Advanced Studies in Biology, Vol. 5, 2013, no. 1, 37 - 50
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Studies on *Bacillus subtilis*, as Potential Probiotics, on the Biochemical Parameters of Rainbow trout, *Oncorhynchus mykiss* (Walbaum) to Challenge Infections

Maryam Kamgar[1], Reza Pourgholam[2], M. Ghiasi[2] and Masood Ghane [3*]

1. Department of Microbiology, Tonekabon Branch, Islamic Azad University Tonekabon, Iran, P.O. Box: 468416-1167

2. Caspian Sea Ecology Research Center, Sari, Iran, P.O. Box: 961

*3. Young Researchers club, Tonekabon Branch, Islamic Azad University Tonekabon, Iran

Corresponding author: Masood Ghane
Masoodghane@toniau.ac.ir

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 and Treatment) 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}. In the day of forty five, 0.1 ml intraperitoneally injection of *S.iniae* with 2×10^7 cells ml^{-1} dosage was done for both of 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 biochemical 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 Control(C) and Treatment(T) groups considering parameters such as glucose and aspartate aminotransferase ($p>0.05$). But significant difference was seen in the serum total protein, serum albumin, IgM, lysozyme,

urea, Alanin aminotransferase and alkaline phosphatase in both groups ($p<0.05$). The serum total protein, serum albumin, IgM and lysozyme were higher in T group and urea, Alanin aminotransferase and alkaline phosphatase were 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.

Keywords: Probiotic; *Bacillus subtilis*; Rainbow trout; *Streptococcus iniae*; Biochemical parameters

1. 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 greating agent is the various species of the *streptococcus* [1, 2, 3]. This disease was reported for the first time in the rainbow trout in Japan in 1958 [4]. After that, the disease was reported in different fresh and saline water fishing, including stripedbass, sturgeon, tilapia, sea trout, eel, pinfish, golden shiner, mullet and salmon [5].

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" [6] out of consideration for the sever 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 [7]. After many years these drugs, by the selves, have created several problems, including the Antibiotic resistant of the pathogenic bacteria and bio environmental problems etc [8].

Therefore application of the Probiotics as a replacement for the former methods has been set rose which it seems that it can prevent many problems [9]. 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 [10-15]. 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 [16-19]. From amongst these probiotics, the *Bacillus* species can be referred to, for example [20, 21, 22]. 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 circulance* and *Bacillus licheniformis* [23, 24, 25].
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 biochemical factors of the rainbow trout was studied in this research followed by the experiential infection with *streptococcus iniea.*

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. 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 [26] at 2.4% of body weight daily.

2.2 Experimental diets

The feed contained 10^7 cells g^{-1} [27], 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) [28]. 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}.

2.3 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) [28]. The resultant suspension adjusted to 2×10^7 cells ml^{-1}.

2.4 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* [29].

$$\text{Mortality \%} = \frac{\text{No. of death in a specified period}}{\text{Total population during that period}} \times 100$$

2.5 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). Whole blood was collected from the caudal vein [30] of each fish at day 60 using syringes (5-ml) and 2.5 ml were rinsed in Eppendorf tubes without anticoagulant [31]. 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 (Tp), Albumin (Alb), Glucose, Urea, Alanin aminotransferase (ALT), Aspartate aminotransferase (AST) and Alkaline phosphatase (ALP) [30].

Lysozyme assay which is based on lysis of lysozyme-sensitive gram positive bacterium *Micrococcus lysodeikticus* [32]. Lysozyme activity of rainbow trout plasma was measured using a modified turbidimetry method described by Ellis [33]. Briefly, a standard suspension of 0.375 g ml^{-1} *Micrococcus 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).

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

Serum Total protein, Albumin, Glucose, Urea, Alanin aminotransferase, Aspartate aminotransferase and Alkaline phosphatase were measured by Pars Azmon Kite (Pars Azmon, Iran) in Autoanalyser (hoshmand-fanavar Co, Tehran, Iran).

2.6 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).

3. Results

Results showed that after feeding of *B.subtilis* treatment group for forty five days then injection of *S.iniae* to both of Control(C) and Treatment(T) groups, the death rates in C group was 54-60% and in T group was 25-31%.
After injection of *S.iniae,* there were no statistically significant differences among T and C groups considering parameters such as glucose and aspartate aminotransferase ($p>0.05$). Serum glucose concentration for the probiotic treated and control fish were 34.67 ± 2.80 mg dl^{-1} and 37.53 ± 3.50 mg dl^{-1}, respectively. The aspartate aminotransferase in *Bacillus subtilis* -fed fish was 185.33 ± 16.48 UL^{-1} and in control fish was 233.29 ± 20.80 UL^{-1}. But significant difference was seen in the lysozyme, IgM, serum total protein, serum albumin, urea, alanin aminotransferase and alkaline phosphatase in both groups ($p<0.05$). The serum lysozyme activity was recorded as 2.92 ± 0.31 mg ml^{-1} and 1.75 ± 0.26 mg ml^{-1} for *Bacillus subtilis* treated and control fish, respectively. There were statistically significant differences in the IgM from fish which received probiotics (38.05 ± 4.57 mg dl^{-1}) as compared with the controls (22.78 ± 2.18 mg dl^{-1}). fish fed with *Bacillus subtilis* showed a higher total protein (3.31 ± 0.22 g dl^{-1}) as compared with the controls (2.55 ± 0.20 g dl^{-1}). Serum albumin was significantly higher in treatment group (1.92 ± 0.03 g dl^{-1}) as compared with the controls (1.55 ± 0.10 g dl^{-1}). The urea in treatment group was 4.53 ± 0.36 mg dl^{-1} and in control group was 8.57 ± 0.36 mg dl^{-1}. Moreover, the alanin aminotransferase from *Bacillus subtilis* fed fish (39.93 ± 2.65 UL^{-1}) was significantly lower than that of controls (52.47 ± 4.63 UL^{-1}). The alkaline phosphatase in treatment group was 69.27 ± 6.11 UL^{-1} and in control group was 97.87 ± 5.45 UL^{-1}. These results are illustrated in 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 which one of the most significant diseases is streptococcosis. Streptococcosis can cause irreparable damages to the industry of the fish culture in case of the prevalence [34]. 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 iniea* 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*. [27], Raida *et al*. [35], Brunt and Austin [36], Kumar *et al*. [37], Aly *et al*. [38], Vendrell *et al*. [39] and Abbass *et al*. [40].

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 glucose and AST. Also addition of the *Bacillus subtilis* in the nutrient ration of the rainbow trout was led to the increase of the lysozyme, IgM, serum total protein and serum albumin. With regard to the increase of the total protein and albumin in the treatment group, it can be concluded that the immuno- modulatory effect of *Bacillus subtilis* on the liver cells which activate the anabolic capacity of the hepatocytes to produce blood proteins [41]. In the confirmation of the above findings, the similar results were obtained by the other researchers, including Brunt *et al*. [29], Newaj *et al*. [27], Sharifuzzaman and Austin [42], Bandyopadhyay and Das Mohapatra [23] and Farzanfar *et al*., [25]. The rate of IgM in the treatment group was higher than control group which the obtained results corresponded with the results of the Panigrahi *et al*. [43] and Al-Dohail *et al*. [44]. IgM is the main immunoglobulin present in fish [45]. 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 [46]. 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 [47]. 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, including Brunt *et al*. [29], Newaj-Fyzul *et al*. [27], Salah Mesulhy *et al*. [48], Tavakoli and Akhlaghi [47], Sharifuzzaman and Austin [42] and Panigrahi *et al*. [43] achieved the similar results in this regard. Lysozyme is an important humoral innate defence parameter, and is widely distributed in invertebrates and vertebrates [49]. 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 [33]. An increase in the lysozyme concentration in fish blood can be caused by infections or invasion by foreign material [50].

The results of urea showed a significant decrease in the treatment group in compared with control group. The elevation of urea in control group was reported in this study. Urea in fish is produced by liver and excreted by kidney [51]. The

elevation of urea in our research might be attributed to kidney dysfunction. In the same aspect, El-boshy *et al*. [52] reported increase of urea in diseased *Oreochromis niloticus* experimentally infected with Aflatoxin B_1. ALT and ALP activities were significantly increased in the control in compared with treated fish groups with probiotic. This elevation could be attributed to liver damage. Our result in accordance with the results of hepatic enzymes analysis which decreased in *O. niloticus* which received probiotics in comparison to control group indicating a positive and beneficial effect of probiotics on the maintenance of the integrity of hepatocytes and their roles in improvement of liver histology. These results were supported by several authors Jessus et al. [53], Nayak et al. [54] and Safinaz [55]. El-boshy *et al*. [52] showed that ALT activities were significantly decreased in the treated fish groups with β-glucan in compared with Aflatoxin B1 (AFB1) treated group, and Pepeljnjak *et al*. [56] who observed elevation in liver transaminase enzymes in *Cyprinus carpio* fed 5.0 mg Fumonisin B1 (FB1)/kg body weight for 42 days. In the same line, Hussein *et al*. [57] reported elevation of liver transaminase of *O. niloticus* that had been intoxicated with AFB1.

In conclusion, the results of the present study indicate that *B.subtilis* used probiotics are able to improve biochemical parameters of rainbow trout. This probiotic 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 immunostimulator.

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Table 1: Effect of *Bacillus subtilis* on biochemical factors of rainbow trout after challenge with *Streptococcus iniae.* The values are presented as mean ± SE

Biochemical factors	**Treatment**	**Control**
TP (g dl^{-1})	3.31 ± 0.22^{a}	2.55 ± 0.20^{b}
Alb (g dl^{-1})	1.92 ± 0.03^{a}	1.55 ± 0.10^{b}
IgM(mg dl^{-1})	38.05 ± 4.57^{a}	22.78 ± 2.18^{b}
Lys (mg ml^{-1})	2.92 ± 0.31^{a}	1.75 ± 0.26^{b}
Glu (mg dl^{-1})	34.67 ± 2.80^{b}	37.53 ± 3.50^{b}
Urea(mg dl^{-1})	4.53 ± 0.36^{b}	8.57 ± 0.36^{a}
ALT(UL^{-1})	39.93 ± 2.65^{b}	52.47 ± 4.63^{a}
AST(UL^{-1})	185.33 ± 16.48^{b}	233.29 ± 20.80^{b}
ALP(UL^{-1})	69.27 ± 6.11^{b}	97.87 ± 5.45^{a}

- TP: Total protein, Alb: Albumin, Lys: Lysozyme, Glu: Glucose, ALT: Alanin aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase
- The difference alphabet, indicate the significant ($P<0.05$) in two sample groups

Received: October, 2012