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Research Article

Comparative effect of different vaccines on immune-related gene expressions of rainbow trout (*Oncorhynchus mykiss*) and experimentally infected with *Streptococcus iniae* and *Lactococus garvie*

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Abstract

To understand how different vaccines, i.e., bivalent formalin-inactivated vaccine (*Lactococus garvie* and *Streptococcus iniae*), could affect the immune-related gene expressions, the TNF-alpha, IL-1β and IL-8 genes of head kidney were measured in *Oncorhynchus mykiss*. To address this, 630 juvenile fish weighing 26±3 g were initially (day 0) injected by three different treatments, including control group (normal saline only), AquaVac^{TM} Garvetil (Merck) and Iranian vaccine (Jehad-e-Daneshagahi) and followed by bath immersion into the same vaccine on day 30. The head kidney was withdrawn to measure immune-related gene expressions at the beginning of experiment (day 0), and following 14, 30, 45 and 60 days of post-vaccination. In the next step, control and both vaccinated groups were also subjected to either *L. garvie* or *S. iniae* challenge. Similar samples were taken immediately following bacteria injection (time= 0) and 12, 48, 72 h, and following 7 and 10 days of challenged test. The real-time PCR indicated up-regulation of all cytokine genes following vaccination at day 14 as compared to initial day ($p<0.05$). In pre-challenged experiment, the higher induction in the level of those genes in the head kidney was related to AquaVac vaccine ($p<0.05$). Injection by *S. iniae* and *L. garviae* induced the level of immune-related gene expression in the head kidney within the first few days with higher intensity in case of unvaccinated control group. Although the AquaVac produces higher up-regulation of inflammatory gene expression rather than Iranian vaccine, the immunostimulatory effects of both vaccines is a time-restricted.

Keywords: Cytokine genes, Pro-inflammatory genes, Bacterial infection, Passive vaccination, *Oncorhynchus mykiss*

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Introduction

Oncorhynchus mykiss is considered as one of the most important commercial fish species for aquaculture in many countries, including Iran. Bacterial infections induced heavy economic losses on this species all over the world (Vendrell *et al*., 2008; Raissy *et al*., 2018). Although the antibiotic treatment was futile to some extent, the aquaculturists are still eager to apply different types of antibiotic even for prevention. Moreover, the emergence of antibiotic-resistance for these pathogen strains (Park *et al*., 2009; Marti *et al*., 2018) negated the success of this method for outbreak control (Sakai *et al*., 1995), and consequently, suggesting the necessity of an alternative way such as vaccination to protect fish against the infections (Dimitroglou *et al*., 2011; Faber *et al*., 2019).

Amongst trout infectious diseases, *Lactococcus garvieae* and *Streptococcus iniae*, the causative agent for lactococcosis and streptococcosis, respectively accounted as massive outbreaks in the aquaculture industry especially during warm seasons (Diler *et al*., 2002; Didinen *et al*., 2014; Raissy *et al*., 2016). It is also considered to be responsible for more than 50% of losses in trout farms (Vendrell *et al*., 2006; Shiry *et al*., 2019). These gram-positive non-motile bacteria affect different freshwater and marine species (Nakai *et al*., 1999; Chen *et al*., 2001; Evans *et al*., 2006; Bastardo *et al*., 2012). The lactococcal septicemia not only produces clinical signs but also some lesions (such as fibroplasia) could be observed at the serous membrane of different organs (Chang *et al*., 2002). The infection caused by *S. iniae* is responsible for several pathological changes, including meningitis, lesions in internal organs as well as sepsis among different species (Eldar *et al*., 1999; Eldar and Ghittino, 1999). It is believed that they can threat the public health by producing some chronic diseases like skin lesions in human (Elliott *et al*., 1991; see the review written by Novotny *et al*., 2004; Wang *et al*., 2007).

Besides the costs forced by drug treatments in large aquaculture systems, bacterial resistance and lack of efficiency could constrain antibiotics application to aquaculture industry (Harikrishnan *et al*., 2010). Protection of aquatic species against lactococcosis and streptococcosis were already examined both in experimental and field studies (Eldar *et al*., 1997; Ooyama *et al*., 1999; Soltani *et al*., 2019). Previously, it has been shown that the i.p. vaccination with formalin-killed *Streptococcus* sp. in the presence of adjuvant could protect *O. mykiss* against this pathogen while the other kind of immunization, i.e., bath immersion did not act the same (Akhlaghi *et al*., 1996). The combined vaccines are also developed to prevent fish from bacterial and viral pathogens (Sakai *et al*., 1995). However the efficacy of combined vaccines for lactococcosis and streptococcosis did not investigate well on *O. mykiss*.

Currently, the importance and success of vaccinations against bacterial

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diseases in fish are obvious; however, little attempts are fulfilled to understand the mechanisms of vaccine-induced disease resistance (Harun *et al*., 2011). One of the possible mechanisms for pursuing how immune system was altered after vaccination is, however, to examine the immune-related gene expression. Activation of macrophages by fish vaccination results in releasing various cytokines such as IL-1, IL-6 and TNF-alpha and enhancing T-cell mediated cytotoxicity and proliferation (Djeraba and Quere, 2000; Evans *et al*., 2004; Mohammadian *et al*., 2019). It typically determined following vaccination against infectious haematopoietic necrosis virus and yersiniosis in *O. mykiss* by Purcell *et al*. (2004) and Harun *et al*., (2011), respectively, but similar studies on lactococcal or streptococcal vaccinations are quite rare and limited to other fish species (Sun *et al*., 2012).

In this paper, we aimed to examine how *O. mykiss* can be immunized following i.p injection with repetitive bath immersion of two different bivalent formalin-inactivated vaccines (*L. garvie* and *S. iniae*) in a time-course manner. To do this, some immune-related gene expression was evaluated to obtain an insight into the possible underlying mechanisms that immunized fish might respond to vaccination. The ability of i.p and bath vaccinations was also followed up by experimental infections with either *L. garvie* or *S. iniae*.

Materials and methods

Fish maintenance

Juvenile rainbow trout, *O. mykiss* (n=630) weighing 26±3 g were purchased from a local supplier in Loresatn province (Iran) and transferred to the Aquatic Animal Health Lab at Shahid Chamran University of Ahvaz. All fish were acclimated for at least 2 weeks before experiments was commenced in 300 l tanks. Acclimation tanks were consisted air supplier to remain dissolved oxygen higher than 10 mg/l and located in a temperature-controlled room (16±2°C). The fish were initially examined for any bacterial pathogens and parasites. All fish were fed three times a day *ad libitum* with diet containing 36.81% crude protein, 11.33% crude fat, 11.58% crude fiber and 3.50% ash (GFT1- Fardaneh Company).

Treatments

O. mykiss juveniles were randomly divided into three groups (treatments). Each group containing 210 fish (each group included three tanks and each tank contained 70 fish). The first group was only received normal saline (similar to vaccine volume) and considered as control. The second group was injected by AquaVac^{TM} Garvetil (Merck, Germany) and third group was injected by Iranian vaccine (Jehad-e-Daneshagahi). The vaccines contained 1×10^9 *S. iniae* cells /mL and 1×10^9 *L. garviae* cells /mL. The injected dose was similar for both vaccines and included 0.1 mL per fish (according

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manufacture manual). All above-mentioned treatments were followed by bath immersion into the same vaccine on day 30. To do this, 1000 g fish were immersed into 9 l water containing 1 l of vaccine for 5 min (all procedures were done according to manufactures' protocol). All fish were deprived of food for two days before either injection and/or immersion bath.

Sample collection

Three fish from each tank were randomly selected and anesthetized by 2-phenoxyethanol (0.3 mL/l, Merck, Germany). The head kidney was withdrawn and stored at -70°C (Matsuyama *et al*., 2007) for further measuring of immune-related gene expression, including tumor necrosis factor alpha (TNF-alpha), interleukin 1 beta (IL-1β) and interleukin 8 (IL-8) at the beginning of experiment (day 0), and following 14, 30, 45 and 60 days. In the next step, the control group, AquaVac and Iranian vaccine treatments were also subjected to either *L. garvie* or *S. iniae* challenge test. Similar samples were taken immediately following bacteria injection (time= 0) and 12, 48, 72 h, and following 7 and 10 days of challenged test.

Gene expression

RNA isolation and cDNA synthesis

At each exact time, the head kidneys' RNA was isolated (see the sampling section), using the Tri Pure isolation reagent according to the manufacturer's procedure (Roche, Canada). Fifty mg of each sample was taken under sterile condition. The RNA concentration was determined by using nano-drop (Eppendorf, Germany) and the purity of the RNA was determined by the optical density (OD) absorption ratio at 260/280 nm. Reverse transcription was carried out with the Rocket Script RT PreMix Kit using 1 μg of RNA and oligo dT based on the manufacturer's protocol (Bioneer Corporation, South Korea).

Real-time quantitative PCR

To evaluate the expression levels of TNF-alpha, IL-1β and IL-8 (Inflammatory genes) (Secombes*et al*., 1998; Secombes*et al*., 2011) mRNA in the head kidneys, real-time PCR was performed using qPCRTM Green Master Kit for SYBR Green I® (Jena Biosciense, Germany) on a Light cycler® Detection System (Roche, USA). Relative expression levels of the all transcripts were compared to β-actin. Specific sets of primers (Bioneer Co., South Korea) were designed based on *O. mykiss* (Table 1). Reactions were performed in a 12.5 μL mixture containing 6.25 μL qPCR TM Green Master Mix (2X), 0.25 μL of each primer (10 μM), 3 μL (100 ng) cDNA, and 2.75 μL nuclease-free water. The PCR protocol consisted of a 5 min denaturation at 94 °C followed by 45 cycles at 94 °C and 60° C for 30 sec (in triplicate). Two separate reactions without cDNA or with RNA were performed as control groups in parallel

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with experimental groups. According to the comparative $2^{-\Delta\Delta Ct}$ method, the relative quantification was performed using Light cycler 96® software (Mohammadian *et al*., 2018). All qPCR analysis was performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guideline (Bustin *et al*., 2009).

Table 1: Primers used for detection of target genes.

Genes	Product size	Primer (5′-3′)	Access Number
EF1α	327bp	Forward CAAGGATATCCGTCGTGGCA ReverseACAGCGAAACGACCAAGAGG	AF498320
TNF-alpha	102 bp	Forward TGTGTGGGGTCCTCTTAATAGCAGGTC ReverseCCTCAATTTCATCCTGCATCGTTGA	AJ277604
IL-8	226 bp	Forward GAATGTCAGCCAGCCTTGTC ReverseTCCAGACAAATCTCCTGACCG	HG917307.1
IL1-β	239 bp	Forward CCTGGAGCATCATGGCGTG ReverseGCTGGAGAGTGCTGTGGAAGAACATATAG	AJ278242

Bacterial challenge

The *L. garvieae* and *S. iniae* strains were originally isolated from naturally infected trout and cultured routinely on tryptone soy agar (Difco) plates, at 27ºC for 48 h. The isolates were identified as *L. garvieae* and *S. iniae* by standard methods like colony and cell morphology, gram staining and biochemical characteristics.

Prior to challenge test of *O. mykiss* with either *L. garviae* or *S .iniae*, the lethal dose (LD) of each bacterium was determined. First, each bacterium was grown on TSA at 27°C for 48 h. The culture medium was then centrifuged at 3500 rpm for 10 min. The bacteria were washed twice with normal saline and concentration of bacteria was adjusted to 1×10^9 CFU/mL with McFarland tubes. Serial dilutions (1×10^5 up to 1×10^9 CFU/mL) were selected for i.p injection. The mortality was recorded daily for 4 continuous days and the obtained data was subjected to probit regression analysis to calculate LD_{50}(Sun *et al*., 2011). The amounts of LD_{50} for each bacterium, i.e., *L. garviae* and *S. iniae* were injected (10^7 CFU/mL and5 10^7 CFU/mL, respectvely) as i.p. doses for further experiments.

Statistical procedure

If data approved for normality test (Kolmogorov–Smirnov) and homogeneity of variance (Levene), further statistical test have been performed. Two-way Analysis of Variance was applied to determine the effects of treatments and time on different parameters in the first experiment (before challenge test). Multi-way Analysis of Variance (MANOVA) was performed to determine the combined effect of treatments, time and bacterial strains on gene expressions after challenge test. The multiple comparisons (Duncan) were followed if the *p value* was statistically significant (SPSS, 18). Logarithmic transformations of

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bacterium doses were used to calculate the LD_{50} at exact time durations using the probit regression analysis (SPSS, 18). All experimental data were presented as the mean±SD, and the level of significance for all tests was set at $p<0.05$.

Results

In order to evaluate whether different vaccines and time interval may composed of any changes in immune-related gene expression of *O. mykiss* prior to the challenge test, the data were subjected to Two-way ANOVA. In spite of any possible statistical difference originated from above-mentioned single factor, the significant combined effects were observed in case of TNF-alpha, IL-1β and IL-8 genes of head kidney. Moreover, in case of challenged test, the MANOVA revealed significant combined effects for all measured parameters (Tables 2 and 3).

Table 2: Two-Way ANOVA performed for each parameter with *P value* in pre-challenge test.

	Time	Treatment	Time× Treatment
TNF-alpha	<0.001	<0.001	<0.001
IL-1β	<0.001	<0.001	<0.001
IL-8	<0.001	<0.001	<0.001

Table 3: MANOVA performed for each parameter with *P value* in post-challenge test.

	Time	Treatment	Bacterial Strain	Time× Treatment	Time× Bacterial Strain	Treatment× Bacterial Strain	Time× Treatment × Bacterial Strain
TNF-alpha	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
IL-1β	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
IL-8	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Pre-challenge test The TNF-alphagene expression in the head kidney of fish, vaccinated with AquaVac showed a significant increase at different time interval as compared to day 0 ($p<0.05$). The higher TNF-alpha was also observed following 14 days of vaccination as compared to other sampling times ($p<0.05$). This gene was restored to its initial level on day 45 and remained unchanged even on day 60. This pattern was also observed in TNF-alpha gene expression in the head kidney of fish vaccinated by Iranian treatments. Regarding that, the only significant increase was observed on days 14 and 30 as compared to other sampling times ($p<0.05$), indicating a peak at those time intervals. Although there was a significant difference in TNF-alphaamong different treatments at the beginning of experiment, this difference was much obvious on day 14 with higher level for AquaVac ($p<0.05$). The higher TNF-alphagene expression was also observed in both vaccine treatments on day 30 as compared with control but this change did not last following 45 and 60 days of experiment (Fig. 1).

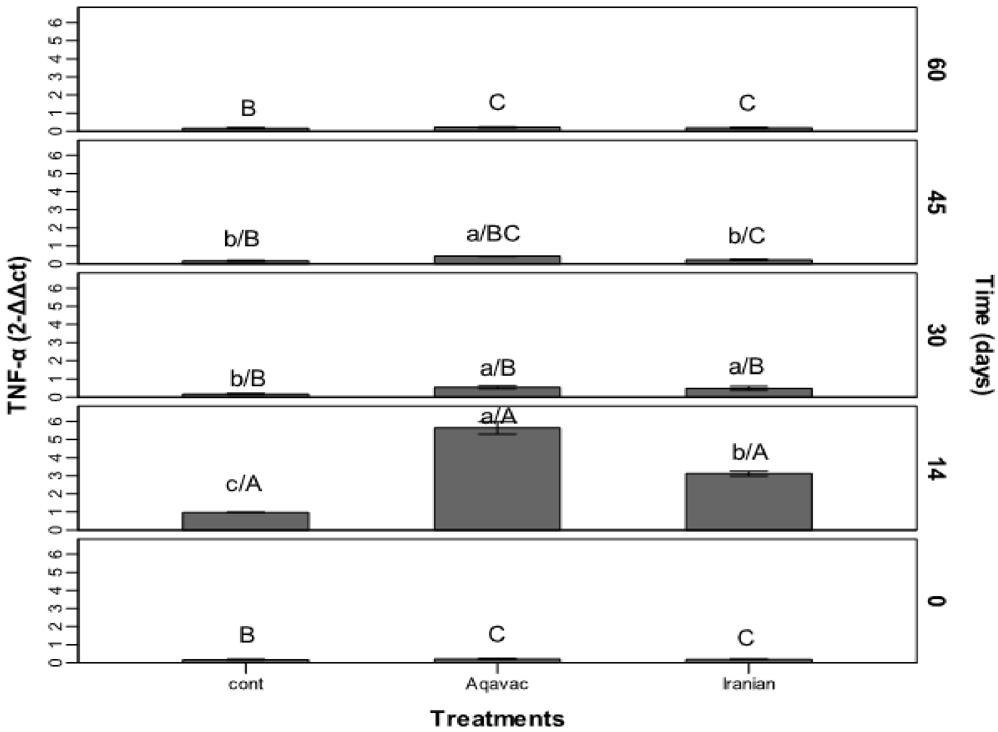

Figure 1: The effects of different vaccines on head kidney TNF- alpha gene expression at different time intervals. All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD. Different lowercase alphabetic letters on each bar indicate significant difference among different treatments and different capital letters express significant difference among different sampling times (*p*<0.05).

IL-1β gene expression in the head kidney showed a significant increase in all vaccinated and unvaccinated groups following day 14 ($p<0.05$). As it is shown in Figure 2, the only significant increase in IL-1β gene expression was related to day 14 for Iranian and AquaVac vaccines in comparison with control group ($p<0.05$).

The results obtained from IL-8 gene expression exhibited a similar trend for both sampling time and treatment as we observed for IL-1β (Fig. 3). In this regard, an up-regulation of IL-8 was observed in all groups following 14 days of experiment. The higher level of this gene was also observed at that time in treated fish with AquaVac ($p<0.05$).

Post-challenge test

The real-time PCR shown that TNF-alphagene expression was elevated following 12 h after i.p. injection of *S. iniae* in control untreated group and this parameter was declined significantly following 48 and 72 h as compared to 12 h ($p<0.05$). This decreasing trend was last for even 7 and 10 days after *S.*

iniae injection in control group and reached the value similar to injection time (day 0).

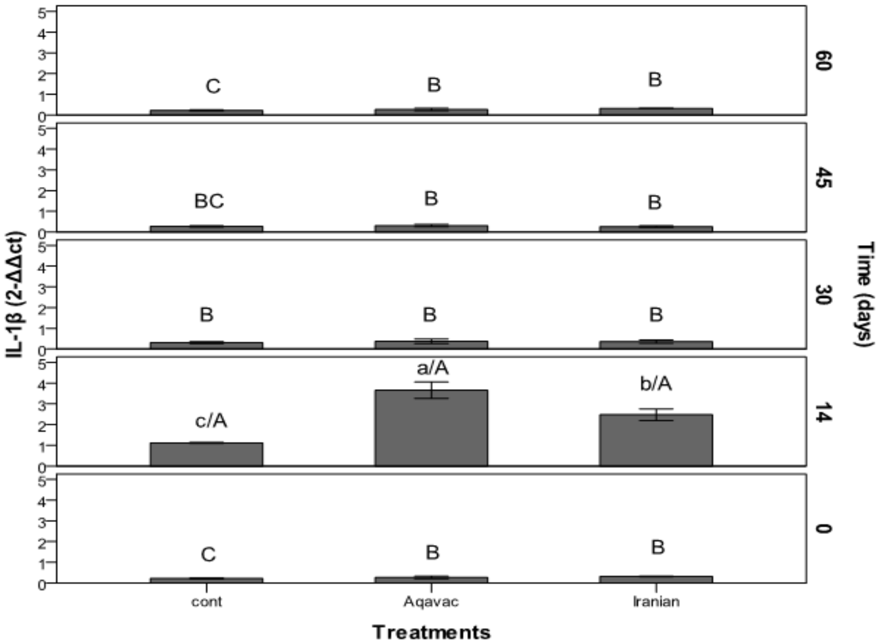

Figure 2: The effects of different vaccines on head kidney IL-1B gene expression at different time intervals. All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD. Different lowercase alphabetic letters on each bar indicate significant difference among different treatments and different capital letters express significant difference among different sampling times (*p*<0.05).

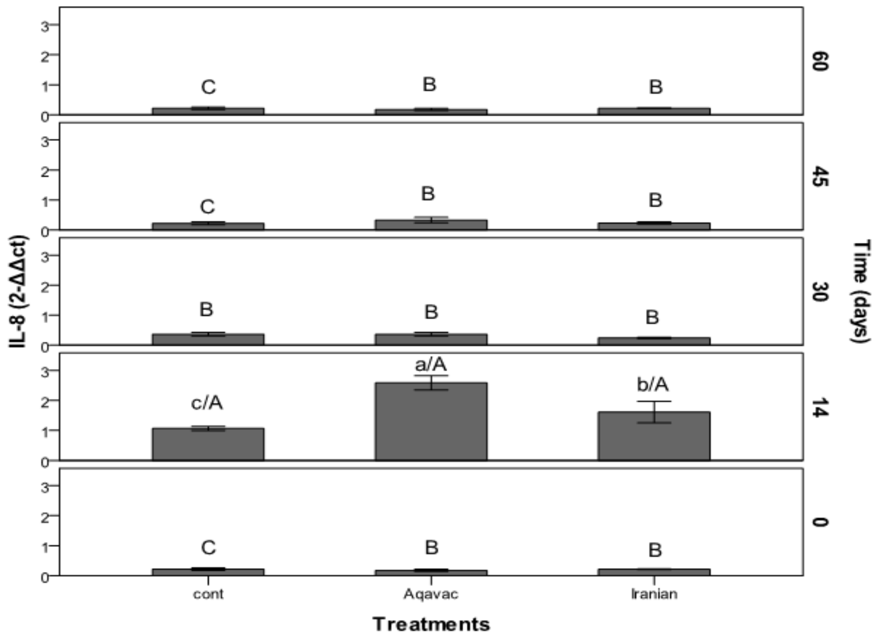

Figure 3: The effects of different vaccines on head kidney IL-8 gene expression at different time intervals. All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD. Different lowercase alphabetic letters on each bar indicate significant difference among different treatments and different capital letters express significant difference among different sampling times (*p*<0.05).

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A significant increase ($p<0.05$) in TNF-alpha gene was also observed in both vaccinated groups (especially Iranian vaccine) in which they reached a peak at 48 and 72 h after injection. The up-regulation of this gene in the head kidney did not last for 10 days and came back to their initial level. Interestingly, the higher ($p<0.05$) level of TNF- alpha gene expression was observed in control challenged group (*S. iniae*) as compared with two vaccinated groups in all sampling times, except for time 0. This difference was also attenuate on day 10 in which a slight decrease ($p<0.05$) was only for Iranian vaccine (Fig. 4).

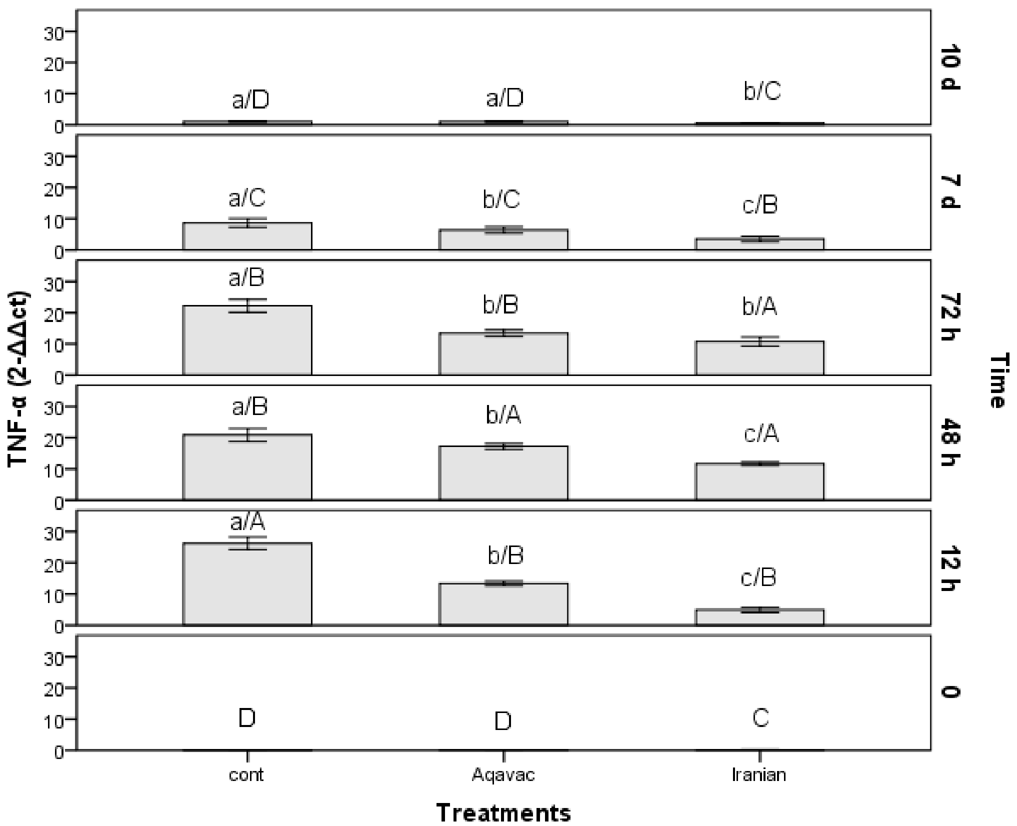

Figure 4: The effects of different vaccines on head kidney TNF- alpha gene expression following challenge test with *S. iniae* at different time intervals. All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD. Different lowercase alphabetic letters on each bar indicate significant difference among different treatments and different capital letters express significant difference among different sampling times (*p*<0.05).

Similar to *S. iniae* challenge test, the control group in *L. garviae* was also showed a significant up-regulation in all sampling points except for day 10 as compared with injection time ($p<0.05$). Similar trend was also observed for other treatments but with lesser intensity. Although the up-regulation of this gene was mostly higher in control group (i.e., at 12 and 48 h), significant increase was also found in AquaVac treatment in which showed similar or even higher (p<0.05) level of TNF-alpha as compared to control (Fig. 5).

The pattern observed for IL-1β was similar to what we observed for TNF-alphafollowing *S. iniae* or *L. garviae* i.p. injections in which the higher level was obtained following 48 h of injection (p<0.05). In addition, the vaccine treatments resulted in significant down-regulation (p<0.05) of this gene approximately at all sampling times with much lower level for Iranian vaccine (Figs. 6 and 7).

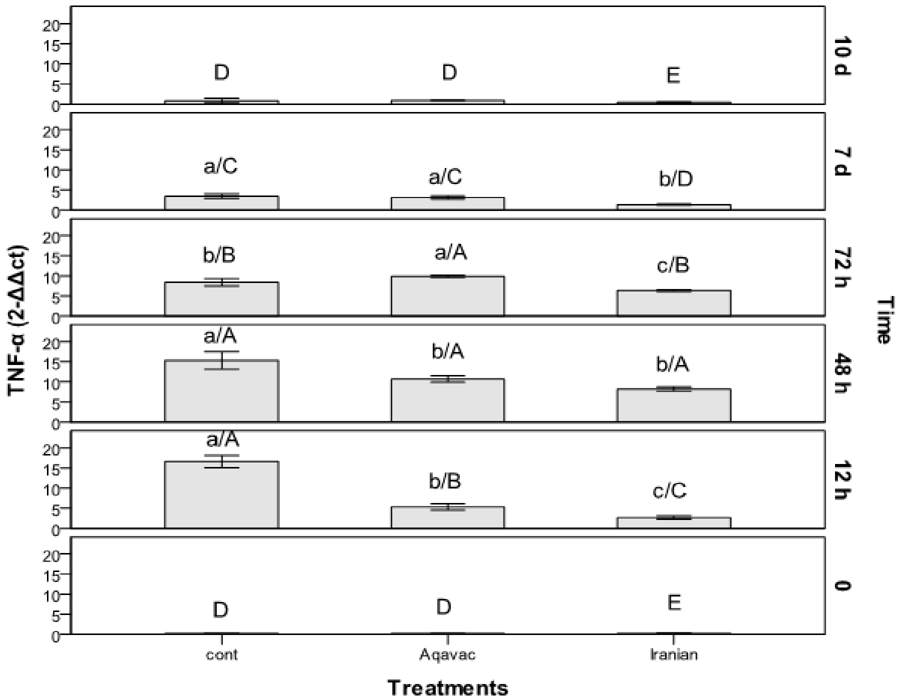

Figure 5: The effects of different vaccines on head kidney TNF- alpha gene expression following challenge test with *L. garviae* at different time intervals. All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD. Different lowercase alphabetic letters on each bar indicate significant difference among different treatments and different capital letters express significant difference among different sampling times (p<0.05).

Similar to other measured genes, the IL-8 was also shown the same trend following *S. iniae* injection (Fig. 8). The control group indicated the higher (p<0.05) level of this gene at all sampling points, except for day 10.

The *L. garviae* injection resulted in significant rise in IL-8 gene expression in the head kidney in all treatments following 48 and 72h of injection as compared with time 0 (p<0.05) and this increase did not continue until the end of sampling time. The down-regulation of IL-8 was much more obvious in the case of Iranian vaccine in all sampling points as compared to other groups (p<0.05). Although the higher level of this gene was mostly observed in control group, there was no significant change at 48 h between AquaVac and control (Fig. 9).

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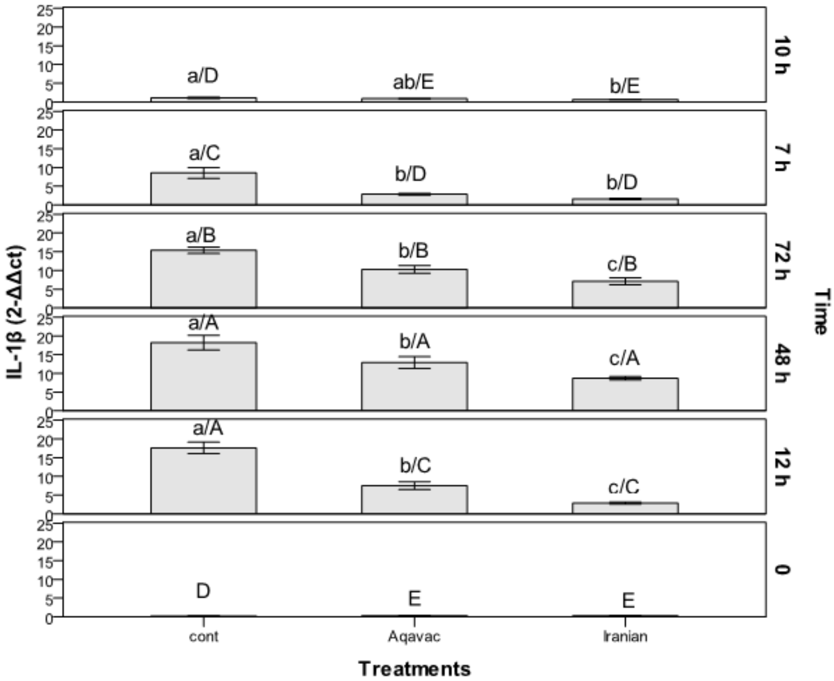

Figure6: The effects of different vaccines on head kidney IL-1B gene expression following challenge test with *S. iniae* at different time intervals. All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD. Different lowercase alphabetic letters on each bar indicate significant difference among different treatments and different capital letters express significant difference among different sampling times (*p*<0.05).

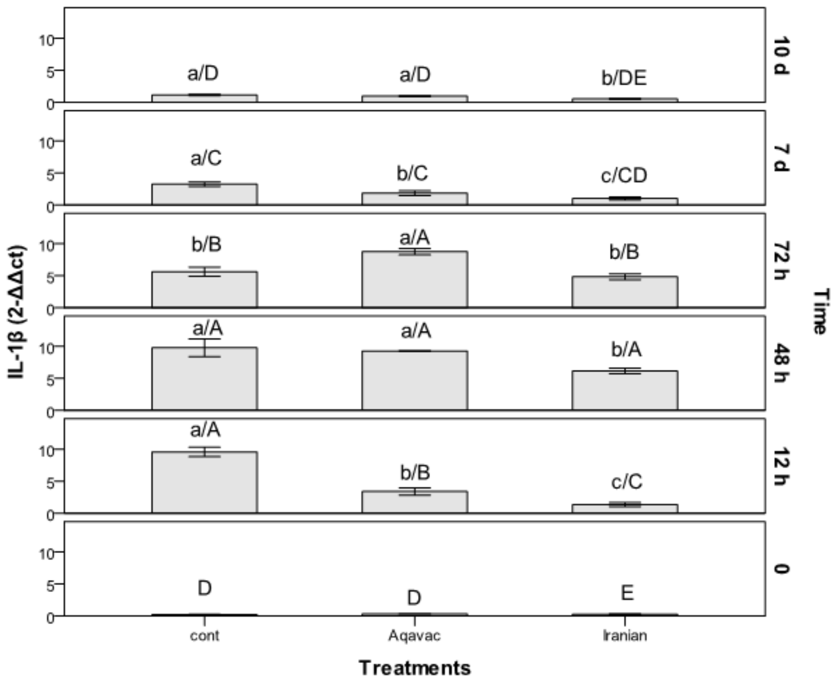

Figure 7: The effects of different vaccines on head kidney IL-1B gene expression following challenge test with *L. garviae* at different time intervals. All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD. Different lowercase alphabetic letters on each bar indicate significant difference among different treatments and different capital letters express significant difference among different sampling times (*p*<0.05).

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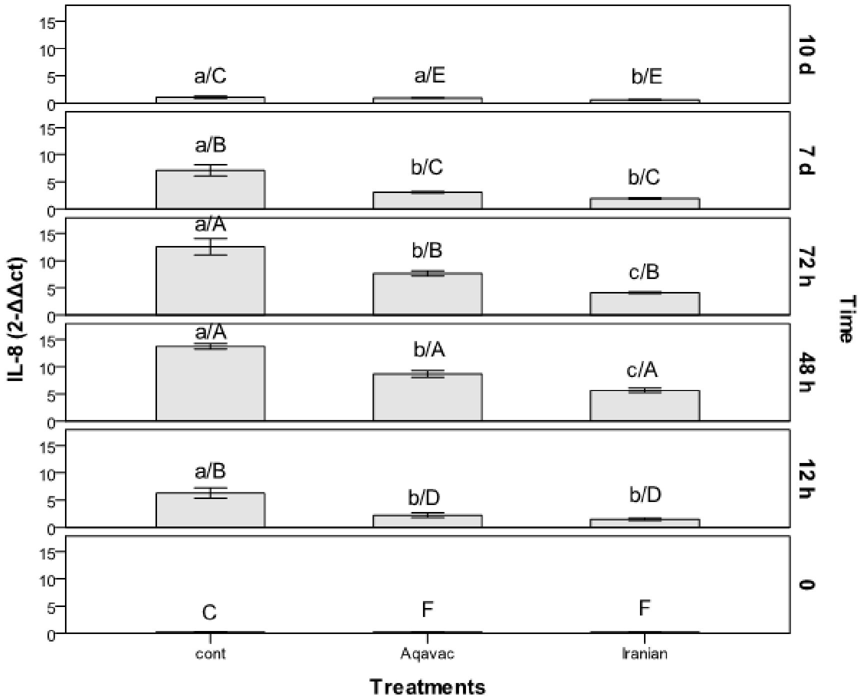

Figure 8: The effects of different vaccines on head kidney IL-8 gene expression following challenge test with *S. iniae* at different time intervals. All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD. Different lowercase alphabetic letters on each bar indicate significant difference among different treatments and different capital letters express significant difference among different sampling times ($p<0.05$).

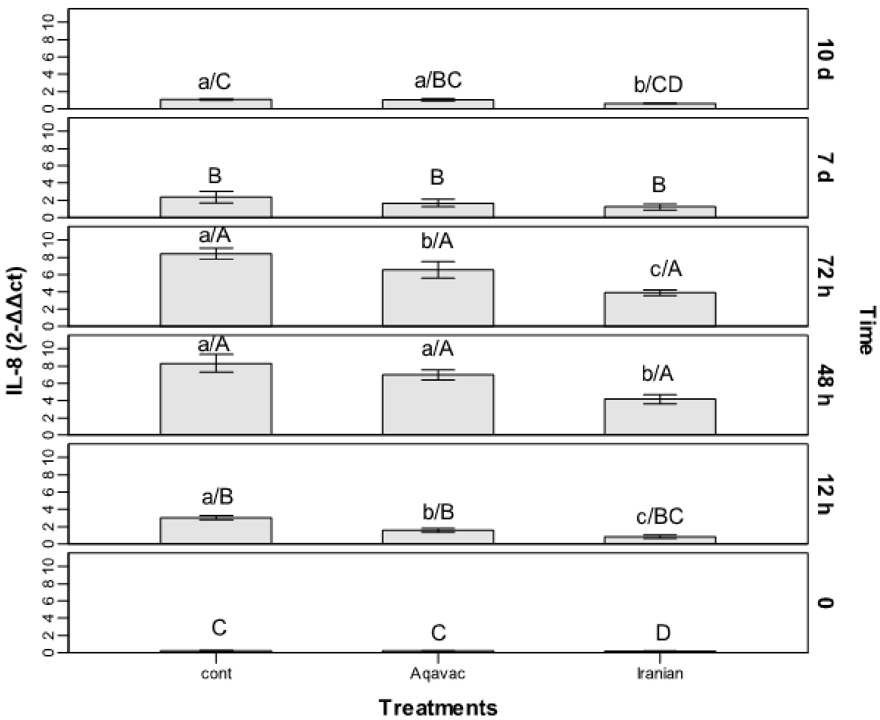

Figure 9: The effects of different vaccines on head kidney IL-8 gene expression following challenge test with *L. garviae* at different time intervals. All values were obtained from 9 individual fish (3/replicate) and expressed as mean±SD. Different lowercase alphabetic letters on each bar indicate significant difference among different treatments and different capital letters express significant difference among different sampling times ($p<0.05$).

Discussion

In aquaculture, passive vaccines are routinely used to immunize fish against bacterial infections owing to several beneficial effects such as their cost-benefit advantages (Vinitnantharat *et al*., 1999; Munang'andu and Evensen, 2019). Protective efficacy of vaccines is strongly related to their ability to stimulate the immune system with wide-ranging effects (Bercovier *et al*., 1997). The immunostimulatory effects of vaccines are thought to be related to their high antigenlevels (Leong *et al*., 1997). It, however, reflects the important status of vaccination against bacterial diseases in fish though little attempt was performed to understand the molecular mechanisms of vaccine-induced disease resistance or immunization (Harun *et al*., 2011). Therefore, identifying genes related to immune functions can basically perceive the level of immune response following vaccination (Huang *et al*., 2014b).

In the current study, although the expression of TNF-alphain control group was slightly elevated on day 14, the increases were much higher in AquaVac and Iranian vaccines at the same sampling point, supporting our hypothesis that bivalent formalin-inactivated vaccinecan stimulate *O. mykiss* immune system after two weeks of vaccination. Similar trends were also observed for IL-1β and IL-8 gene expressions. These increases are probably due to increased phagocytosis and chemotaxis activity and production of effective molecules, free radicals of oxygen and nitrogen (Ma, 2001), which, in turn, can help degradation of extracellular pathogens and therefore, increasing the non-specific defense mechanisms. In addition, an increase in pro-inflammatory response might be the results of up-regulation of IL-1β, antimicrobial activity of leukocytes, and induction of nitric oxide production by monocytes/ macrophages (Yang *et al*., 2013). TNF-alphaand IL-1β genes are capable of induce inflammatory response by regulating the expression of other cytokines (Whyte, 2007). Similar increases in the expression of other cytokines, including IL-8 expression were reported in different studies in *Paralichthys olivaceus* with a peptide subunit vaccine (Sun and Hu, 2015), *Cyprinus carpio* in the early post-vaccination period (Jiang *et al*., 2016) and in the liver of commercially vaccinated trout fed with ergosan immune stimulant after the introduction of antigen into the body (Gioacchini *et al*., 2008). IL-8 gene expression was found to increase significantly in *Gadus morhua* during vaccination with *Listonella anguillarum* (Caipang, 2013).

Nonetheless, the up-regulation of immune gene no longer continued, i.e., following day 30 the immunization has been weakened, suggesting the immunization is restricted to the history of vaccination. This might, however, manifest the ineffectiveness of the booster applied here since antigen absorption is a vital process for activation of the immune system (Joosten *et al*., 1997). Furthermore, the lower level of immune-related gene on day 30 and afterward demonstrated the lack of effectiveness or even negligible effects for bath immersion, which has been done at the same day, i.e., day 30. Repeated injection of vaccine in *O. mykiss* could raise a secondary immune response whereas similar immersion vaccination against yersiniosis did act the same (Jaafar *et al*., 2018). Similarly, Akhlaghi *et al*. (1996) examined the passive immunization with mammalian antistreptococcal antibodies in *O. mykiss*. They found only one month protection in this species by measuring the relative survival rate, confirming our findings that exhibited a peak in immune-related gene expression of head kidney after immunization. In support of the present finding, Soltani *et al*. (2007) reported higher antibody production in i.p immunized *O. mykiss* than fish immunized by immersion and oral routes. In another study, the effect of i.p. injection of formalin-inactivated vaccine on *S. iniae* infection in *Epinephelus coioides* was performed. It was observed that expression of IL-1β and TNF- alpha genes reached to the peak following 14 days of post-immunization (Huang *et al*., 2014a). Bastardo *et al*. (2012) reported high serum antibody level in *O. mykiss* immunized by two bivalent vaccines against *L. garvieae* and *A. hydrophila* 15 day after immunization. Moreover, the only initial up-regulation of IL-1β gene expression was reported in different organs of *Salmo salar* and *Danio rerio*(Haugland *et al*., 2005, Zhang *et al*., 2012). This might however be the results of low antigen uptake through bath immersion that diminished the efficiency of vaccination procedure as compared to only one-step injection (Jaafar *et al*., 2018). Klesius *et al*. (2000) hypothesized that efficiency of vaccines following to different administration routes could be related to the antigenic composition of pathogen, especially in *S. iniae*.

In the next part of the study, we focused on the immune-related gene expression of post-challenged vaccinated fish. Our findings indicated that TNF- alpha, IL-1β and IL-8 expression in head kidney was up-regulated in all challenged vaccinated and unvaccinated control treatments in which the control was shown higher amount of those with more steady level over the measured times. Similarly but with lesser extent, these increased levels could be observed up to 72 h after *L. garvieae* and *S. iniae* challenge test in most treatments. Yang *et al*. (2016) reported that when fish were subjected to challenge test with *Edwardsiella ictaluri*, lower level of IL-1β gene was observed as compared with control infected fish. Similarly, *O. mykiss* may also increase their immune-related gene expression, especially TNF- alpha and IL-1β in the spleen and gill of vaccinated group after 6 h of challenging with *Yersinia ruckeri*. This increasing trend reached the maximum level following 24 and 48 h post-infection (Harun *et al*., 2011). However, this increase was not as strong as our

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observation in control infected fish. Reduction in the expression of TNF-alpha and IL-1β genes were reported in the intestine of *O. mykiss* after challenging with *Aeromonas salmonicida* in comparison to non-infected fish (Mulder *et al*., 2007). Down-regulation of immune–related gene expression after *E. tarda* challenge was observed in vaccinated *P. olivaceus* when compared with control (Matsuyama *et al*., 2007). Similar to our findings, it is also known that infected catfish with *E. ictaluri* can increase the IL-8 gene expression of different organs while it was lower than that of the control group (Chen *et al*., 2005). In addition, IL-1β gene expression was up-regulated following 6 and 12 h post-challenging with *Edwarssiella tarda* in *Labeo rohita*, but after 7 days it returned to its normal value, although its value was lower than that of the control group (Mohanty and Sahoo, 2010), confirming our findings. It seems that the lower expression of TNF- alpha, IL-1β, and IL-8 genes in the vaccine treatment in comparison with the control treatment is only subjected to experimental challenge with *S. iniae* and *L. garvieae* bacteria, which might probably manifest the effective role of the used vaccine in the reduction of the microbial load of kidney cells. This consequently induces a reduction in the anti-inflammatory responses in vaccine treatment, which ultimately provides better protection for vaccinated treatments (Ballesteros *et al*., 2015). Other reports on the effects of viral and bacterial challenges in other infectious agents as well as species were seldom revealed no significant changes or even a slight increase when compared the vaccinated fish to control. The results of these studies are not consistent with the present study, which is probably because of the positive effects of the vaccine due to the presence of antigens to activate macrophages and white blood cells, resulting in the initiation of pro-inflammatory reactions, increased TNF- alpha, IL-1β and IL-8 cytokines in the body of fish. Ultimately, it increases the host's protection against *S. iniae* and *L. garviea*e bacteria. Thus, results in this kind of immunological responses are indeed different among species as well as experimental procedure.

In summary, the selected immune genes in the present study revealed an initial up-regulation in both vaccinated groups with higher intensity in AquaVac treated fish. However, this suggests that the inflammatory gene response might be changed in line with the immunity response, nominating them as marker to prove the efficiency of vaccination in *O. mykiss* instead of conventional parameters. Although different vaccination methods, applied here, indicated significant gene up-regulation, the i.p. injection is much efficient rather than bath immersion in case of inactivated *S. iniae* and *L. garvieae*vaccines. According to the obtained results, the higher amount of TNF-alpha, IL-1β and IL-8 gene

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expressions in unvaccinated control fish was discerned with more stabilized level over the measured times. However, the conclusion cannot be generalized for either lower and/or higher ranges of vaccines in other species. More investigation needs to be addressed to elucidate the possible effects of other pathogenic bacteria in fish.

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