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Studies on *Bacillus subtilis*, as Potential Probiotics, on the Hematological and Biochemical Parameters of Rainbow trout, *Oncorhynchus mykiss* (Walbaum)

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Abstract The probiotic activity of *Bacillus subtilis* was evaluated by its effect on the hematological and biochemical factors of rainbow trout. The experience was carried out in 2 groups (Control and Treatment) and 3 replicates. In Control group, probiotic was not applied in diet but in Treatment group, *B. subtilis* was administered in feed at a concentration of 10^7 cells/g. In the day of thirty, 5 blood samples were caught from every replicate for biochemical and hematological experiments. Results showed that *B. subtilis* addition to diet had not effect on erythrocyte count, hemoglobin, hematocrit, mean cell volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) and there was no significant difference among two groups ($p>0.05$). But the leucocyte count, percent of lymphocyte, serum total protein, serum albumin, IgM and lysozyme of T group was significantly higher than that of C group ($p<0.05$), whereas percent of neutrophile and monocyte of C group was significantly higher than that of T group ($p<0.05$). The results suggest that *B. subtilis* can stimulate immune parameters in rainbow trout.

Keywords: *probiotic, Bacillus subtilis, rainbow trout, Hematological and Biochemical factors*

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1. Introduction

Rainbow trout (*oncorhynchus mykiss*) is one of the most significant trading species of the salmons which are cultured extensively in the many countries of the world. This species is the most important water-cool culturing fish in IRAN. The bacterial infections are decreasing factors of the reproduction and culture farms which one of the commonest method of the treatment of these infections is to use the antibiotics [[1]] but, application of the antibiotics in order to treatment the fish diseases has been criticized extensively [2,3,4,5]. Today, probiotics or microbial supplements are to be placed against the antibiotics [6]. Probiotics are supplemental microorganisms such as bacteria, fungi and yeasts which increase the health of the host through balancing the microbial flora of the gastrointestinal tract [7-12]. The species which are to be used more inclued the *Bacillus coagolans*, *Bacillus cereus*, *Bacillus subtilis*, *Bacillus claisey* and *Bacillus licheniformis.* Which when they are used as the probiotics in form of the feeding supplement, they will lead to the stimulation of the immune system and have the antimicrobial activity and competitive prevention [13,14,15,16]. Since the genus of the *Bacillus* in the water organisms has not been reported as the pathogen, it is used widely in the aquatic culture. *Bacillus* is able to produce the antibiotics, amino acides and enzymes and *B. subtilis* produces and number of the peptide, including the subtillin and bacitracin [8]. As a result, in this research, the effect of the *B. subtilis* as the probiotic on the hematological and biochemical factors of the rainbow trout was studied.

2. Materials and Methods

2.1. Fish

This study was carried out in winter 2011 at Rainbow trout, *Oncorhynchus mykiss* (Walbaum) of 60 gram average weight were obtained from a commercial fish farm in north of Iran for a period of 60 days. Samples used, the number of rainbow trout was 120, which includes 2 groups (Control and Treatment) and 3 replicates. As the number of fish per pool 20 released 60 days were examined. The fish, in groups of 60, were maintained in continuously aerated free-flowing dechlorinated freshwater at 14.5°C, and fed with commercial pelleted diet at 2.4% of body weight daily [17].

2.2. Experimental Diets

The feed contained 10^7 cells/g [18] and the fish were fed to satiation three times a day for 30 days. For this, *B.subtilis* PTCC 1720 cultures were grown for 48 h at 25°C in blood agar. The culture was centrifuged at 4000 g for 10 min at 4°C, was washed three times in 0.9% (w/v) saline, and was prepared a suspension in 0.9% (w/v) saline to achieve an absorbance of 0.132 at 600 nm (0.5 McFarland Standard) [19]. The resultant suspension adjusted to 10^7 cells/g.

2.3. Blood Sampling

15 fish were randomly collected from each groups. The fish were anesthetized by immersion in water containing 0.1 ppm tricaine methane sulfonate (MS-222) [20]. Whole blood (3 ml) was collected from the caudal vein [21] of each fish at day 30 using syringes (5-ml) and 0.5 ml were rinsed in Eppendorf tubes with heparin (15 unit ml^{-1}) [20], to determine hematological factors include Red Blood Cells (RBC), White Blood Cells (WBC), Hemoglobin (Hb), Hematocrit (Hct), Mean Cell Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC) and Differential Count Leukocyte (Diff). For separation of serum, blood samples (2.5 ml) were withdrawn from the fish caudal vein, as before, and transferred to Eppendorf tubes without anticoagulant. The blood samples were centrifuged at 3000 g for 15 min and the supernatant serum was collected and stored at -20°C until used for biochemical factors include Lysozyme, IgM, Total protein and Albumin [21].

2.4. Red Blood Cells Count and White Blood Cells Count

The blood was used to determine the number of erythrocytes by means of a Neubauer hemocytometer slide at a magnification of ×400. The blood was diluted to 1:50 in 0.9% (w/v) saline. Count the erythrocytes occurring in five small squares at the centre of the grid, a total area of 0.02 mm^3 (1/50 of 1 mm^3). The total area counted here (0.02mm^3, at a dilution of 1:50) should be sufficient for an accurate count to be obtained. The dilution is 1:50, therefore the number of cells occurring per mm^3 may be calculated as follows:

Number of cells occurring per mm^3 = Number of cells counted in 0.02 mm^3 × 50 (area counted) × 50 (dilution) [22].

The blood was used to determine the number of leucocyte by means of a Neubauer hemocytometer slide at a magnification of ×400. The blood was diluted to 1:50 in Dacies fluid [21,22]. Count the leucocytes occurring in the four corner squares marked on the grid, a total area of 0.1 mm^3. The total area counted here (0.1 mm^3, at a dilution of 1:50), should be sufficient for an accurate count to be obtained. The dilution is 1:50, therefore the number of cells occurring per mm^3 may be calculated as follows:

Number of cells occurring per mm^3 = Number of cells counted in 0.1 mm^3 × 10 (area counted) × 50 (dilution) [22].

2.5. Hemoglobin Level

The precision of various methods for estimating hemoglobin concentration may differ considerably. The cyanohemoglobin method has been the standard method used in hematological studies for a number of decades. Used a pipette add a sample of 20 µl of blood to 5 ml of Drabkin's solution in a test tube and mix thoroughly. Place approximately 2 ml of the resulting solution into a cuvette, and read the absorbance values in a spectrophotometer at 540 nm. The hemoglobin concentration of the blood sample can be calculated from a curve prepared from known standards [22].

2.6. Hematocrit Level

Hematocrit capillary tubes were two-third filled with the whole blood and centrifuged in a hematocrit centrifuge for 5 min at 13500 rpm and the percentage of the packed cell-volume was determined by the hematocrit tube reader [22].

2.7. Red Blood Cell Indices

Red blood cell indices are blood tests that provide information about the hemoglobin content and size of red blood cells [23]. Mean corpuscular volume (MCV) is the average size of a red blood cell and is calculated by dividing the hematocrit by the red blood cell count.

$$MCV = \frac{Hct}{RBC}$$

Mean corpuscular hemoglobin (MCH) is the average amount of hemoglobin (Hb) per red blood cell and is calculated by dividing the hemoglobin by the red blood cell count.

$$MCH = \frac{Hb}{RBC}$$

Mean corpuscular hemoglobin concentration (MCHC) is the average concentration of hemoglobin per red blood cell and is calculated by dividing the hemoglobin by the hematocrit.

$$MCHC = \frac{Hb}{Hct}$$

2.8. Differential Leucocyte Counts

Blood films from duplicate samples were prepared on glass icroscope slides with fixation for 5 min in 96% methanol. After air-drying for a few minutes at room temperature, staining was by Giemsa's method [24], and the preparations were examined at ×1000 to determine the proportion of neutrophile, monocytes and lymphocytes [23]. Triplicate groups of 100–200 cells were counted on each of the slides.

2.9. Serum Lysozyme

Lysozyme assay which is based on lysis of lysozyme-sensitive gram positive bacterium *Micrococcus lysodeikticus* [25]. Lysozyme activity of rainbow trout plasma was measured using a modified turbidimetry method described by Ellis [26]. Briefly, a standard suspension of 0.375 g ml^{-1} *M. lysodeikticus* (Sigma) was prepared in 1 mL PBS (pH 5.8). Rainbow trout serum (25 mL) was added to 175 mL of bacterial suspension, and the optical density was measured after 15 and 180 seconds by

spectrophotometer at 670 nm. One unit of lysozyme activity was defined as reduction in absorbance of 0.001/min. The units of lysozyme present in sera were obtained from a standard curve made with hen egg white lysozyme (Sigma).

2.10. IgM

IgM concentration was determined by nephelometry method (MININEPH TM Human Kit, the binding Site Ltd, Birmingham, UK).

2.11. Serum Total Protein & Serum Albumin

Serum total protein and serum albumin were measured by Pars Azmon Kite (Pars Azmon, Iran) in Autoanalyser (hoshmand-fanavar Co, Tehran, Iran).

2.12. Statistical Analysis

Significant differences among treatment groups were tested by one-way analysis of variance (ANOVA) and the comparison of any two mean values was made by Duncan's multiple range tests. A significance level of $P < 0.05$ was used. The statistical analysis was performed by using the software program Statistical Package for the Social Sciences (SPSS).

3. Results

Hematology and biochemistry experiments showed *B.subtilis* addition to diet had not effect on erythrocyte count, hemoglobin, hematocrit, mean cell volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration and there was no significant difference among two groups.

The erythrocyte counts for the probiotic treated and control fish were 1.15 ± 0.05 $\times 10^9$/ml and 1.09 ± 0.06 $\times 10^9$/ml, respectively. The hemoglobin in *B. subtilis* fed fish was 7.67 ± 0.15 g/dl and in control fish was 7.73 ± 0.18 g/dl. The percent of hematocrit for the probiotic treated and control fish were 39.77 ± 0.69 and 40.83 ± 1.26, respectively. The mean cell volume in treatment group was 353.65 ± 14.13 fl and in control group was 381.64 ± 16.36 fl. The mean corpuscular hemoglobin for the probiotic treated and control fish were 68.44 ± 3.34 and 72.69 ± 3.43 pg, respectively. The mean corpuscular hemoglobin concentration in *B. subtilis* fed fish was 19.33 ± 0.38 g/dl and in control fish was 19.02 ± 0.28 g/dl (Table 1).

Table 1. Effect of *Bacillus subtilis* on hematological factors of rainbow trout (The values are presented as mean ± SE)

Hematological factors	Treatment	Control
Erythrocytes ($\times 10^9$ ml^{-1})	1.15 ± 0.05	1.09 ± 0.06
Hct (%)	39.77 ± 0.69	40.83 ± 1.26
Hb (g/dl)	7.67 ± 0.15	7.73 ± 0.18
MCV (fl)	353.65 ± 14.13	381.64 ± 16.36
MCH (pg)	68.44 ± 3.34	72.69 ± 3.43
MCHC (g/dl)	19.33 ± 0.38	19.02 ± 0.28
Leucocytes($\times 10^6$ /ml)	37.133 ± 1.24	31.467 ± 2.38
Lymphocyte(%)	97.93 ± 0.36	96.33 ± 0.69
Neutrophile(%)	2.00 ± 0.34	3.47 ± 0.64
Monocyte(%)	0.07 ± 0.07	0.20 ± 0.11

Hct: Hematocrit, Hb: Hemoglobin, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration

But the leukocyte count, percent of lymphocyte, serum total protein, serum albumin, IgM and lysozyme of T group was significantly higher than that of C group, whereas percent of neutrophile and monocyte of C group was significantly higher than that of T group(Table 1& Table 2).

Table 2. Effect of *Bacillus subtilis* on biochemical factors of rainbow trout (The values are presented as mean ± SE)

Biochemical factors	Treatment	Control
TP (g/dl)	4.75 ± 0.11	4.44 ± 0.10
Alb (g/dl)	2.63 ± 0.10	2.29 ± 0.12
IgM(mg/dl)	41.28 ± 5.01	28.55 ± 3.53
Lys (mg/ml)	2.94 ± 0.45	1.52 ± 0.29

TP: Total protein, Alb: Albumin, Lys: Lysozyme

Generally, there was stimulation of the immune system (both cellular and humoral immune) after administering *B. subtilis* to rainbow trout. Specifically, the number of leucocytes increased from 31.467 ± 2.38 $\times 10^6$ /ml in the control group to 37.133 ± 1.24 $\times 10^6$ /ml in *B. subtilis* fed fish. The percent of lymphocyte of *B. subtilis* fed fish (97.93 ± 0.36) was significantly higher than that of controls (96.33 ± 0.69).

In addition, the serum lysozyme activity was recorded as 2.94 ± 0.45 mg/ml and 1.52 ± 0.29 mg/ml for *B. subtilis* treated and control fish, respectively.

There were statistically significant differences in the IgM from fish which received probiotics (41.28 ± 5.01 mg/dl) as compared with the controls (28.55 ± 3.53 mg/dl).

fish fed with *B. subtilis* showed a higher total protein (4.75 ± 0.11 g/dl) as compared with the controls (4.44 ± 0.10 g/dl). Serum albumin was significantly higher in treatment group (2.63 ± 0.10 g/dl) as compared with the controls (2.29 ± 0.12 g/dl) (Table 2).

The percent of monocyte in treatment group was 0.07 ± 0.07 and in control group was 0.20 ± 0.11. Moreover, the percent of neutrophile from *B. subtilis* fed fish (2.00 ± 0.34) was significantly lower than that of controls (3.47 ± 0.64) (Table 1).

4. Discussion

With regard to the existence of the ideal condition in the mazandaran province in order to culture the trout fish has been developed much quickly within recent years, new farms have been established and the rate of production has been increased noticeably. Nevertheless, varieties of the infectious diseases develop among the population of the fish [27]. Considering this fact that generating agent of the disease exists in the aquatic environments all the year, it seems that the protection from the fish against the pathogenic agents is the most significant, easiest and the most inexpensive way in order to prevent from the damages and losses resulting from the occurrence of the diseases in the cultures of the aquatics.

The results of the present study indicate that *B. subtilis* stimulated both cellular and humoral immune responses. The role of probiotics in influencing immune responses in fish has been previously reported as having important regulatory effects on the innate and adaptive immune responses of the host [28,29]. The addition of the *B. subtilis* in the nutrient ration of the rainbow trout had no effect on the rate of hematocrit, hemoglobin, number of the red blood cell, MCV, MCH and MCHC. Which the similar results were also obtained by other authors

[30,31,32,18]. Also addition of the *B. subtilis* in the nutrient ration of the rainbow trout was led to the increase of the white blood cells which the similar results were also obtained by other reports [18,31,33,34,35]. Also showed that the usage of the probiotic in the nutrient ration was led to the increase of with blood cells.

The above mentioned results indicate that *B. subtilis* leads to the increase of the percent of the lymphocyte while reducing the percent of the neutrophile and monocyte which is because of the effect of the probiotic on the immunity system and its stimulation so that they can cause to increase the B lymphocytes in the fish [28,36]. Also, lymphocytes are one of the most important protective factors of the fish against the microbial agents so that Th2 cell, while stimulating, secrete cytokines, including interleukin 4 which leads to the reinforcement of the growth of the precursor cells of the hematopoietic and more distinction of the cellular families of the myeloid and intensifies noticeably the activity of the fatality of the macrophages [37]. Increase of the percent of the lymphocyte trout racing the potential of the Immune system leads to the increase of the resistance against the pathogenic agents, the environmental stimulations and stresses which this affair can cause to improve to the growth, decrease the rate of the mortality and increase the survival [36].

With regard to the increase of the total protein and albumin in the treatment group, it can be concluded that the usage of the increase of the mentioned factors. In the confirmation of the above findings, the similar results were obtained by the other researchers [16,18,38,39,40]. The rate of IgM in the treatment group was higher than control group which the obtained results corresponded with the results of the other authors [41,42].

Various probiotics or one species or a few species together can cause to increase the phagocytosis, lysozyme, respiratory explosion and also to produce different cytokines in the fish and they can stimulate the immunity system of the fish's stomach through increasing the cells of the immunoglobulin and acidophil granulocyte [43]. The process of the production of the immunoglobulins in the fish is the occurrence of a collection of the reactions among the antigen presenting cells, the activated T helper cells and interleukins which stimulates the B lymphocytes. These lymphocytes produce the plasma cells as a result of the stimulation which are able to secrete the immunoglobulin [33]. The result obtained from the rate of the lysozyme serum suggest that the lysozyme serum in the treatment group receiving the probiotic was higher than the control group which other researchers achieved the similar results in this regard [18,33,36,38,39,41]. Lysozyme is an important humoral innate defence parameter, and is widely distributed in invertebrates and vertebrates [44]. Lysozyme has an antibacterial activity by attacking peptidoglycan in the cell wall of bacteria, predominantly Gram-positive bacteria, thereby causing lysis and stimulation of phagocytosis of bacteria by phagocytic cells. An increase in the lysozyme concentration in fish blood can be caused by infections or invasion by foreign material [45].

It can thus be concluded that *B. subtilis* can stimulate immune parameters in rainbow trout. The immune responses of rainbow trout to *B. subtilis* included a significant increase in the number of leukocyte count, percent of lymphocyte, serum total protein, serum albumin, IgM and lysozyme. It is also suggested that some more experiments may be conducted using some other species of fishes and other doses of *B. subtilis* in order to establish the role of *B. subtilis* as an immunostimulator.

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