Effect of the probiotic *Entrococcus faecium* on hematological and non-specific immune parameters and disease resistance in zander (*Sander lucioperca)*

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Abstract

The current study evaluated effects of dietary administration doses of *Entrococcus faecium* on the hematological factors, and disease resistance of *Sander lucioperca* against *Aeromonas hydrophila* infection. Fish were fed with dietary administration containing *E. faecium* doses including of (10^{10}, 10^{8}CFU/ g) for diet A_1 and A_2, respectively in a commercial diet as basal diet were used for 6 weeks**.** The control group was basal diet with serum. The hematological and immunity parameters were measured and fish were challenged with *A.hydrophila* (4.5×10^{8}CFU/mL). Fish were monitored daily and the mortality rates were recorded over 8 days post – challenge. The results indicated that HCT in A_1 treatment (10^{10}CFU/g) had significantly increased in compare to the control and A_2 (10^{8} CFU/g) treatments (p<0.05). MCV in A_1(10^{10}CFU/g) and control groups were significantly higher than A_2 (10^{8} CFU/g) Treatment (p<0.05). However, other parameters e.g. RBC, HB, MCH, MCHC had no significant different. The serum lysozyme, alternative complement activity (ACH50) and IgM levels were significantly enhanced in dietary administration *E. faecium*(10^{8},10^{10}CFU/g) in feeding period. Survival rate of fish by dietary administration *E. faecium* (10^{10}CFU/g) was significantly higher (p<0.05) than the other groups (86.6%).

Keywords: *Entrococcus faecium*, *Aeromonas hydrophila*, Hematology, Immunity

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Introduction

Aquaculture is one of the fastest growing industries for supplying nutritional and food safety to the human consumption which contains high-quality protein and good nutritional value (Mohapatra *et al.,* 2012). Global demand is rising for the supply of sea foods that Aquaculture production showed growing of 32.4 million metric tones' in 2000 to 66.6 million MT 2012 (FAO, 2011). *S. lucioperca* is one of the main species of economical fish in Iran. Despite its importance, few studies have been conducted on this species. The administration of antibiotic has been prohibited in many countries for animal products which are using as food for human due to bacterial resistance and the chemicals in the animal bodies. Probiotics are a good alternative for the chemotherapy (Giri *et al.*, 2013). Probiotics in aquaculture have significantly improved feed efficiency, host immunity, growth pathogenic microorganisms inhibitors and also improved the stress tolerance (Son *et al*., 2009; Eissa *et al*., 2011; Sun *et al.*, 2011; Giri *et al*., 2013). Probiotics decrease potential pathogens in the intestinal tract and reduce the mucosal damage, repair damaged tissue, improve tissue structure and proper function (Eissa *et al.*, 2014). Probiotics used in aquaculture belong to species of microorganisms such as *Saccharomyces, Lactobacillus, Bacillus, Clostridium, Entrococcus, Shewanella, Leuconostoc, Lactococcus, Carnobacterium, Aeromonas* and several other species (Kim *et al.*, 2010; Abumourad., 2014). *E. faecium* is a lactic acid bacterium present in the intestinal microflora of many animals and human showing inhibitory effects on enteropathogens. *E. faecium is* a probiotic used in animal nutrition especially in farmed aquatic species (Sun *et al*., 2011). *S. lucioperca* is one of the major fish species of substantial economic importance in the Caspian Sea especially in the Anzali Wetland and Aras dam. Only a few studies have been performed on this species of fish (Gharibkhani *et al.,* 2014). According to FAO reports, *S.lucioperca* is susceptible to diseases like *A. hydrophila infection* (FAO, 2001). *A. hydrophila* is a gram- negative aquatic bacterium and an opportunistic pathogen that causes hemorrhagic septicemia in several fish species, especially those growing in warm waters. The objective of the present study was to evaluate the effects of the dietary administration using two doses of *E. faecium (*10^8, 10^{10} CFU/g) on the hematological parameters and innate immune system of *S. lucioperca* for 6 weeks.

Materials and methods

Bacterial strain

In this study, *E. faecium* was isolated and identified by biochemical test and 16 SrDNAgen sequencing (Gen bank Accession number KP869848) of the intestinal tract of juvenile *S. lucioperca.* (*E. faecium* as evaluated as probiotic).

Diet preparation

The basal diet was prepared, Gammarid powder was mixed with trout pellet (Faradaneh, Iran) containing 44.8% crude protein, 22.5% crud fat and 11.2% ash Poroximate analysis of diet were determined according to the AOAC method (AOAC, 1995). The bacterial suspension with two doses (10^8, 10^{10}CFU/ g) were sprayed and mixed. The control group was without probiotic and was sprayed serum to basal diet. The diet was air dried by air blower at room temperature and under sterile conditions for 2 h. and stored at 4 °c until used. The diets were prepared weekly (Talas and Gulhan, 2009).

Experimental design

S. lucioperca with average weight, 14 g were collected from a private farm in Guilan Province. The selected fish had no signs of bacterial disease, parasitical and viral infections in different organs (e.g. skin, gills, kidney and liver) and acclimatized in tanks for two weeks. Fish density was 10 pieces in 150 L. tank in triplicate per treatments(30 per treatment).All stocks were fed with basal diet during the acclimatisation time and water temperature was 21±2° C and approximately 30% of the illumination was natural during hours of feeding was artificially dark. Water quality was monitored at the experiments period and important factor such as pH(7.2 to 7.8), the O_2(7.5 to 9) mg O_2 L^{-1}, NH_3 and NO_2^- at 0.04-0.09mgL^{-1} and 0.03 -0.07 mgL^{-1}ppm were recorded, respectively.

Haematology test

At the end of feeding with probiotics, blood samples were collected by cutting the tail of the fish.

The hemoglobin (Hb), hematocrit (Ht) (Larsen 1964; Goldenfarb *et al.*1971).

The red blood cell (RBC) abundance was evaluated by hemocytometer Neubauer and microheamatocrit (Leonard and Cormick, 2005). MCV, MCH and MCHC were calculated as formulas below:

Mean corpuscular volume (MCV) = (Ht × 10) /RBC,

Mean corpuscular hemoglobin (MCH) = (Ht ×10)/Hb

Mean corpuscular hemoglobin concentration (MCHC) = (Hb×100)/ Ht) (Benfey and Sutterlin,1984).

Immunological measurements

Serum lysozyme activity

Serum lysozyme activity was evaluated on lyse gram positive bacteria (*Micrococcus lysodeikticus*) that was sensitive to lysozyme enzyme (Clerton *et al.,* 2001).

Alternative complement pathway activity assay

Alternative Complement pathway activity (ACH50) was determined as described (Yanno *et al.,* 1992)

The volume yielding 50% hemolysis was used for determining the complement activity of the sample as follows:

ACH50 (unit/mL) = K × (reciprocal of the serum dilution) × ½

(K= the rate of serum (mL) giving 50% hemolysis and ½ is the correction factor since the assay was performed on half scale of the original method).

IgM levels

Serum total IgM levels were measured using ELISA, samples were read at 450 nm in plate reader, the mean absorbance of the negative controls were subtracted from the optical density at 450nm (Negative control was no biotin antibody) (Magnadottir *et al.*,1992).

Challenging the bacteria

Areomonas hydrophila (ATCC7966) was grown in brain heart infusion agar (BHI). After growing of bacteria, the cells were centrifuge at 4000 rpm for 15 min at 4°C. Letal dose50 (LD_{50}) of *A. hydrophila* (10^7,10^8,10^9CFU per fish) were determined by intraperitoneal injection on 20 fish for 7 days. And LD_{50} obtained (4.5×10^8 CFU/mL). the bacterial suspension was injected with 100µL PBS containing 4.5×10^8 live *A.hydrophila* to all treatments (10^8,10^{10}) and were injected 0.5 ml of saline serum to control treatment. The treatments were kept the condition for 8 days. The survival and mortality rates were recorded individually for the treatments (Andani *et al.*, 2011)

Statistical analysis

The obtained data were analyzed under one- way test of variance. The significant differences between treatment were analyzed at 95% confidence level (p<0.05) and mean separation was conducted by Duncan's multiple range test. All statistical analyzes were done using the SPSS software package version 15.

Results

Hematological parameters

Hematological parameters including RBCS, Hb, MCH, MCHC, monocytes and Eosinophils, had no significant difference between dietary administration *E. faecium* (Table1). Hematological parameters including WBC, HCT and MCV in fed *E. faecium* supplemented at 10^{10}cfu / g was significantly increased (p<0.05) in compare to other treatment. Rate of lymphocytes in probiotic groups were significantly higher than control treatment and the neutrophil**s** in control treatment was higher than fed *E. faecium* supplemented at (10^8 and 10^{10} CFU/g) after 6 weeks (p<0.05).

Humoral immune parameters

During feeding with probiotic

The results showed that the serum lysozyme alternative complement activity (ACH50), Serum IgM levels and serum lysozyme activities of dietary administration *E. faecium* at (10^8 and 10^{10} CFU/g) were higher than control treatment during 6 weeks (See Fig.1).

Tabel 1: Hematological parameters of *Sander luciperca* fed with different doses of *Entrococcus faecium* and control treatment at 6 weeks*.

Hematological parameters	Treatment		
	Control	A1	A2
RBC(X106 /µL)	1.56±0.6[a]	1.57±0.25[a]	1.53±0.34[a]
Hb%(g/dl)	6.03±0.35[a]	6.2±0.057[a]	5.9±0.1[a]
Hct(%)	33.3±1.52[b]	37.66±0.57[a]	32±1.732[b]
MCV(fl)	231.66±3.5[a]	237.66±3.05[a]	214.66±5.5[b]
MCH(Pg)	38±1.7[a]	38±0.00[a]	37±0.00[a]
MCHC(Pg)	16.33±0.577[a]	16.33±0.577[a]	16.66±0.577[a]
Lymphocytes(cells/mm-3)	75±1.000[b]	77.5±1.154[b]	78±0.577[a]
Neutrophils(cells mm-3)	22±0.577[a]	20±0.577[b]	20±0.577[b]
Eosinophils (cells mm-3)	0.66.±0.577[a]	0.33±0.577[a]	-
Monocytes (cells mm^{-3})	1.66±0.577[a]	1.154±1.33[a]	1.33±0.577[a]

*The mean values with different superscripts are significantly (p<0.05)

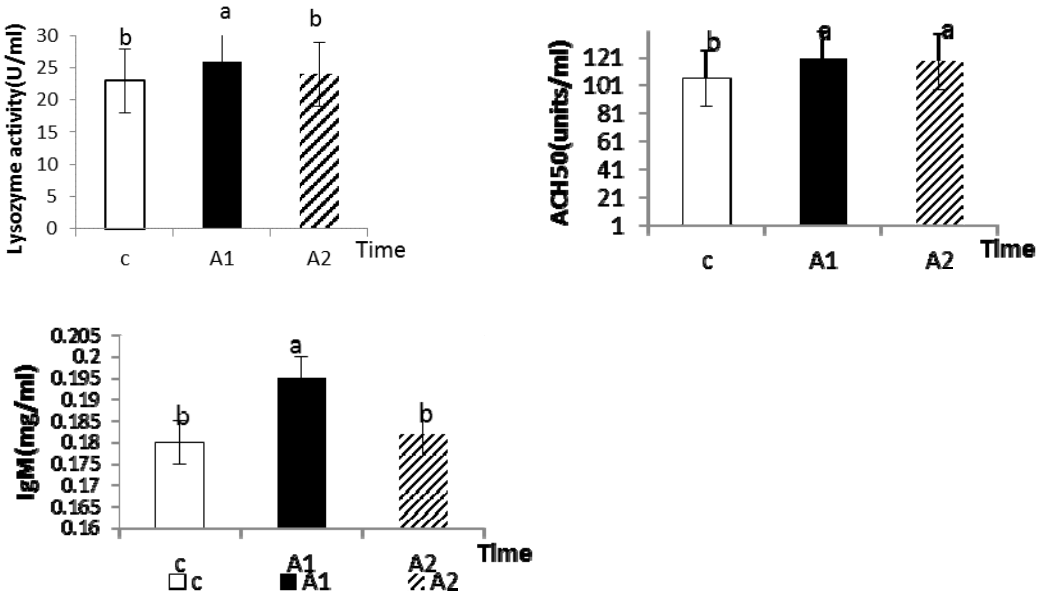

Figure 1: Humoral immune parameters from *Sander lucioperca* fed with different doses of *Entrecoccus faecium* and control treatment at 6 weeks. (A_1= 10^{10} , A_2= 10^8CFU/g) control means at the sampling day with different letters are significantly different (p< 0.05). Each value (mean±SE) is the average performance of fish per treatments for a period of 6 weeks.

Challenge test

The survival rate of *S. lucioperca* challenged with *A. hydrophila* after 6 weeks feeding in *E. faecium* (10^{10} CFU/g) diet was higher than *E. faecium* (10^8CFU/g) diets and control treatment (Fig.2).

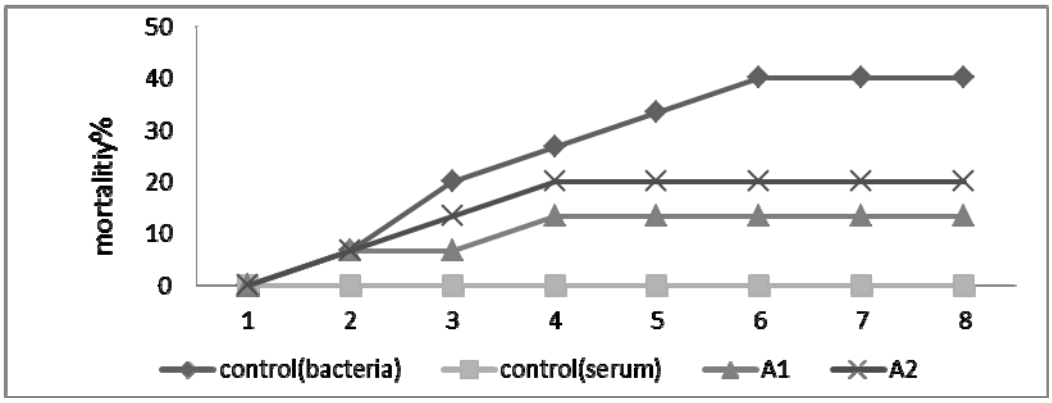

Figure 2: The survival rate of *Sander lucioperca* challenged with *A.hydrophila* after 6 weeks feeding with different doses of *E. faecium*(0,10^8,10^{10} CFU/g) diets.

Discussion

Currently, due to the indiscriminate use of antibiotics and resistant bacteria in aquatic animals, the use of antibiotics have been limited or banned. Rezgui *et al.* (2010) showed several species of genus *Pseudomonas, Aeromonas, Vibrio* and *Enterobacteriacea* belong to sea bream and sea bass which had antibiotic resistant to tetracycline and penicillin. Probiotics are a suitable alternative to antibiotics and chemotherapy which cause intestinal microbial balance, stimulation effects of non- specific defense system in host, digestive and enzyme activities, increase the disease resistance and improve the growth rate are used in aquaculture (Balaczar., 2006; Giri *et al.,* 2011). The hematological parameters generally proper perspectives for fish health and well-being monitoring such as temperature and dissolved oxygen and other environmental factors (Eissa and Wang., 2013; Eissa and Abou.,2014).

In the present study, hematological parameters including, HCT and MCV parameters in the dietary administration of *E. faecium* (10^{10}-10^8 CFU/g) were higher than control treatment. The lymphocytes had been significantly increase in fed *E. faecium* spp(10^8 CFU/g) supplemented to other treatments. Signs of increasing the percentage of blood lymphocytes are including strengthen the immune system, increasing resistance to pathogens and environmental stress conditions, improve growth, reduce mortality and increase the survival rate (Nayak, 2010).

The results agreed with data of some scientists who recorded feeding to probiotics (Irianto and Austin, 2003; Aly *et al.*, 2008; Eissa and El-Ghiet, 2011; Giri *et al.* 2012; Giri *et al.* 2013; Giri *et al.*, 2014).

The complement system is non - specific humoral immune response, that has essential role as alert for immune system when occurred pathogen attack to body and can directly lyse pathogens (Panigrahi *et al.*, 2005; Nutr., 2007). Panigrahi *et al* (2005) recorded considerable increase in the alternative

complement activity (ACH50) and lysozyme activity in *O. mykiss* dietary supplements of *L. rhamnosus,* containing dose at 10^{11} CFU/g for 4 weeks.

Lysozyme causes lysis of peptidoglycan cell walls, gram- positive bacteria and can kill Gram- negative bacteria (Hjelmel *et al.,* 1983). Giri *et al.* (2013) noted that rohita fed diet supplemented with *L. plantarum* VSG3 (10^8 CFU/g) during a period of 60 days indicated higher serum lysozyme activity levels in contrast *L. plantarum* (10^{10}CFU/ g).

In the present study, significantly increased ACH50 in the both doses of probiotic treatments and serum lysozyme especially dose 10^{10}CFU/g of *E. faecium* during 6 weeks feeding with probiotic. The results supported the data of grouper *Epinephilus coioids* (Son *et al.,* 2009; Sun *et al.,* 2011) Nile tilapia (Eissa and Abou., 2014) and *Labeo rohita* (Giri *et al.,*2013).

Balcazar *et al*. (2006) studied on fed supplemented *L. lactis* and *L. mesenteroides* dose at 10^6 CFU/g in *Salmo trutta* during 14-21 days indicated significant promotion ACH50 and lysozyme actives. Kim and Austin (2006) noted in *O. mykiss* fed *Carnobacterium maltaromaticum* containg diet at 10^7 CFU/g showed increased lysozyme activity. Son *et al.* (2009) studied on dietary supplement *L. plantarum* at the values of (10^6,10^8, and 10^{10}CFU/g) in grouper *Epinephilus coioides* demonstrated enhanced ACH50 in the 10^8CFU/g treatment and higher significant lysozyme activity of *L. plantarum* at the dose 10^{10}CFU/g compared to other treatments (Son *et al.,* 2009). Becerril *et al.* (2012) recorded on dietary supplemented *L. sakei* (10^6 CFU/g) with marine silages enriched (Humbold- squid silage) in pacific red snapper at 6 weeks and post challenge with *A. veroni* showed significant increased in lysozyme concentration in fish blood. Sharifuzzaman and Austin (2009) noted fed probiotic diet in rainbow trout after 2, 4 and 6 weeks caused significant increased in lysozyme activity. IgM is a basic antibody that is the most important immunoglobulin in fish (Walts *et al.,* 2001). Nikoskelainen *et al* (2001) noted probiotic bacteria (*L.rhamnosus*) is as stimulating immunoglobulin. Sun *et al* (2010) demonstrated the serum IgM levels in *Epinephalus coioides* that dietary supplemented *E. faecium and Lactococcus lactis* during 60 days were higher than of control treatment. The present study, the serum IgM level of dietary supplemented *E. faecium* (10^{10}CFU/g) was considerably higher than *E. faecium* (10^8 CFU/g) and control treatment after 6 weeks of feeding (p<0.05) but there were not significant difference between *E. faecium* (10^8 CFU/g) and control treatment (p>0.05).

Abumourad *et al.* (2014) recorded that efficient use of *E. faecium* as nutritional supplements increased growth promoting effect and stimulate the immune response of *O. niloticus.*

Becerril *et al.* (2012) noted effects of marine silages enriched with

Lactobacillus sakei increased immune activity and disease resistance of pacific red snapper *(Lutjanus peru*).

Current study, dietary supplemented of *E. faecium* at 10^8 -10^{10}CFU/g during 6 weeks enhanced the survival rate of *S. lucioperca* challenged with *A.hydrophila*. Bogut *et al* (2000) recorded that dietry supplemented *E. faecium* in fish had adhesion ability to intestine epithelium and decrease of pathogen bacteria. The most and the least survival rate was indicated in the fish treatment of dietary administration *E. faecium* at 10^{10}CFU/g (86.6%) and control treatment (66.6%), respectively. Some scientists studied on antagonistic effects of *E. faecium* against *A. hydrophila* invitro conditions (Bannai., 2013; Abumourad *et al* ., 2014).

Present study indicated that *E.faecium* supplementation was able to providing resistance to *A. hydrophila* infection by increasing the non-specific immune system in *S. lucioperca*. This study supported earlier data in *tilapia* (Panigrahi *et al*., 2004) *rainbow trout* (Nikoskelainen *et al.,* 2001; Panigrahi *et al.,* 2004; Balcazar *et al*., 2007). African catfish and Al- Dohail *et al.,* 2009). Thereby, dietary administration of *E. faecium* clearly showed positive effect on hematological parameters, non- specific immune system and survival of *S. lucioperca* against *A. hydrophila* infection. The optimal dose of dietary *E. faecium* administration is at 10^{10} CFU/g. This probiotic can be used as a bio- control agent effective in aquaculture strengthen and increase the *S. lucioperca* immune system response against *A. hydrophila.*

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References

Abumourad, I.M.K., 2014. *Enterococcus faecium* probiotic as a growth promoter and its impact on the expression of the host innate immune in cultured *Oreochromis niloticus*. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, 63(**1**), 1-6.

Al-Dohail, M.A., Hasim, R., Aliyu-Paiko, M., 2009. Effects of the probiotic, *Lactobacillus acidophilus*, on growth performance, hematology parameters and immunoglobulin concentration in African catfish (*Clarius gariepinus*, Burchell 1882) fingerlings. *Aquaculture*, 40(**2**), 1642-52.

Aly, S.M., Ahmed, Y.A.G., Ghareeb, A.A.A., Mohamed, M.F., 2008. Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of *Tilapia nilotica (Oreochromis niloticus)* to challenge infections. *Fish and Shellfish Immunology*, 25(**1**), 128-36.

Andani, H., Tokmechi, A., Meshkini, S., Ebrahimi, H., 2011. Rainbow

trout increased resistance against infection with *Aeromonas hydrophila* and Yersinia Rocker using Lactobacilli from intestines Carp. *Iranian Veterinary Journal*, 7(**2**), 26-35.

A0AC, 1995. Official methods of analysis, 16th ed, Association of Official Analytical Chemists. AOAC, Arlington,VA.

Balcazar, J.L., Blas, ID., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., Muzquiz, J.L., 2006. The role of probiotics in aquaculture. *Veterinary Microbiology*, 114(**1**), 173-86.

Balcàzar, J.L., Blas, I.d., Ruiz-Zarzuela, I., Vendrel, D., Gironés, O., Muzquiz, J.L., 2007. Enhancement of the immune response and protection induced by probiotic lactic acid bacteria against furunculosis in rainbow trout (*Oncorhynchus mykiss*). *FEMS Immunology & Medical Microbiology*, 51(**1**), 185-93.

Banni, M.A., 2013. Diagnosis of *Aeromonas hydrophila* infection carassiuegibelo fish in shatt Al-arab, south of Iraq. *International Journal of Aquaculture*, 20(**1**), 115-116.

Becerril, M.R., Ascencio-Valle, F., Macias, M, E., Maldonado, M., Rojas, M., Esteban, M, A., 2012. Effects of marine silages enriched with *Lactobacillus sakei* 5-4 on haemato immunological and growth response in *Pacific red snapper (Lutjanu speru)* exposed to *Aeromonas veronii*, *Fish and Shellfish Immunology*, 33(**1**), 984-992.

Benfey, T.J., Sutterlin, A.M., Thompson, R.J., 1984. Use of erythrocyte measurments to identify triploid salmonid.,*Aquaculture*, 41(**1**), 980-984.

Bogut, I., Milakovic, Z., Brkic, S., Novoselic, D., Bukvic, Z., 2000. Effects of *Enterococcus faecium* on the growth rate and content of intestinal microflora in sheat fish (*Silurus glanis*). *Veterinární Medicína*, 45(**1**), 107-109.

Clerton, P., Troutaud, D., Verlhac, V., Gabraudan, J., Deschaux, P., 2001. Dietary vitamin E and rainbow trout (*Oncorhynchus mykiss*) phagocyte functions: effect on gut and on head kidney leucocytes. *Fish and Shellfish Immunology*, 11(**1**), 1-13.

Eissa, N., El-Ghiet,E., Shaheen, A., Abbass, A., 2010. Characterization of Pseudomonas Species Isolated from Tilapia" *Oreochromis niloticus*" in Qaroun and Wadi-El-Rayan Lakes, Egypt. *Global Veterinary*, 5(**1**), 116-121.

Eissa, N., Abou, E., El-Ghiet, E., 2011. Efficacy of *Pseudomonas fluorescens* as biological control agent against *Aeromonas hydrophila* Infection in *Oreochromis niloticus*. *World Journal Fish Marine Science*, 3(**1**), 564-569.

Eissa, N., Wang, H.P., 2013. Physiological stress response of yellow perch subjected to repeated handlings and salt treatments at

different temperatures. *Aquaculture*, 75(**1**), 449-454.

Eissa, N and Abou-ElGheit, E., 2014. Dietary supplementation impacts of potential non-pathogenic isolates on growth performance, hematological parameters and disease resistance in *Nile Tilapia (Oreochromis niloticus*), *journal of veterinary advances*, 25(**1**), 712-719.

FAO, 2001. Cultured Aquatic Species Information Programme *Sander lucioperca* (Linnaeus, 1758), 13P.

FAO, **2011**. Statistics and information service of the fisheries and aquaculture department. FAO year book of fishery and aquaculture statistics, 2009. FAO, Rome.

Gharibkhani, M., Pourkazemi, M., Rezvani Gilkolai, S., Tatina, M., Azizzadeh, L., 2014. Genetic analysis of pike-perch, *Sander lucioperca* populations revealed by microsatellite DNA markers in Iran, *Caspian Journal of Environmental Science*, 12(**1**), 99-108.

Giri, S.S., Sukumaran, V., Sen, S.S., Vinumonia, J., Banu, B.N., Jena, P.K., 2011. Antagonistic activity of cellular components of potential probiotic bacteria, isolated from the gut of *Labeo rohita*, against *Aeromonas hydrophila.* Probiotics Antimicrobial, *Fish and Shellfish Immunology*, 3(**2**), 214-222.

Giri, S.S., Sukumaran, V., Oviya, M., 2013. Potential probiotic *Lactobacillus plantarum* VSG3 improves the growth, immunity, and disease resistance of tropical freshwater fish, *Labeo rohita*, *Fish and Shellfish Immunology*, 34(**1**), 660-666.

Giri, S.S., Sukumaran, V., Sen, S.S., Jena, P.K., 2014. Effects of dietary supplementation of potential probiotic *Bacillus subtilis* VSG1 singularly or in combination with *Lactobacillus plantarum VSG3* or/and Pseudomonas aeruginosa VSG2 on the growth, immunity and disease resistance of *Labeo rohita*. *Aquaculture Nutrition*, 20(**1**), 163-169.

Goldenfarb, P.B., Bowyer, F.P., Hall, E., 1971. Reproductibility in the hematology laboratory: the microhematocrit determination. *Clinical Pathology*, l56(**1**), 35-39.

Irianto, A., Austin, B., 2003. Use of dead probiotic cells to control furunculosis in rainbow trout, *Oncorhynchus mykiss (Walbaum). Journal Fish Diseases*, 26(**1**), 59-62.

Kim, D.H., Austin, B., 2006. Innate immune responses in rainbow trout (*Oncorhynchus mykiss*) induced by probiotics. *Fish and Shellfish Immunology*, 21(**1**), 513–24.

Larsen, H.N., 1964. Comparison of various methods of hemoglobin detection of channel catfish blood. Prog. *Progressive Fish-Culturist*, 26(**1**), 11-15.

Leonard, J.B.K. and Cormick, S. D. M.C., 2005. Changes in haematology during upstream migration to American. *Fish Biology*, 54(**2**), 1218-1230.

Magnadottir, B and Bjarnheiður, K., 1992. A comparison of total and

specific immunoglobulin levels in healthy Atlantic salmon (*Salmo salar* L.) and in salmon naturally infected with *Aeromonas salmonicida* subsp. *achromogenes*. *Veterinary Immunology and Immunopathology*, 32(**1**), 179–189.

Mohapatra, S., Chakraborty, T., Prusty, A.K., Das, P., Paniprasad, K., Mohhanta, K.N., 2012. Use of different microbial probiotics in the diet of rohu, *Labeo rohita* fingerlings: effects on growth nutrient digestibity and retention, digestive enzyme activities and intestinal microflora. *Aquaculture Nutrition*, 18(**1**), 1-11.

Nayak, S. K., 2010. Probiotics andimmunity: A fish perspective. *Fish and Shellfish Immunology*, 29, 2-14.

Nikoskelainen, S., Ouwehand, A., Salminen, S., Bylund,G., 2001. Protection of rainbow trout (*Oncorhynchus mykiss*) from furunculosis by *Lactobacillus rhamnosus*. *Aquaculture*, 198(**1**), 229–36.

Nutr, B.R.J., 2007. Changes in intestinal microbiota and humoral immune response following probiotic administration in brown trout (*Salmo trutta*), *Fish and Shellfish Immunology*, 97(**1**), 522–527.

Panigrahi, A., Kiron, V., Kobayashi, T., Puangkaew, J., Satoh,S., Sugita, H., 2004. Immune responses in *rainbow trout Oncorhynchus mykiss* induced by a potential probiotic bacteria *Lactobacillus rhamnosus* JCM 1136. *Veterinary Immunopathology*, 102(**1**), 379–88.

Panigrahi, A., Kiron, V., Kobayashi,T., Puangkaew, J., Satoh, S., Sugita, H., 2005. The viability of probiotic as a factor influencing the immune response in rainbow trout *Oncorhynchus mykiss*. *Aquaculture*, 243(**1**), 241-53.

Rezgui, I., 2010. Etude de l'antibioresistance chez deux espèces de poisons aquacoles en Tunisie: le loup (*Dicentrarchus labrax*) et la daurade (*Sparus aurata*). Master of Biology. University of Sciences of Tunis. Institut des Sciences et Technologies de la Mer. Tunis, Tunisia.

Sharifuzzaman, SM., Austin, B., 2009. Influence of probiotic feeding duration on disease resistance and immune parameters in rainbow trout. *Fish and Shellfish Immunology*, 27(**1**), 440-445.

Son, V.M., Chang, C.C., Wu, M.C., Guu, Y.K., Chiu, C.H.W., Cheng, W., 2009. Dietary administration of the probiotic, *Lactobacillus plantarum*, enhanced the growth, innate immune responses, and disease resistance of the *grouper Epinephelus coioides*, *Fish and Shellfish Immunology*, 26(**1**), 691–698.

Sun, Y.Z., Yang, H. L., Ma, R.L., Lin, W.Y., 2010. Probiotic applications of two dominant gut Bacillus strains with antagonistic activity improved the growth performance and immune responses of grouper *Epinephelus*

coioides, *Fish and Shellfish Immunology*, 29(**1**), 803-809.

Talas, Z.S and Gulhan, M.F., 2009. Effects of various propolis concentrations on biochemical and hematological parameters of rainbow trout (*Oncorhynchus mykiss*). *Ecotoxicology*, 72(**1**), 1994-1998.

Watts, M., Munday, B.L., Burke, C.M., 2001. cDNA sequences and organization of IgM heavy chain genes in two holostean fish. *Developement Immunology*, 9(**1**), 153-64.

Yano, T., 1992. Assays of hemolytic complement activity, *Techniques in Fish Immunology*, 2(**1**), 131-141.