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Effects of dietary *Bacillus subtilis* on growth performance and immune responses, in rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792)

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Abstract

Four hundred and eighty five rainbow trout (76 ± 6.44 g mean weight) were acclimated to laboratory conditions and then randomly divided into four groups of tanks in triplicate. The first group (Group 1) was fed on a commercial diet (control) without *Bacillus subtilis* supplementation. The second, third and fourth groups (Group 2, Group 3 and Group 4, respectively) were given a diet supplemented with 1, 5 and 10 g probiotic powder (containing 8×10^7 CFU g^{-1} *Bacillus subtilis*) per kg commercial feed. Growth performance, immune responses and glucose levels were analyzed on days 0, 22 and 44. The results showed that dietary *Bacillus subtilis* supplementation significantly ($p<0.05$) reduced final weight in treated groups compared with that in the control group. No significant difference ($p>0.05$) was observed in weight gain rate (WGR) between the control and group 2. On the effect of dietary *Bacillus subtilis* on serum lysozyme, it was observed that group 3 and group 4 did not show any significant differences in serum lysozyme activity and serum total antibody on day 44. Results of alternative complement activity, showed significant increase during the experimental days ($p<0.05$). Results on glucose assay showed that group 3 had the lowest glucose level (13.71 mg/dL) which was not significantly different than that in other groups on day 44. Fish diet supplementation with 5 g probiotic powder (Group 3) is preferable for immune system responses; however, high dose of *Bacillus subtilis* may be helpful to improve growth performance in rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792).

Keywords: *Rainbow trout*, *Bacillus subtilis*, Dietary supplementation, Growth factors, Immune function

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Introduction:

Fisheries is one of the richest and fastest food producing sector in the world. It is estimated that people gain about 25% of their animal protein from fish and shellfish (Nayak, 2010). Obviously, disease can be a significant limiting factor in aquaculture because of high mortality and its contagious nature (Zhou *et al.,* 2010). In recent years, use of antibiotics as a preventive factor caused several problems such as development of antibiotic-resistant strains because of the overuse of antibiotics and the reduced efficacy of the remaining antibiotics in fish. It is well known that the normal indigenous microbiota play an important role in the health of animals. Probiotics are defined as live microorganisms including many yeast and bacteria, which when administered in adequate amounts could enhance the growth and health of the host. These effects can be related to a number of mechanisms such as producing some inhibitory compounds, competing with pathogenic microbes for chemicals and adhesion sites, modulating and stimulating the immune function and improving the intestine microbial balance (Balcazer *et al.*, 2007, Heo *et al.*, 2013). A wide range of microalgae yeasts (*Debaryomyces*, *Phaffia*, and *Saccharomyces*), gram-positive bacteria (*Bacillus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Micrococcus*, *Streptococcus*, and *Weissella*) and gram-negative bacteria (*Aeromonas*, *Alteromonas*, *Photorbodobacterium*, *Pseudomonas* and *Vibrio*) have been applied as probiotics to improve aquatic animal growth, survival, health, and disease prevention (Chiu *et al*., 2010). *B. subtilis*, one of the most studied probiotics in fish, is able to grow at a vast range of temperatures (10 - 50°C), pH values (5 - 10), and NaCl levels (0% - 9%) suggesting that it can be used for various purposes in aquaculture, such as probiotics for both freshwater and marine species, immunostimulation and disease resistance (Liu *et al*., 2012).

Oncorhynchus mykiss(Walbaum, 1792)is one of the most famous fish species in Iran. It has been widely used as a farmed fish in many provinces because of its rapid growth and high value as food. Its aquaculture production dramatically increased from 500 tons in 1994 to 30000 tons in 2005 (Nafisi Bahabadi and Falahati marvdoost, 2007). However, fish diseases have been a serious problem in most intensive aquaculture systems for this fish species. Non-specific immune systems are very important in the defense mechanisms of fish against pathogens and microorganisms, so the objective of this study was to examine innate immune parameters such as serum lysozyme activity, total serum antibody, serum alternative complement activity, also glucose level and growth performance following dietary supplementation of *B.subtilis* in *rainbow trout*.

Materials and methods

Fish

Four hundred and eighty five healthy rainbow trout at an average weight of 76 ± 6.44 g were obtained from a private fish farm at Urmia, Iran. Fish were kept in an indoor cement tank for 10 days for acclimation to laboratory conditions. Fish were maintained in continuously aerated free-flowing dechlorinated freshwater at 14.7 ± 0.5°C, and fed on a commercial pellet diet (crude protein: 38%, crude lipid: 14%, moisture:11%) (Faradaneh, Iran) based on the water temperature and their body weight. Daily weight measurements were done by random selection of fish per tank and using a digital balance.It should be noted that the commercial pellets were first checked for the presence of bacteria by culture on blood agar and confirmation by biochemical tests (catalase enzyme, oxidase enzyme, citrate utilization, melt the gelatin, motility, production of indole, glucose fermentation, reduce nitrate) before application.

Diet preparation and feeding trial

Vibrio control 7 (VC-7) probiotic was purchased from Team aqua corporation (Taiwan). In order to achieve accurate counts of *B.subtilis*, the number of bacterial cells present per g of culture medium was measured by plating on blood agar. After 24 hours incubation of plates at 37°C, the measured population levels of *B. subtilis* in the test diets were 8×10^7 CFU g^{-1}. Food pellets supplemented with probiotic suspension were prepared by slowly spraying 10 mL of suspension (1, 5 and 10 g probiotic powder added to 10 mLdistilled water) onto a clean plate containing the dry pellets for 3–5 min and drying at room temperature for 2 h, to make 1 kg of experimental feedstuff. The control group had only 10 mLdistilled water added to the fish feed.

Effect of B. subtilis on the grow-out of rainbow trout

15 fish from each group (5 fish per tank) were randomly sampled and anaesthetized with clove powder (200 mg L^{-1}) (Tukmechi and Bandboni, 2014). Body weights and lengths (total length, standard length and fork length) were measured on days 0, 22 and 44 of the growing-out trial. Growth effects were determined by calculating weight gain rate (WGR), specific growth rate (SGR) and condition factor (CF) according to the formula below:

$$\text{WGR (\%)}: \left[(W_f - W_i)/ W_i\right] \times 100$$

$$\text{SGR (\%)}: \left[(\ln W_f - \ln W_i)/T\right] \times 100$$

$$\text{CF (\%)} \left[W_f / (L_f)^3\right] \times 100$$

Where W_f refers to the mean final weight, W_i is the mean initial weight, L_f is the mean final length of fish and T is the feeding trial period in days (Al-Dohail *et al*., 2009).

Immunological assays

Five fish from each tank (fifteen per each group) were sampled 1 day before and after feeding began, and then on days 0, 22 and 44. Using syringes coated with heparin, blood was collectedfrom caudate vein and transferred immediately into sterile tubes and allowed to clot at room temperature for 1 h. Samples were then kept at 4°C for 5 h. The sera were separated by centrifugation (3500 rpm for 5 min at 4°C) and stored at -80°C until analyses.

Serum lysozyme activity

Lysozyme activity in serum was determined according to the method of Clerton *et al*. (2001) and Kim and Austin (2006) based on the lysis of the lysozyme sensitive gram-positive bacterium *Micrococcus lysodiekticus* (Sigma, St. Louis, MO). Lysozyme acts upon susceptible bacteria by combining with and breaking down a mucopolysaccharide. This mucopolysaccharide has been shown to be situated in the bacterial cell wall. *M.lysodeikticus*, one of the gram positive bacteria, is normally highly sensitive to lysozyme.3 dilutions of hen egg white lysozyme (Sigma) ranging from 0 to 25 µg mL^{-1} (in 0.1 M phosphate-citrate buffer, pH 6) (Sigma, USA) were used as the standard. Prepared standard solutions were placed along with the undiluted serum sample (25 µL) in the wells of a 96-well plate in triplicate, 175 µL of M. *lysodiekticus* suspension (750 µg mL^{-1}) was prepared in the same buffer then added to each well. After rapid mixing, the change in turbidity was measured at 0 and 4 min at 450 nm at approximately 20°C using a microplate reader (Statt facts, Germany). The equivalent unit of activity of the sample as compared to the standard was determined and expressed as µg mL^{-1} serum.

Serum total antibody level

Serum total immunoglobulin was assayed with a total protein kit (Parsazmoon, Iran). After mixing 20 µL serum samples with 1000 µLl Biuret indicator and incubating at 37°C for 5 min, total protein content was determined by the Biuret method and the optical density (OD) of the supernatant determined at 546 nm using a spectrophotometer (Awareness, USA). Then 50 µL of total serum samples were mixed with an equal volume of 12% solution of polyethylene glycol (Sigma) in wells of a 96-well microtiter plate. After 2 h incubation at room temperature, the plate was centrifuged at 3800 rpm at 4°C; 20 µL of the supernatant was mixed with 1000 µLof Biuret indicator and the protein content determined by the Biuret method, as mentioned above. This value was subtracted from the total protein level, with the result equal to the total immunoglobulin concentration of the serum was expressed as mg mL^{-1}(Tukmechi and Bandboni, 2014).

Serum complement activity

Alternative complement activity was assayed based on the hemolysis of rabbit red blood cells (RaRBC)as described by Amar *et al*. (2000). The RaRBC were washed three times in ethylene glycol tetra acetic acid-magnesium-gelatin veronal buffer (0.01

M EGTA-Mg-GVB, pH 7) and the cell numbers adjusted to 2 × 10^8 cells per mLby microscopic counting of cells on a neobar slide in the same buffer. At first, the 100% lysis value was obtained by adding 100 µL of the above RaRBC to 2.9 mL distilled water. The hemolysate was centrifuged at 3800 rpm for 10 min at 4 °C, pellet was discarded and the OD of the supernatant was determined at 414 nm using a spectrophotometer (Awareness, Palm city, FL). The test sera were then diluted (24 times) and then different volumes ranging from 50 to 250 µL were poured in test tubes (total volume adjusted to 250 µL with the buffer) and allowed to react with 100 µL of RaRBC in small test tubes. This mixture was incubated at 20°C for 90 min with intermittent mixing, following which 3.15 mL of 0.85% NaCl solution was added and the tubes were centrifuged at 3800 rpm for 5 min at 4°C, and the OD of the supernatant measured as mentioned above. A lysis curve was plotted on a graph paper. It was obtained by plotting the percentage of rabbit red blood cells (RaRBC) hemolysis against the volume of serum added on a log-log graph. The volume yielding 50% hemolysis was used to determine the complement activity of the sample as:

ACH50 (Units mL^{-1}) = 1/K × (reciprocal of the serum dilution) × 0.5

Where K is the amount of serum (mL) giving 50% lysis and 0.5 is the correction factor since the assay was performed at half scale of the original method (Tukmechi and Bandboni, 2014).

Glucose assay

Samples of blood were collected from fish into eppendorf tubes and centrifuged for 5 min at 23000 rpm. The serum (10 µL) was taken and incubated with a glucose reagent (Pars azmoon, Iran) (1mL) at room temperature for 20 min. The test based on the coupling of the enzymatic oxidation of glucose by glucose oxidase, resulting in hydrogen peroxide which is subsequently used for the generation of a colored quinoneimine product (Teuscher and Richterich, 1971). The glucose value was calculated (mg dL^{-1}) according to the following formula(Al-Dohail *et al*., 2009):

Glucose (mg dL^{-1})= (ΔA sample / ΔA Standard) × concentration of the standard (mg dL^{-1})

Statistical analysis

The data were subjected to ANOVA and Tukey's HSD test using SPSS software Version 19. Differences were considered significant when $p<0.05$.

Results

Growth performance

Data on the growth performance of the rainbow trout, including initial weight, final weight, WGR, SGR and CF are given in Table 1. Fish treated with *B.subtilis* at a dose of 10 g per kg feed (Group 4) showed the lowest WGR (88.49%), followed by the group treated with a dose of 5 g per kg feed (98.25%) while there was no significant differences between them.*B. subtilis* at a dose of 1 g per kg feed (Group 2) resulted in better WGR and SGR (107.87% and 0.72 % respectively) between treated groups, while SGR showed no significant differences between all treated groups (p>0.05).

Immune response

The effects of probiotic treatment on lysozyme activity of fish are shown in Fig. 1. Fish fed diets with *B.subtilis* showed a significant increase in lysozyme activity on days 22 and 44 of the treatment, while there were no significant differences (p>0.05) between group 3 and 4 on days22 and 44 of the treatment.

The total antibody content of the control and probiotic treated groups are shown in Fig. 2. Similar to the lysozyme activity, dietary supplementation with *B. subtilis* showed a significant increase in total antibody (p<0.05) on days 22 and 44 of the treatment, while there was no significant differences (p> 0.05) between group 3 and 4 on day 44 of treatment (18.06 and 18 mg/dL,respectively). Clearly, the feed supplemented with *B. subtilis* at a dose of 5 g per kg feed appeared to be sufficient to enhance the lysozime activity and total antibody during 44 days.

The effects of probiotics on the complementary activity of rainbow trout are shown in Fig 3. As it can be seen complementary activity increased at higher doses of *B.subtilis* (Group 4) on days 22 and 44 rather than other groups (64 and 72 units/ mL, respectively), but there were no significant differences (p>0.05) between all of the groups at each time.

Glucose assay

The effect of different probiotic treatments on glucose levels of rainbow trout are shown in Fig 4. Probiotic treatment with 5 g per kg feed (Group 3) showed the lowest but not significantly different (p>0.05) glucose levels on day 22 and 44 than other groups.

Table 1: Growth performance of rainbow trout treated with (Group 2: diet supplemented with 1 g probiotic, Group 3: diet supplemented with 5 g probiotic, Group 4: diet supplemented with 10 g probiotic) or without (Group 1) probiotic for 44 days.

Group	Initial weight (g)	Final weight (g)	WGR (%)	SGR (%)	CF (%)
1	81.64 ± 1.44^{a}	179.51 ± 3.68^{a}	119.87 ± 7.02^{a}	0.77 ± 0.07^{a}	1.44 ± 0.23^{a}
2	75.18 ± 1.54^{b}	156.25 ± 3.03^{b}	107.87 ± 6.05^{ac}	0.72 ± 0.06^{ac}	1.29 ± 0.21^{a}
3	74.70 ± 2.20^{b}	148.10 ± 2.40^{c}	98.25 ± 7.08^{bc}	0.68 ± 0.06^{ac}	1.32 ± 0.15^{a}
4	79.63 ± 2.04^{c}	150.10 ± 2.30^{c}	88.49 ± 7.21^{b}	0.61 ± 0.07^{bc}	1.33 ± 0.19^{a}

Results are presented as means ± SD of triplicate observations.
Means in the same column with different superscripts are significantly different (p<0.05).

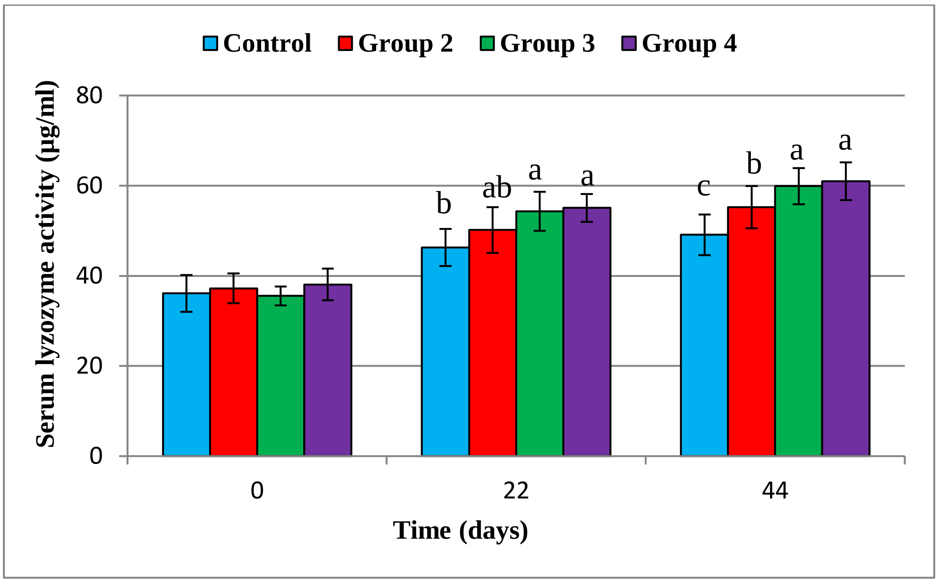

Figure 1: Lysozyme activity of rainbow trout treated with (Group 2: diet supplemented with 1 g probiotic, Group 3: diet supplemented with 5 g probiotic, Group 4: diet supplemented with 10 g probiotic) or without (Group 1) probiotic for 44 days. Different letters above the columns are significantly different (*p*<0.05).

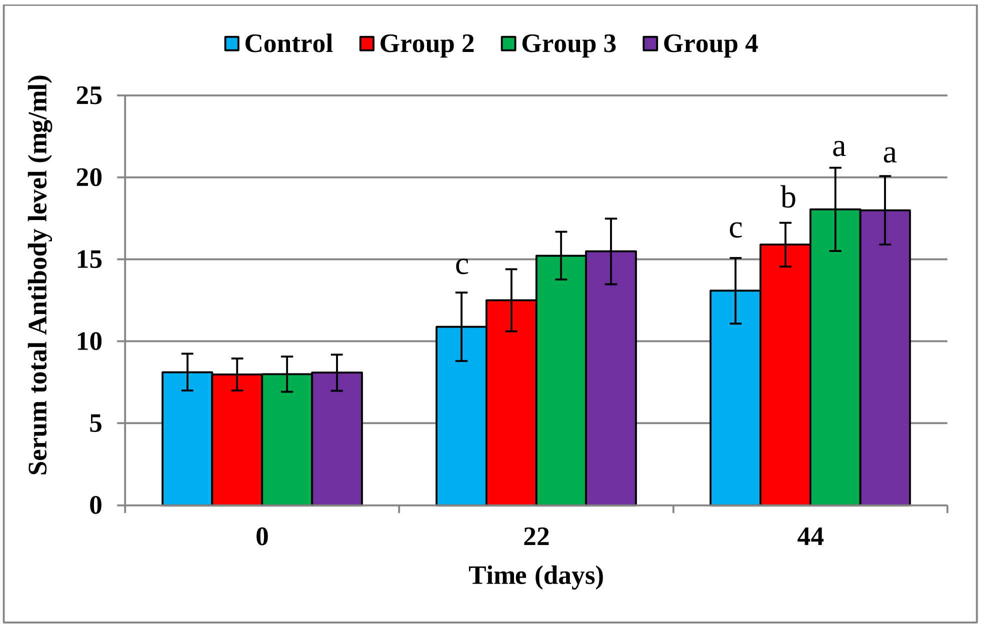

Figure 2: Serum total antibody level of rainbow trout treated with (Group 2: diet supplemented with 1 g probiotic, Group 3: diet supplemented with 5 g probiotic, Group 4: diet supplemented with 10 g probiotic) or without (Group 1) probiotic for 44 days. Different letters above the columns are significantly different (*p*<0.05).

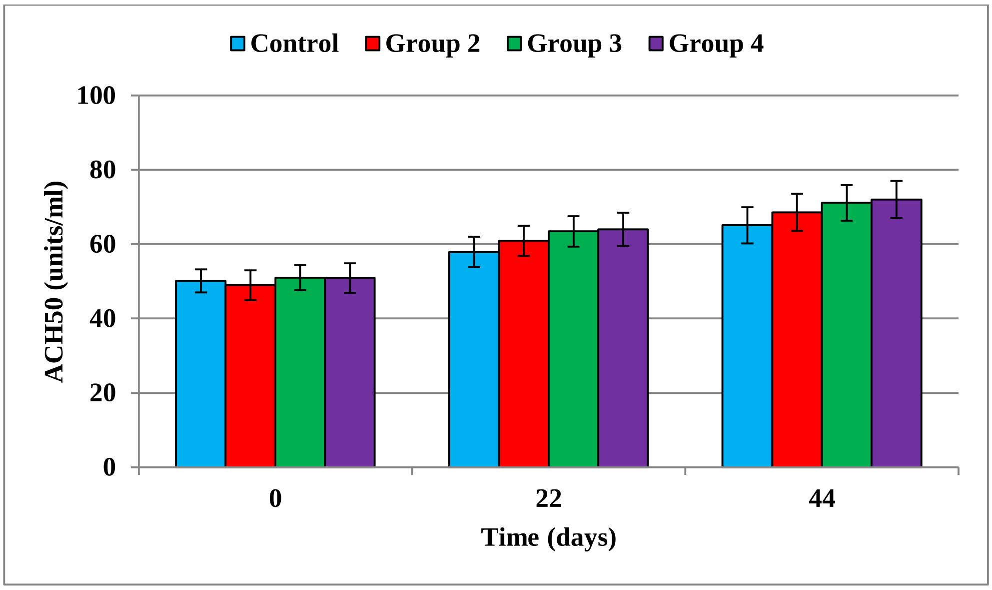

Figure 3: Alternative complement activity of rainbow trout treated with (Group 2: diet supplemented with 1 g probiotic, Group 3: diet supplemented with 5 g probiotic, Group 4: diet supplemented with 10 g probiotic) or without (Group 1) probiotic for 44 days. Different letters above the columns are significantly different (*p*<0.05).

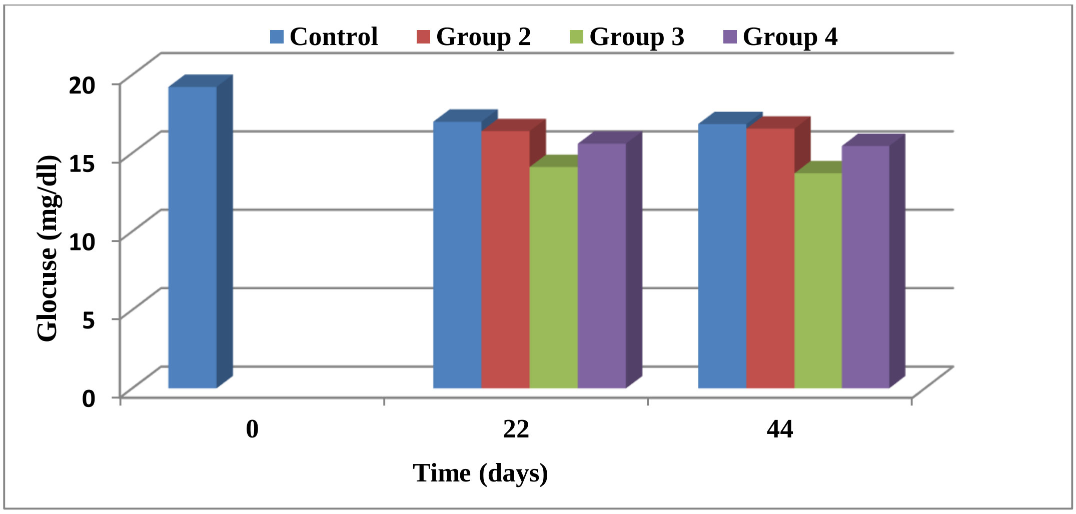

Figure 4: Blood glucose level of rainbow trout treated with (Group 2: diet supplemented with 1 g probiotic, Group 3: diet supplemented with 5 g probiotic, Group 4: diet supplemented with 10 g probiotic) or without (Group 1) probiotic for 44 days. Different letters above the columns are significantly different (*p*<0.05).

Discussion

Probiotics are known as living microbial cells that enhance the health of their host by improving the microbial balance of the gut (Al-Dohail *et al*., 2009). In the present study, it was shown that probiotic supplementation with *B.subtilis* after a 44 day feeding trial was ineffective on WGR, SGR and CF. Similarly, Shen *et al.* (2010) reported that the shrimps fed on*B.subtilis* at doses of 1×10^4 and 5×10^4 CFUg $^{-1}$ feed showed significantly better growth factors than that of the control, but a dose of 1×10^5CFUg $^{-1}$ had no effect on WGR and final weight. In another study it was shown that supplementation of either citrus by-product fermented (CBF) with several probiotic bacteria or graded levels of CBF fermented with *B. subtilis* did not exhibit any significant difference on the growth performance of juvenile olive flounder in optimum or low water temperature conditions (Lee *et al*., 2013). Ridha & Azad (2012) evaluated the effects of two bacteria, *Bacillus amyloliquefaciens* and the dairy yogurt (DY) *Lactobacillus* sp., on improving the growth performance of Nile tilapia *Oreochromis niloticus.* They results showed that at the end of the probiotic-feeding phase (day 99), no significant difference was observed in all parameters (including mean the body weight, specific growth rate, percentage increase in body weight and feed conversion ratio) between the three treatments (treatment 1: diet supplemented with *B.amyloliquefaciens*, treatment 2: diet supplemented with *Lactobacillus* sp, treatment 3: control). According to these results, ineffective performance of *B.subtilis* supplementation on growth parameters in our study may be attributed to requirements for a longer period of time or requirements to higher concentrations of the probiotic supplements in diets to obtain a significant improvement in growth factors(Shen *et al*., 2010, Ridha and Azad, 2012).

Among the different beneficial effects of probiotics, modulation of immune system is one of the most commonly intended benefits of the probiotics. The results achieved as illustrated in Fig. 2 show that serum total protein increased insignificantly (p>0.05) in groups 3 and 4 on the 44th day of the evaluation process. B lymphocytes are the main cells involved in the secretion of immunoglobolins including serum IgM levels that were found to be stimulated in fish fed probiotics (Kaattari *et al.*, 2009). Elevation of immunoglobulin levels by probiotics supplementation is reported by many authors (Nayak, 2010) that are in agreement with our findings.

Lysozyme, being a cationic enzyme with antimicrobial activity, can split the peptidoglycan layer in bacterial cell walls, especially of the gram-positive species and, in conjunction with complements, even some gram-negative bacteria, and can cause lysis of the cell wall (Balcazer *et al*., 2007). Our results showed that lysozyme activity significantly increased during the experimental days. It showed higher and nosignificant differences between group 3 and 4 on each evaluation day. These findings were in agreement with Liu *et al.* (2012) who studied the effects of *B. subtilis* in grouper, *Epinephelus coioides*. They found fish fed diets containing *B. subtilis* at 10^4, 10^6 and 10^8 cfu g^{-1} were significantly and dose-dependently higher lysozyme activity than those of fish fed the control diet for 28 days, i.e. 10^8 cfu g^{-1} of *B. subtilis*showed significant and highest lysozyme activity in grouper, *E.coioides*. On the contrary, many studies showed dietary supplementation of fish with *B.subtilis* failed to activate lysozyme activity. For example Zhou *et al*. (2010) found serum lysozyme content of tilapia was not affected by treatment with probiotics as water additives.

The complement system is composed of more than 35 soluble plasma proteins that are in zymogene forms and play key roles in innate and adaptive immunity. Complement is started by one or a combination of three pathways, including the alternative, lectin and classical. The alternative complement pathway is antibody independent and can be directly activated by the lipopolysaccharide of Gram-negative bacteria, and can result in lysis of the bacterial cell (Balcazer *et al*., 2007). This system as a component of the non-specific defense mechanism is more important in fish than in mammals;hence, higher levels could indicate better fish resistance to diseases (Tukmechi and Bandboni, 2014). Probiotics can increase natural complement activity of fish and many studies reported that dietary as well as water treatment of many probiotics stimulate the complement components(Nayak, 2010). Our results showed that probiotic supplemented diet have increased the complement system activity.However the control group showed no significant difference with each of the treated groups during evaluationdays. Balcazer *et al*. (2007) investigated the effects of different *Lactococcus* and *Lactobacillus* species on the cellular and humoral immune responses of rainbow trout. According to their results serum alternative complement activity increased significantly and lysozime activity decreased significantly in all probiotic treated groups after 2 weeks. Our result can be attributed to antibody independency of alternative complement pathway, however, different variables such as the probiotic dosage, the treatment duration, and/or the fish species may be affective on *B. subtilis* effects on complement activity (Cerezuela *et al*., 2012).More studies are needed to understand the cause of such observed effects on this important humoral immune component.

Increased levels of glucose in the blood are commonly used as indicators of stress. Dias *et al*. (2012) investigated the effects of *B.subtilis* as a dietary supplement on several hematological aspects of female matrinxa (*Brycon* *amazonicus*) during reproduction period. Results showed that plasma glucose levels increased significantly in probiotic treated group in comparison to the time- zero group, but this elevation in females was insignificant and in males was significant as compared to that in the control group. Their observation showed that probiotic treatment was not strongly affective on reduction of serum glucose in femalesduring reproduction period and the reproduction period was stressful for the fish (Dias *et al.,* 2012). In our study group 3 exhibited best and lowest glucose during evaluation period. Similarly Bandyopadhyay and Mohapatra (2008) reported that 2 × 10^5 cells of *Bacillus circulans* per 100 g feed was more affective in glucose reduction rather than high dose of this probiotic.

The findings of the present study demonstrated that the dietary administration with different concentrations of *Vibrio* control *B.subtilis* does not affect growth performance during 44 days of experiment, whereas it significantly enhanced the immune responses. This strain is a good candidate feed additive to improve fish immune response. However, further extensive investigations, including different bacterial doses, longer period of experiment, combination with other probitic bacteria also herbal extracts or other additives and a full immune system analysis, are needed prior to its widespread application in aquaculture.

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