Int. J. Aquat. Biol. (2019) 7(4): 211-217
ISSN: 2322-5270; P-ISSN: 2383-0956
Journal homepage: www.ij-aquaticbiology.com

Original Article

Effects of florfenicol on skin mucus immune parameters and immune related genes expression in zebrafish (*Danio rerio*)

Narmin Ezatrahimi[1], Siyavash Soltanian[*1], Mostafa Akhlaghi[1], Seyed Hossein Hoseinifar[2]

[1]Department of Clinical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran
[2]Department of Fisheries, Faculty of Fisheries and Environmental Sciences, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran.

Abstract: Florfenicol (FF) is a common, inexpensive antibiotic with relatively low toxicity. The present study investigates the possible effects of Florfenicol on cutaneous mucus immune parameters and immune related genes expression in *Danio rerio*. After two weeks adaptation, fish were stocked in experimental units at density of 20 fish per aquaria and fed with experimental diets containing 0, 10, 30 and 50 mg/kg FF per for 11 days. Evaluation of immune parameters at the end of trial showed that the control group had a significantly lower level of lysozyme activity when compared with those treated with antibiotic. However, the skin mucus total immunoglobulins level of fish fed diets containing antibiotics did not show any significant difference compared to the control group. The highest expression level of TNF-α, IL1β and lysozyme was observed in fish treated with 10 mg/kg FF while the lowest expression was noticed in those fish treated with 30 and 50 mg/kg FF. The present results indicate the relatively positive effects of this antibiotic on the immune system of zebrafish, and it seems that the appropriate dosage of the drug can serve as an immunostimulant for zebrafish.

Article history:
Received 3 June 2019
Accepted 24 August 2019
Available online 25 August 2019

Keywords:
Florfenicol
Immune
Mucus
Gene expression
Zebrafish

Introduction

Fishes are vulnerable to a vast variety of pathogenic and non-pathogenic micro-organisms. As aquaculture is developing and new methods of culture are developed, there is an increase in incidence rates of bacterial, viral, parasitic diseases as well as environmental problems. To deal with stressful conditions, fish develop adaptation mechanism. In fact, through development of such mechanism, natural or homeostatic status of body can be maintained (Makvandi et al., 2011). A complex set of specific and non-specific immune parameters concerning stress response in fish have been identified (Ellis, 2001). Primary response to pathogens is formed by the primary defense system which include the skin mucus, skin, gills, gastrointestinal tract as well as blood components, including natural killer cells and phagocytes (Anbarasu and Chandran, 2001). The mucus layer consists of glycoprotein, proteoglycan and proteins that, besides providing physical protection, contain secretory compounds such as agglutinins, lysines, lysozymes, non-specific precipitators, acute phase protein. Also, the presence of natural anti-bodies provide defensive-chemical role against environmental micro-organisms (Soltani, 2008). Fish are commonly exposed to chemicals released into aquatic system which *per se* can disrupt innate and adaptive immune system (Yousefi and Hoseinifar, 2018).

Antibiotics have significant roles in controlling diseases. Besides, in aquaculture they have formerly been used as growth stimulators, antibacterial agents, and for a variety of other purposes (Masahiro et al., 1999; Li et al., 2012). However, due to their residual effects on the fish and shrimp tissues and consumers' lack of interest, application of antibiotics is not recommended in aquaculture (Khajeali, 2012). The interaction between medications and lymphoid tissues may affect the performance and balance of immune system causing unintentional negative effects such as

[*]Correspondence: Siyavash Soltanian
E-mail: siyavashsoltanian@yahoo.com
DOI: https://doi.org/10.22034/ijab.v7i4.631

immunosuppression, uncontrolled cell proliferation, changes in defensive mechanisms of host against pathogens, and even neoplasia (Ren et al., 2014). Several immunomodulatory effects of anti-bacterial and other medications have been reported (Lunden et al., 1999). In recent years, several reports on antibiotic resistance in pathogens raised a concern about fisheries and public health, hence, emphasizing identification and application of new antibacterial compounds to deal with such bacterial diseases (Park et al., 2008).

Florfenicol (FF) is a broad-spectrum antibiotic, uses for treatment of various infections, which has been confirmed in food-producing animals due to low price and relatively low toxicity (Carty et al., 2008). In addition to merits of chloramphenicol such as wide spectrum effects and high tissue infiltration, FF has a longer elimination half-life. On the other hand, due to substitution of fluorine with a hydroxyl group in its chemical structure, florfenicol is more resistant to enzymatic inactivation, putting effects on a wider spectrum of chloramphenicol-resistant bacterial strains (Anadon et al., 2008). Despite an increase in use of FF, some studies document its toxicity (Hu et al., 2014). Therefore, it is essential to determine its effects on mucosal immune level of fishes as their primary defense mechanism against pathogens.

Zebrafish *(Danio rerio*) has recently been introduced as a model for rapid analysis of gene performance (Zon et al., 2005). It is widely used in different laboratories for genetic research (Haffter et al., 1996; Driever et al., 1996). Hence, the present study aims to investigate the effects of florfenicol on the fish mucosal immunity as the primary defensive mechanism against pathogens.

Materials and Methods

Fish and experimental design: A total of 240 zebrafish were purchased from a private farm. Each experimental unit (84 L aquarium) contained 20 fish. Prior to the study, a 2-week adaptation period with laboratory conditions was considered. They were fed a commercial food Biomar (BioMar SAS, Nersac, France). Throughout the study, water temperature, pH, and dissolved oxygen were monitored daily and maintained at optimum rates.

Table 1. The proximate composition of the basal diet.

Proximate analysis (%)	
Dry matter	93.2
Crude protein	38.6
Crude lipid	15.1
Ash	11.0

FF was purchased from the Rooyan Company and added to the basal diet. The experiment was designed as completely randomized with four treatments (three experimental and one control) repeated in triplicates. The feeding trial was lasted for 11 days, and during this period, fish fed 3% of body weight, twice a day.

Experimental diet preparation: In this study, commercial food, Biomar (BioMar SAS, Nersac, France) (Table 1) was used as the basal diet and different doses (10, 30 and 50 mg/kg) of FF were added.

Skin mucus immune parameters: Skin mucus samples were obtained based on Ross et al. (2000). Accordingly, three fish from each tank were randomly selected and anaesthetized. The mucus samples were kept in -80°C before determining the lysozyme activity and the total Ig level. Enzymatic assay of lysozyme was performed using turbidimetric method (Subramanian et al., 2007). In this procedure, *Micrococcus Luteu*s (ATCC4698) was used as substrate. The mucus total Ig levels was measured based on the method suggested by Siwicki and Anderson (1993).

Determination of immune genes expression: The intestinal tissue samples were obtained from samples and immediately transferred to liquid nitrogen tank. To extract total DNA, 50-100 milligrams of the tissue was homogenized in 1 ml of RNX-Plus for 15 minutes in room temperature according to company guidelines.

After drying the product, it was dissolved in 50 ml of distilled water and then kept in the refrigerator at -80°C. Prior transferring samples to refrigerator, the concentration enzyme activity of RNA were checked using spectrophotometer in 260/280 nanometers.

Table 2. Sequences and accession numbers of primers used.

Primer name	Primer Sequence	Application	Accession number
i1b q-PCRF	CGTCTCCACATCTCGTACTCA	Immune	AY340959.1
il1b q-PCRR	GTGTCTTTCCTGTCCATCTCC		
tnf-alpha q-PCRF	CTGCTTCACGCTCCATAAGA	Immune	AY427649.1
tnf-alpha q-PCRR	CTGGTCCTGGTCATCTCTCC		
lyz q-PCRF	GGCAGTGGTGTTTTTGTGTC	Immune	NM_139180.1
lyz q-PCRR	CGTAGTCCTTCCCCGTATCA		
β-actin q-PCRF	AGCAGATGTGGATCAGCAAG	Housekeeping	NM_131031.1
β-actin q-PCRR	TACCTCCCTTTGCCAGTTTC		

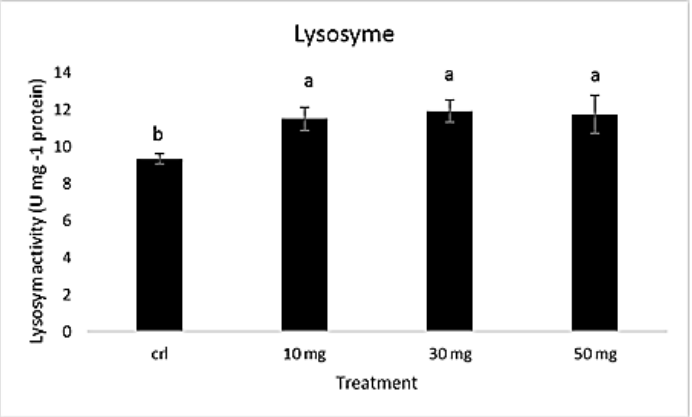

Figure 1. The effect of different doses of florfenicol on mucus lysozyme activity in zebrafish. Values (n=9) are presented as the mean ± standard deviation, obtained by dividing each sampling value by the mean control value at the same sampling time. Different letters above the bars denote a significant difference between treatments (P<0.05).

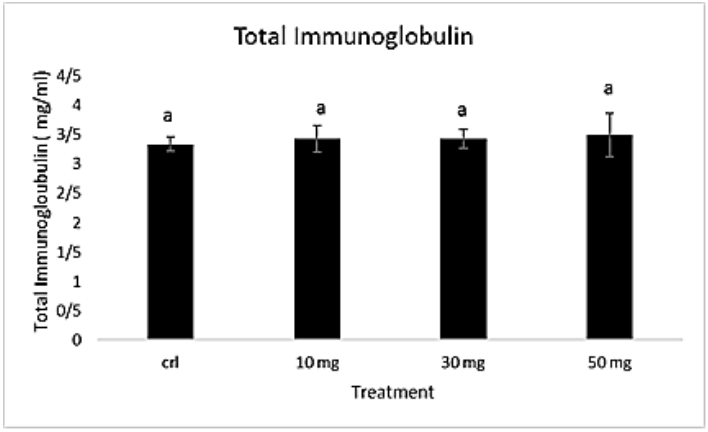

Figure 2. The effect of different doses of florfenicol on mucus total Ig in zebrafish. Values (n=9) are presented as the mean ± standard deviation, obtained by dividing each sampling value by the mean control value at the same sampling time. Different letters above the bars denote a significant difference between treatments (P<0.05).

Also, the quality of RNA was assessed using agarose gel electrophoresis as well as dyeing with ethidium bromide (Miandare et al., 2013). One ml of total RNA was used to build cDNA using bio rad kit. Copying process was conducted according to the manufacturer's instructions. The resulted cDNA was kept in -20°C (Gioacchini et al., 2010).

The primers of qPCR were designed for TNF-∝, IL-1 and Lys genes in gene bank based on protected areas of DNA sequences (Table 2). Then, the primers were added to the samples for analyzing gene expressions. Copying process was conducted according to a specific protocol. Cyber green method was used to investigate gene expression and the samples were put in Real Time PCR instrument to measure gene expression.

Statistical Analysis: SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The statistically significant differences (P<0.05) were determined using one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests.

Results

Skin mucus immune parameters: The skin mucus lysozyme activity of fish in control group's was significantly lower than that of the experimental groups treated by various doses of antibiotic (P<0.05) (Fig. 1). The results showed the total immunoglobulin in fish mucus was not significantly different between treatments (P>0.05) (Fig. 2).

Immune related genes expression: gene expression studies revealed the highest expression of TNF-α gene in 10 mg of FF treatment (Fig. 3), whereas the others did not have significant differences compared to control. In addition, in 10 mg of FF treatment the expression of IL-1β gene was the highest (Fig. 4), and those of 30 and 50 mg had the lowest level, and the control one showed an intermediate levels (P<0.05). The expression of Lysozyme gene in 10 mg/g FF was

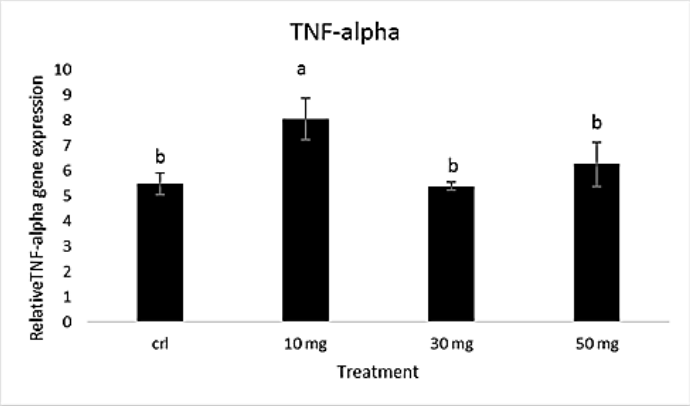

Figure 2. The effect of different doses of florfenicol on relative expression of TNF-α gene in zebrafish. Values (n=9) are presented as the mean ± standard deviation, obtained by dividing each sampling value by the mean control value at the same sampling time. Different letters above the bars denote a significant difference between treatments (P<0.05).

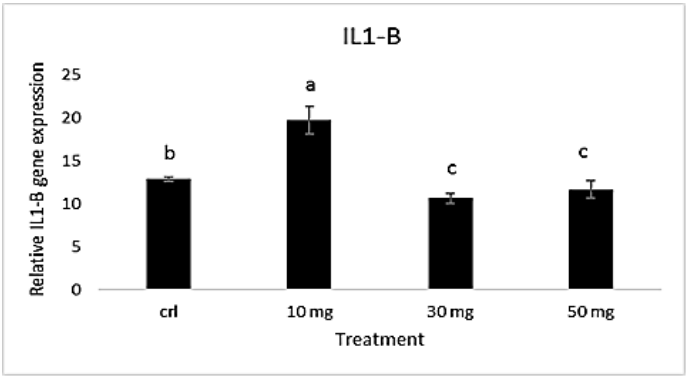

Figure 4. The effect of different doses of florfenicol on relative expression of IL-1β gene in zebrafish. Values (n=9) are presented as the mean ± standard deviation, obtained by dividing each sampling value by the mean control value at the same sampling time. Different letters above the bars denote a significant difference between treatments (P<0.05).

significantly higher than others (Fig. 5). However, no significant difference was noticed when compared with control group.

Discussions

Improvement and protection of fish health is one of the main factor in the aquaculture industry (Mahghani et al., 2014). Increasing the productivity of fish farming is associated with stressful conditions, which provides a sensitive environment for infectious diseases (Dehghan et al., 2016). Lysozyme is one of the most important parameters of the non-specific humoral immune system. It is a polypeptide with a

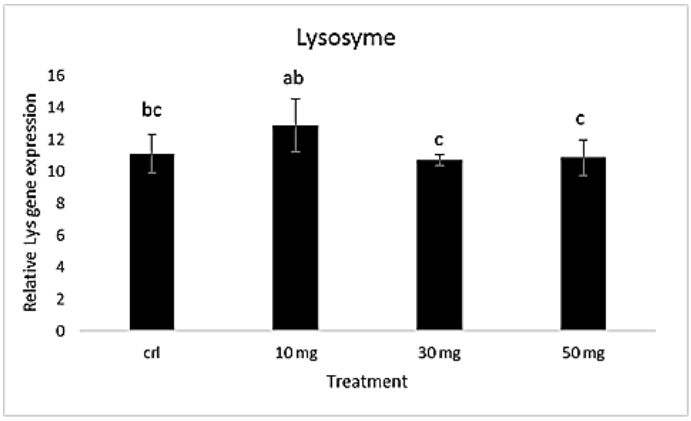

Figure 5. The effect of different doses of florfenicol on relative expression of lysozyme gene in zebrafish. Values (n=9) are presented as the mean ± standard deviation, obtained by dividing each sampling value by the mean control value at the same sampling time. Different letters above the bars denote a significant difference between treatments (P<0.05).

molecular weight of 14 to 18 kDa. It exists in a wide range of vertebrates, including freshwater and marine species, and is known as an antibacterial agent (Itami et al., 1992). This enzyme is secreted from white blood cell granules (mostly by neutrophils, monocytes and less by macrophages) as well as leukocyte-rich tissues such as kidneys, mucus membranes, spleen, gills and digestive tract since these organs are highly susceptible to invasion by pathogenic bacteria (Holloway et al., 1993). The enzyme can break down the glycosidic bond of peptidoglycan layer in the bacterial cell wall. Lysozyme has direct effects on gram-positive bacteria; and can eliminate the gram-negative bacteria with the help of complement system (Yano, 1996).

In the present study, evaluation of skin mucus lysozyme activity revealed significant increases in all experimental groups compared to the control group. However, the treatment containing 30 mg FF showed more activity, which was not statistically significant. Reda et al. (2013) reported that lysozyme activity is significantly increased in tilapia after FF administration, but had no significant effect on IgM, phagocytosis and ALT as seen in the present study. In contrast, administration of FF in hybrid tilapia diet at rate of 0.02 g kg^{-1} had no effect on serum lysozyme. It may be due to different conditions used in previous studies, fish species, temperature, antibiotic dose and

route of administration which can affects drug absorption (Bjorklund and Bylund, 1990). According to Ren et al. (2014), juveniles of *Litopenaeus vannamei* revealed no significant differences in antibacterial activities in plasma following administration of 100 mg kg^{-1} FF, whereas 200 mg kg^{-1} FF treatment suppressed them, the total hemocyte count and phagocytic activity decreased significantly in both treatments. In the present study, the total Ig level in the skin mucus of fish fed diet containing different antibiotic doses showed no significant difference compared to control.

Since the function of a gene at the mRNA level varies from that of the protein after translation, the evaluation of the expression of lysozyme gene can be reliable in evaluation of the effect of immunostimulants on this gene. The level of lysozyme or its activity is viewed as an important indicator in the primary defense of fish. There are changes in levels of lysozyme activity or mRNA expression in response to infectious diseases or stress. Based on the results, the highest expression of lysozyme was in fish treated with 10 mg/g FF. However, there was no significant differences when compared with the control. IL1β and TNF-α expression in the 10 mg/kg FF were highest; however, 30 and 50 mg/kg of FF had the lowest expression level, and the fish in control group showed an intermediate level. The tumor necrosis factor (TNF) is a signaling protein (cytokine) participating in systemic inflammation as one of the cytokines forming the reaction of the acute phase. TNF-α serves as intermediate proteins in immune cells and puts effects on them (Gruss, 1996). TNF-α is the main mediator of acute inflammatory response to Gram-negative bacteria and other bacterial microbes and accounts for most of the systemic disorders of acute infections. The results showed that the expression of this gene in fish treated with 10 mg/kg was highest. In contrast, suppressing effects of FF on rat immune responses showed that FF could suppress humoral and cellular immune responses in mice (Guan et al., 2011). Similarly, some antibiotics have a suppressive effect on increased levels of TNF-α (Er et al., 2010; Er and Yazar, 2012). In this study, the expression of this gene decreased in a dose-dependent manner.

In brown trout, serum levels of TNF-α in fish treated with FF were not suppressed following LPS injection. This may be due to the dose of FF or differences in the species type (Ayse and Burak, 2014). According to Caipang et al. (2009), oral administration of FF and oxolinic acid significantly increase the anti-inflammatory cytokines, including IL-1β and IL-8 in Atlantic cod (*Gadus morhua*). Changes in the immune response and antioxidant defense of the Atlantic cod have also been documented in relation to the oral administration of oxolinic acid and FF. These antibiotics could modulate some components of the innate humoral immune responses, bacterial pathogen proliferation in the sera, antioxidant defense genes and transcription of selected immune response when given at therapeutic concentration (Caipang et al., 2009).

Based on the present results, it can be concluded that FF had the relatively positive effects on the immune system of zebrafish. It seems that the appropriate dose of drug in the diet can act as an immunostimulant which preventing stressors and disease occurrence.

References

Anadon A., Martinez M. A., Martinez M., Rios, A., Caballero V., Ares I., Martinez-Larranaga R. M. (2008). Plasma and tissue depletion of florfenicol and florfenicol-amine in chickens. Journal of Agricultural and Food Chemistry, 56: 11049-11056.

Anbarasu K., Chandran M.R., (2001). Effect of ascorbic acid on the immune response of the catfish, *Mystus gulio* (Hamilton), to different bacterins of *Aeromonas hydrophila*. Fish and Shellfish Immunology, 11(4): 347-355.

Awad E., Mitchell W.J., Austin B. (2011). Effect of dietary supplements on cytokine gene expression in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Diseases, 34: 629-634.

Ayse E., Burak D. (2014). The Effects of florfenicol on the values of serum tumor necrosis factor- and other biochemical markers in lipopolysaccharide-induced endotoxemia in brown trout. Mediators of Inflammation, ID 464373.

Bjorklund H., Bylund G. (1990). Temperature-related absorption and excretion of oxytetracycline in rainbow trout (*Salmo gairdneri* R.). Aquaculture, 84: 363–372,

Caipang C.M.A., Lazado C.C., Brinchmann M.F., Berg I., Kiron V. (2009). In vivo modulation of immune response and antioxidant defense in Atlantic cod *Gadus morhua* following oral administration of oxolonic acid and florfenicol. Comparative Biochemistry and Physiology, 150: 459-464.

Catry B., Duchateau L., Van De Ven J., Laevens H., Opsomer G., Haesebrouck F., De Kruif A. (2008). Efficacy of metaphylactic florfenicol therapy during natural outbreaks of bovine respiratory disease. Journal of Veterinary Pharmacology and Therapeutics, 31: 479-487.

Dehghan F., Vazirzadeh A., Soltanian S., Karami A., Akhlaghi M. (2016). Mortality rate and immune responses of rainbow trout (*Oncorhynchus mykiss*) infected with *Yersinia ruckeri* subsequent to feeding on diet supplemented with *Ducrosia anethifolia* essential oil. International Journal of Aquatic Biology, 4(5): 340-344.

Driever W., Solnica-Krezel L., Schier A.F., Neuhauss S.C., Malick J., Stemple D.L. (1996). A genetic screen for mutations affecting embryogenesis in zebrafish. Development, 123: 37-46.

Ellis A.E. (2001). Innate host defense mechanisms of fish against viruses and bacteria. Developmental and Comparative Immunology, 25(8): 827-839.

Er A., Yazar E., Uney K., Elmas M., Altan F., Cetin G. (2010). Effects of tylosin on serum cytokine levels in healthy and lipopolysaccharide-treated mice. Acta Veterinaria Hungarica, 58(1): 75-81.

Er A., Yazar E. (2012). Effects of tylosin, tilmicosin and tulathromycin on inflammatory mediators in bronchoalveolar lavage fluid of lipopolysaccharide-induced lung injury. Acta Veterinaria Hungarica, 60(4):465-76.

Gioacchini G., Maradonna F., Lombardo F., Bizzaro D., Olivotto I., Carnevali O. (2010). Increase of fecundity by probiotic administration in zebrafish *(Danio rerio)*. Reproduction, 140: 953-959.

Gruss H.J. (1996). Molecular, structural, and biological characteristics of the tumor necrosis factor ligand superfamily. International Journal of Clinical and Laboratory Research, 26(3): 143-159.

Guan S., Song Y., Gao W., Wang D., Zhang Z., Lu J., Deng X. (2011). Immunosuppressive activity of florfenicol on the immune responses in mice. Immunological Investigations, 40: 356-366.

Haffter P., Granato M., Brand M., Mullins M.S., Hammerschmidt M., Kane D.A. (1996). The identification of genes with unique and essential function in the development of the zebrafish, *Danio rerio*. Development, 123:1-36.

He S., Zhou Z., Meng K., Zhao H., Yao B., RingØ E., Yoon I. (2011). Effects of dietary antibiotic growth promoter and Saccharomyces cerevisiae fermentation product on production, intestinal bacterial community, and nonspecific immunity of hybrid tilapia *(Oreochromis niloticus* female · *Oreochromis aureus* male). Jounnal of Animal Science, 89: 84-92.

Holloway Jr. H.L., Shoemaker C.A., Ottinger C.A. (1993). Serum lysozyme levels in paddlefish and walleye. Journal of Aquatic Animal Health, 5: 324-326.

Hu D., Zhang T., Zhang Z., Wang G., Wang F., Qu Y., Niu Y., Liu S. (2014). Toxicity to the hematopoietic and lymphoid organs of piglets treated with a therapeutic dose of florfenicol. Veterinary Immunology and Immunopathology, 162: 122-131.

Itami T., Takehara A., Nagano Y., Suetsuna K., Mitsutani A., Takesue K., Takahashi Y. (1992). Purification and characterization of lysozyme from ayu skin mucus. Nippon Suisan Gakkaishi, 58: 1937-1944.

Khajeali Y., Kheiri F., Rahimian Y., Faghani M., Namjo A. (2012). Effect of use different levels of caraway (*Carum carvi*) powder on performance, some blood parameters and intestinal morphology on broiler chickens. World Applied Science Journal, 19: 1202-1207.

Li L., Yang Y., Hou X., Gu D., Ba H., Abdulla R., Aisa H.A. (2013). Bioassay-guided separation and purification of water-soluble antioxidants from *Carthamus tinctorius* L. by combination of chromatographic techniques. Separation and Purification Technology, 104: 200-207.

Lunden T., Miettinen S., Lonnstrom L.G., Lilius E.M., Bylund G. (1999). Effect of florfenicol on the immune response of rainbow trout (*Oncorhynchus mykiss*). Veterinary Immunology and Immunopathology, 67: 317-325.

Mahghani F., Gharaei A., Ghaffari M., Akrami R. (2014). Dietary synbiotic improves the growth performance, survival and innate immune response of Gibel carp (*Carassius auratus gibelio*) juveniles. International Journal of Aquatic Biology, 2(2): 99-104.

Makvand H., Khodadadi M., Keyvanshokooh S.,

Mohammadi Makvandi Z. (2011). Effect of salinity stress on cortisol and glucose levels of grass carp (*Ctenopharyngodon idella*). Aquaculture and Fisheries, 2(8): 77-84. (In Persian)

Masahiro S. (1999). Current research status of fish immunostimulants. Aquaculture, 172: 63-92.

Miandare H.K., Farahmand H., Akbarzadeh A., Ramezanpour S., Kaiya H., Miyazato M., Rytkönen K.T., Nikinmaa M. (2013). Developmental transcription of genes putatively associated with growth in two sturgeon species of different growth rate. General and Comparative Endocrinology, 182: 41-47.

Park B.K., Lim J.H., Kim M.S., Hwang Y.H, Yun H.I. (2008) Pharmacokinetics of florfenicol and its metabolite, florfenicol amine, in dogs. Research in Veterinary Science, 84: 85-89.

Polinski M., Bridle A., Neumann L., Nowak B. (2014). Preliminary evidence of transcriptional immune-modulation by praziquantel in bluefin tuna and Atlantic salmon in vitro cultures. Fish and Shellfish Immunology, 38: 42-46.

Reda R.M., Ibrahim R.E., Ahmed E.N.G., El-bouhy Z.M. (2013). Effect of oxytetracycline and florfenicol as growth promoters on the health status of cultured *Oreochromis niloticus*. Egyptian Journal of Aquatic Research, 39: 241-248.

Ren X., Pan L., Wang L. (2014). Effect of florfenicol on selected parameters of immune and antioxidant systems, and damage indexes of juvenile *Litopenaeus Vannamei* following oral administration. Aquaculture, 432: 106-113.

Ross N.W., Firth K.J., Wang A., Burka J.F., Jojnson S.C. (2000). Changes in hydrolytic enzyme activities of Atlantic salmon (*Salmo salar*) skin mucus due to infection with the salmon louse (*Lepeophtheirus salmonis*) and cortisol implantation. Disease of Aquatic Organisms, 41: 43-51.

Siwicki A.K., Anderson D.P. (1993). Nonspecific defense mechanisms assay in fish: II. Potential killing activity of neutrophils and macrophages, lysozyme activity in serum and organs and total immunoglobulin level in serum. Fish Disease Diagnosis and Prevention Methods Olsztyn, Poland. 1993: 12-105.

Soltani M. (2008). Immunology of fish and crustaceans. Tehran University Publishing House. 80 p. (In Farsi)

Subramanian S., MacKinnon S.L., Ross N.W. (2007). A comparative study on innate immune parameters in the epidermal mucus of various fish species. Comparative Biochemistry and Physiology, Part B, 148: 256-263.

Yano T. (1996). Non-specific immune system: humoral defense. In: The Fish Immune System. G. Iwama, T. Nakanishi (Eds). Academic Press. pp: 106-159.

Yousefi S., Hoseinifar S.H. (2018). Protective effects of prebiotic in zebrafish, Danio rerio, under experimental exposure to Chlorpyrifos. International Journal of Aquatic Biology, 6(2): 49-54.

Zon L.I., Peterson R.T. (2005). In vivo drug discovery in the zebrafish. Natural Review Drug Discovery, 4: 35-44.