Iranian Journal of Fisheries Sciences 22(2) 278-302 2023
DOI: 10.22092/ijfs.2023.128921

Research Article

The effects of probiotics and prebiotics on growth, stress reponses, blood and immune parameters of rainbow trout

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Received: May 2018 Accepted: January 2019

Abstract

Regarding to the roles of prebiotics and probiotics in feeding the aquatic animals, the aim of this study was to evaluate the effects of *Pediococcus Acidilactici*, Mannan-oligosaccharide prebiotic and Beta-glucan on hematologic and immunologic parameters of the trout. 720 healthy rainbow trout were divided into four groups as the control group and groups treating with prebiotics, probiotics and both. After the treatment, immunologic, hematologic, growth-nutritional parameters were measured with various stressors indicating the positive effects of simultaneous probiotic and prebiotic treatment on all above-mentioned parameters and stress prevention in the trout.

Keywords: Trout, *Pediococcus Acidilacitc*, Mannan-oligosaccharide and beta-glucan, Hematology, Immunology

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Introduction

Aquaculture is a developing basis of agriculture and animal husbandry all over the world. Increasing demand for fish due to fast population growth, the income, preferring fish protein to other animal proteins and cultural/ health reasons expedite the growth of this industry. This necessitates developing aquaculture activities and increasing the production. While aquaculture industry should be effective and profitable, its ecological effects have to be minimized. Feeding with appropriate feed ingredients to provide essential nutrients is determinant in profitability and productivity of modern aquaculture since feed ingredients are responsible for 30- 70 % of total costs in this industry. In addition, nutrition clearly plays an important role in immunomodulation and disease's resistance. As a result, feed quality and nutrition management are critical parameters (Ebrahimi, 2006).

Rainbow trout is one of the most important economical species rearing in the most parts of the world. In the recent years, aquaculture has been developed fast with establishing new farms and increasing the production rate per area. Extensive culture methods of this species causes many types of stresses making the trout susceptible to various viral, bacterial, fungal and parasitic diseases (Bahram, 2005).

Many ways, such as administering antibiotics ~~to~~ for controling diseases, have been proposed to solve these problems with limited success, though. Antibiotics are medicinal additives that have been used in fish feed since 1950 which due to causing antibiotic resistance in the host as one of the limitations of antibiotic administration resulted in many issues such as pathogen resistance and ecological issues.

Regarding to this fact that pathogens are available in the aquatic environments all around the year, it seems that protecting fish against pathogens is the most important, easiest and the cheapest way to prevent damages and their consequences of diseases in aquaculture animals.

There have been several reviews recently on the use of probiotic in aquaculture (Balcazar *et al*., 2006; Vine *et al*., 2006). These authors have been reviewed a comprehensive overview of the application of probiotic in aquaculture, with some discussions on the mechanisms of action (MOA). Mucosal adhesion has become one of the criteria for selection of prebiotic and probiotic in fish (Nikoskelainen *et al*., 2001). A long residence time of prebiotic and probiotic in the intestinal tract is supposed to extend their potential beneficial effects on the site (Lee *et al*., 2004). It is accepted that the major routes of infection in fish are through the skin, gills, and gastrointestinal tract. The ability of a bacterial pathogen to attach to the mucosal surface of the skin or gut epithelium has been related to its virulence (Bruno, 1988) and is considered the first step of bacterial infection (Bengmark, 1998). The transfer of a pathogen, i.e., its

movement from the intestinal lumen through the epithelial mucosa to infect sterile tissues, is also important in the infection process and pathogenesis (Ringo *et al*., 2007). Therefore, competition for attachment sites may serve as the first barrier of defense against invading pathogenic bacteria (Vine *et al*., 2004). The ability of a strain to colonize the gut and adhere to the mucus layer is considered a good criterion for preselection of probiotics in aquaculture (Balcazar *et al*., 2006; Vine *et al*., 2006). However, there is a little information on bacterial colonization on mucosal surfaces in fish larvae, apart from a few reports. Ringo and Birkbeck (1999) provided a comprehensive review of the adhesion ability of bacteria to the gut mucosal surfaces, and the establishment of intestinal bacteria in fish larvae, which can be influenced by the microbiota of the eggs and the live feed and the bacteria present in the rearing water. The introduction of a strain of lactic acid bacteria (*Lactobacillus plantarum*) into the rearing water of cod larvae (*Gadus morhua*) changed the bacterial composition in the fish gut by reducing the colonization with opportunistic bacteria (Strom and Ringo 1993). However, these observations do not unambiguously demonstrate that physical exclusion from adhesion sites by the probiotics was the MOA involved. Moreover, adhesion sites might change during the early larval stages, as the maturation of the GI tract might result in modifications in the chemical composition of the mucus, and among other physicochemical characteristics, its hydrophobicity, which is an important factor in adhesion (Lee and Puong, 2002).

Immunostimulants are used to prevent disease's occurrence in the aquaculture being safer and more reliable than chemicals with vaster effects than vaccines (Geney, 1994).

The main aim of aquaculture is to maximize the efficacy and productivity. Not only probiotic and prebiotic-supplemented diet could provide all essential nutrients, but also they are one of the best ways to keep the cultured aquatic healthy and to increase their resistance to stress and pathogens (Gatlin, 2002).

The functions of probiotics could be divided into immunonutrients and immunostimulants being categorized based on their mechanisms of action. While immunonutrients are nutrients for the immune system, immunostimulants affect this system by acting on neuroimmune-endocrine axis of the animals. Probiotics and prebiotics are supplements to rations with positive effects on growth and activity of helpful micro-biota of the digestive system (Gibson and Roberfroid, 1995). Probiotics act by improving the aqueous environment conditions and decreasing the pathogens (Gatlin, 2002). Positive effects of probiotics and prebiotics in the aquaculture have been approved by different views such as improving physical and chemical parameters of environment, prevention and control of pathogens and promoting the growth of the aquatic animals in numerous studies by researchers (Irianto and Austin, 2002). Most studies

concluded into valuable results being used as practical principles in the aquaculture (Gomez-Gill *et al.*, 1998).

These probiotics and prebiotics are used with two main reasons as to improve the ecology of the aquatic animals and to introduce helpful gut flora. Nowadays, probiotics are used in many fields such as agriculture, aquaculture and environment. They have recently introduced to aquaculture with many uses in all aspects (Karim Zadeh *et al.*, 2009).

Effective administration of prebiotics requires studying the population and types of the aquatic micro flora of the digestive system. Gut flora could ferment the prebiotics, selectively. Gut's sugar fermentation improves the growth and energy level of these bacteria resulting in promoting gut micro-flora and preventing colonization of pathogenic bacteria (Bosscher 2006). These bacteria secrete ingredients to stimulate the immune system increasing the resistance of the host against the pathogens (Flickinger *et al.*, 2003).

It was suggested that probiotics and prebiotics could improve gut micro flora by increasing the population of useful bacteria. They could also improve the health status, fasten disease's recovery, promote the growth, prevent the gross anomalies, help to digestion and balance the micro-flora.

The aim of this study was to supplement trout's fed with Bactocell, as the probiotic, and mannan oligosaccharide, as the prebiotic, to promote the growth and some immune parameters.

Supplementing the rations of the aquatic animals with probiotics resulted in improving digestive and enzymatic activities, stimulating the appetite (Irianto and Austin, 2002), balancing the micro flora in the gut of the host, producing useful ingredients such as vitamins and some enzymes, stimulating the immune system with improving the efficacy, promoting the growth, developing the nutrient levels (Merrifield *et al*, 2009) and improving the water quality (Panigrahi *et al*, 2007).

Limited studies were performed on these fields recently in Iran. Rainbow trout (*Oncorhynchus mykiss*) is one of the most important Salmonidae fish in Iran. Proper management and feeding are critical to culture this species with higher viability. Some growth, nutrition and immune response parameters of rainbow trout together with total weight and length, feed conversion rate (FCR), special growth rate (SGR), the levels of lysozyme, complement and total immunoglobulin and blood parameters related to white blood cells, red blood cells and some stressors were measured and analyzed in this study to check the effects of Bactocell probiotic and mannan-oligosaccharide prebiotic.

Materials and methods

720 healthy rainbow trout with the average weight of 15±2 g were selected for this study and then transferred to fish tank.

1×25×2.5 cement fish tanks were disinfected with 3% NaCl solution for ten minutes and then the fish were

released and kept there based on the standard method. Also, all the fishes were checked and examined for any possible ectoparasites/ diseases and then kept for 7 days to adapt with the new environment. After the adaptation, the fish were divided into 4 groups based on commercial feed, prebiotics and probiotics as followed.

Group 1: feeding with commercial feed entitled as FFT1 (Faradaneh Company, Iran) (3 repetitions)

Group 2: 300 ppm probiotics administration (3 repetitions)

Group 3: 2 ppt prebiotics administration (3 repetitions)

Group 4: 150 ppm probiotics and 1 ppt prebiotics administration (3 repetitions)

Each repetition included 60 trout and the fish tanks were selected randomly. The feeding was based on the biomass and wate temperature using the feeding chart for 8 weeks 4 times a day. The water was also provided from a well. Aeration, water flow and the outlets were based on trout's normal standard.

Feed preparation

Bactocell probiotics (Lallemand, France) containing *Pediococcus acidilactici* and Agrimos mannan oligosaccharides and beta-glucan (Lallemand, France) were provided for this study. To add these ingredients into the commercial feed, first the amount needed for each group was calculated and then the selected levels of probiotics and prebiotics were suspended into sunflower seed oil and mixed with the feed. After that, the feed were dried in the room temperature for two hours in a clean environment. It is necessary to mention that the control groups only receive oil and NaCl solution.

Evaluating growth parameters

To evaluate the effects of administering probiotics and prebiotics on the growth of the trout and to compare the treatment groups with the control ones, growth parameters were assessed 5 times in 15 days.

Hematology assays

After anesthetizing the fish with 200 ppm dianthus extract, blood samples were taken from the caudal vein on days 28 (week 4) and 54 (week 8/ end of the treatment period) and then transferred into heparinized anti-coagulant tubes (1 droplet heparin/ 1 cc blood). Then the samples were quickly shipped to the laboratory on the ice and blood parameters including complete blood count (Klontz, 1994), hemoglobin and hematocrit measurements (Klontz 1994), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) (Klontz 1994) were checked.

Biochemical and immunological serum assays

The blood samples were taken from the caudal vein on days 28 (week 4) and 54 (week 8) without any anti-coagulants and transferred into 2 ml tubes. The samples were quickly shipped to the hematology laboratory on the ice and after centrifugation in 4000 g for 15 minutes at -4 ^{o}C; the sera were

separated and kept at -20 ^{o}C for the final test. Eventually, immune parameters were evaluated.

Lysozyme (Merrifield *et al.*, 2010), immunoglobulin M (Khoshbavar-Rostami *et al.*, 2006) and total serum antibody and complement system activities (Siwicki, 1994) were checked on days 0, 30 and 60 of this study as the parameters of the immune response. To do so, 3 trout were selected each time to take the blood samples from the caudal vein after anesthetizing and to evaluate the above-mentioned parameters.

Heat stress

3 trout from each group were selected randomly in every repetition, totally 9 trout of each group, and then transferred to separate tanks with the temperatures of 20 and 25 ^{0}C and the swimming activity and the mortality rate of the fish~~es~~ were recorded for 24 hours.

Anorexia stress

Just like heat stress, 9 fish from each group were selected and kept separately in non-oxygenated water for 3 and 6 minutes in a net and then returned to the flowing water tanks. Eventually, the swimming activity and mortality rate were observed and recorded for 12 hours.

Salinity stress

3 trout from each repetition, totally 9 of each group, were selected randomly and transferred to separate tanks with the salt concentration of 10, 20 and 30 ppt for an hour and the swimming activity and mortality rate were observed and recorded for 24 hours.

Formalin stress

3 trout from each repetition, totally 9 of each group, were selected randomly and transferred to separate tanks with the formalin concentration of 20, 40 and 60 ppt for an hour and the swimming activity and mortality rate were observed and recorded for 24 hours.

Statistical analysis

To analyze the results, Two-way analysis of variance (ANOVA), SPSS Software (Version 21) and Tukey's honest significant test were used. In all studies, the significance level was assigned at (p<0.05). Also, the figures were drawn with Excel 2007 Software.

Results

Immunoassay results

Lysozyme

There is no significant difference between the lysozyme levels of treatment groups; only the increment of lysozyme level of group 4 was significantly higher than other groups on day 60.

Table 1: Average lysozyme levels (µg ml^{-1}).

	Day 0	Day 30	Day 60
Group I (Control)	23.03 [a]	24.98 [a]	29.02 [a]
Group II (Probiotics)	23.49 [a]	25.14 [a]	29.75 [a]
Group III (Prebiotics)	24.06 [a]	26.13 [a]	31.11 [a]
Group IV (Prebiotics and Probiotics)	25.83 [a]	27.1 [a]	34.91 [b]

Results were presented as means ±S.E. of triplicate observations.

The values in the same column compared with the control group did not significantly differ showed with (a) or significantly differ showed with (b), (p<0.05).

There was no significant difference in total immunoglobulins in different treatments and the average total immunoglobulin level of group 4 was significantly different (p<0.05) on day 30 and 60.

Total serum antibody (total immunoglobulin)

Table 2: Total serum antibody levels (µg ml^{-1}).

	Day 0	Day 30	Day 60
Group I (Control)	2.97 [a]	6.97 [a]	7.15 [a]
Group II (probiotics)	2.97 [a]	7.03 [a]	7.35 [a]
Group III (prebiotics)	2.98 [a]	7.84 [a]	7.91 [a]
Group IV (Prebiotics and probiotics)	2.98 [a]	8.91 [b]	9.92 [b]

Results were presented as means ±S.E. of triplicate observations.

The values in the same column compared with the control group did not significantly differ showed with (a) or significantly differ showed with (b), (p<0.05).

Complement

There was no significant difference between the complement levels of various groups except day 60 with complement levels of groups 2, 3 and 4 being significantly higher than the control group.

Table 3: Complement activity (µg ml^{-1})

	Day 0	Day 30	Day 60
Group I (Control)	19.95 [a]	43.13 [a]	45.11 [a]
Group II (Probiotics)	18.91 [a]	43.45 [a]	56.34 [b]
Group III (Prebiotics)	19.76 [a]	43.78 [a]	57.79 [b]
Group IV (Probiotics and prebiotics)	19.43 [a]	44.08 [a]	58.14 [b]

Results were presented as means ±S.E. of triplicate observations.

The values in the same column compared with the control group did not significantly differ showed with (a) or significantly differ showed with (b), (p<0.05).

Immunoglobulin Massay

After checking immunoglobulin M levels on days 0, 30 and 60, It was indicated that the levels increased with aging. Though group four's level of this

immunoglobulin was the highest on days 30 and 60, the difference was not significant.

Table 4: IgM levels.

Group	Day 0	Day 30	Day 60
Group I (Control)	88.6 [a]	91.8 [a]	95.4 [a]
Group II (Probiotics)	87.1 [a]	92.1 [a]	95.6 [a]
Group III (Prebiotics)	89.3 [a]	91.6 [a]	94.6 [a]
Group IV (Probiotics and prebiotics)	87.4 [a]	93 [a]	97.3 [a]

Results were presented as means ±S.E. of triplicate observations.

The values in the same column compared with the control group did not significantly differ showed with (a), (p<0.05).

Growth and nutrition results

Weight

No significant difference was found between the groups in any periods.

Total length

There was no significant difference among the groups on days 15, 30 and 45 but there was between group four with other groups on day 60 (p<0.05).

Table 5: Average total length of trout in various treatments during the study (cm).

Group	Sampling days			
	Day 15	Day 30	Day 45	Day 60
Group I	12.04±0.13 [a]	14.49±0.56 [a]	15.32±0.55 [a]	17.2±0.81 [a]
Group II	12.13±0.33 [a]	14.6±0.94 [a]	15.40±0.26 [a]	14.47±1 [a]
Group III	12.19±0.25 [a]	14.40±0.37 [a]	15.67±0.98 [a]	17±0.9 [a]
Group IV	12.94±0.79 [a]	14.78±0.80 [a]	15.91±0.88 [a]	18.58±0.83 [b]

Results were presented as means ±S.E. of triplicate observations.

The values in the same column compared with the control group did not significantly differ showed with (a) or significantly differ showed with (b), (p<0.05).

Special growth rate (SGR)

SGR of groups 2, 3 and 4 were significantly higher than group 1 on day 15 while no significant difference has been found on day 30. On day 45, group 4's SGR was significantly different than group 1's. Finally on day 60, all groups were significantly different than group 1.

Table 6: The averages of special growth rate of trout under various treatments during the study.

Group	Sampling days			
	Day 15	Day 30	Day 45	Day 60
Group I	0.66±0.11 [a]	0.95±0.50 [a]	0.82±0.05 [a]	0.86±0.05 [d]
Group II	0.73±0.06 [b]	1.03±0.04 [a]	0.93±0.06 [a]	0.98±0.09 [d]
Group III	0.86±0.03 [b]	1.06±0.05 [a]	0.93±0.08 [a]	0.96±0.09 [d]
Group IV	0.86±0.09 [b]	1.06±0.02 [a]	1±0.03 [c]	1±0.04 [d]

Results were presented as means ±S.E. of triplicate observations.

The values in the same column compared with the control group did not significantly differ showed with (a) or significantly differ showed with (b, c, and d), (p<0.05).

Feed Conversion Rate (FCR)

The best average of feed conversion rate was belonged to group IV on day 15 with significant difference with other groups (p<0.05). On day 30, groups 2 and 4 had the best FCR while the difference of group 4 was significant. On day 45, no significant difference was found between the groups. Finally on day 60, the best average FCR was belonged to group 4 with a significant difference.

Table 6: Average feed conversion rates of trout under various treatments during the study.

Group	Sampling days			
	Day 15	Day 30	Day 45	Day 60
Group I	0.77±0.10 [a]	0.85±0.07 [a]	0.98±0.07 [a]	0.96±0.06 [a]
Group II	0.58±0.03 [a]	0.69±0.06 [a]	0.88±0.03 [a]	0.87±0.06 [a]
Group III	0.66±0.51 [a]	0.78±0.09 [a]	0.97±0.08 [a]	0.94±0.06 [a]
Group IV	0.49±0.04 [b]	0.57±0.10 [c]	0.85±0.05 [a]	0.77±0.02 [d]

Results were presented as means ±S.E. of triplicate observations.

The values in the same column compared with the control group did not significantly differ showed with (a) or significantly differ showed with (b, c, and d), (p<0.05).

Stress assays

Heat stress at 20 ^{o}C

Heat stress-induced Mortality's onset began sooner in group I, after 6 hours, with the highest rate. The least mortality rate was belonged to group 4 with 1 in the last hours.

Table 7: Mortality rate induced by 20 degrees Celsius heat stress in 24 hours.

Group	No. of fishes	No. of mortality	No. of remaining	Mortality rate (%)
Group I	9	5	4	55.55 [a]
Group II	9	4	5	44.44 [a]
Group III	9	3	6	33.33 [a]
Group IV	9	1	8	11.11 [b]

Results were presented as means ±S.E. of triplicate observations.

The values in the same column compared with the control group did not significantly differ showed with (a) or significantly differ showed with (b), (p<0.05).

Heat stress at 20 ^{o}C

Heat stress-induced Mortality's onset began in two hours in all groups except group IV. Though the mortality began later in group IV, after 5 hours, all fishwere died.

Anoxia stress

No mortality was recorded in fishes which were out of the water for 3 minutes. However, the morality rate of fishes were as below with insignificant differences.

Table 8: Mortality rate induced by 6 minutes anoxia in 12 hours.

Group	No. of fishes	No. of mortality	No. of remaining	Mortality rate (%)
Group I	9	2	7	22.2 [a]
Group II	9	2	7	22.2 [a]
Group III	9	2	7	22.2 [a]
Group IV	9	1	8	11.11 [a]

Results were presented as means ±S.E. of triplicate observations.

The values in the same column compared with the control group did not significantly differ showed with (a), (p<0.05).

Salinity stress

After exposing fish to different salt's concentrations, no mortality was recorded in 10 and 20 ppt salt after 24 hours. However, similar mortalities were seen in all groups with 30 ppt salt while the onset of mortality was later in groups 2, 3 and 4.

Table 9: Mortality rate due to salinity stress induced by 30 ppt salt.

Group	No. of fishes	No. of mortality	No. of remaining	Mortality rate (%)
Group I	9	3	6	33.33 [a]
Group II	9	3	6	33.33 [a]
Group III	9	3	6	33.33 [a]
Group IV	9	3	6	33.33 [a]

Results were presented as means ±S.E. of triplicate observations.

The values in the same column compared with the control group did not significantly differ showed with (a), (p<0.05).

Formalin stress

After exposing fish to different formalin's concentrations, no mortality has been recorded in 20 ppm formalin after 24 hours. However, similar mortalities were seen in all groups with 40 ppm formalin while the onset of mortality was later in groups 2, 3 and 4. In 60 ppm formalin, all fish of all groups were died.

Table 10: Mortality rate due to stress induced by 40 ppm formalin.

Group	No. of fishes	No. of mortality	No. of remaining	Mortality rate (%)
Group I	9	4	5	44.44 [a]
Group II	9	3	6	33.33 [a]
Group III	9	3	6	33.33 [a]
Group IV	9	4	5	44.44 [a]

Results were presented as means ±S.E. of triplicate observations.

The values in the same column compared with the control group did not significantly differ showed with (a), (p<0.05).

White blood cell (WBC) count

Based on Two-way analysis of variance (ANOVA), no significant difference was observed between the number of white blood cells of the control group's fish and other groups in both periods (p>0.05). The results indicated that the number of white blood cells was

increasing during the study but it was not significant in all groups except day 60 of group 4.

Table 11: Average white blood cell count (10^3 mm^{-3}) of fries in control and various treatment groups.

Groups	Day 0	Day 30	Day 60
Group I	11.8 [a]	13.3 [a]	15.4 [a]
Group II	12.2 [a]	12.9 [a]	16.1 [a]
Group III	11.6 [a]	14.1 [a]	16.2 [a]
Group IV	11.9 [a]	13.9 [a]	17.8 [b]

Results were presented as means ±S.E. of triplicate observations.

The values in the same column compared with the control group did not significantly differ showed with (a) or significantly differ showed with (b), (p<0.05).

Red Blood Cell (RBC) count

The number of RBCs of trout's fries of all groups was checked in different time periods separately. There was no significant difference between various treatment and control groups in 3 time periods based on Two-way ANOVA (p>0.05).

Also no significant difference has been found in the red blood cells count's average of various treatment and control group's flies based on independent T-test (p>0.05).

Effects on blood hemoglobin

No significant difference has been found in any periods. However, the hemoglobin rate of the control group was less on day 60 while the rate of group 4 was the highest; not significant, though.

Effects on blood hematocrit

The results of Two-way ANOVA test indicated no significant difference among the groups (p>0.05).

Mean corpuscular volume (MCV)

The results of Two-way ANOVA test indicated no significant difference among the groups (p>0.05).

Mean corpuscular hemoglobin concentration (MCHC)

The results of Two-way ANOVA test indicated no significant difference among the groups (p>0.05).

Mean corpuscular hemoglobin (MCH)

The results of Two-way ANOVA test indicated no significant difference among the groups (p>0.05).

Lymphocyte count

The results of Two-way ANOVA test indicated no significant difference among the groups (p>0.05).

Table 12: The average blood lymphocyte count of fries in treatment and control groups.

Groups	Day 0	Day 30	Day 60
Group I	82.1 [a]	83.6 [a]	85.2 [a]
Group II	80.3 [a]	84.7 [a]	85.9 [a]
Group III	84.1 [a]	85.7 [a]	86.4 [a]
Group IV	85.3 [a]	86.3 [a]	87.9 [a]

Results were presented as means ±S.E. of triplicate observations.

The values in the same column compared with the control group did not significantly differ showed with (a), (p<0.05).

The results of Independent T-test indicated no significant difference in blood lymphocyte percentage of the fries between the groups (p>0.05). The lymphocyte counts of all treatment groups were increased at the end of the study with significant difference recorded for the increment of group 4.

Neutrophil count

The results of Two-way ANOVA test indicated no significant difference in blood neutrophil count of the groups except on day 60 with group 4 (p>0.05).

Table 13: The average blood neutrophil count of fries in treatment and control groups.

Groups	Day 0	Day 30	Day 60
Group I	7.3 [a]	7.9 [a]	10.1 [a]
Group II	6.9 [a]	8.8 [a]	9.8 [a]
Group III	7.1 [a]	9.3 [a]	10.4 [a]
Group IV	7.4 [a]	10 [a]	15.1 [b]

Results were presented as means ±S.E. of triplicate observations.

The values in the same column compared with the control group did not significantly differ showed with (a) or significantly differ showed with (b), (p<0.05).

The results of Independent T-test indicated no significant difference in blood neutrophil percentage of the fries between the groups except on day 60 with group 4 (p>0.05).

Eosinophil and monocyte count

Eosinophil and monocyte count were limited in all treatment and control groups with no significant differences in any periods based on Two-way ANOVA test (p>0.05). Changes in eosinophil and monocyte count of all treatment groups were checked in the middle and at the end of the study using Independent T-test showing no significant difference (p>0.05).

Basophil count

No significant difference has been found in blood basophil count between treatment and control groups (p>0.05) based on Two-way ANOVA test. The basophil count was decreased in all groups of which the decrement of group 4 on day 60 was significant due to the increment of neutrophil and lymphocyte counts.

Discussion

Analyzing the results indicated no significant difference in weight between the treatment groups on days 15 and 30.

Also on days 45 and 60, no difference found between treatment and control groups and between treatment groups themselves. The lengths of group 4 fish were significantly higher on day 60 (p<0.05).

Special growth rates of treatment groups, groups 2, 3 and 4, were higher than the control one on day 15 (p<0.05). While only the special growth rate of group four was higher than group 1 on day 45, the rates of all treatment groups became significantly higher than the control group in day 60. The best feed conversion rate recorded on day 15 was belonged to group 4 while on day 30, the rate of groups 2 and 4 were the best ones (p<0.05). no significant different has been found in the feed conversion rateof the groups on day 45 and finally, the best rate recorded on day 60 again belonged to group 4 (p<0.05).

No significant difference has been found in trout's body condition of the groups on days 15 and 30. However, the body conditions of groups 2, 3 and 4 were significantly higher than the control group on days 45 and 60. As indicated above, administering probiotics, and especially with prebiotics simultaneously, could improve the nutritional parameters of the trout.

The results of the previous studies indicated that probiotics could improve the appetite and also the feeding of the hosts by producing vitamins and breaking up toxins and non-digestible particles of the feed (Salminen *et al.*, 1999).

It was indicated in a study that supplementing rainbow trout fed with *Pediococcus Acidilactici* could result in FCR<1 and SGR>2.00 with survival rate of >95%. However, no growth promotion has been observed compared with feeding without any probiotics (Merrifield et al, 2010a). Previous studies indicated that supplementing trout's feed with Pediococcus acidilactici could improve the weight (Aubin *et al*, 2005). Also, this effect was approved in Nile tilapia (Shelby *et al.*, 2006) and Channel catfish (Shelby *et al.*, 2007).

Also feeding with *Pediococcus acidilactici*-supplemented brine shrimp increased the weight of Pollack's larvae (Gatesoupe, 2002).

Merrifiel *et al.* (2010) concluded that supplementing trout's ration with probiotics could improve feed efficacy rate, feed conversion rate and the balance, the health and the survival of gut's flora (Merrifield *et al.*, 2010b).

Sohail *et al.* (2013) suggested that some probiotics would enhance the appetite and total growth parameters such as weight. Irianto and Austin (2002) claimed that adding probiotics into fish feed could increase digestive and enzymatic activities resulting in appetite stimulation and growth improvement.

Adding 3 g mannan oligosaccharide to each kilogram of the diet did not result any significant differences in the growth and nutrition parameters of Gulf sturgeon caviar (*Acipencer oxyrinchus desotoi*) compared to the control group (Pryor *et al.*, 2003). Supplementing 2% prebiotics such as inulin, oligofructose

and lactosucrose to the *Psetta maxima* larvae's feed indicated that the average final weight and special growth rate of the group fed with oligofructose were higher than other treatments but no difference was found in survival rate and the control group's rate was the highest (Mahious and Gatesoupe, 2006).

Daniels (2006) studied the effects of brine shrimp supplementation with 2, 20 and 200 ppt mannan oligosaccharide on European lobster (*Homarus gamarus*) and reported higher survival rate and growth with adding 2 and 20 but not 200 ppt mannan oligosaccharide.

Gence *et al.* (2007) has been found no significant difference in growth and nutrition parameters by supplementing 1.5, 3 and 4.5 g mannan oligosachharide per kilogram feed of tilapia (*Oreochromis Niloticus* × *O. Aureus*). Also in a similar study on green tiger shrimp (*Penaeus semisulcatus*), it was concluded that after 48 days feeding with 3 g mannan oligosaccharide kg^{-1} ration could result in growth promotion compared with other treatments. In this study, significant difference has been observed in fish length when simultaneously supplementing feed with Bactocell probiotic and mannan oligosaccharide prebiotic but not with the weight, except on day 60 in group 4 ($p<0.05$).

According to the results of the current study, it could be concluded that Bactocell and mannan oligosaccharide are synergists and supplementing trout's ration with both could improve growth and nutrition parameters. Hence, supplementing the feed with both is recommended, in case of being cost-effective.

The efficacy of the feed and the quality of the cultured fish could be generally determined by a simple measuring the survival rate and resistance of the fish~~es~~ against different kinds of stress. In fact, the physiological conditions of the fishes could determine the survival rate of the fishes.

In this study, different levels of Bactocell's probiotic and mannan oligosaccharide's prebiotic has increased the resistance of rainbow trout against heat stress at 20 ^{o}C with 11.11% mortality rate in group 4 which fed with Bactocell and mannan oligosaccharide and had the highest survival rate with the lowest mortality rate.

For the heat stress at 25 ^{o}C, the mortality has begun in all groups except for group 4 in the first two hours. Though the mortality of group 4 was begun later, all fish were finally died.

In 3 minute-anoxia's stress, no mortality was recorded in all groups. However, the mortality rates with 6 minutes' anoxia were as followed:

Mortality's of groups 2, 3 and 4 started later than then group 1 but finally there was no significant difference in the mortality of the groups except that only one fish was died in group 4 while 2 of each other groups were died.

After exposing the fish to different concentrations of the salt, no mortality was found in 10 and 20 ppt salt while in

the concentration of 30 ppt, similar mortalities were found later in groups 2, 3 and 4.

After exposing fish to different concentrations of formalin, no mortality has been found in 20 ppm formalin after 24 hours while in the concentration of 40 ppm, similar mortalities have been found later in groups 2, 3 and 4. In the concentration of 60 ppm, all fishes were similarly died in all groups.

In general, probiotics could resist fishes against all kinds of stress and improving their health and growth.

Several reports indicated that probiotics could increase aquatic larvae resistance against environmental stresses (Gatesoupe, 1999; Verschuere *et al.*, 2000).

Probiotics could also stimulate the immune system, causing to tolerate environmental stressors and stimulators better (Irianto and Austin, 2002; Sharifuzzaman, 2009).

Gatlin (2002) indicted the efficacy and potential of adding 2 g kg^{-1} or 200 ppm mannan oligosaccharide in improving the growth and survival rate and increasing the resistance against environmental stresses of cultured trout. Supplementing hybrid striped bass's feed with 1-2% of a prebiotic such as GroBiotic-A could improve immune response promoting the health and resistance against stresses and pathogens (Gatlin, 2002).

As mentioned above, probiotics could improve the resistance and the survival of fishes against all types of stresses improving the health and the growth of fishes.

Complement system is one of the most important serum factors due to its activating effects on cellular immunity. The activity level of antibody-independent alternate pathway is higher in fish than mammals. The proteins of this system play several roles in defending against microorganisms.

Lysozyme is one the main immune system compartments of both vertebrates and invertebrates. Although the physiological role of lysozyme is not clear, it participates in defending against the microorganisms. The level of this enzyme is significantly high in blood serum and mucus of the fishes. Besides serum level of this enzyme increased followed by pathogenic injection and in response to bacterial infections and probiotic fortified-ration.

The immune system of fish disruption by environmental stresses and could result in sensitize fish to all kinds of diseases limiting economic growth of the aquaculture. Adding supplements to the ration could promote the growth and the immune system and it is one of the solutions to improve the health, and resisting to stressors and pathogens.

No significant difference has been found in lysozyme level of the groups on day 0. On day 30, groups 2, 3 and 4 with 13, 26 and 27.1 g ml^{-1} lysozyme had the highest average rate but the difference was not significant. On day 60, increasing average levels of lysozyme have been found in all groups of which group 4 was significantly higher. High serum activity level of lysozyme might be correlated with high

levels of mannan oligosaccharide and probiotics.

On days 0 and 30, no significant differences have been found in complement levels of the groups. On day 60, the complement levels of groups 2, 3 and 4 were significantly higher than the control group. This indicated the positive effects of probiotics and prebiotics in studied groups.

Regarding to total immunoglobulins, no significant differences have been found between the groups on day 0. The average of total globulin of all groups was 2.98 mg ml^{-1} on day 0. On days 30 and 60, the average total immunoglobulins of group 4 was significantly different than other groups ($p<0.05$).

After measuring the level of immunoglobulin M on days 0, 30 and 60, it was found that its level would be increased with aging. IgM level of group 4 was higher than other groups on days 30 and 60, not significant though.

High immune parameters such as total immunoglobulin, lysozyme and complement indicated the positive effects of probiotics and prebiotics in the treatment groups. Supplementing fish feed with probiotics, could stimulate and improve the efficacy of the immune system, e.g. lysozyme serum activity increment (Merrifield *et al.,* 2010a).

Song *et al.* (2006) indicated lysozyme activity increment in *Miichthys miiuy* fish fed with 10^7-10^9 CFU g^{-1} of *Clostridium butricum* on 8th week.

Tokmehchi (2007) claimed the activity level of alternate complement pathway and the level of lysozyme of the trout supplemented with *Lactobacillus delbrueckii* were significantly higher than the control group 20 days after the supplementation ($p<0.05$). Also, the activity level of complement system was decreased after the supplementation period. Some studies indicated that probiotics could increase the survival rate by stimulating the immune system (Irianto and Austin, 2003).

Panigrahi *et al.* (2004) indicated that the activity levels of complement system, lysozyme and phagocytosis of leukocytes of trout supplemented with lactobacilli were increased in the ventral part of the kidneys. Also, the serum activity levels of complement system and lysozyme were decreased after the supplementation (Panigrahi *et al.,* 2004).

Austin and Sharifuzzman (2009) have been reported the level of blood lysozyme and total serum immunoglobulin of trout supplemented with 10^8 cell Kocuria Sm1 probiotics g^{-1} diet were increased compared with the control group significantly ($p<0.05$).

Nikoskelainen *et al.* (2003) claimed that supplementing trout's diet with *Lactobacillus rahmnosus* could increase total serum immunoglobulin level compared to the control group. Also, the researcher mentioned the level of total immunoglobulin was significantly

decreased after the supplementation period.

In a study on *Cherax tenuimanus* supplemented with 0.2 and 0.4% mannan oligosaccharide, the resistance against stressors and bacterial infections were increased significantly and resulting in immune system stimulation (Sang *et al.*, 2009).

Torrecillas *et al.* (2007) studied the effects of 0, 2 and 4 g mannan oligosaccharide kg^{-1} diet on *Dicentrachus labrax* and indicated that those received the prebiotic had increased lysozyme activity level, *Vibrio alginolyticus* resisteance and immune system stimulation (Torrecillas *et al.*, 2007).

Cerezuela *et al.* (2008) added 5 or 10 g inulin kg^{-1} diet of *Spaus aurata* for 2 weeks and found out that inulin could significantly prevent immune parameters and hence it is not a good immunostimulator for this species (Cerezuela *et al.*, 2008).

The results of analyzing immune parameters indicated the positive effects and synergism between Bactocell and mannan oligosaccharide in improving lysozyme, complement and total immunoglobulin activity and hence the immune system response of rainbow trout.

Blood parameters are used as physiological parameters in response to internal or external changes of fishes (Cataldi *et al.*, 1998). Changing in blood and biochemical parameters is documented in various seasons, feeding with various diets and diseases (Aldrin *et al.*, 1984).

Two-way to diagnose the diseases in fishes is hematology. Hence, hematology is also considered a way to control physiologic condition of the fishes in growth, disease and even fertility (Bahmani, 2001). Basically, blood parameters indicate the current physiological condition affecting by feed (Waagbo *et al* 1998; Kumar *et al.*, 2006; Klinger *et al.*, 1996).

In this study, no significant difference was found in red blood cell count ($p>0.05$). However, the hemoglobulin rate of the control group was the least on day 60 and the rate of group 4 was the highest.

The results of Two-way ANOVA test indicated no significant difference in blood hematocrit of the groups ($p>0.05$).

No significant difference was also found in the average volume of red blood cells.

Based on Two-way ANOVA test on the average cellular hemoglobulin concentrations, no significant difference was found between treatment and control groups ($p>0.05$). As such, Independent T-test indicated no significant difference in the averages of cellular hemoglobulin concentrations of the fries in the middle and at the end of the treatment period ($p>0.05$).

The average hemoglobin of red blood cells showed no significant difference between the treatment and control groups ($p>0.05$).

The results of this study indicated that supplementing rainbow trout's diet with Bactocell could increase hematocrit, hemoglobin, red bloods cells, mean corpuscular hemoglobin

and mean hemoglobin concentration in the time checked compared with the control group but the difference was not significant.

Since mostly oxygen is transferred through hemoglobin attached to the red blood cells (Val, 2000), similar results for the hemoglobin concentration and red blood cell count is based on a logic correlation and on the same way with the results of Raida *et al.* (2003), Brunt and Astin (2005), Newaj-Fyzul *et al.* (2007). Hematocrit is subsidiary compartment of red blood cells and it is directly correlated with (Tangestani *et al.*, 2011).

Studying red blood cells, blood hemoglobin and hemoglobin at the end, of period, and also cellular hemoglobin concentration of the blood and average hemoglobin of red blood cells at both periods indicated that the rates of the above-mentioned parameters were higher in all three treatment groups indicating respiratory priority of the treatment groups to the control group, though the difference was not significant.

The levels of hematocrit were not significantly different between control and treatment groups at the middle of the treatment period, indicating that the synergism between Bactocell and the prebiotic could not necessarily effect on hematologic parameters in a significant way (Tangestani *et al.*, 2011).

Cellular immunity of the fishes is mostly depended on leukocytes (Magnadottir, 2010). White blood cells play important roles in phagocytosis and immune response to parasitic, bacterial and viral pathogens and healing injured tissues. Based on studies done on some fish species, the range of white blood cells changes are widely different in the blood (Feldman *et al.*, 2000). Number and type of white blood cells are the important criteria in checking the health and immune system of the animals (Shalaby *et al.*, 2016). Immune stimulators are components activate white blood cells (Raa, 2000). The numbers of white blood cells of the fries were not significantly different between control and treatment groups (p>0.05). The results indicated that the count of white blood cells increased by time but these changes were not significant except on day 60 in group 4. Regarding to lymphocyte count, no significant difference was found between control and test groups. Lymphocyte rates of all treatment groups have been increased at the end of the treatment period compared to the middle. This increment was significant in group 4 on day 60.

No significant difference has been found in blood neutrophil ratios at the middle and end of the treatment period, except for group 4 on day 60 (p>0.05). It was also true about the average percentage of blood neutrophils in the fries.

Eosinophil and monocyte rates were so limited in all treatment and control groups with no significant differences between (p>0.05). Basophil counts of all groups were decreased, especially significant in group 4 on day 60, which was due to the increments of neutrophil and lymphocyte counts.

Supplementing rainbow trout diet with Bactocell and prebiotics increased white blood cell count like Brunt and Austin (2005) in which GC2 strain of *Aeromonas sobria* increased the number of leucocytes and their phagocytosis potential in the trout. Other studies such as Newaj *et al.* (2007), Tavakoli and Akhlaghi (2009) and Ali *et al.* (2010) also indicated that supplementing the feed with probiotics could increase white blood cells count.

Lymphocytes are important fish immune compartments against pathogens with the ability to phagocyte and to produce antibodies. Measuring the number and rate of lymphocytes is one of main assays to evaluate the health of the immune system (Feldman *et al.*, 2000). Lymphocyte increment results in stimulating macrophage stimulators and phagocytosis (Sakai, 1999; Motero-Rocha, 2006).

The above-mentioned results indicated that Bactocell and prebiotics could simultaneously increase the lymphocyte rate, especially lymphocyte B, in fishes (Nayak, 2010). Lymphocyte count increment results in improving the immune system and resistance against the pathogens, environmental stimulants and stresses. This caused promoting the metabolism and hence growth promotion, mortality decrement and increasing the survival rate (Nayak, 2010).

The results indicated that supplementing rainbow trout diet with Bactocell and mannan oligosaccharide might increase the growth, survival and the immune system.

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