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Enterococcus faecium probiotic as a growth promoter and its impact on the expression of the host innate immune in cultured *Oreochromis niloticus.*

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ABSTRACT

This study was planned to investigate the effect of *Enterococcus faecium* probiotic on the growth performance and to assess its impact on the expression of some immunomodulating genes in *Oreochromis niloticus*. *E. faecium* was administered orally in a level of 8g/kg feed to fish for 45 days. At the end of the feeding period, fish were I/P challenged with pathogenic *Aeromonas hydrophila* (0.2 ml of 2 x10^8 CFU / ml).The fish group fed the diet containing *E. faecium* showed significant increase in weight gain, SGR and PER. Also it showed low mortality rate, less severe clinical pictures and histopathological changes during challenge test against *A.hydrophila* compared to the control group. Semi-quantitative PCR was employed to determine the mRNA levels of IL-8 and TNF-α genes of the control and probiotic groups. IL-8 and TNF-α were significantly up-regulated by *E. faecium*. Moreover these genes expression were significantly higher in the *E. faecium* group after *A. hydrophila* infection, suggesting that *E. faecium* can stimulate the immune response of *Oreochromis niloticus*. It could be concluded that the proper and efficient use of *E. Facium* probiotic as feed additive is valuable for cultutred *O. niloticus* because of its growth promoting effect and its immunostimulant activity.
Keywords: *Enterococcus faecium*; probiotic; *Aeromonas hydrophila*; Histopathological changes; immune genes response.

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INTRODUCTION

Fish farming industries are constantly under threat due to the outbreaks of infectious diseases as a consequence of the rapid developments in aquaculture. *Aeromonas hydrophila* has been recovered from a wide range of freshwater aquarium and cultured fish species worldwide (Nielsen *et al*., 2001). The extensive use of antibiotics has led to an increase in antibiotic resistance in *A. hydrophila* (Ansary *et al*., 1992**)**. As an alternative strategy to these antimicrobial compounds, the prophylactic use of beneficial bacteria (probiotics) has emerged to improve health and zoo technical performances such as survival, production, feed conversion and growth rates of cultured aquatic species. Probiotics are live microorganisms, which when consumed in adequate amounts; confer beneficial effects on the health and well-being of the host by modifying the host-associated or ambient microbial community of the gastrointestinal tract (Geng *et al*., 2012).

Probiotic agents exert a beneficial effect via a wide array of actions. These include competition for adhesion sites and resistance to colonization; competition for essential nutrients; production of antagonistic compounds against pathogens; enhancement of the immune response and diseases resistance. In addition, probiotics improving enzyme activity, feed digestibility and feed utilization, fish health and performance (Balcázar *et al*., 2006; Denev *et al*., 2009). The benefits of the supplements include improved feed value, enzymatic contribution to digestion, inhibition of pathogen, antimutagenic and anticarcinogenic activity, growth promoting effects, and increase immune response (Balcazar, 2003; Wang *et al*., 2008). The common probiotics used in aquaculture industry include a wide range of species belong to *Saccharomyces*, *Lactobacillus*, *Bacillus*, *Clostridium*, *Enterococcus*, *Shewanella*, *Leuconostoc*, *Lactococcus*, *Carnobacterium*, *Aeromonas* and several other species (De Rodriganez *et al.*, 2009; Kim *et al.*, 2010).

Lactic acid bacteria (LAB) are potential probiotic candidates in aquaculture and are also known to be as a normal inhabitant in the intestine of healthy fish. *Enterococcus faecium* is one of the most commonly used lactic acid producing bacteria in animal nutrition and has become a focus of attention for use in commercially farmed aquatic species. Enterococci are Gram-positive, facultative anaerobic bacteria which are widely distributed in nature and considered as bacteria of low pathogenicity (Klare *et al.,* 2003). Several important ways in which probiotics producing lactic acid (such as *E. faecium*) can provide a performance benefit are improving intestinal microbial balance (Fuller, 1989), stimulating the immune system and decreasing pH as well as the release of bacteriocins (Rolfe, 2000). Bacteriocins are small peptides which are characterized by their ability to inhibit pathogenic bacteria; whereas, some have a narrow spectrum of activity while others inhibit a wide variety of bacteria.

Aeromonas hydrophila is a ubiquitous Gram-negative aquatic bacterium and opportunistic pathogen causing fatal hemorrhagic septicemia in several fish species including warm water and temperate aquaculture species (Handfield *et al.*, 1996).

The expression of a wide number of genes responsible for innate immune responses towards microbes in fish has been evaluated (Lieschke and Trede, 2009). A recent study demonstrated the distribution of important innate antibacterial immunity mediators such as

peptidoglycan recognition protein (pglyrp) and a factor that regulates neutrophilic cell densities and cytokines in the entire intestine of healthy zebrafish (Oehlers *et al*., 2011). The aim of this study was, therefore, to assess the role of *Enterococcus faecium* bacteria as a probiotic with special emphasize on its role in cultured *Oreochromis niloticus* as a growth promoter and immune stimulant agent beside its role during challenge with *Aeromonas hydrophila*.

MATERIALS AND METHODS

- **Experimental fish:** *Oreochromis niloticus* were collected from a governemental fish farm in Sharkya province, Egypt.
- **Bacterial probiotic**: *Enterococcus faecium* was activated in M17 broth (Terzaghi and Sandine, 1975) and incubated at 37°C for 48h. Microencapsulation of the collected cells was done using alginate with sodium chloride (Klinkenberg *et al*., 2001). The total counts of *Enterococcus faecium* was detected as 88 X 10^9 CFU/ml.
- **Fish diet:** A basal diet was prepared at Dept. of Animal Production, Faculty of Agriculture, Cairo University. It contained 30% crude protein, 3.7 Kcal/g of metabolizable energy, 3.4% fiber and 7.03% fat as well as vitamins and minerals in the form of dry pellets.. Fish were fed daily on 3% of the body weight.
- ***Aeromonas hydrophila* :** Well identified pathogenic isolate of *Aeromonas hydrophila* was kindly supplied by the Hydrobiology lab., Veterinary division, NRC.

Experimental design

Fish groups

O. niloticus with an average weight (20±2.0) were acclimatized in indoor glass aquaria (75×40×50 cm) for two weeks prior to the experiments. These aquaria were supplied with aerated dechlorinated tap water at 28±2 °C, under a natural photoperiod (light/dark hours = 12/12).

The fish were classified into two groups. The first group was a control group: fed on basal fish diet and the second group: fed on basal diet supplemented with probiotic cell suspension at the level of 8gm / kg feed. Fish were distributed at a rate of 25 fish / aquarium in triplicate treatment. The experimental fish were weighed at the beginning of the experimental period. Fish were fed on *Enterococcus faecium* supplemented feed once daily for 45 days at a fixed feeding rate of 3% body weight of fish per day. At the end of the experiment fish were collected and weighed. Blood samples were collected from the caudal vessels .Serum and plasma samples were separated and stored at -20 for further analysis.

Growth parameters

- Body weight gain**:** Final fish weight (g) - Initial fish weight (g) (Annet, 1985).
- 2 -Specific growth rate (SGR) = 100 (ln W_2 – ln W_1) T^{-1}
- Where W_1 and W_2 are the initial and final weight, respectively, and T is the number of days in the feeding period according to Pouomonge and Mbonglang (1993).

- Feed Conversion Ratio (FCR): FCR = Total feed consumed by fish (g) / Total weight gain by fish (g) according to Pouomonge and Mbonglang (1993).
- Protein efficiency ratio (PER) = (B2 – B1) PI^{-1}

Where B_1 and B_2 are the biomass at the start and the end of the experiment, and PI is the protein intake (Pouomonge and Mbonglang, 1993).

Screening for the antimicrobial activity

Pure cultures of *Enterococcus faecium* were examined for inhibitory effects against the pathogenic bacteria, *A. hydrophila.* The *in vitro* antimicrobial activity was assessed using agar diffusion method and the inhibition zones were determined according to Ruiz *et al.* (1996). The probiotic bacteria (*Enterococcus faecium*) was inoculated in the petridishes, containing Muller – Hinton agar (MH agar) R. Parthasarathy and D. Ravi *89* and incubated at 30 °C for 24 h. Subsequently, fresh inoculums of the pathogenic *A. hydrophila*, were spread over the plates. The plates were further incubated at 30 °C for 24 h, and then checked for the appearance of inhibition zone (Abd El-Rhman *et al.*, 2009).

Challenge test

After 45 days of feeding, 60 fish of each group were divided into two sub groups (30 fish for each), the first subgroup of each treatment was challenged I/P with pathogenic *Aeromonas hydrophila* (0.2 ml dose of 24-h saline from virulent bacterial pathogen of *A. hydrophila* (2×10^8 CFU/ml) was given by interperitoneal (IP) route (Badran and Eissa 1991). The second subgroup was injected I.P. (0.2 ml of saline) as control. Three days after infection, RNA was extracted from 10 individuals from each group and the remained fish were kept under observation for 15 days post challenge to record the mortality rates, clinical signs and post-mortem findings (Schäperclaus *et al*.,1992).

Histopathological studies

Tissue specimens from skin, gills, liver, kidneys, spleen, brain, ovary and testis were taken from *Oreochromis niloticus* groups at the end of the challenge test. The samples were fixed in 10% formal saline, processed by conventional method, sectioned at 4 µm and stained with Haematoxylin and Eosin (Roberts, 2001).

Examination of tissue specific expression of IL-8 and TNF immune genes after the bacterial infection using RT-PCR

Total RNA Isolation

Total RNA was isolated from livers of some individuals 3 days after infection; it was purified from lysates using a spin column kit purchased from Fermentas life science Co. (Invitrogen Corporation) (Van Allen Way, Carlsbad, Canada) according to the manufacturer's instructions. RNA samples were treated with DNase I (Ambion, UK) to remove contaminating genomic DNA and re-purified by spin column. Then RNA samples were stored in –80 ^{o} C until the process of reverse-transcriptase.

Smart cDNA synthesis

2 µg RNA was reverse transcribed with Revert Aid First Strand cDNA Synthesis Kit™ (purchased from Fermentas life science Co., Invitrogen Corporation, using hexanucleotides and used as templates for RT semi-quantitative PCR. Positive and negative control reactions were used to verify the results of the first strand cDNA synthesis steps. Beta actine gene was used as positive control.

Primers design& semi-quantitative Polymerase chain reaction

Primers for target genes (IL8 and TNF) were designated following the web site NCBI and target genes sequence, table 1 illustrates primers corresponds to target genes sequence. The resulting cDNA was subjected to PCR for 35 cycles with respective primers designated from the sequence of interleukin 8 (IL-8) and Tumor necrosis factor (TNF**)** genes (table 1) and were purchased from Invitrogen Corporation (Van Allen Way, Carlsbad, Canada). Dream Taq™Green PCR Master Mix (Invitrogen Corporation) was used in the PCR. Products of PCR were then displayed on an appropriate agarose gel (1.5%) and examined for yield and specificity. Analysis of gel images was done using Gel analyzer Pro (version 3.1) software.

Table (1): Sequences of the 5' and 3' synthetic primers used in PCR.

Gene & its accession number	Primers(sense and antisense 5'→3'	Annealing Temp.
TNF-α NM_001279533	Sense: 5'- ACACACTGGCCTGTACTTCG-3' Antisense: 5'- ATCTGGCTCTGTGCAGCTTT-3'	53 ℃
IL-8 NM_001279704	Sense: 5'- AGAGAACAGAGGAGACCGGG A-3' Antisense: 5'- CTCCACCTTCTCGATGTGGC -3'	55℃

Statistical analysis

The results obtained in this study were statistically analyzed according to Petrie and Watson (1999). All data were subjected to one-way analysis of variance (ANOVA) followed by Duncans multiple range test for comparison among treatment means using SAS, Version 8.2 (2001).

RESULTS

Effect of *Enterococcus faecium* on the growth parameters

The effect of addition of *E.faecium* cells into fish feed on the growth parameters were shown in table (2).There was a significant increase in final weight, weight gain and specific growth rate and PER while there was a significant decrease in FCR in group of fish which fed on basal diet supplemented with *E. faecium* when compared with the control group.

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Table (2) Growth parameters of cultured *Oreochromis niloticus*

Items	Control group fed on basal diet	Fish group Fed on *Enterococcus faecium*
Initial weight (g/fish)	18.6 ± 1.03	18.55 ± 1.17
Final weight (g/fish)	26.09 ± 0.141 [a]	29.053 ± 0.02 [b]
Weight gain (g/fish)	6.03 ± 0.474 [a]	9.943 ± 0.042 [b]
SGR (g/day)	0.757±0.03 [a]	0.997±0.04 [b]
PER	2.54± 0.309 [ab]	2.906± 0.412 [a]
FCR	1.324± 0.42 [a]	1.103± 0.021 [b]

Data represented as means± SE (n =15). Within rows, values with different superscripts a,b,c & d are significantly different at ($p<0.05$).

Antimicrobial Effect of *E. faecium* on *A. hydrophila in Vitro* and during the challenge with *A. hydrophila* :

In vitro antagonistic test: ***E.faecium*** developed large inhibition zone (17mm) against ***A. hydrophila*** on MH agar plates.

Mortality rates, clinical signs and post-mortem findings: At the end of the challenge test, the mortality rate was 25 % in fish group challenged I/P with pathogenic *A. hydrophila* and fed on basal diet supplemented with *E. faecium* while it was 60 % in fish group challenged I/P with pathogenic *A. hydrophila* and fed on basal diet. Infected fishes showed the same clinical picture with varied degree in the form of loss of the appetite exophthalmia , abnormal swimming behavior, detachment of scales, skin erosion and ulcer, darkening of the body, fin and tail rot , external haemorrhage, abdominal distension with prolapse of the vent in some cases. Post- mortem examination showed swollen and congested spleen, kidney and liver with distension of the gall bladder. The body cavity was distended with fluid.

Histopathological results

Figure A illustrates the histopathological changes in the fish group fed on basal diet supplemented with *Enterococcus faecium* and challenged I/P with pathogenic *Aeromonas hydrophila* revealed slight congestion in the gills branchial and lamellar blood vessels with beginning of hyperplasia at the base of the secondary lamellae (1).The kidney revealed slight congestion in the renal blood vessels with infiltration of chronic inflammatory cells and melanomacrophage cells in between the heamopiotic tissues, peri-tubular and peri - glomerular edema also detected but in less extent (2). The liver have a normal hepatocytes but there was slight infiltration of chronic inflammatory cells in between the hepatic parenchyma (3).The spleen showed congestion with excessive increase in the melanomacrophage cells in between the heamopiotic tissues(4), The ovary showed Ovary showed different oocyte developmental stages with edema in between, decrease in the number of mature oocytes also detected (5). In the fish group fed on basal diet and challenged I/P with pathogenic *Aeromonas hydrophila* the changes more severe where the skin showed increase in the melanin carrying cells in the epidermal layer with zenker necrosis in the muscular layer (6), the gills revealed severe congestion, telangiectasis (7), hyperplasia and fusion of the secondary lamellae associated with degenerative and necrotic changes in the respiratory epithelium (8). The liver showed severe congestion in the hepatic

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blood vessels with degenerative changes in the hepatocytes in focal areas(9), hyperplasia in the wall of bile duct also detected and excessive infiltration of melanomacrophages in between the hepatic parenchyma and surrounding the bile ducts, chronic inflammatory cells infiltration also appeared in between the hepatocytes (10). The kidney revealed congestion and excessive infiltration of chronic inflammatory cells and melanomacrophages in between the heamopiotic tissues associated with peri-tubular and peri-glomerualer edema (11), degenerative changes also detected in the tubular epithelium and in endothelium lining the glomerular tuft, focal areas of necrosis also detected in the heamopiotic tissues (12), The spleen showed severe congestion with excessive increase the number of melanomacrophage centers(13), The testis revealed degeneration in the lobules with regression of spermatozoal amount in lobular lumen (14), The brain showed congestion and edema (15) associated with neuronal degeneration (16).

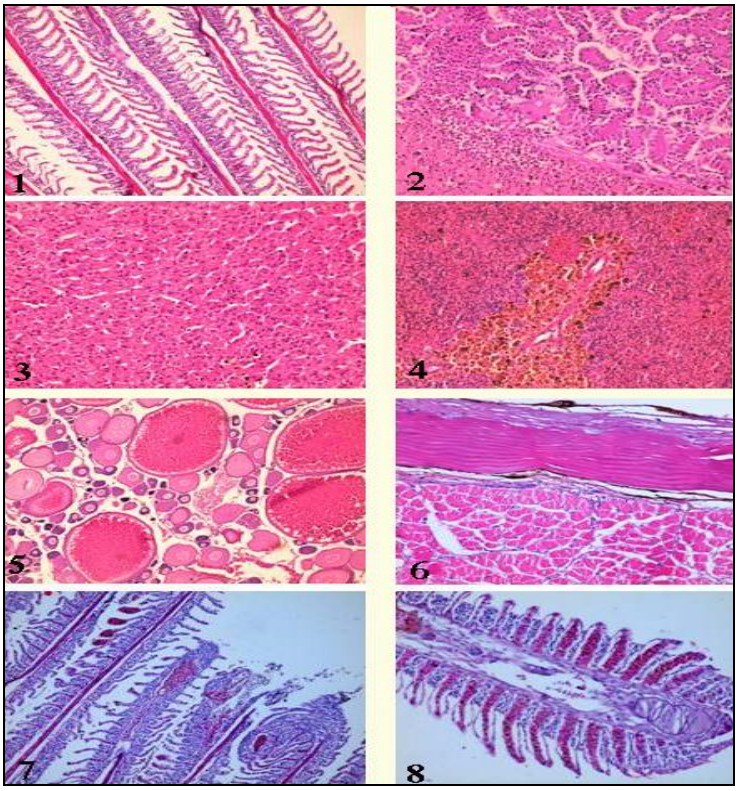

Figure A: Histopathological changes in the fish group fed on diet supplemented with *Enterococcus faecium* and challenged I/P with pathogenic *Aeromonas hydrophila:*

1: gills showed slight congestion and hyperplasia in the respiratory epithelium at the base of secondary lamellae (H&E, X200).

2: Kidney showed congestion in the renal blood vessels, peri-tubular edema and slight degenerative changes in the tubular epithelium (H&E , X400)

3: liver showed normal hepatocytes (H&E, X400).

4: spleen showed congestion and increase in the melanomacrophages cells (H&E, X400)

5: Ovary showed different oocyte developmental stages with aedema in between (H&E, X400).

6: skin showed increase in the melanin carrying cells in the epidermal layer, sub dermal edema and zenkers necrosis in the muscular layer (H&E, X400).

7: Gills showed severe congestion, telangectasis, hyperplasia associated with degenerative and necrotic changes in the respiratory epithelium lining the secondary lamellae (H&E, X200).

8: Gills showed congestion and hyperplasia associated with degenerative and necrotic changes in the respiratory epithelium lining the secondry lamellae. (H&E, X400).

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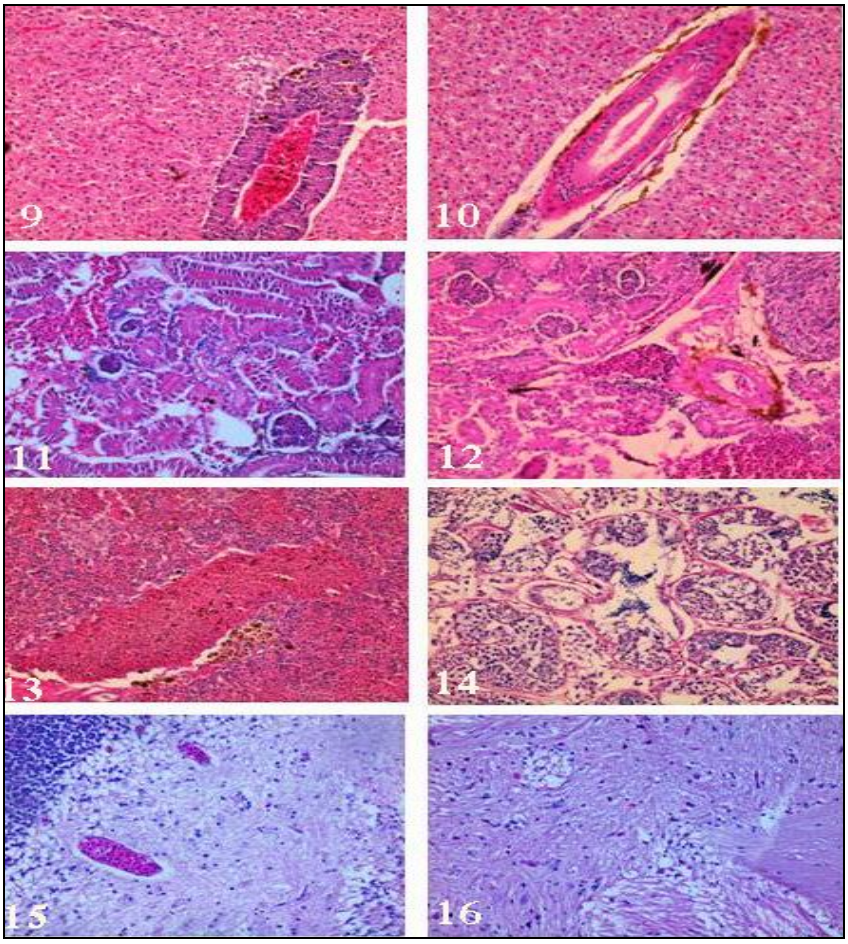

Figure B: Histopathological changes in the fish group fed on diet not supplemented with *Enterococcus faecium* and challenged I/P with pathogenic *Aeromonas hydrophila:*

9: Liver showed dilatation and severe congestion in the hepatic blood vessels and sinusoids and in between the pancreatic tissues with infiltration of chronic inflammatory cells in between the hepatic parenchyma (H&E , X400).

10: Liver showed the same changes before with hyperplasia in the wall of the bill duct with infiltration of melanomacrophages surrondig it (H&E , X400).

11: Kidney showed peritubular and periglmerullar edema, congestion in the renal blood vessels, degenerative changes in the tubular epithelium and in the endothelium linng the glomerular tuft (H&E , X400).

12: Kidney showed the same picture before with infiltration of melanomacrophage cells and chronic inflammatory cells in between the heamopiotic tissue (H&E , X400).

13: Spleen showed severe congestion and increase number of melanomacrophage centers (H&E , X400).

14: Testis showed degeneration in the lobules with regression of spermatozoal amount in lobular lumen in (H&E , X400).

15: Brain showed congestion and aedema (H&E , X400).

16: Brain showed degeneration in the neuronal cells with edema (H&E , X400).

Results of tissue specific expression of IL-8 and TNF immune genes

Molecular investigation and RT-PCR revealed that IL8 gene was highly expressed in fish livers in the infected fish fed on the Enterococcus faecium supplemented basal diet (Fig. C-1-Lane 2,3) compared to the internal control beta actin(C1-Lane 1) and to the fish group fed on basal diet (C 1-Lane 4,5). The obtained band molecular weight of IL8 gene was 386 bp.

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Figure C-2 illustrates the expression of TNF∞ gene which was also highly expressed in fish livers in the infected fish fed on the Enterococcus faecium supplemented basal diet (Lane 2,3) compared to the internal control beta actin(Lane 1) and to the fish group fed on basal diet (Lane 4,5). The band molecular weight of TNF∞ gene was 405 bp.

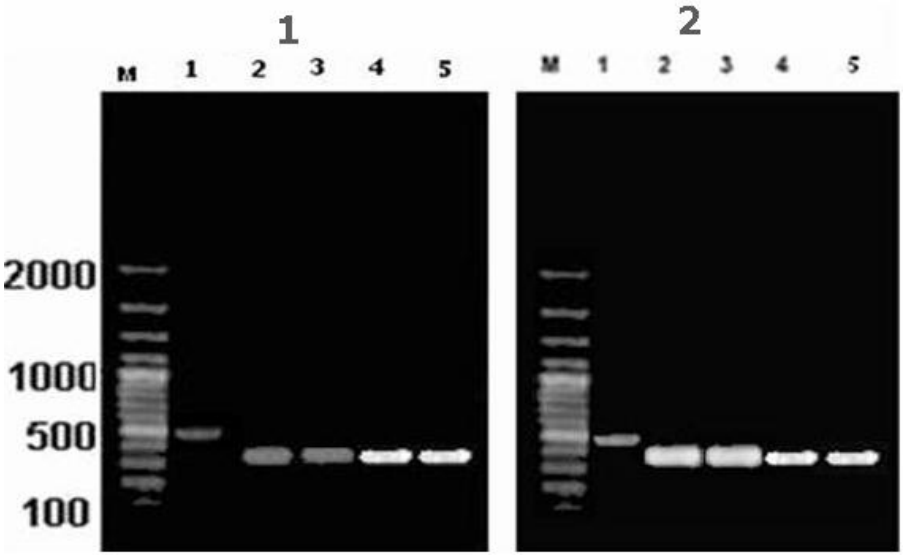

Fig. C: tissue specific gene expression of IL-8 (A) and TNF-α (B) by semi-quantitative reverse transcriptional-PCR.M: marker, 1: beta actin, 2&3: *E.faecium* group, 4&5: basal diet group.

DISCUSSION

Rezgui *et al*., (2010) in his study showed that, the abundance of antibiotic resistant bacteria isolated mainly from gills and intestinal tract of sea bre*am* and sea bass which belong to several species of the genus *Pseudomonas, Aeromonas, Vibrio* and *Enterobacteriaceae*, theses isolated bacteria were resistant essentially to tetracyclin and penicillin (antibiotics commonly used respectively in veterinary and human clinical).

The use of antibiotics as disease controllers and growth promoters is currently restricted or forbidden in many countries (Verstegen, 2001), and a growing concern about the high consumption of antibiotics in aquaculture has initiated a definite need in which both consumer and manufacturer are looking for the alternative health management strategy, which can be accomplished by microbial intervention (Panigrahi and Azad, 2007). There has been heightened research in developing new dietary supplementation strategies in which various health and growth-promoting compounds as probiotics, prebiotics, synbiotics, phytobiotics and other functional dietary supplements have been evaluated (Denev, 2008).

Verschuere *et al.* (2000)) suggested a new definition of a probiotic for aquatic environments: 'a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host's response towards disease, or by improving the quality of its ambient environment', or that 'a probiotic is an entire microorganism or its components that are beneficial to the health of the host' (Irianto & Austin,2002).The use of probiotics or beneficial bacteria, which control pathogens

through a variety of mechanisms, is increasingly viewed as an alternative to antibiotic treatment. They have begun to be applied in aquaculture (Bache`re, 2003). Enterococci are Gram-positive, facultative anaerobic bacteria which are widely distributed in nature and considered as bacteria of low pathogenicity (Klare *et al*., 2003). Probiotic enterococci infections haven´t been reported in the veterinary medicine, so the risk appears to be limited (Rinkinen *et al.,* 2003). Eaton and Gasson (2001) found that *E. faecium* strains were also generally free of virulence determinants. *E. faecium* (strain IMB 52) is considered as safe and advantageous potential probiotic for aquatic species (BIOMIN Holding GmbH, 2013). Results of this study revealed that the addition of *Enterococcus faecium* cells into fish feed at the level of 8gm ⁄ kg feed resulting in significant increase in final weight , weight gain, survival rate and PER while there was a significant decrease in FCR. Similar results concerning significant increase in body weight of *Silurus glanis* by addition of *E. faecium* to fish diet(Bogut et al.,2000).Also, many studies are focused on the immunological stimulation of fish defense mechanisms by probiotic bacteria (Díaz-Rosales *et al*., 2006 a,b). These findings could be explained by those of Wang *et al.* (2008) who demonstrated that the addition of *E. faecium* (1 x 10^7 CFU/mL) in aquaria water could significantly increase final weight and daily weight gain (DWG) of tilapia. Since certain immunological parameters (myeloperoxidase and respiratory burst activity) of tilapia were improved as well, the increased growth performance might be attributed to less bacterial challenge, confirming the benefit for the non-specific innate immunity of this kind of fish. Several authors have suggested that organisms in aquaculture are primarily affected by beneficial bacteria through the enhancement of host nutrition due to the stimulation of digestive enzymes resulting in a higher growth and FCR (Suzer *et al*. 2008). Furthermore, the presence of beneficial bacterial cells in the intestine improves microbial balance, which in turn improves nutrient absorption and utilization (Lara-Flores 2003).

In this study, *Enterococcus faecium* developed large inhibition zone (7 cm) against *Aeromonas hydrophila* on MH agar plates. Concerning this aspect, *In vitro* studies using the agar spot method (Rosskopf, 2010) have shown that *Enterococcus faecium* (strain IMB 52) has inhibition properties against a wide spectrum of aquatic pathogens including *Yersinia ruckeri*, *Vibrio harveyi*, *Streptococcus agalactiae* and *Aeromonas veronii.*. Also, agar spot method was used to detect the antagonistic effects toward *Aeromonas hydrophila* (Gopalakannan & Venkatesan, 2011). In the studies that are conducted by *E. faecium*, antagonistic effects against *A. hydrophila* were observed by cross-streaking and the agar spot method. The strain's probiotic effects were confirmed and it was checked for non pathogenicity to fish (Gopalakannan & Venkatesan, 2011).The observed clinical signs and post-mortem findings during challenge test in this study were similar to that reported by Plumb (1999); Azad *et al*. (2001) and Bannai (2013). The mortality rate was 25 % in fish group challenged I/P with pathogenic *Aeromonas hydrophila* and fed on basal diet supplemented with *Enterococcus faecium* while it was 60 % in fish group challenged I/P with pathogenic *Aeromonas hydrophila* and fed on basal diet. The reduction of mortality rate due to the effect of *Enterococcus faecium* could be explained by Bogut *et al*. (2000).They recorded that the lactic acid bacterium *Enterococcus faecium* consumed by the fish through pelleted diet possesses high adhesive ability in the digestive tract epithelium. It resulted in a reduction of harmful bacteria with complete elimination two weeks after the fish received pelleted diet with probiotic addition thus better body weight were observed also could be as a result of immune system activation against pathogens which recorded in this study. The

histopathological findings in infected fishes were similar to those observed by Plumb (1999); Aly *et al.* (2000); Azad *et. al.* (2001) and Aydin and Ciltas (2004). It was cleared that diminish the pathogenic effect of *Aeromonas hydrophila* infection and this could be explained by the foundation of Bogut *et al*.(2000). The recorded clinical signs and histopathological changes in challenged *Oreochromis niloticus* could be attributed to the different types of toxins as haemolysin (Kanai and Takagi,1986), Acetylcholinestrase (Nieto *et al*.,1991), protease (Reily and Day,1983) and enterotoxin (Bernheimer and Avigad,1974) produced by *Aeromonas hydrophila*.

Immunostimulants like probiotic agents have a beneficial effect via a wide array of actions including enhancement of the immune response and diseases resistance. on the other side, aquaculture fish mortality due to severe bacterial infections has emphasized the need for development of new therapeutic and prophylactive strategies. The outcome of an infection depends on two factors: the growth of the microorganisms (including the effect of antibacterial drugs), and the host's defensive response to the invading organism. It is known that injection of bacterial products into experimental animals leads to enhanced nonspecific resistance to a variety of microorganisms, The discovery of the specific mediators responsible for modulation of host defense has created new possibilities for the development of alternative treatment strategies. Liver is considered to be the central organ regulating the acute phase response by releasing specific acute phase proteins like the pro-inflammatory cytokines including interleukin 1 beta (IL-1β), interleukin 6 (IL-6), interleukin 8(IL-8) and Tumor necrosis factor (TNF) (Panigrahi *et al.* (2007).

Molecules such as interleukins, interferons, tumor necrosis factors and hematopoietic growth factors have become available in recombinant form, and their therapeutic potential in various infectious diseases has been tested in various experimental models of infections. Initial data in various immunized groups indicated that adjunctive therapy with recombinant proinflammatory cytokines may have beneficial effects in the treatment of bacterial and fungal infections. So, identification of mediators regulating the process of the host immune function could prove to be a good tool to improve the aquaculture fish productivity. In these concerns, this study was designated to assess the role of *E. faecium* probiotic as a growth promoter and in enhancing tilapia immune function during experimental infection with *A. hydrophila* through examination of some immune genes expression exemplified by IL8 and TNF-α. In the current study *E. facium probiotic* resulted in a clear increase in expression of the genes encoding the pro-inflammatory cytokine and chemokine ; TNF α and IL-8 in contrast to fish fed on basal diet without the probiotic. These cytokines are important inducers of Acute phase reactants (APR) resulting in increased production of Acute Phase Proteins (APPs) and consequently the elimination of bacterial threats. The fact that IL-8 and TNF-α are highly induced and expressed is consistent with the expected expression profile at the early stages of infection (3 days in this study). So, a most remarkable finding was the impact of the *E. faecium* bacterial probiotic on the expression of innate immune genes, expressed in the tilapia fish's liver during the challenge test. Panigrahi *et al.* (2007) examined the immune modulation including some cytokine gene expressions of rainbow trout (*Oncorhynchus mykiss*) and demonstrated that these parameters were improved by probiotic feeding of freeze-dried *Lactobacillus rhamnosus, Enterococcus faecium* or *Bacillus subtilis* (10^9 CFU/g) after 45 days. Fish fed particularly the *E. faecium* strain showed better performance which could possibly correlated with the suitable

ambient temperature conditions of this strain. Pandit *et al.* (2013) recorded an increase of TNF-α expression in association with bacterial and viral infections and they suggested that it plays a potentially important role in the immune function of fish. Also, Cantas *et al*. (2012) found that there was a strong induction of selected inflammatory and immune response genes (IL 1 beta, IL 8 and TNF13b) which was evident in fish subjected to ineffective treatment protocols. These results were in accordance with our findings in tilapia fish, thus we can consider that *E. Facium* strengthens and enhances the *O. niloticus* immune system response against *A. hudrophila* .

CONCLUSION

Collectively, the result of this study clearly shows that, proper and efficient use of *E. Facium* probiotic could be used as a feed additive to improve *O. niloticus* growth rates and immune function and is a valuable for cultured *O. niloticus*.

Conflict of Interest

The authors declare that there is no conflict of interest.

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