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Immuno-Protective Efficiency of the Bivalent Inactivated Vaccine Against *Vibrio scophthalmi* and *Aeromonas salmonicida* Infections in Turbot (*Scophthalmus maximus* L.)

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Abstract *Vibrio scophthalmi* and *Aeromonas salmonicida* can cause high turbot mortality and huge economic losses. Presently, vaccination is the most promising method for preventing communicable diseases. In this study, we used formalin to kill *V. scophthalmi* and *A. salmonicida* cells, and mixed with the mineralized oil adjuvant (Montanide^{TM} ISA 763 AVG) to prepare the bivalent inactivated vaccine. The results showed that turbot inoculated with the bivalent inactivated vaccine exhibited strong tolerance to the infection of *V. scophthalmi* and *A. salmonicida*, and no obvious clinical symptoms and pathological changes were observed. The activities of enzymes lysozyme, acid phosphatase and complement C3 had significantly increased after the vaccination. The antibody titer response of vaccinated turbot was greatly boosted, which was positively connected with the immunological impact according to ELISA results. Simultaneously, the expression levels of immune-related genes such as *MHC-IIα*, *MHC-IIβ*, *CD4*, *CD8*, *TNF-α* and *IL-1β* were up-regulated, demonstrating that it might stimulate humoral and cellular immunological response in turbot. These findings highlight the potential of the bivalent inactivated vaccine for controlling *V. scophthalmi* and *A. salmonicida* infections in turbot.

Key words *Aeromonas salmonicida*; *Vibrio scophthalmi*; bivalent inactivated vaccine; immune response

1 Introduction

The turbot (*Scophthalmus maximus* L.) is a lucrative farmed fish species in China, and is mainly distributed in Shandong, Liaoning and Hebei Provinces. It is also widely cultured in other Asian and European countries (Bao *et al*., 2019). Since 2016, turbot farming production and output have reached 130000 tons and 50000 tons respectively (Sun *et al*., 2020). With the development of intensive aquaculture, various diseases like ascites, vibriosis and exophthalmic disease have become a restriction to the turbot culture. Moreover, complex infection of multiple pathogens has made the situation worse. *Edwardsiella tarda* (Castro *et al*., 2006) and *Vibrio harveyi* (Wang *et al*., 2008) are major pathogens, and other pathogens include *Aeromonas salmonicida subsp* (Farto *et al*., 2011), *Vibrio scophthalmi* (Zhang *et al*., 2020), and even viral haemorrhagic septicaemia virus (Lopez-Vazquez *et al*., 2007). Effective measures need to be taken to combat thenotorious pathogens (Gudding and Van Muiswinkel, 2013; Pei *et al*., 2019).

V. scophthalmi was first separated from the gut of the turbot larva by Spanish scientists in 1997 and was defined as an early species of *Vibrio* genus (Cerda-Cuellar *et al*., 1997). This bacterium was successively isolated from infected *Paralichthys olivaceus* (Qiao *et al*., 2012), *Paralichthys dentatus*, *Ruditapes philippinarum*) and *Thunnus maccoyii*, which proved its pathogenicity to aquatic animals. The classic symptoms of *V. scophthalmi* infection in fish include blackeningbody surface, ascites, inflammation and visceral congestion. It results in 30% to 90% of the infected fish death and huge pecuniary losses. *A. salmonicida* is the causative agent of salmon furunculosis in breeding farms, and has been considered as one of the most vital fish causative agents for more than 100 years (Marana *et al*., 2017). *A. salmonicida*, which is known as the only non-motile species in the genus *Aeromonas* (Marana *et al*., 2017), can induce bacterial septicemia that results in momentous economic losses due to fish morbidity and mortality (Thompson *et al*., 2005). *V. scophthalmi* and *A. salmonicida* have caused serious economic losses to the turbot breeding industry.

Traditionally, antibiotic therapy is a common method for the treatment of pathogenic diseases. Nevertheless, with the development of multidrug-resistant pathogens in aquaculture, more effective treatment measures are urgently needed. Vaccination is one of the most effective methods of disease management, helping to significantly reduce disease outbreaks and antibiotic use in aquaculture (Zhang *et al*., 2019). Vaccination is extensive applied in aquaculture industry because of its low cost. Usually, formalin-killed cells (FKC) is a good candidate vaccine to achieve the desired

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protection (Nguyen *et al*., 2017). At the same time, it maintains safety and high availability and strong immune protection in aquaculture. Actually, commercial vaccines are usually mixed with some adjuvants such as mineral or non-mineral oil. However, usually more than one pathogen were isolated from the infected fish in recent years, which is considered as multiple-pathogen infection (Sun *et al*., 2020). Consequently, the bivalent inactivated vaccine which can simultaneously resist the infection of two kinds of bacteria plays a vital role in treating the infection of pathogens.

Currently, bivalent inactivated vaccine against *V. scophthalmi* and *A. salmonicida* of turbot has not been reported so far. In our study, we prepared the bivalent inactivated vaccine against *V. scophthalmi* and *A. salmonicida*. The effectiveness of the vaccine was represented by relative percentage survival (RPS) which was assessed by measuring survival rates after intraperitoneal injection of the bivalent inactivated vaccine. Moreover, some immune parameters like antibody titer, lysozyme (LZM) activity, acid phosphatase (ACP) activity and complement C3 activity in sera from inoculated and uninoculated turbot were measured. Ultimately, RT-qPCR assay was applied to detect the transcription level of multiple immune-related genes in inoculated and uninoculated turbots.

2 Materials and Methods

2.1 Fish

Turbots with an average length of 10 cm±3 cm were purchased from a fish farm in Haiyang, China. Fish were cultured in aquatic animal culture system with circulating seawater, and the water temperature was maintained at 17.0℃ ± 1.0℃ two weeks before the experiment. Ten healthy turbots were randomly selected for bacteria detection to ensure that they were free of *V. scophthalmi* and *A. salmonicida* or other pathogens. Healthy internal organs (liver, spleen and kidney) were isolated from healthy turbots for mRNA expression analyses by RT-qPCR.

2.2 Preparation of Inactivated Vaccine

V. scophthalmi and *A. salmonicida* were isolated from infected fish in Haiyang, Shandong Province, China. The bacterial strains were developed with Tryptic Soy Broth (TSB, Hopebio) and cultured at 28℃ for 24 h. A single colony of *V. scophthalmi* and *A. salmonicida* was selected and inoculated in 200 mL of TSB medium with shaking at 200 r min^{-1} for 24 h at 28℃. Then the culture media were centrifuged at 5000 r min^{-1} for 15 min at 4℃, and the supernatant was abandoned. Then 5‰ formaldehyde was mixed with the resuspended bacteria and kept at 4℃ for 24 h. Then the same amount of PBS was added to wash the formaldehyde, and the suspension was centrifuged. It was repeated for three times until formaldehyde was washed away. The bacteria was resuspended in sterile PBS, its concentration was measured with ultra micro spectrophotometer, and the volume of PBS was adjusted to make the concentration of bacteria was 2×10^8 CFU mL^{-1}. Inactivated *V. scophthalmi* and *A. salmonicida* were mixed with the same amount to oscillate evenly. The bacteria did not grow after 5 days in the TSB plate, which were determined to be inactivated. The vaccine was prepared by mixing the dead bacteria suspension with the Montanide^{TM} ISA 763 AVG adjuvant (Seppic, France) (30%/70%, v/v) (Nguyen *et al*., 2017; Lim and Hong, 2020). Vaccines were kept at 4℃.

2.3 Fish Immunization and Challenge

The fish were divided into two groups: vaccination and control (each with 150 fish). Each fish in the vaccination group received 100 μL of 1×10^9 CFU mL^{-1} bivalent inactivated vaccine of *A. salmonicida* and *V. scophthalmi* intraperitoneally. In the control group, the same amount of PBS was intraperitoneally injected into each fish. The blood and internal organs (liver, spleen, and kidney) of nine fish in the vaccination and control groups were obtained at 0, 7, 14, 21, and 28 days post-vaccination (dpv), and the tissues were kept at a temperature of −80℃. The sera were collected and kept at 80℃ after centrifugation at 3000 r min^{-1} for 10 min. The concentrations of *A. salmonicida* and *V. scophthalmi* were 2.63×10^6 CFU mL^{-1} and 2.13×10^8 CFU mL^{-1}, respectively (LC50 based on preliminary works). A total of 30 fish were randomly selected for intraperitoneal challenge with 200 μL of 1:1 mixed bacterial suspension of two pathogens after 30 days of post-vaccination. The mortality was monitored within 15 days after the challenge, and the relative percentage survival calculation formula is:

$$\mathrm{RPS} = (1-\frac{\text{Vaccinated group mortality}}{\text{Control group mortality}})\times100\% .$$

2.4 Analysis of Enzyme Activity

The activities of enzymes LZM, ACP and C3 were determined by the reagent kits according to the product specification. LZM assay kit and ACP assay kit were purchased from Nanjing Jiancheng Bioengineering Institute in China. C3 assay kit was purchased from Zhejiang Yilikang Bioengineering Institute in China. Each sample was repeated in triplicate.

2.4.1 LZM activity

The unit of LZM activity was defined as the amount of sample resulting in a decrease in absorbance of 0.001 min^{-1} (Jiang *et al*., 2019). In brief, 200 μL of serum samples, standard solutions, and distilled water were placed on the ice, mixed with 2 mL of bacterial suspension (*Micrococcus lysodeikticus*), respectively. They were incubated at 37℃ for 15 min. Double distilled water was applied as a blank. The reaction was carried out at 25℃ and absorbance was determined at 530 nm with a spectrophotometer.

2.4.2 ACP activity

The unit of ACP activity was defined as the reaction with matrix at 37℃ for 30 min to engender 1 mg of phenol in 100 mL serum, and the absorbance was determined at 520 nm.

2.4.3 C3 activity

When cultured at the optimum temperature, complement C3 in serum and complement C3 antibody in reagent can combine to form a macromolecular immune complex. Briefly, 3 μL serum samples were well-mixed with 225 μL Tris buffered saline (100 mmol L^{-1}) and polyethylene glycol (40 g L^{-1}), and the absorbance value at 340 nm was read as A1. Complement C3 antibody and sodium azide (0.95 g L^{-1}) were then added to suspend the reaction, and the absorbance value at the same wavelength was read as A2. As a control and comparison, double distilled water and various standard complement C3 antigens were used.

2.5 Specific Antibody Titer

The specific antibody titer of fish serum was determined by ELISA following the methods of previous studies (Gwab *et al*., 2020). The 96-hole ELISA plate was coated overnight at 4℃ with 100 μL of formalin-killed *V. scophthalmi* and *A. salmonicida*. In a 96-hole plate blocked with 2% BSA, two-fold continuous dilutions of fish serum samples were placed. The antigen-antibody combination was investigated using a turbot IgM monoclonal antibody. The plate was cleaned again before being incubated for 0.5 h at 37℃ with a 1:2000 diluted horseradish peroxidase (HRP) labeled with goat anti-mouse IgM antibody. The reaction was carried out with hydrogen peroxide and stopped by adding 50 μL of 2 mol L^{-1} sulfuric acid to the 3,3,5,5-tetramethylbenzidine (TMB) substrate. Using a microplate reader, the absorbance of the solution was determined at an OD of 450 nm (Chen *et al*., 2019). Each sample was repeated in triplicate.

2.6 Real-Time Quantitative PCR (RT-qPCR)

Total RNA was extracted from samples using TransZol up (TransGen, China) according to the manufacturer's instruction. The cDNA was synthesized using One-step gDNA removal and cDNA Synthesis SuperMix kit (TransGen, China). The mRNA transcript levels of the immune-related genes such as *MHC-IIα*, *MHC-IIβ*, *CD4*, *CD8*, *TNF-α* and *IL-1β* were detected by RT-qPCR using the Light Cycler Real-time PCR detection system. All primers were designed by NCBI and shown in Table 1, in which *β-Actin* gene was employed as the internal reference.

RT-qPCR was conducted in triplicate on the basis of standard protocols with the use of SYBR® Green qPCR superMix kit (TransGen, China). The final reaction volume of 10 μL included 0.8 μL of cDNA, 5 μL of 2×SYBR Green qPCR superMix, 0.4 μL of each primer (10 μmol L^{-1}), and 3.4 μL of ddH_2O. The reaction included initial activation at 94℃ for 30 s, and denaturation at 94℃ for 0.05 s, optimal annealing at 55℃ for 60 s, extension at 72℃ for 0.05 s for 40 amplification cycles. Gene expression levels were analyzed with the $2^{-\Delta\Delta Ct}$ method (Pulpipat *et al*., 2020).

2.7 Statistical Analysis

All data of relative mRNA expression level are shown as means ± SE. The date were subjected to analysis of *t*-test, and the *P* values less than 0.05 were considered statistically significant.

Table 1 Primers used in RT-qPCR

Primer	Sequence (5'–3')
MHC-IIα-F	ACTGGACTTCACCCCACAGT
MHC-IIα-R	CATCAACCAATCAGCTGCACTC
MHC-IIβ-F	ACTGTCTCAACAACGTGGGA
MHC-IIβ-R	GTACCAATCACTGTCCGCCA
CD4-F	ACAGACAATCACTTGTGACTACGA
CD4-R	CGACCGTATACCACCTCAGC
CD8-F	AACGCTCTGCTGCAATGTTC
CD8-R	TCCCCCTCTTTCACGACTCT
TNF-α-F	CAGCCGAGTGTTTTAGCTGC
TNF-α-R	CCCCGTAGCCGACTTCTTTT
IL-1β-F	CGCTTCCCCAACTGGTACAT
IL-1β-R	ACCTTCCACTTTGGGTCGTC
β-actin-F	AATGAGCTGAGAGTTGCCCC
β-actin-R	AGCTTGGATGGCAACGTACA

3 Results

3.1 RPS of Bivalent Inactivated Vaccine

The RPS of bivalent inactivated vaccine was shown in Fig.1. The results indicated that the survival rate of vaccination group was obviously improved. After *A. salmonicida* and *V. scophthalmi* challenges, from 1 to 7 days, seven fish were dead in both the vaccination and control groups. However, from 7 to 15 days, the mortality rate of the vaccination group was significantly lower than that of the control group. Finally, the relative survival of the bivalent inactivated vaccine against *A. salmonicida* and *V. scophthalmi* was 64.06%.

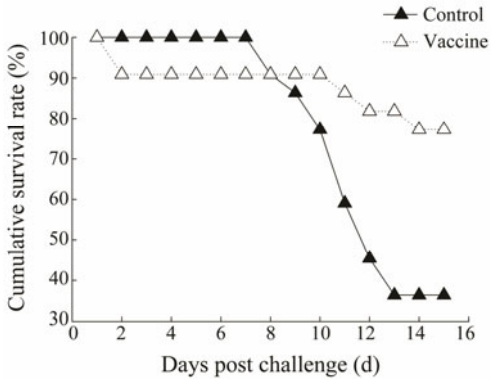

Fig.1 Cumulative survival rate of turbot challenged with live bacteria within 15 days after challenge.

3.2 Analyses of Enzymes' Activities

3.2.1 Analysis of LZM activity

The activities of LZM in fish serum at different time were shown in Fig.2A. The experimental results indicated that the vaccination group had significantly higher activity than the control group ($P<0.05$). The maximum activity of LZM was 42.44 μg mL^{-1} at day 7 after immunization in vaccination group. During 7 to 28 days, the overall fluctuation of LZM activity range was not obvious, and there was always significant difference among the vaccination group and the

control group.

3.2.2 Analysis of ACP activity

The ACP activity in fish serum determined in different immune periods were shown in Fig.2B. Within 14 days after immunization, ACP activity in the control group was decreased slightly, while ACP activity in the vaccination group increased slightly on day 14. After 21 days, ACP activity in the control group did not change significantly, but ACP activity in the vaccination group increased significantly ($P<0.05$). After 28 days, ACP activity in vaccination group decreased but still exhibited higher expression level compared to the original activity.

3.2.3 Analysis of C3 activity

The C3 activity in fish serum determined at different time were shown in Fig.2C. The concentration of C3 in the vaccination group increased significantly and reached the maximum after 7 days of vaccination ($P<0.05$), and the difference was significant from day 14 to day 28. As for the control group, there was no obvious fluctuation from 0 to 28 days.

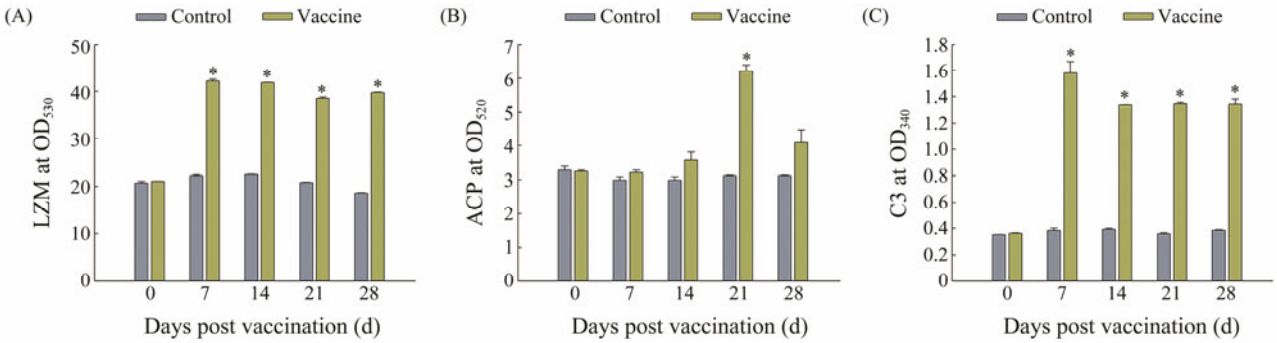

Fig.2 The activities of LZM (A), ACP (B) and C3 (C) in serum of turbot after vaccination. The asterisk (*) indicates that there is a significant difference between 0 dpv of control group and vaccination group ($P<0.05$).

3.3 Analysis of Antibody Titers

The production of specific antibody in serum after inoculation of turbot was measured by ELISA. As shown in Fig.3, antibody titers of vaccination group during the whole experimental period were obviously higher than control group during the period of 28 days post-vaccination ($P<0.05$). The results indicated that the antibody level in the vaccination group increased obviously from day 7 to day 28 ($P<0.05$).

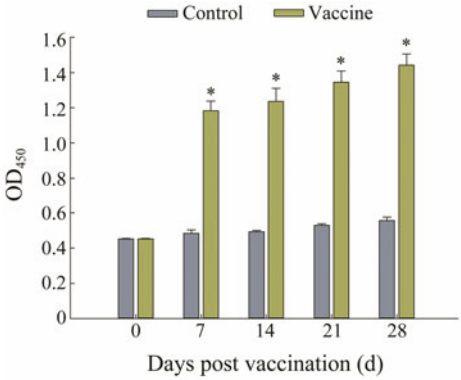

Fig.3 Serum antibody titer of turbot at different time after vaccination. The asterisk (*) indicates that there is a significant difference between the 0 dpv of control group and vaccination group ($P<0.05$).

3.4 Expression Pattern of Immune-Related Genes After Vaccination

RT-qPCR was conducted to analyze the mRNA transcript levels of *MHC-IIα*, *MHC-IIβ*, *CD4*, *CD8*, *TNF-α* and *IL-1β* in the liver, spleen and kidney of turbot within 28 days after vaccination. As shown in Fig.4, the results indicated that all the detected immune-related genes were obviously up-regulated after vaccination. Transcription of *MHC-IIα* increased obviously in the liver, spleen and kidney of turbot at 7 dpv, 14 dpv and 14 dpv, respectively ($P<0.05$), and then decreased, but all were obviously higher than those of the control group. The expression of *MHC-IIβ* was increased significantly in the liver, spleen and kidney at 7 dpv, 7 dpv and 14 dpv ($P<0.05$). The *CD4* and *CD8* transcript levels in the spleen and kidney were gradually up-regulated to the highest expression levels at 14 dpv, and then decreased gradually. However, they were still higher than that of the control group until the end of the experiment. The expression levels of *TNF-α* in the liver, spleen and kidney began to rise significantly at 7 dpv, 21 dpv and 14 dpv ($P<0.05$). The expression of *IL-1β* in liver, spleen and kidney reached the highest value at 14 dpv ($P<0.05$).

4 Discussion

Turbot has been successfully introduced into China from Europe for 28 years, and the breeding area in China reached 6.06 million square kilometers at the end of 2019. However, turbot are usually infected by bacteria, viruses, and parasites due to intensive cultivation (Khezerlou *et al*., 2018). The bivalent inactivated vaccines can simultaneously control diseases caused by multiple pathogens.

As far as we know, there is no commercial vaccine for both *V. scophthalmi* and *A. salmonicida* in China, despite the huge economic losses caused by these two bacteria in the aquaculture industry. In this research, the protective efficacy of the bivalent inactivated vaccine of *V. scophthalmi* and *A. salmonicida* was evaluated in turbot, then we

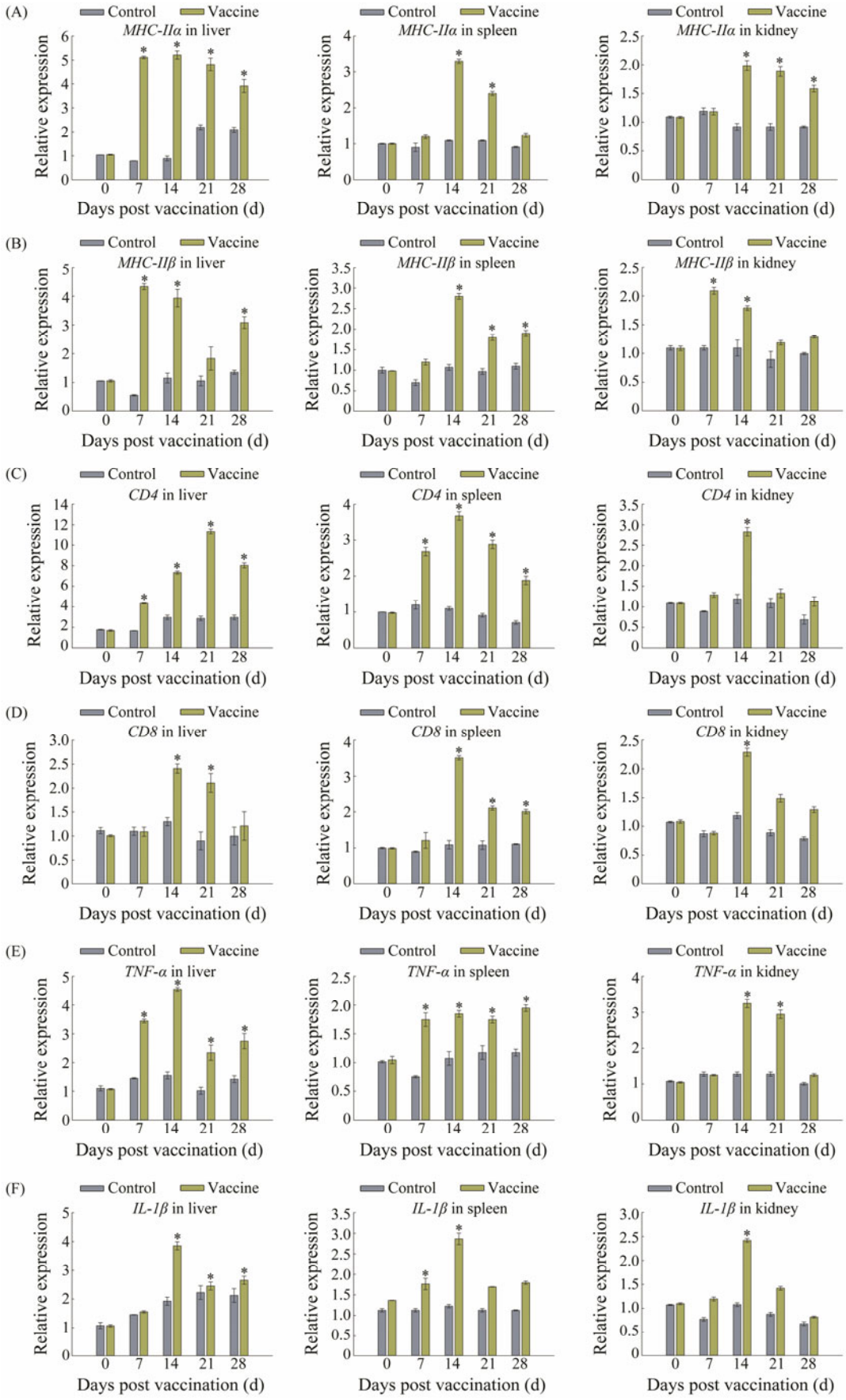

Fig.4 The relative mRNA expressions of *MHC-IIα* (A), *MHC-IIβ* (B), *CD4* (C), *CD8* (D), *TNF-α* (E) and *IL-1β* (F) in the liver, spleen and kidney tissues, respectively. The expression profiles were analyzed by quantitative RT-qPCR at days 0, 7, 14, 21 and 28 after injected with the bivalent inactivated vaccine or PBS. For each gene, the mRNA level of the fish injected with PBS was set as 1. Each bar represents the mean of three biological replicates and error bars represent standard deviation. Data are presented as means ± SE ($n = 3$). Significant differences (* $P < 0.05$) are indicated by asterisks.

made further efforts to study the immunological processes induced by the bivalent inactivated vaccine. Inactivated vaccines show relatively poor immunogenicity compared to live vaccines. Therefore, adjuvant is essential to improve vaccine efficacy (Ramos-Espinoza *et al*., 2020). Compared with aqueous vaccines, oil adjuvanted vaccines are better protected, so we chose the mineral adjuvant Montanide™ ISA 763 AVG to enhance immune response coadministered with the bivalent inactivated vaccine. In addition, Montanide™ ISA 763 AVG contains many immunomodulatory molecules that can induce the maturation of antigen presenting cell (APC) and supply a pro-inflammatory surroundings (Song *et al*., 2018). In emulsions, antigenic components are the main inducers of specific acquired immune response, while adjuvants enhance and extend this response (Bui *et al*., 2014).

The inactivated vaccine was treated with chemicals such as formaldehyde or phenol, which can kill the powerful wild causative agents but persist their immunogenicities. Other inactivated vaccines also showed high levels of protection for fish. For example, the RPS values of the formalin-kill ed vaccines of against the infectious hematopoietic necrosis virus in rainbow trout (*Oncorhynchus mykiss*) was 79% (Tang *et al*., 2016), The RPS value against the Betanodavirus in European sea bass (*Dicentrarchus labrax*) and scale drop disease virus (SDDV) in Asian sea bass (*Lates calcarifer*) were 81.9% and 74%, respectively (Dehghani, 2012). Furthermore, compared with other vaccines, the bivalent inactivated vaccine has no infectious components and can induce the host to engender a humoral immune response. It is safe after injection and can be used in combination with other vaccines. In the safety test, we found that the bivalent inactivated vaccine was safe, enough bacteria were inactivated, and there was no acute or chronic side effects from residual formaldehyde on fish.

In this study, a powerful antibody response and significant protection was obtained by the inactivated bivalent vaccine against *A. salmonicida* and *V. scophthalmi* in turbot. Some previous researches have also revealed that fish vaccinated with different vaccines showed higher antibody titers. Our results are consistent with that using formalin inactivated *Edwardsiella tarda* vaccine to enhance immune protection of turbot (Liu *et al*., 2017). The results of this study were also similar to those obtained with *S. agalactiae* Nile tilapia vaccinated with other types of vaccine (Ramos-Espinoza *et al*., 2020). The findings prove that the antibody level (IgM) of the immunized fish was increased significantly than the unvaccinated fish when checked with bacterial antigens. Thus the antibodies are very reliable factor for protective immunity in immunized fish (Caipang *et al*., 2009).

The innate immune response is triggered promptly after the antigen enters the host. In this research, several immunological parameters like the activities of LZM, ACP and C3 enzymes were measured, which showed that the fish in vaccination group had a considerably higher levels of activity than control group ($P<0.05$). It indicated that a powerful innate immune response was induced by the bivalent inactivated vaccine. Lysozyme is a single chain polypeptide with 120 amino acids and a molecular weight about 14.4 kDa, which can activate the complement system and phagocytes, so as to prevent diseases caused by bacteria (Taju *et al*., 2015; Xu *et al*., 2019). Lysozyme provides nonspecific defense to fish by dividing the connection between n-acetylcytomic acid and n-acetylglucoside in the Gram-negative peptide polyscan layer, resulting in cell death. This important immune substance is vital for the primary immune defence of turbot to ensure their survival (Saurabh and Sahoo, 2008; Abu Nor *et al*., 2020). ACP, one of the most representative hydrolases of macrophages, is a marker enzyme of macrophage lysosomes, and it participates in the transfer and metabolism of phosphate groups. In this study, after vaccination, ACP activity of turbot was higher than that of the control group, indicating that the bivalent inactivated vaccine also enhanced the non-specific immunity in turbot. The complement system is the main effector system of innate immunity and plays a vital role in the recognition and removal of potential causative agents (Yuan *et al*., 2017). C3 is a vital part of the innate immune system, and it is a vital and traditional terminal index of fish innate immunity that is liable for various immune effector functions (Torres *et al*., 2012). The increase of C3 complement content after the injection of the bivalent vaccines also proved the effectiveness of the bivalent inactivated vaccines against *A. salmonicida* and *V. scophthalmi* in turbot.

The major immune tissues (liver, spleen and kidney) were chosen to study the mRNA expression levels of immune-related genes (*MHC-IIα*, *MHC-IIβ*, *CD4*, *CD8*, *TNF-α* and *IL-1β*) at different time points following vaccination. *MHC-IIα*, *MHC-IIβ*, *CD4* and *CD8* were significantly increased in all investigated tissues after vaccination, suggesting that *A. salmonicida* and *V. scophthalmi* might be presented *via* the *MHC-II-CD4* pathways. It has been confirmed that the inactivated antigen were presented to T cells *via MHC-II* molecules, inducing a broad variety of specific antibacterial response by activating $CD8^+$ cytotoxic T lymphocytes (CTLs) and $CD4^+$ T helper cells (Th1) (Li *et al*., 2017). *CD8* is a subgroup of T lymphocytes, which plays a very vital role in antigen identification and representation in specific immune response. *CD8* binds to *MHC-II* molecules on its surface and binds to *MHC-I* molecules on the surfaces of other immune cells to identify antigens attached to other immune cells (Yan *et al*., 2018). Moreover, the expression level of *IL-1β* also increased, indicating that nonspecific immune responses of macrophages were activated following vaccination. *IL-1β* is a classic proinflammatory cytokine and can be produced in a variety of cell types, including macrophages and neutrophils (Chen *et al*., 2013; Zhou *et al*., 2017). Classic proinflammatory cytokine gene *IL-1β* plays a key role in the supervision of the inflammatory process at the early stages of infection in fish, supplying the first step of host defense. Proinflammatory cytokines can activate neutrophils and lymphocytes, increase the permeability of vascular endothelial cell, and be extensively involved in inflammatory response and immune regulation (Nascimento *et al*., 2007; Wang *et al*., 2013). The results showed *TNF-α* was significantly up-regulated after vaccination. A large number of studies in fish directly prove

that *TNF-α* is a significant macrophage-activating factor (MAF) produced by leukocytes, which induces the expressions of a number of genes in immune response (Shona and Fish, 2007).

5 Conclusions

This study shows that the bivalent inactivated vaccine of *A. salmonicida* and *V. scophthalmi* using Montannite™ ISA 763 AVG as adjuvant exhibits a good immune protection effect. The relative percentage survival, enzyme activities, antibody titer and the expression of immune-related genes indicate that the bivalent inactivated vaccine can effectively activate the immune response of turbot. Additionally, we found that the bivalent inactivated vaccine could activate antigen processing and presentation processes. These findings established a foundation for the future application of this bivalent inactivated vaccine of *A. salmonicida* and *V. scophthalmi*, which is of great significance for fish disease prevention and control.

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