)	Monocyte (%)
T1 (control)	1	5.12±0.45^{c}	32.72±0.91^{a}	42.45±1.65^{d}	4.9 ±0.38^{c}
	2	5.35±0.43^{c}	31.33±1.51^{a}	42.65±1.18^{d}	5.8 ± 1.02^{bc}
T2 (protexin)	1	7.70±0.29^{ab}	19.46±0.51^{bc}	62.37±1.45^{bc}	7.8±0.94^{ab}
	2	8.7±0.56^{a}	18.07±0.59^{c}	70.55±1.82^{a}	9.1±0.29^{a}
T3 (Biogen-S)	1	6.65±0.55^{b}	21.03±1.15^{b}	58.03±1.43^{c}	7.36±0.64^{ab}
	2	8.44±0.38^{a}	17.75±1.07^{c}	67.08±2.43^{ab}	6.8±0.33^{abc}
T4 (Diamond-V)	1	6.75±0.26^{b}	20.29±0.28^{bc}	59.5±1.02^{c}	7.4±0.49^{ab}
	2	7.93±0.42^{ab}	18.85±0.32^{bc}	67.87±1.94^{a}	7.1±86^{ab}
Two way ANOVA	P Value				
Prob.		0.0001	0.0001	0.0001	0.003
Month		0.003	0.009	0.0001	0.435
Prob. × Month		0.373	0.655	0.055	0.465

The values were given as means (±S.E.) of three replicates. Means values with different superscript letters within the same columns are differ significantly at (P ≤ 0.05). Means with same letters within column non-differ significantly, P ≤ 0.05 (a- c).

Table 6: Effect of probiotics on some liver function tests of Nile tilapia fed different probiotics for the first and the second month.

Groups	Month	ALT (u/l)	AST (u/l)	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A/G Ratio
T1 (control)	1	32.73±0.93^{a}	57.28±0.51^{a}	2.61±0.14^{c}	0.92±0.029^{bc}	1.68±0.11^{b}	0.55±0.03^{a}
	2	29.91±0.33^{a}	55.46±1.07^{a}	2.79±0.10^{bc}	1.05±0.026^{ab}	1.74±0.11^{b}	0.60±0.05^{a}
T2 (protexin)	1	23.00±0.96^{b}	40.44±0.32^{c}	3.26±0.04^{ab}	0.92±0.023^{c}	2.34±0.07^{a}	0.39±0.02^{b}
	2	24.61±0.53^{b}	44.18±1.40^{bc}	3.63±0.14^{a}	1.08±0.046^{a}	2.54±0.13^{a}	0.43±0.03^{b}
T3 (Biogen-S)	1	26.00±2.50^{b}	46.00±2.02^{b}	3.22±0.14^{ab}	0.93±0.019^{bc}	2.32±0.17^{a}	0.38±0.04^{b}
	2	25.01±0.68^{b}	46.30±3.06^{b}	3.48±0.17^{a}	1.13±0.079^{a}	2.38±0.06^{a}	0.46±0.03^{b}
T4 (Diamond-V)	1	23.84±1.35^{b}	48.14±1.68^{b}	3.16±0.13^{ab}	0.83±0.021^{c}	2.26±0.14^{a}	0.44±0.03^{b}
	2	24.73±1.17^{b}	47.39±0.68^{b}	3.56±0.33^{a}	1.05±0.037^{ab}	2.56±0.28^{a}	0.38±0.01^{b}
Two way ANOVA	P Value						
Prob.		0.0001	0.0001	0.001	0.171	0.0001	0.0001
Month		0.712	0.747	0.023	0.0001	0.16	0.25
Prob. × Month		0.305	0.36	0.905	0.699	0.826	0.2

The values were given as means (±S.E.) of three replicates. Means with different letters within column differ significantly, P ≤ 0.05, Means with same letters within column non-differ significantly, P ≤ 0.05 (a- c).

3.3. Immune parameters

After the 4th week and the 8th week, NBT assay and lysozyme activity showed significant increase in T2, T3 and T4 in comparison with T1 Table 7. After the 4th and the 8th week, NBT assay showed significant increase in T2 than the values of T3 and

T4. The highest value of NBT assay obtained at T2 (2.11±0.13) after the 4th week and the lowest value obtained at T4 (1.6±0.09) after the 4th week in comparison with T1. No statistic difference (P > 0.05) was noticed between the 1st and 2nd month of the experiment in all treatments. On the contrary, lysozyme activity (Table 7) showed significant increase (P < 0.05) all over the experiment period in T2, T3 and T4 compared to control group. The highest value of lysozyme obtained at T3 (1.87±0.12) after the 8th week in comparison with T1 (control) and the lowest value of lysozyme obtained at T4 (1.29±0.06) after the 4th week. Statistics showed that duration of feeding was of significant importance for lysozyme activity but not for NBT.

Table 7: Effect of probiotics on some immunological parameters in *O. niloticus* after the first and second month of experiment.

Groups	Month	NBT (mg/ml)	Lysozyme (µg/ml)
T1 (control)	1	1.02±0.04^{d}	0.83±0.09^{d}
	2	1.00±0.06^{d}	1.13±0.10^{cd}
T2 (protexin)	1	2.11±0.13^{a}	1.55±0.09^{ab}
	2	1.97±0.07^{a}	1.75±0.17^{a}
T3 (Biogen-S)	1	1.66±0.05^{c}	1.41±0.08^{bc}
	2	1.73±0.04^{bc}	1.87±0.12^{a}
T4 (Diamond-V)	1	1.6±0.09^{c}	1.29±0.06^{bc}
	2	1.90±0.03^{ab}	1.74±0.07^{a}
Two way ANOVA	P Value		
Probiotic		0.0001	0.0001
Month		0.272	0.0002
Prob × Month		0.037	0.547

The values were given as means (±S.E.) of three replicates. Means with different letters within column differ significantly, P ≤ 0.05, Means with same letters within column non-differ significantly, P ≤ 0.05 (a- c).

3.4. Gene expression of some proinflammatory cytokine (IL-1β and TNF-α) in response to probiotics

Fold changes in quantitative real time PCR for proinflammatory cytokine genes TNF-α and IL1-β in the head kidneys of the Nile tilapia were evaluated at the end of the feeding trials. The transcript level of all these genes relative to the house- keeping gene β-actin was indicated in Figure 1. Dietary probiotics (protexin, biogen and diamond) supplementation caused significant up-regulation in IL-1 and TNF α mRNA levels in the pattern of T2 > T3 > T4 compared with the control β-actin levels.

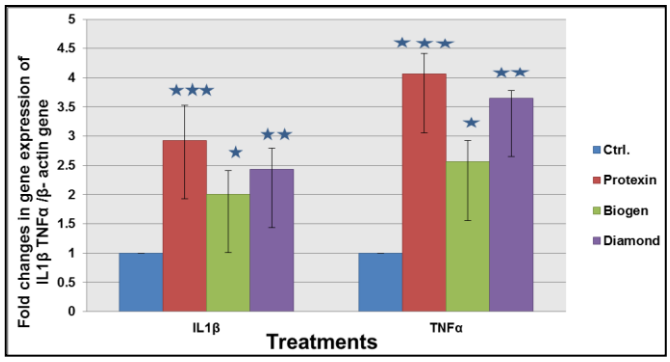

Fig 1: Graphical presentation of real time quantitative PCR analysis of the expression of proinflammatory cytokine TNFα and IL1β genes of Nile tilapia fed different probiotics for two months. *showed significant difference between treatments and control.

3.5. Challenge test

From Table 8, It was noticed that, the highest mortality percentage was in T1 (70%) while the lowest mortality observed in T2 (30%). Relative level of protection (RLP) was higher in T2 (47.6%).

Table 8: Mortality rates and RLP among the experimentally infected Nile tilapia fed different probiotics for 14 days.

Groups	Treatment	Number of fish	Number of dead fish	Mortality %	RLP%
T1 (control)	A. hydrophila	30	21	70	0.0
T2	A. hydrophila + PROTEXIN	30	9	30	47.6
T3	A. hydrophila + BIOGEN	30	11	36.67	33.3
T4	A. hydrophila + DIAMOND	30	16	40	23.8

3.6. Antibody titer

Results of Table 9 showed that the highest level of the antibody titre to *A. hydrophila* infection was obtained with T2 (9 log10). Also high values were obtained in T3 and T4 as compared to T1.

Table 9. Effect of prebiotics supplemented diet on the antibody titre level due to *A. hydrophila* infection.

Antigen	Antibody titre (log10)			
	T1	T2	T3	T4
A. hydrophila	4	9	7	6

4. Discussion

Nile tilapia, *Oreochromis niloticus* is one of the most important species, and its growth performance and diseases resistance are critical keys for its culture expansion (Abdel-Tawwab *et al*., 2008) [3]. Probiotic supplementation enhanced growth performance in the first and second month of the experiment over control diet. The noticed increase in the body weight may be due to high food utilization and the increase in digestibility of different diet components. A similar enhancement of growth performance was reported in Nile tilapia supplemented with *Enterococcus faecium* (Wang *et al*., 2008a) [67], *S. cerevisiae* (Abdel-Tawwab *et al*., 2008; Goda *et al*., 2012) [3, 18] and with bacterial cocktail of (*Lactobacillus acidophilus, Streptococcus thermophilus, and Bifidobacterium bifidum*) (Ayyat *et al*., 2014) [7]. On the contrary, Iwashita *et al*. (2015) [23] mentioned that administration of a combination of probiotics of *B. subtilis, S. cerevisiae and A. oryzae* had no effect on the growth rates of Nile tilapia.

The hematological and biochemical parameters usually give a good picture for fish health and well-being monitoring (Eissa and Abou-El Gheit 2014) [13]. In the present work, there was significant improvement in fish fed probiotic-enriched diets in (T2, T3 and T4) compared to the control (T1). This indicates that all types of probiotics have positive impact on erythrogram. Consequently, there was a significant increase in blood indices like Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin (MCH) but not for MCHC ($P > 0.05$). This confirm the findings of Abd Elaziz *et al*. (2007) [1] who observed significant increases in RBCs count, WBCS count, Hb concentration and PCV % in Nile tilapia received two types of probiotics (*Bacillus subtilis and saccharomyces cerevisiae*). The enhancement of the erythrogram parameters may be attributed to the hepato-stimulatory and hepato-protective effects of probiotics (Sarma *et al*., 2003) [51].

Leukocytosis with neutrophilia and lymphocytosis were observed in treated groups when comparing with the control fish. In the same line, Tantawy *et al*. (2009) [61] and Mehrim (2011) [34] also noticed increases in total and differential leukocytic counts (lymphocytes, heterophils and monocytes) in probiotic-supplemented fish.

The innate immunity is correlated with serum protein, albumin and globulin levels (Wiegertjes *et al*. 1996) [69]. Fish administrated probiotics revealed hyperproteinemia and hyperglobulinemia. The obtained results validate those of Kumar *et al*. (2006) [28] and Nayak *et al*. (2007) [38] who found higher total serum protein and globulins in Nile tilapia fed probiotic in diet while albumin was not affected by probiotic supplementation. As the source of antibody in blood serum is globulin, the total serum globulin level probably reflects the level of specific immunoglobulin (antibody). Otherwise, Ayyat *et al*. (2014) [7] reported that, highest serum albumin level was obtained in the group of fish fed the three-bacteria cocktail (*Lactobacillus acidophilus, Streptococcus thermophilus, and Bifidobacterium bifidum*).

A significant decrease ($p< 0.05$) in the serum levels of Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and glucose along the experimental periods were noticed in probiotics-supplemented groups. Nile tilapia also revealed the same behavior of transaminases decrease under the effect of dead *Saccharomyces cerevisiae* yeast (Abdel-Tawwab *et al*., 2008) [3] and both of live *Bacillus subtilis and S. cerevisiae* (Marzouk *et al*., 2008) [32], *B. licheniformis* (Soltan and El-Laithy, 2008) [57] and *E. faecium and B. coagulans* (El-moghazy Mohamed *et al*., 2015) [15]. Talib (2004) [59] indicated that probiotics has no hepatotoxic or nephrotoxic effects in *Oreochromis niloticus* and *Mugil cephalus*. Silva *et al*. (2015) [55] noticed that *B. amyloliquefaciens* seems to have positively influenced the glucose levels of fish in cage-reared Nile tilapia.

In the present study, fish fed one of the tested probiotics (Protexin, Biogen-S and Diamond V) showed higher immunity as indicated by respiratory burst and lysozyme activities than those fed the control diet. Similar results were reported when Nile tilapia fed baker yeast (Abdel-Tawwab *et al*., 2008) [3], *B. subtilis* and *L. acidophilus* or each one alone (Aly *et al*., 2008) [4], *E. faecium* ZJ4 (Wang *et al*., 2008a) [67], *Spirulina* (Abdel-Tawwab and Ahmad, 2009) [2] and *B. amyloliquefaciens* (Selim and Reda, 2015) [53].

Lysozyme is an indispensable bactericidal cationic enzyme that hydrolyzes the peptidoglycan layers of bacterial cell walls by splitting glycosidic bonds between N-acetylmuramic acid and N- acetylglucosamine and is increased in the sera of fish during infection with various variable microorganisms (Magnadottir, 2006) [30]. High nonspecific immunity was developed in Nile tilapia that was fed diets supplemented with probiotics (T2, T3 and T4) which resulted in an increase in the serum lysozyme. Dietary supplementation with different probiotic strains enhances lysozyme activity in tilapia (Ridha and Azad, 2012) [49], rainbow trout, *Oreochromis mykiss* (Panigrahi *et al*., 2005; Kim and Austin, 2006; Ramos *et al*., 2013) [41, 27, 46] and seabream (Picchietti *et al*., 2007) [45]. In contrast, dietary or water supplemented with *Bacillus* spp. fails to increase serum lysozyme levels in tilapia and rainbow trout (Zhou *et al*., 2010) [72].

Cytokines are simple polypeptide or glycoprotein mediators that are produced by immune cells and act as signaling molecules for the immune system and contribute to cell growth and differentiation and defense mechanisms of the host (Ellis, 2001; Peddie *et al*., 2002) [14, 42]. Proinflammatory cytokines, including interleukin-1 (IL-1β) and tumor necrosis factor-alpha (TNF-α), are biomarker immune- regulators and activate lymphocytes, macrophages and natural killer cells, which results in subsequent enhancements of phagocytosis, respiratory burst activity and nitric oxide production (Mulero and Meseguer, 1998) [36]. In the present study, probiotics administration modulated the production of IL-1β and TNF-α in tilapia following 2 months of feeding trials. He *et al*. (2013) [21] reported that the expression of the immune response genes IL-1 and TNFα significantly increased in tilapia fed *B. subtilis* after 56 days of feeding. Similar modulations have been reported in rainbow trout fed *Bacillus subtilis, Lactobacillus rhamnosus or Lactococcus garvieae* (Awad *et al*., 2011, Nayak, 2010, Pérez-Sánchez *et al*., 2011) [6, 37, 43]. In contrary, cytokines IL-1 and TNF-α have been reported to be down-regulated by *Lactobacillus delbrueckii* supplementation through live carriers in Dicentrarchus labrax (Picchietti *et al*., (2009) [44]. Probiotic bacteria colonize in the

gut and are involved with the gut-associated lymphoid tissue to stimulate systemic signals that end with cytokine production (Zhigang, 2011) [71].

Fish prophylacted by probiotics then challenged with *A. hydrophila* showed a statistical improvement in survival percent between treated groups than control one. These findings are substantiated by Abdel-Tawwab *et al*. (2008) [3] who found that Nile tilapia fed baker yeast and challenged by *A. hydrophila* showed lower mortality than those fed the control diet. Villamil *et al*. (2014) [64] found that survival percent was 80.1% in *L. acidophilus* fed groups compared with 46.6% in Nile tilapia fed the control diet and infected with *A. hydrophila.*

The highest level of the antibody titer after i/p with *A. hydrophila* was obtained with T2 and the lowest value was obtained with the control fish. Statistical increase in total serum immunoglobulin levels was reported in rainbow trout, *Oncorhynchus mykiss* (Panigrahi *et al*., 2004) [40] and the Indian major carp, *Labeo rohita*, fed diet containing *B. subtilis* (Nayak *et al*., 2007) [38].

5.1. Conclusion

Improvement in growth performance was achieved when Nile tilapia fed diets containing Protexin, Biogen-S or Diamond V) for 2 months. Also, fish health and innate immunity was significantly improved by the dietary administration of any of the probiotics. The interleukin-1 (IL-1) and tumor necrosis factor a (TNFα) mRNA levels in the anterior kidney tissues increased significantly in the supplemented groups. These increases in the examined immune parameters following the administration of the probiotic were correlated with an improvement in the fish survivability of inoculations with *A. hydrophila*.

6.1. References

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