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 A1 and A2, 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 A1 treatment (10^{10}CFU/g) had significantly increased in compare to the control and A2 (10^{8} CFU/g) treatments (p<0.05). MCV in A1(10^{10}CFU/g) and control groups were significantly higher than A2 (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% crude fat and 11.2% ash Poroximate analysis of diet were determined according to the AOAC method (AOAC, 1995). The bacterial suspension with two doses \(10^8, 10^{10} \text{CFU/g}\) 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

*Lebistes reticulatus* 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 \(O_2 \text{ L}^{-1}\), \(NH_3\) 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) = \(\frac{\text{Ht} \times 10}{\text{RBC}}\),
Mean corpuscular hemoglobin (MCH) = \(\frac{\text{Ht} \times 10}{\text{Hb}}\)
Mean corpuscular hemoglobin concentration (MCHC) = \(\frac{(\text{Hb} \times 100)}{\text{Ht}}\) (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:

\[ \text{ACH50 (unit/mL)} = K \times \left(\frac{1}{\text{serum dilution}}\right) \times \frac{1}{2} \]
(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 (LD50) of *A. hydrophila* (10^7,10^8,10^9CFU per fish) were determined by intraperitoneal injection on 20 fish for 7 days. And LD<sub>50</sub> 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 10^10cfu / 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 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 lucioperca* fed with different doses of *Entrococcus faecium* and control treatment at 6 weeks*.

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

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

Figure 1: Humoral immune parameters from *Sander lucioperca* fed with different doses of *Entrococcus faecium* and control treatment at 6 weeks. (A1= 10^{10}, A2= 10^{8}CFU/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^{8}CFU/g) diets and control treatment (Fig.2).
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 coioides* (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* containing 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 10^8 CFU/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* (10^8 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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