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Evaluation of *Bacillus subtilis* Effect as Probiotic on Hematological Parameters of Rainbow Trout, *Oncorhynchus mykiss* (Walbaum) Following Experimental Infection with *Streptococcus iniae*

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ABSTRACT

In this study, *Bacillus subtilis* is used for control of streptococcosis with the agent of *Streptococcus iniae* in rainbow trout. The experience was carried out in 2 groups (Control (c) and Treatment (T)) and 3 replicates. In control group, probiotic was not applied in diet but in treatment group, *Bacillus subtilis* was administered in feed at a concentration of 10^7 cells g^{-1}. At 45th day, 0.1 mL intraperitoneally injection of *S. iniae* with 2×10^7 cells mL^{-1} dosage was given to both groups and were checked for the rest of the survey duration (2 weeks). At the end of the time which took about two months, blood samples were caught for hematological experiments for realizing the effect of *B. subtilis* feeding on resistance of fish against *S. iniae* infection. After injection of *S. iniae,* there was no significant difference among T and C groups considering parameters such as erythrocyte count, hemoglobin, hematocrit, Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC) and percent of monocyte ($p>0.05$). But significant difference was seen in the leucocyte count, percent of lymphocyte and neutrophile in both groups ($p<0.05$). The leucocyte count and percent of lymphocyte was higher in T group and neutrophile percent was lower in comparison with the control. The results of the present study indicate that *B. subtilis* can be used as an agent for the control of streptococcosis in rainbow trout hatchery and culture farms for decreasing economical disasters.

Key words: Probiotic *Bacillus subtilis*, rainbow trout, streptococcosis, *Streptococcus iniae*

INTRODUCTION

As the sole water-cool culturing species being available in the country, rainbow trout is of very much significance. In 2009, Iran, by producing almost 73000 tons of the rainbow trout, a located the first position of the world to itself regarding the production of the rainbow trout in the freshwater. One of the significant bacteria diseases in the rainbow trout is streptococcosis whose causal agents are the various species of the *Streptococcus* (Garedaghi *et al*., 2011; Haghighi *et al*., 2010; Yanong and Francis-Floyd, 2002). This disease was reported for the first time in the rainbow trout in Japan in 1958 (Hoshina *et al*., 1958). After that, the disease was reported in different fresh and saline water fishing, including striped bass, sturgeon, tilapia, sea trout, eel, pinfish, golden shiner, mullet and salmon (Inglis *et al*., 1993).

Streptococcus iniae is one of the most important species of cause streptococcosis which, for the first time, isolated in 1979 from the subcutaneous abscesses of a sample of the Amazon river's dolphin being afflicted by an infection under the topic of "golf ball disease" (Austin and Austin, 2007). Out of consideration for the severe damages losses and high mortality in the fish population, streptococcosis have converted in to the most significant bacteria disease of the farms for fish culture which is of a particular healthful important considering its transfer to human. Hence, its control is necessary affair and many measures have been taken in recent years in order to control and treat the infection origination from the *Streptococcus iniae* including it can refer to the usage of the antibiotics such as furazolidon, oxytetracycline, erythromycin and amoxicillin (Agnew and Barnes, 2007). After many years these drugs, by the selves, have created several problem, including the resistant-becoming (resistance) of the pathogenic factors bio environmental problems etc (WHO, 2006).

Therefore, application of the probiotics as a replacement for the former methods has been set raised which it seems that it can obviate many problems (Deeseenthum *et al*., 2007). 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 (Vivas *et al*., 2004; Bagheri *et al*., 2008; Hung *et al*., 2008; Zhou *et al*., 2009; Son *et al*., 2009; Agouz and Anwer, 2011). Usage of the probiotics, in fact, is considered as a new technology of the aquaculture being in sync with the bioenvironmental. Using these materials, both the production can be increased and the quality of the water can be corrected and they can be taken into consideration as a biological fight. The effect of the probiotics in the nutrition, resistance against the diseases and the other useful activities has been confirmed which out of the useful effects they have on the health, the effect is one the immune system and stimulation of the immune system (Abraham *et al*., 2008; Dalmo and Bogwald, 2008; Soundarapandian and Sankar, 2008; Vijayabaskar and Somasundaram, 2008). From amongst these Probiotics, the *Bacillus* species can be referred to, for example (Mohamed and Refat, 2011; Wang *et al*., 2008; Lakshmanan and Soundarapandian, 2008). The *Bacillus* species have been using as the probiotics for less than 50 years. Of the species that have been most extensively examined these are *Bacillus subtilis*, *Bacillus clausii*, *Bacillus cereus*, *Bacillus coagulans*, *Bacillus circulans* and *Bacillus licheniformis* (Cutting, 2010; Bandyopadhyay and Mohapatra, 2009; Farzanfar *et al*., 2009).

As genus of *Bacillus* has not been reported as the pathogen in the water organisms, it is thus used widely in the aquaculture and the effect of the *Bacillus subtilis* on the hematological factors of the rainbow trout was studied in this research followed by the empirical infection with *Streptococcus iniae*.

MATERIALS AND METHODS

Fish: This study was carried out in winter 2011 at rainbow trout, *Oncorhynchus mykiss* (Walbaum) of 60 g average weight were obtained from a commercial fish farm in north of Iran for a period of 60 days. 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 (NRC, 1993) at 2.4% of body weight daily.

Experimental diets: The feed contained 10^7 cells g^{-1} (Newaj-Fyzul *et al*., 2007) and the fish were fed to satiation three times a day for 45 days before challenge with *S. iniae*. For this, *Bacillus 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) (MacFarland, 2000). The resultant suspension adjusted to 10^7 cells g^{-1}. In control group, probiotic was not applied in diet but in treatment group, *Bacillus subtilis* was administered in feed at a concentration of 10^7 cells g^{-1}.

Bacterial pathogen: *Streptococcus iniae* isolated from diseased rainbow trout, *Oncorhynchus mykiss* (Walbaum) and identified by phenotyping tests. Bacterial isolates were grown for 48 h at 25°C in blood agar, The culture was grown for 48-75 h at 25°C in tryptic soy broth, harvested by centrifugation at 4000 g for 10 min at 4°C and 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) (MacFarland, 2000). The resultant suspension adjusted to 2×10^7 cells mL^{-1}.

Challenge test: Forty five days after the start of the feeding experiments, 60 fish were collected from each of the treated and control groups. Fishes were challenged intraperitoneally injection with 0.1 mL of fresh culture suspension containing 2×10^7 bacteria mL^{-1} *Streptococcus iniae* (Brunt *et al*., 2007).

Blood sampling: Fifteen fish were randomly collected from each groups. The fish were anesthetized by immersion in water containing 0.1 ppm tricaine methane sulfonate (MS-222). Whole blood was collected from the caudal vein (Abdelhamid *et al*., 2009) of each fish at day 60 using syringes 2 and 0.5 mL were rinsed in Eppendorf tubes with heparin (15 unit mL^{-1}) (Larsen, 1964), 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 Leucocyte (Diff).

Red blood cells count: The blood was used to determine the number of erythrocytes by means of a Neubauer hemocytometer slide at a magnification of x400. 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 (0.02 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 (IMBC, 1998):

$$\text{No. of cells occurring mm}^{-3} = \text{No. of cells counted in 0.02 mm}^{-3} \times 50 \text{ (area counted)} \times 50 \text{ (dilution)}$$

White blood cells count: 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 (IMBC, 1998; Abdelhamid *et al*., 2009). 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 (IMBC, 1998):

$$\text{No. of cells occurring mm}^{-3} = \text{No. of cells counted in 0.1 mm}^{-3} \times 10 \text{ (area counted)} \times 50 \text{ (dilution)}$$

Hemoglobin level: 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 (IMBC, 1998).

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 (IMBC, 1998).

Red blood cell indices: Red blood cell indices are blood tests that provide information about the hemoglobin content and size of red blood cells (Benfey and Sutterlin, 1984).

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:

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

Differential leucocyte counts: Blood films from duplicate samples were prepared on glass microscope 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 (Thrall, 2004) and the preparations were examined at ×1000 to determine the proportion of neutrophile, monocytes and lymphocytes (Benfey and Sutterlin, 1984). Triplicate groups of 100-200 cells were counted on each of the slides.

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 SPSS (version 18).

RESULTS

Results showed that after feeding of *B. subtilis* treatment group for forty five days then injection of *S. iniae* to both of Control and Treatment groups, the death rates in C group was 54-60% and in T group was 25-31%.

Table 1: Effect of *Bacillus subtilis* on hematological factors of rainbow trout after challenge with *Streptococcus iniae*

Hematological factors	Treatment	Control
Erythrocytes ($\times 10^9$ mL^{-1})	0.56±0.06^b	0.48±0.05^b
Hematocrit (%)	25.83±2.25^b	24.90±2.55^b
Hemoglobin (g dL^{-1})	4.37±044^b	4.10±0.41^b
Mean corpuscular volume (fl)	480.06±24.12^a	539.16±30.99^a
Mean corpuscular hemoglobin (pg)	79.51±3.75^{ab}	89.75±5.91^a
Mean corpuscular hemoglobin concentration (g dL^{-1})	16.66±0.39^b	16.61±0.34^b
Leucocytes ($\times 10^6$ mL^{-1})	36.87±4.22^a	16.37±2.10^b
Lymphocyte (%)	95.53±0.98^a	91.93±1.28^b
Neutrophile (%)	4.07±0.92^b	7.47±1.18^a
Monocyte (%)	0.40±0.13^{ab}	0.60±0.19^a

The values are presented as Mean±SE, Means with the same letter for each parameter are not significantly different at p>0.05

After injection of *S. iniae,* there were no statistically significant differences among T and C groups considering parameters such as erythrocyte count, hemoglobin, hematocrit, mean cell volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and percent of monocyte (p>0.05). The erythrocyte counts for the probiotic treated and control fish were 0.56±0.06×0^9 mL^{-1} and 0.48±0.05×10^9 mL^{-1}, respectively. The hemoglobin in *Bacillus subtilis* fed fish was 4.37±044 g dL^{-1} and in control fish was 4.10±0.41 g dL^{-1}. The percent of hematocrit for the probiotic treated and control fish were 25.83±2.25 and 24.90±2.55, respectively. The mean cell volume in treatment group was 480.06±24.12 femtoliter and in control group was 539.16±30.99 femtoliter. The mean corpuscular hemoglobin for the probiotic treated and control fish were 79.51±3.75 and 89.75±5.91 picogram, respectively. The mean corpuscular hemoglobin concentration in *Bacillus subtilis* -fed fish was 16.66±0.39 g dL^{-1} and in control fish was 16.61±0.34 g dL^{-1}. The percent of monocyte in treatment group was 0.40±0.13 and in control group was 0.60±0.19 (Table 1). But significant difference was seen in the leucocyte count, percent of lymphocyte and neutrophil in both groups (p<0.05). Generally, there was stimulation of the immune system after administering *Bacillus subtilis* to rainbow trout. Specifically, the number of leucocytes increased from 16.367±2.10×10^6 mL^{-1} in the control group to 36.867±4.22×10^6 mL^{-1} in *Bacillus subtilis* fed fish. The percent of lymphocyte of *Bacillus subtilis* fed fish (95.53±0.98) was significantly higher than that of controls (91.93±1.28). In addition, the percent of neutrophil from *Bacillus subtilis* -fed fish (4.07±0.92) was significantly lower than that of controls (7.47±1.18) (Table 1).

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 which one of the most significant diseases is streptococcosis which can cause irreparable damages to the industry of the fish culture in case of the prevalence (Akhlaghi and Keshavarz, 2002). 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. As a results it was tried in this research that, trout

the probiotic prescription together which the feeding ration, to make the fish resistant against the agent of the disease and to reach a minimum the damages and losses resulting from the mentioned infection the obtained results confirm this subject so that, after the edible prescription of *Bacillus subtilis* for 45 days and then, injection of the bacterium *Streptococcus iniae* to the fish of the test and control groups, it was observed that the rate of the losses, within 14 days, in the control group which had not received probiotic was 54-60%, while it was 25-31% in the treatment group. This result was in conformity with the results of other researchers, including Newaj-Fyzul *et al.* (2007), Raida *et al.* (2003), Brunt and Austin (2005), Kumar *et al.* (2006), Aly *et al.* (2008), Vendrell *et al.* (2008) and Abbass *et al.* (2010).

Furthermore, the above findings showed that the addition of the *Bacillus 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 Newaj-Fyzul *et al.* (2007), Raida *et al.* (2003), Brunt and Austin (2005) and De Carla Dias *et al.* (2010). Also, addition of the *Bacillus subtilis* in the nutrient ration of the rainbow trout was led to the increase of the white blood cells which, in harmony with these results, Brunt and Austin (2005), Newaj-Fyzul *et al.* (2007), Tavakoli and Akhlaghi (2009), Ali *et al.* (2010), Sihag and Sharma (2012). 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 *Bacillus subtilis* leads to the increase of the percent of the lymphocyte while reducing the percent of the neutrophil 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 (Aly *et al.*, 2008; Trachoo and Boudreaux, 2006). 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 (Pangrahi *et al.*, 2005; Hoseinifar and Pooramini, 2007). 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 (Sharifuzzaman and Austin, 2009).

CONCLUSION

The results of the present study indicate that *B. subtilis* can be used as an agent for the control of streptococcosis in rainbow trout hatchery and culture farms for decreasing economical disasters and Immune system in fish can be stimulated with *B. subtilis*. It is also suggested that some more experiments may be conducted using some other doses of *B. subtilis* and finding the effect of *B. subtilis* in control of streptococcosis in different stages of fish life in order to establish the role of *B. subtilis* as an immuno-stimulator.

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