JOURNAL OF NUTRITION FASTING AND HEALTH

Effect of *Pediococcus Acidilatici* on Intestinal Microbiota and the Oxidative Parameters of Blood and Muscles in Common Carp (*Cyprinus Carpio*)

Morteza Mirshahpanah[1], Amir Salari[2*], Davar Shahsavani[2], Hasan Baghshani[3], Mohsen Nematy[4]

1. Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
2. Department of Food Hygiene and Aquaculture, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
3. Department of Basic Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
4. Department of Nutrition, Metabolic Syndrome Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

ARTICLEINFO

Article type:
Research Paper

Article History:
Received: 10 Nov 2018
Accepted: 06 Mar 2019
Published: 09 Apr 2019

Keywords:
Pediococcus acidilactici
Common Carp
Lipid Peroxidation
Protein Carbonyls
Intestinal Microbiota

ABSTRACT

Introduction: The present study aimed to assess the effects of *Pediococcus acidilactici* as a dietary supplement on some oxidation parameters and intestinal microbiota in common carp (*Cyprinus carpio*).

Methods: In this study, 60 carps (weight: 75±5 g) were randomly divided into two groups of 30. In the first group (control), the fish received a basic dietary plan, and the second group (treatment) received a basic dietary plan supplemented with 0.9×10^7 CFU of *Pediococcus acidilactici* per gram of diet for 30 days. At the end of the trial and after blood sampling, the fish were dissected, and muscle and intestinal samples were obtained. Some oxidative status biomarkers were measured in the blood samples (superoxide dismutase, glutathione peroxidase, and glutathione) and muscle samples (malondialdehyde [MDA], protein carbonyls, and total antioxidant status) using validated spectrophotometric methods. Moreover, the microbial culture of the intestinal samples was performed.

Results: Measurement of the erythrocytic antioxidants showed no significant difference between the treatment and control groups. However, muscle MDA levels significantly decreased in the treatment group compared to the control group ($P<0.05$). In addition, muscle protein carbonyls significantly decreased in the treatment group compared to control group. Total antioxidant status was evaluated based on ferric-reducing antioxidant power and increased significantly in the treatment group compared to the control group. Microbial culture also indicated that the level of lactic acid bacteria increased in the intestinal microbiota of the probiotic group.

Conclusion: According to the results, supplementation with 5% *Pediococcus acidilactici* was effective in enhancing the antioxidant system against oxidative stress, while it also had remarkable effects on the intestinal microbiota of common carp.

► *Please cite this paper as*:
Mirshahpanah M, Salari A, Shahsavani D, Baghshani H, Nematy M. Effect of *Pediococcus acidilatici* on Intestinal Microbiota and the Oxidative Parameters of Blood and Muscles in Common Carp (*Cyprinus carpio*). J Nutrition Fasting Health. 2019; 7(1): 37-43. DOI: 10.22038/jnfh.2019.36151.1157

Introduction

Within the past decade, use of probiotics in aquaculture has developed as an environmentally friendly approach (1). Probiotics are specific, vivid microorganisms with beneficial effects on the health of the host (2). These microorganisms colonize in the intestine, preventing the action of pathogenic bacteria. In addition, they could produce various antioxidant metabolites that increase the level of antioxidant enzymes in the host (3). Recently, several studies have confirmed the beneficial effects of probiotics on the host health and growth performance of aquatic animals (4-7).

Reactive oxygen species (ROS) are naturally

* *Corresponding author:* Amir Salari, Department of Food Hygiene and Aquaculture, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran. Tel: 00985138805594; Email: a-salari@um.ac.ir

produced in living cells during normal aerobic metabolism. In some condition (e.g., hypoxia, heat stress, and some infections), ROS production increases, causing oxidative stress (4, 8). ROS degrade all biomolecules, including proteins, lipids, DNA, and carbohydrates. However, ROS production is part of the immune defense system and plays a pivotal role in antipathogenic activity in some immune cells, such as phagocytes. Nevertheless, ROS over-production or residual ROS may cause oxidative stress (9).

Due to the high levels of polyunsaturated fatty acids, fish are highly sensitive to oxidative stress (10). The antioxidant defense system decreases the production of ROS by antioxidant enzymes (e.g., superoxide dismutase [SOD] and glutathione peroxidase [GPx]) and with the help of some minerals and vitamins (e.g., vitamin E and selenium). The antioxidant defense system and immune system closely combat pathogens, while the antioxidative defense system potential of cultured fish is relatively poor (4, 11, 12).

The immunological effects of probiotics support the antioxidative defense system and immune system.

One of the main routes through which probiotics affect the immune function in fish is the effect of probiotics on gut immunity (5). Gut immunity is composed of macrophages, granulocytes, numerous diffusely organized lymphoid cells, and mucus IgM (13). Probiotics improve gut immunity through the augmentation of the immune response while in adherence to the gut-associated lymphoid tissue (GALT), thereby directly affecting the immune cells (e.g., leukocytes), enhancing the number of Ig^+ cells and acidophilic granulocytes (AGs), and increasing T-cells (14-16).

Pediococcus acidilactici (strain MA18/5M) is among the most important probiotic bacteria. Several studies have indicated the beneficial effects of this lactic acid bacteria, such as the improvement of weight gain, survival, feed conversion ratio, and hemolymph total antioxidant status in shrimps (17), modulation of intestinal bacterial communities in growing red tilapia, stimulation of some aspects of nonspecific immune response (18), reduction of infection levels, and improving the antioxidant status in shrimps (4). However, the mechanisms of these benefits have not been well recognized.

The present study aimed to assess the effects of *Pediococcus acidilactici* as a dietary supplement on some oxidation parameters and intestinal microbiota in common carp (*Cyprinus carpio).*

Material and methods

Experimental Design and Sampling

Probiotic Bactocel® (Duncan, Germany) was used in this study, which contained $1*10^{10}$ CFU/g of *Pediococcus acidilactici* (strain MA18/5M). Dietary formulation and proximate composition of the basal diet are presented in Table 1.

Table 1. Composition and Proximate Analysis of Diets

Ingredient	(g/kg)
Fishmeal	300
Soybean Meal	160
Corn Meal	240
Wheat Flour	180
Rice Bran	80
Fish Oil	20
Soybean Oil	20

In total, 60 common carps (*Cyprinus carpio*; weight: 75±5 g) were obtained from a local farm in Mazandaran, Iran and randomly divided into two equal groups. The samples were preserved in two glass aquaria, each of which contained 250 liters of fresh water. The fish were acclimatized for seven days before the experiments and received a commercial pellet diet at the rate of 2% body weight day^{-1}. During the experiments, the physicochemical conditions of water were the dissolved oxygen of 5.5-6 ppm, temperature of 25±1ºC, and pH of 7±0.5. The photoperiod was a 12:12 light-dark cycle, and the water in the aquaria was renewed every 48 hours.

The fish in the first group received a basic dietary plan (control), and the fish in the second groups received a basic dietary plan supplemented with 0.9×10^7 CFU of *Pediococcus acidilactici* per gram of diet (17). The fish in each group were fed three times daily at 8:00, 13:00, and 19:00 throughout the experiments (30 days).

At the end of the experiments, five fish were selected randomly from each aquarium and anesthetized in diluted MS-222. Blood samples were collected by cardiac puncture using heparinized syringes and tubes. After plasma separation by centrifugation at 1000×g for 20

minutes, erythrocyte pellet was washed three times with normal saline solution. The washed and centrifuged erythrocytes were hemolyzed with the addition of an equal volume of ice-cold redistilled water, and the prepared plasma hemolysate aliquots were stored at the temperature of -70°C until analysis.

The fish were sacrificed by cutting the spinal cord. Afterwards, the dorsal and abdominal surfaces of the samples were disinfected with 70% ethanol. The abdominal cavity was opened using a sterile surgical knife, and the intestine was separated from the esophagus to 0.5 centimeter prior to the anus. Muscle samples were collected from the middle-dorsal region, located below the dorsal fin of the dark muscles on each side of the body. The muscle samples were rapidly thawed and homogenized in 10 volumes (w/v) of ice-cold 0.05 M phosphate buffer (pH: 7.4) for five minutes and centrifuged at 4,000 g at the temperature of 4˚C for 15 minutes. The supernatant was preserved in ice until assayed.

Microbial Culture

In order to investigate the effect of *Pediococcus acidilactici* on the intestinal microbiota, the intestinal samples (gastrointestinal wall and its contents) were cut longitudinally with a sterile surgical knife at the end of the experiments. Following that, the samples were diluted using sterile distilled water and placed in a bag mixer for 10 minutes in order to make them identical. At the next stage, the dilutions were prepared and cultivated in total vial count (TVC) on plate count agar to determine the total count of the intestinal bacteria, as well as De Man, Rogosa, and Sharpe (MRS) agar (Merck, Germany) in order to specify the count of intestinal lactic acid bacteria.

Biochemical Assays and Analysis

The ferric-reducing antioxidant power or ferric-reducing ability (FRAP assay) of the extracts was performed as described previously (19). The prepared stock solutions included acetate buffer (300 mM), pH of 3.6, 2, 4, 6-tri (2-pyridyl)-s-triazine (TPTZ; 10 mM) solution in 40 mM of HCl, and FeCl3·6H_2O solution (20 mM).

The plant extracts or standard methanolic Trolox solutions (150 µL) were incubated at the temperature of 37°C with two milliliters of the FRAP solution, which had been prepared by mixing 25 milliliters of acetate buffer, five milliliters of the TPTZ solution, and 10 milliliters of the $FeCl_3 \cdot 6H_2O$ solution, for 30 minutes in the dark. Afterwards, the absorbance of the formed blue ferrous tripyridyltriazine complex was read at 593 nanometers.

Malondialdehyde (MDA) concentration was determined based on the spectrophotometry of the pink-colored product of thiobarbituric acid-reactive substances as described by Latha and Pari (20). The concentration of MDA was calculated using the molar extinction coefficient value of 156,000 M^{-1} cm^{-1}.

The carbonyl groups of proteins were detected through the reaction with 2, 4-dinitrophenylhydrazine, which led to the formation of a stable 2, 4-dinitrophenylhydrazone product (21). The resulting 2, 4 dinitrophenylhydrazones were quantified spectrophotometrically at 370 nanometers using the molar extinction coefficient of 22,000 M^{-1} cm^{-1}.

Measurement of some endogenous antioxidants (SOD, GPx, and GSH) in the erythrocyte samples was performed using RANDOX® kits (Randox, UK).

Statistical Analysis

Data were expressed as mean and standard deviation, and data analysis was performed using one-way analysis of variance (ANOVA). In addition, student's t-test was used to compare the mean values in the study groups, and the differences were considered significant at the P-value of less than 0.05.

Results

Biochemical Assays and Analysis

As is depicted in Figures 1-3, the levels of the measured antioxidants (SOD, GPx, and GSH) had no significant differences between the treatment and control groups.

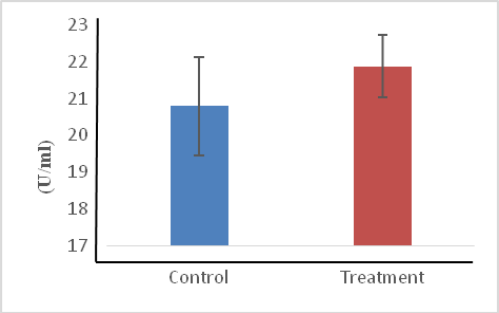

Figure 1: Effect of *P. acidilactici* on superoxide dismutase activity in erythrocyte hemolysate of common carp. Data are mean ± SEM (n = 5 in each group). (Asterish significantly different from control)

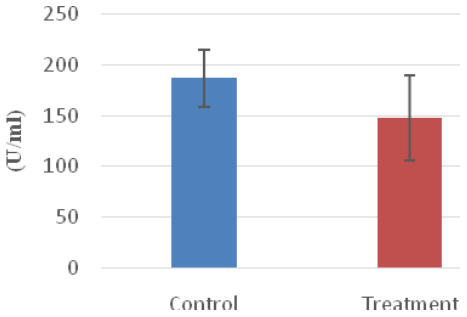

Figure 2: Effect of *P. acidilactici* on glutathione peroxidase activity in erythrocyte hemolysate of common carp. Data are mean ± SEM (n = 5 in each group). (Asterish significantly different from control)

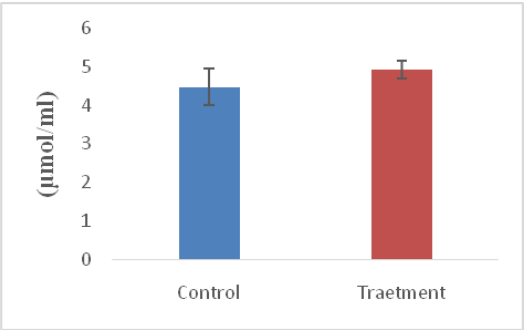

Figure 3: Effect of *P. acidilactici* on glutathione concentration in erythrocyte hemolysate of common carp. Data are mean ± SEM (n = 5 in each group). (Asterish significantly different from control)

With regard to the MDA levels in the muscle tissues, the obtained results indicated that the MDA levels in the treatment group were significantly lower compared to the control group (Figure 4).

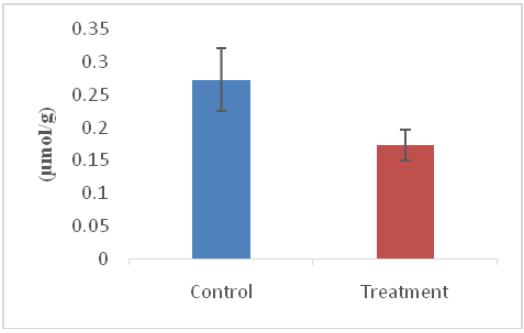

Figure 4: Effect of *P. acidilactici* on malondialdehyde in muscle of common carp. Data are mean ± SEM (n = 5 in each group). (Asterish significantly different from control)

Furthermore, the protein carbonyl contents significantly decreased in the treatment group compared to the control group (Figure 5).

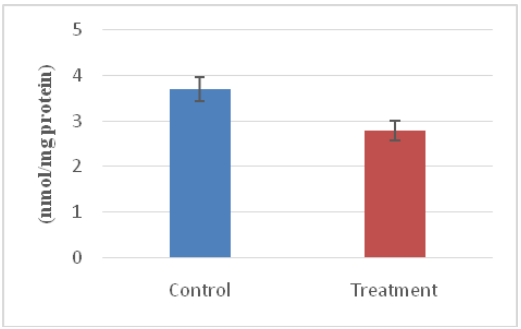

Figure 5: Effect of *P. acidilactici* on carbonyl groups of proteins in muscle of common carp. Data are mean ± SEM (n = 5 in each group). (Asterish significantly different from control)

As is shown in Figure 6, the muscle FRAP values significantly increased in the treatment group compared to the control group.

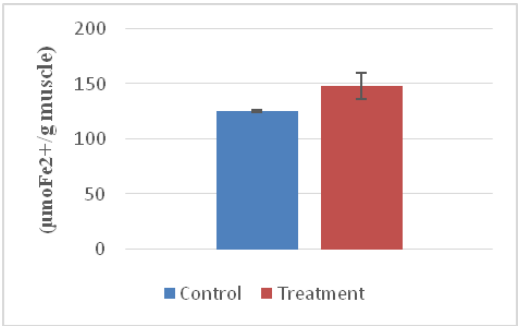

Figure6: Effect of *P. acidilactici* on FRAP values in muscle of common carp. Data are mean ± SEM (n = 5 in each group). (Asterish significantly different from control)

Microbial Culture

The results of microbial culture in the MRS agar and TVC are presented in Table 2. Accordingly, the number of the *Lactobacillus* bacteria in the probiotic group significantly increased compared to the control group (P<0.05).

Table 2. Viable Counts (CFU g^{-1}) of Intestinal Microbiota after Trial (day 30) (data expressed as mean±SEM; n=5 per group)

Group	MRS Agar	TVC
Control	2.4233±0.05239a	0.7167±0.05239[a]
Treatment	2.6333±0.03756b	1.3400±0.06928[b]

[a, b] Different superscript letters in the same column indicate significant differences (P<0.05).

Discussion

Aerobic organisms require molecular oxygen (O_2) to provide energy, which is completely restored to water during cellular respiration. However, 2-4% of the oxygen is consumed in defective resuscitation and ROS production (22). Organisms neutralize ROS and counteract their harmful effects using some enzymatic systems and the low-weight molecules that act as antioxidants. The formation of ROS could also cause oxidative stress (23), and oxidative stress may cause protein degradation (22), inactivation of enzymes, lipid peroxidation, cell damage, and apoptosis (24).

In the present study, the status of oxidative stress was evaluated by measuring the products or macromolecules that were damaged by ROS (e.g., MDA and carbonyl protein levels) in the muscles and measuring the primary defense against ROS using antioxidants (e.g., SOD, GPx, and GSH) in erythrocyte hemolysate. According to our findings regarding antioxidants (SOD, GPx, GSH), *Pediococcus acidilactici* had no significant effects on the concentrations of these enzymes in the comparison of the treatment and control groups. Similarly, Castex et al. (17) reported no significant difference in the concentrations of SOD and GSH between the treatment and control groups, while the concentration of GPx in the digestive glands increased in shrimps (*Litopenaeus stylirostris*).

In another research, Castex et al. (4) observed that *Pediococcus acidilactici* significantly increased the activity of SOD and GPx, while significantly decreasing the concentration of GSH in the digestive glands of *Litopenaeus stylirostris*. The differences in the levels of antioxidant enzymes could be due to the fact that enzymes have higher activity in digestive tissues. Therefore, our findings indicated that probiotics may have significant effects on the levels of the antioxidants in erythrocytes.

Our findings regarding the free radical damage to lipids (MDA) and proteins (carbonyl proteins) indicated a significant reduction in the treatment and control groups. Since MDA levels are considered to be an appropriate indicator for lipid peroxidation (25), the results obtained by Weifen et al. (26) on grass carp *(Ctenopharyngodon idellus)* demonstrated that the use of *Bacillus* in culture water and as a dietary supplement reduced the levels of MDA in the liver. The main reasons for the mentioned finding were the use of *Bacillus* as a pathogen and stimulating the immune system, improvement of nonspecific immune parameters, and enhancement of the antioxidant ability of grass carp. Furthermore, Bita et al. (27) observed that the addition of Synbiotic Biomin Imbo® significantly reduced the concentration of MDA in the muscles of *Mugil cephalus*, which is consistent with the results of the present study. As stated earlier, the concentration of carbonyl proteins significantly decreased in the treatment group compared to the control group in the present study. This is in congruence with the findings of Castex et al. (17).

In the current research, TAS was significantly higher in the treatment group compared to the control group in the evaluation of the muscle samples. This finding is in line with the results reported in the previous studies in this regard (4, 17, 27-29). Therefore, it could be inferred that the use of *Pediococcus acidilactici* as probiotic supplementation could prevent the damage caused by ROS to some macromolecules through improving the total antioxidant status. However, considering the findings of the current research, the bacteria had no effect on the antioxidants (SOD, GPx, and GSH) in the erythrocyte samples. Therefore, the probiotic bacteria might enhance the antioxidant status by affecting the intestinal microbiota.

According to the results of microbial culture in the MRS agar and TVC, lactic acid bacteria increased in the treatment group compared to the control group. This finding could clarify the

possible effects of *Pediococcus acidilactici* on the oxidation status, which could be attributed to the effects of the bacteria on the digestive tract of the fish. Probiotics exhibit such functions through limiting the presence and activity of pathogenic bacteria in the gastrointestinal tract via the production of inhibitory compounds or competition for adhesion sites, energy, and nutrients (30), and stimulating the gut immune system by increasing the number of Ig^+ cells and acidophilic granulocytes (15, 16). T-cells are found in the GALT of many fish and significantly increase by probiotics (16, 31, 32) through the production of vitamins and digestive enzymes (e.g., proteases) and degradation of indigestible compounds, thereby enhancing the nutritional status in fish (33), performing fermented processes in the body of fish, and producing antioxidant metabolites (26).

According to the findings of the current research, *Pediococcus acidilactici* might enhance the antioxidant status by influencing the intestinal microbiota and reducing the damages induced by oxidative stress in the lipids and proteins of the muscle tissues in common carp.

Conclusion

According to the results, *Pediococcus acidilactici* might enhance the antioxidant status by influencing the intestinal microbiota and reducing the damages caused by oxidative stress in the lipids and proteins of the muscle tissues in common carp.

Acknowledgments

Hereby, we extend our gratitude to Ferdowsi University of Mashhad, Iran for the financial support of this study.

Conflict of interest

None declared.

References

1. Yanbo W, Zirong X. Effect of probiotics for common carp (*Cyprinus carpio*) based on growth performance and digestive enzyme activities. Anim Feed Sci Technol. 2006; 127(3-4):283-92.
2. Stanton C, Gardiner G, Meehan H, Collins K, Fitzgerald G, Lynch PB, et al. Market potential for probiotics. Am J Clin Nutr. 2001; 73(2 Suppl): 476S-483S.
3. Tagizadeh A, zargari F, Dehnad A, Habibi P, Ghaderi R. The effect of probiotic *Lactobacillus* on serum antioxidant status in streptozotocin-induced diabetic rats. Razi journal of medical science. 2018; 25(1): 92-9.
4. Castex M, Lemaire P, Wabete N, Chim L. Effect of probiotic *Pediococcus acidilactici* on antioxidant defences and oxidative stress of *Litopenaeus stylirostris* under *Vibrio nigripulchritudo* challenge. Fish Shellfish Immunol. 2010; 28(4): 622-31.
5. Nayak SK. Probiotics and immunity: a fish perspective. Fish Shellfish Immunol. 2010; 29(1): 2-14.
6. Iwashita MK, Nakandakare IB, Terhune JS, Wood T, Ranzani-Paiva MJ. Dietary supplementation with *Bacillus subtilis, Saccharomyces cerevisiae* and *Aspergillus oryzae* enhance immunity and disease resistance against *Aeromonas hydrophila* and *Streptococcus iniae* infection in juvenile tilapia Oreochromis niloticus. Fish Shellfish Immunol. 2015; 43(1): 60-6.
7. Wang YB, Tian ZQ, Yao JT, Li WF. Effect of probiotics, *Enteroccus faecium*, on tilapia (*Oreochromis niloticus*) growth performance and immune response. Aquaculture. 2008; 277(3-4): 203-7.
8. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. 3rd ed. Oxford University Press; 1999.
9. Shacter E. Quantification and significance of protein oxidation in biological samples. Drug Metab Rev. 2000; 32(3-4): 307–26.
10. EFSA. Scientific opinion on dietary reference values for fats, including saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, trans fatty acids and cholesterol. EFSA J. 2010; 8(3): 1461.
11. Nakano T, Kanmuri T, Sato M, Takeuchi M. Effect of astaxanthin rich red yeast (*Phaffia rhodozyma*) on oxidative stress in rainbow trout. Biochim Biophys Acta. 1999; 1426(1): 119-25.
12. Holmblad T, Söderhäll K. Cell adhesion molecules and antioxidative enzymes in a crustacean, possible role in immunity. Aquaculture. 1999; 172: 111-23.
13. Rombout JHWM, Taverne-Thiele AJ, Villena MI. The gut-associated lymphoid tissue (GALT) of carp (*Cyprinus carpio L.*): an immunocytochemical analysis. Dev Comp Immunol. 1993; 17(1): 55-66.
14. De Simone C, Salvadori BB, Negri R, Ferrazzi M, Baldinelli L, Vesely R. The adjuvant effect of yogurt on production of g-interferon by Con A-stimulated human peripheral blood lymphocytes. Nutr Rep Int. 1986; 33: 419-33.
15. Picchietti S, Mazzini M, Taddei AR, Renna R, Fausto AM, Mulero V, et al. Effects of administration of probiotic strains on GALT of larval gilthead seabream: immunohistochemical and ultrastructural studies. Fish Shellfish Immunol. 2007; 22(1-2): 57-67.
16. Picchietti S, Fausto AM, Randelli E, Carnevali O, Taddei AR, Buonocore F, et al. Early treatment with *Lactobacillus delbrueckii* strain induces an increase in intestinal T-cells and granulocytes and modulates

immunerelated genes of larval *Dicentrarchus labrax* (L.). Fish Shellfish Immunol. 2009; 26(3): 368-76.
17. Castex M, Lemaire P, Wabete N, Chim L. Effect of dietary probiotic *Pediococcus acidilactici* on antioxidant defences and oxidative stress status of shrimp *Litopenaeus stylirostris*. Aquaculture. 2009; 294(3-4): 306-13.
18. Ferguson RM, Merrifield DL, Harper GM, Rawling MD, Mustafa S, Picchietti S, et al. The effect of *Pediococcus acidilactici* on the gut microbiota and immune status of on-growing red tilapia (*Oreochromis niloticus*). J Appl Microbiol. 2010; 109(3): 851-62.
19. Benzie IF, Strain JJ. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power: The FRAP assay". Anal Biochem. 1996; 239(1): 70-6.
20. Latha M, Pari L. Preventive effects of *Cassia auriculata L.* flowers on brain lipid peroxidation in rats treated with streptozotocin. Mol Cell Biochem. 2003; 243(1-2): 23-8.
21. Petron MJ, Raes K, Claeys E, Lourenc M, Fremaut D, De Smet S. Effect of grazing pastures of different botanical composition on antioxidant enzyme activities and oxidative stability of lamb meat. Meat Sci. 2007; 75: 737-45.
22. Dean RT, Fu S, Stocker R, Davies MJ. Biochemistry and Pathology of radical – mediated protein oxidation. Biochem J. 1997; 324(Pt 1): 1–18.
23. Welker TL, Congleton JL. Effect of dietary α-tocopherol + ascorbic acid, selenium, and iron on oxidative stress in sub-yearling Chinook salmon (*Oncorhynchus tshawytscha Walbaum*). J Anim Physiol Anim Nutr (Berl). 2009; 93(1): 15-25.
24. Katoch B, Sebastian S, Sahdev S, Padh H, Hasnain SE, Begum R. Programmed cell death and its clinical implication. Indian Journal of experimental Biology. 2002; 40: 513–24.
25. Nogueira CW, Quinhones EB, Jung EA, Zeni G, Rocha JB. Anti-inflammatory and antinociceptive activity of diphenyl diselenide. Inflamm Res. 2003; 52(2): 56-63.
26. Weifen L, Xiaoping Z, Wenhui S, Bin D, Quan L, Luoqin F, et al. Effects of Bacillus preparations on immunity and antioxidant activities in grass carp (*Ctenopharyngodon idellus*). Fish Physiol Biochem. 2012; 38(6): 1585-92.
27. Akbary P, Bita S, Sarhadipour M. Effect of Synbiotic Biomin Imbo on anti-oxidant status and lipid profiles of *Mugil cephalus*. Veterinary Researches & Biological Products. 2017; 30(2): 224-33. (Persian)
28. Mohammadinejad S, Sayari A, Moslehi M, Veysi M. Impact of consumption of a single course of probiotic supplementation on malondialdehyde, total antioxidant capacity and lactic acid in futsalist women. Jun sci med j. 2018; 17(2): 159-67.
29. Kullisaar T, Songisepp E, Mikelsaar M, Zilmer K, Vihalemm T, Zilmer M. Antioxidative probiotic fermented goats' milk decreases oxidative stress mediated atherogenicity in human subjects. Br J Nutr. 2003; 90(2): 449-56.
30. Vershuere L, Rombaut G, Sorgeloos P, Verstraete W. Probiotic bacteria as biologicalcontrol agents in aquaculture. Microbiol Mol Biol Rev. 2000; 64(4): 655-71.
31. Scapigliati G, Romano N, Abelli L, Meloni S, Ficca AG, Buonocore F, et al. Immunopurification of T-cells from sea bass *Dicentrarchus labrax* (L.). Fish Shellfish Immunol. 2000; 10(4): 329-41.
32. Romano N, Rossi F, Abelli L, Caccia E, Piergentili R, Mastrolia L, et al. Majority of TcRβ+ T-lymphocytes located in thymus and midgut of the bony fish, *Dicentrarchus labrax* (L). Cell Tissue Res. 2007; 329(3): 479-89.
33. Imada, C, Simidu U, Taga N. Isolation and characterization of marine bacteria producing alkaline protease inhibitor. Bull Jpn Soc Sci Fish. 1985; 51: 799–803.