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Dietary effects of procyanidin and Bio-Aqua® on hematological and immune indices of rainbow trout (*Oncorhynchus mykiss*)

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Abstract

The role of immunostimulants in reducing diseases and enhancing fish performance has been proven in various studies. In this study, the effects of antioxidant procyanidin and commercial probiotic Bio-Aqua® on some hematological and immune indices of rainbow trout (*Oncorhynchus mykiss*) were investigated. A total number of 525 fingerlings in seven treatments (each treatment in triplicates) with experimental diets, including (1) commercial food without supplements (CTL), (2) 400 mg procyanidin kg^{-1} feed (PC400) , (3) 800 mg procyanidin kg^{-1} feed (PC800), (4) 0.50% probiotic diet (PB0.5), (5) 1.00% probiotic diet (PB1), (6) probiotic and procianidin 0.50% and 800 mg kg^{-1} feed, respectively (PB0.5 + PC800) and (7) probiotic and procianidin 1.00% and 400 mg kg^{-1} feed, respectively, (PB1 + PC400) were fed for eight weeks. The results showed that the use of procyanidin alone did not have any significant effect on blood biochemistry and immune indices. However, the individual use of Bio-Aqua® probiotic could affect the hematological and immune indices of fish, except for monocyte, compared to the control and procyanidin treatments. However, the combined use of the supplements improved the hematological and immune indices of fish. It was concluded that the simultaneous dietary administration of procyanidin and probiotic Bio-Aqua® could improve the general health of rainbow trout.

Introduction

Aquaculture is one of the main food producing sectors in the world which has significantly expanded in the recent years and has become a thriving food producing industry.[1] The main objectives in the aquaculture industry are both to maintain fish health and to improve fish productivity. Due to the limitations in the use of antibiotics and the lower efficiency of vaccines in aquatic animals, the use of immunostimulants, probiotics, prebiotics and plant derivatives (phytogenics) is increasing. The administration of these compounds in livestock has been successful, although their potential to be used in fish diets is still under investigation.

Probiotics are living microorganisms such as fungi and bacteria that are used as food supplements. They affect the bacterial balance of the gut changing the trend in favor of increasing the population of beneficial bacteria.[2] Probiotics can enhance the growth performance of fish by producing digestive enzymes and increasing their nutritional value. The positive effects of probiotics as a feed additive on growth performance, innate immune system, intestinal microbiota, resistance to stress and disease, and improvement of water quality in rainbow trout have been confirmed.[2-5] Probiotics can improve the immune system of fish by regulating pro-inflammatory cytokine genes and increasing the phagocytic activity of leukocytes.[6]

The role of probiotics in preventing various diseases, improving fish performance, immune system and fish growth has been reported previously.[7-9] Bio-Aqua® is a multi-strain probiotic complex consisting of nine bacterial and yeast species including *Pediococcus acidilactici, Enterococcus faecium, Bacillus subtilis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus casei, Lactobacillus rhamnosus, Bifidobacterium bifidum* and *Saccharomyces cerevisiae*. In the study of Akbari Nargesi *et al*., this probiotic demonstrated an improvement in most hematological and immune indices of male breeding rainbow trout. However, it did not have any effect on lymphocytes, neutrophils and monocytes.[10]

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The positive effect of using medicinal plants and their compounds alone or in combination with probiotics in improving the hematological and immune indices of fish has been reported in different studies.[4,11-16] Medicinal plants due to bearing a number of biologically active compounds such as glycyrrhizin and aglycone glycyrrhetic acid, liquityrin, liquiritinapioside, isoliquiritin and glabridine, and plant secondary metabolites including glycosides, terpenoids, alkaloids, terpenes, flavonoids, quinones, etc. possess many properties such as anti-stress, appetite stimulant, antibacterial, antiviral, antifungal, anti-parasitic and growth stimulant.[17] Flavonoids have attracted much attention as feed additives to improve the health and performance of aquatic animals.[18] Procyanidins and proanthocyanidins as a flavonoid are found in different parts of plants with especially high concentrations in stems and seeds.[19] Procyanidins and especially pro-anthocyanidins derived from grape seed are the focus of great interest because of their strong natural antioxidant properties. The grape seed extract (containing procyanidins) indicated a significant effect on biochemical indices, growth performance and body chemical composition of tilapia (*Oreochromis niloticus*).[20] Additionally, Mohammadi *et al*., showed the promising effects of diets supplemented with the grape seed extract on the growth and antioxidant capacity of common carp (*Cyprinus carpio*).[21]

The hematological and biochemical indices are in direct relationship with the changes in the health and these indices are affected by different factors such as fishes' species, size, age, physiological state, environment and nutritional conditions.[22,23] Therefore, the study of hematological indices provides valuable information in evaluating the health status of fish. As there was limited information about the effect of the simultaneous dietary administration of a probiotic and antioxidant on the hematological and immune indices of fish, this study was designed to evaluate the individual effects of procyanidin (as a natural antioxidant) and Bio-Aqua® probiotic (as a set multi-strain bacteria), and their combination on the hematological and immunity indices of rainbow trout.

Materials and Methods

Fish husbandry. The research was carried out in the Department of Aquaculture and Fish Health, Faculty of Veterinary Medicine, Urmia University, using well water. This experiment was carried out in a re-circulatory system and every 12 hr the water in the tanks was refreshed. The average physical and chemical indices of water during the experiment were as follow: temperature 17.00 ± 1.00 ˚C, oxygen 8.10 ± 0.60 mg L^{-1}, and pH 7.40 ± 0.40. The experiment was performed under natural light/dark cycle. A total number of 525 fingerlings with an average weight of 25.00 ± 2.00 g were obtained from a local farm. For two weeks, the fish were allowed to adapt to the new conditions. During this period, they were fed with a commercial diet (Faradaneh Co., Shahrekord, Iran). After adaptation, they were transferred to 21 tanks (300 L) with an equal number of 25 fish in each tank and then for eight weeks they were fed with experimental diets (Table 1). This study was approved by the Urmia University Animal Ethic Committee, Urmia, Iran (IR-UU-AEC-3/3).

Diet preparation. Preparation of ration was done weekly. Procyanidin (Shanghai yuanye Bio-Technology Co., Ltd., Shanghai, China) and Bio-Aqua® probiotic (Aquaforest, Brzesko, Poland) together with gelatin (4.00%) were sprayed on the basal diet (Faradaneh Co., Shahrekord, Iran) and were stored in the refrigerator at 4.00 ˚C after drying at room temperature. The composition of the basal diet used during the experiment was 45.00% protein, 17.00% carbohydrate, 13.00% lipid, 12.00% ash, 10.00% water, 2.00% fiber and 1.00% phosphorus. The feeding was done three times a day at the rate of 2.50 % Body Weight (BW). Feeding rate was recalculated biweekly according to the new average weight.[23]

Blood sampling. At the end of the experimental period, five fish of each replication were randomly selected and anesthetized with 300 mg L^{-1} of clove powder and then blood was taken using a 21-G needle through the caudal vein (one day before blood sampling, feeding was withdrawn). To measure hematological and immune parameters, the blood samples was transferred into tubes containing heparin (500 U heparin mL^{-1}) and stored at 4.00 ˚C. The remaining blood was transferred into heparin-free vials and stood first for 1 hr at room temperature, and then for 4 hr at 4.00 ˚C to clot. After centrifugation for 15 min at 3,000 rpm, the serum was separated and stored at – 20.00 ˚C for further tests.[24]

Hematological parameters. The red blood cells (RBCs) were counted using a Neubauer slide after diluting uncoagulated blood with Riess solution at a ratio of 1:200.[25] Hematocrit was determined by standard micro hematocrit and expressed as percentage.[26] A white Melanjor pipette was used to count the WBC. Furthermore, the differential count of white blood cells (WBC) was done by preparing a smear and Giemsa staining.[27]

Immune parameters. Lysozyme enzyme activity in serum was measured according to the method of Clerton *et al*. and based on the lysis of the Gram-positive bacteria (*Micrococcus lysodiekticus*) sensitive to lysozyme enzyme.[28] Total immunoglobulin was measured by the method of Siwicki and Anderson.[29] In this method, globulins were precipitated using a 12.00% polyethylene glycol solution (400 g mol^{-1}) and the amount of total immunoglobulin was calculated based on the difference in protein content at a wavelength of 590 nm before and after precipitation. The amount of complement activity (ACH50) was determined by a method previously described by Yano.[30]

Table 1. Specifications of the experimental diets used in this study.

Treatments	Diets specification
1	Basic diet (CTL)
2	Basal diet + 400 mg kg^{-1} procyanidin (PC400)
3	Basal diet + 800 mg kg^{-1} procyanidin (PC800)
4	Basic diet + 0.50% per kg of Bio-Aqua® probiotics (PB0.5)
5	Basic diet + 1.00% per kg of Bio-Aqua® probiotics (PB1)
6	Basic diet + 0.50% per kg of Bio-Aqua® probiotics + 800 mg kg^{-1} procyanidin (PB0.5 + PC800)
7	Basic diet + 1.00%/kg of Bio-Aqua® probiotics + 400 mg kg^{-1} procyanidin (PB1 + PC400)

Statistical analysis. Statistical analysis of data was performed using SPSS Software (version 20.0; IBM Corp., Armonk, USA). The normal distribution of the results was checked by the Kolmogorov-Smirnov test. A one-way ANOVA was performed to determine significant differences among treatments. The comparison of means was made according to Tukey's honestly significant difference test ($p < 0.05$).

Results

In this study, the effect of antioxidant procyanidin and Bio-Aqua® probiotics in separate treatments (PC400 and PC800, and PB0.5 and PB1) and as well as combined treatments (PB0.5 + PC800 and PB1 + PC400) were investigated on hematological and immunity indices of rainbow trout. Table 2 shows the results as regards the individual and combined effects of procyanidin and Bio-Aqua® probiotics on hematological parameters.

The results showed that procyanidin (PC400 and PC800) had no significant effect on blood indices ($p > 0.05$). However, Bio-Aqua® probiotic affected all hematological parameters (PB0.5 and PB1) and showed a significant difference with the fish of the control group ($p < 0.05$). Unlike other hematological indices, the monocyte showed a significant decrease in the treatments of Bio-Aqua® probiotics and combined procyanidin and Bio-Aqua® probiotics (PB0.5 + PC800 and PB1 + PC400), ($p < 0.05$). Nonetheless, the combination of procyanidin and Bio-Aqua® probiotics (PB0.5 + PC800 and PB1 + PC400) could affect all the hematological parameters more than the individual treatments and showed a significant difference with the fish of the control group ($p < 0.05$).

Table 3 shows the results of the individual and combined effects of procyanidin and Bio-Aqua® probiotics on immune system indices. The results proved that procyanidin individually (PC400 and PC800) did not have any significant effect on immune system indices ($p > 0.05$). But the Bio-Aqua® probiotic affected the immune system indices and had a significant difference with the fish of the control group ($p < 0.05$). The combination of procyanidin and Bio-Aqua® probiotics (PB0.5 + PC800 and PB1 + PC400) was able to affect the immune system indices more than the individual treatments of each of these substances and showed a significant difference with the fish of the control group ($p < 0.05$).

Table 2. Some blood parameters in rainbow trout fed with different experimental diets. Data are presented as mean ± standard deviation.

Treatments	RBC (× 10^6 mL^{-1})	WBC (× 10^3 mL^{-1})	PCV (%)	Lymphocyte (%)	Monocyte (%)	Neutrophil (%)
1	1.59 ± 0.19^{a}	12.64 ± 0.62^{a}	37.53 ± 1.12^{a}	75.90 ± 5.88^{ab}	3.42 ± 0.71^{d}	14.38 ± 0.69^{b}
2	1.53 ± 0.14^{a}	12.58 ± 0.51^{a}	36.76 ± 1.28^{a}	71.80 ± 4.03^{a}	3.73 ± 0.65^{d}	14.12 ± 0.63^{a}
3	1.93 ± 0.19^{a}	13.02 ± 0.49^{a}	37.31 ± 1.9^{a}	75.03 ± 4.64^{a}	3.64 ± 0.90^{d}	14.50 ± 0.66^{b}
4	3.68 ± 0.14^{b}	16.47 ± 0.40^{b}	39.45 ± 1.75^{b}	78.23 ± 6.18^{abc}	2.73 ± 0.60^{c}	15.79 ± 0.48^{c}
5	4.13 ± 0.14^{b}	16.98 ± 0.80^{b}	40.22 ± 1.60^{b}	78.66 ± 5.90^{bc}	2.54 ± 0.40^{c}	16.81 ± 0.53^{d}
6	5.89 ± 0.15^{c}	19.94 ± 0.81^{c}	43.13 ± 1.70^{c}	79.75 ± 6.02^{c}	1.67 ± 0.11b	17.95 ± 0.33^{e}
7	6.78 ± 0.10^{d}	20.88 ± 0.83^{c}	44.14 ± 1.30^{d}	79.84 ± 6.40^{c}	0.85 ± 0.08^{a}	18.84 ± 0.70^{f}

RBC: red blood cell, WBC: white blood cell, and PCV: packed cell volume.
$^{a-f}$ Means in each column with different superscripts show significant difference ($p < 0.05$).

Table 3. Immune system parameters in rainbow trout fed with different experimental diets. Data are presented as mean ± standard deviation.

Treatments	Immunoglobulin (mg dL^{-1})	Lysosome (µg mg^{-1})	Complement (U mL^{-1})
1	49.85 ± 2.45^{a}	23.19 ± 0.75^{a}	272.99 ± 16.40^{a}
2	50.04 ±2.18^{a}	22.96 ± 0.82^{a}	274.12 ± 18.42^{a}
3	51.03 ± 2.69^{a}	23.26 ± 0.65^{a}	276.42 ± 19.30^{a}
4	53.48 ± 3.20^{b}	27.22 ± 0.49^{c}	308.44 ±22.41^{b}
5	53.60 ±3.11^{b}	28.01 ± 0.90^{c}	315.83 ± 19.06^{b}
6	55.36 ± 4.05^{bc}	29.66 ± 0.90^{d}	341.21 ± 25.52^{c}
7	56.86 ± 4.25^{c}	30.59 ±1.10^{e}	356.07 ± 24.77^{d}

$^{a-e}$ Means in each column with different superscripts show significant difference ($p < 0.05$).

Discussion

One of the most important and reliable indicators in examining the health and physiology of fish is the measurement of hematological parameters. They are affected by nutrition, environmental factors and the age.[31] In the present study, the treatment of fish with procyanidin alone could not change the hematological and immune indices of rainbow trout, however, Bio-Aqua® probiotics were individually able to make significant changes in the same indices. The simultaneous dietary administration of procyanidin and Bio-Aqua® probiotic caused a significant improvement in the hematological and immune indices (except for monocytes). Panahi Sahebi *et al*. found that in the Caspian carp fry (*Cyprinus carpio*) fed with PrimaLac probiotic, WBC, hematocrit and hemoglobin indices were significantly improved and the number of RBCs was not affected.[32] In a previous study on common carp, a dose dependent response of supplementing probiotic *Bacillus cereus* and increased production of immunoglobulin, lysozyme, complement, lymphocyte and WBC were observed.[33] Furthermore, Akbari Nargesi *et al*. showed that, the treatment of rainbow trout with one gram per kilogram feed of Bio-Aqua® probiotics could cause a significant improvement in the level of RBCs, WBC, hemoglobin and hematocrit. However, no significant differences were observed in the count of lymphocytes, neutrophils and monocyte.[10] The positive effect of probiotics supplemented diet on blood and immune indices has been reported many times.[34,35] Also, Nadalizadeh Tabari *et al*. reported that Lactobacillus probiotic bacteria, isolated from the intestines of trout, showed a significantly higher antibacterial activity than the antibiotic enrofloxacin against *Yersinia ruckeri* infection (Yersiniosis).[36] The difference in the properties of probiotics can be explained according to their type, amount of use, method of the application, fish species, physiological status of fish and the environmental conditions.[37]

Procyanidin is a phenolic compound that its anti-oxidant capacity is 20 and 50 times greater than vitamin E and vitamin C, respectively.[38] The effect of procyanidin on hematological and immune indices of fish have been reported.[19,21,24,39] Adel *et al*. reported indices such as the number of WBC, RBCs, neutrophils, hematocrit, hemoglobin, total protein, immunoglobulin M and lysozyme in trout fed with peppermint (*Mentha piperita*) were increased significantly.[4] However, the complement index (C3, C4) was not increased significantly. The feeding of common carp with *Otostegia persica* and goldfish (*Carassius auratus*) with nettle plant (*Utica dioica*) did not increase the number of WBC, RBCs and hematocrit significantly.[13,16]

Tasa *et al*. feeding common carp with diet supplemented with paneer booti extract (*Withania coagulans*) found that the plant significantly increased RBCs, hematocrit, hemoglobin, lysozyme, immunoglobulin and neutrophil indices.[15] Studies have shown that although herbal dietary supplements have positive effects on many biological indices, they exert their effects in a dose dependent manner. So that, the doses more or less than the permissible limit can have no effect or even an inhibitory effect.[40] Therefore, the reason for the differences in the findings of studies can be also related to the differences in doses, duration of using the plant and the method of the application.[13,41] In the present study, the combination procyanidin and probiotic Bio-Aqua® compared to the individual treatments resulted in the highest level of hematological and immune indices, except for monocytes. The promising result of the combined use of probiotics and prebiotics compared to individual treatments has been reported in previous studies.[42] Bajelan *et al*. showed that the combination of probiotic *Entrococcus faecium* and prebiotic inulin improved the level of hematological and immune indices of binni fish (*Mesopotamichthys sharpeyi*).[43] Aftabgard *et al*. showed the combination of beta-plus probiotic and galactoligo-saccharides and isomalto-oligosaccharides prebiotics improved the hematological parameters of Caspian salmon (*Salmo trutta caspius*).[44] In the study of Noori *et al.*, the combination of aloe vera powder and *Pediococcus acidilactici* was able to significantly increase the hematological and immune indices of bluga (*Huso huso*).[45] Plant compounds have the ability to significantly accelerate the growth of probiotics in the intestine.[46] In other words, the combined use of probiotics and plants acts symbiotically exerting a synergistic effect, stimulating the growth of beneficial bacteria, improving intestinal function and increasing resistance against diseases.[47] Probiotics seem to act synergistically with antioxidants through successfully coordinating redox homeostasis in the host cell resulting in increased overall antioxidant capacity. It is also stated that probiotics can influence the redox status of the host by their capacity to: (a) chelate metal ions; (b) activate the host's antioxidant system in addition to having its antioxidant enzyme system and (c) create metabolites with antioxidant activity, such as glutathione (GSH) and butyrate.[48]

In conclusion, the results of the present study indicated that the combination of procyanidin (as a strong plant antioxidant) and Bio-Aqua® probiotic (as a multi-strain probiotic complex) could have the greatest effect in increasing the level of hematological and immunity indices except for monocytes and through the synergistic effect in rainbow trout. As blood and blood cells form an important part of the innate immune system and the specific immunity, their increase not only would improve overall health of the host but it also increases the resistance against pathogens.[49] Therefore, it is suggested to use the combined treatments of these two substances (PB0.5 + PC800 and PB1 + PC400) in rainbow trout to improve hematological and immunity indices.

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Conflict of interest

The authors declare that they have no competing interests.

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