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Evaluation of Immune Protection of a Bivalent Inactivated Vaccine against *Aeromonas salmonicida* and *Vibrio vulnificus* in Turbot

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Abstract: The *Aeromonas salmonicida* is responsible for causing furunculosis in various fish species. Furunculosis is a ubiquitous disease that affects the aquaculture industry and causes the mass mortality of turbot. *Vibrio vulnificus* is a pathogen that causes skin ulcers and hemorrhagic septicemia in fish, resulting in significant mortality in aquaculture. In this study, we have established a bivalent inactivated vaccine against *A. salmonicida* and *V. vulnificus* with Montanide™ ISA 763 AVG as an adjuvant. This bivalent inactivated vaccine was used to immunize turbot by intraperitoneal injection, and the relevant immune indexes were detected. The results demonstrate that the bivalent inactivated vaccine exhibited a relative percent survival (RPS) of 77% following *A. salmonicida* and *V. vulnificus* intraperitoneal challenge. The vaccinated group exhibited higher levels of acid phosphatase activity and lysozyme activity compared to the control group. ELISA results showed a significant increase in serum antibody levels in immunized turbot, which was positively correlated with immunity. In the kidney tissue, related immune genes (*TLR5*, *CD4*, *MHCI* and *MHCII*) were up-regulated significantly, showing that the vaccine can induce cellular and humoral immune responses in turbot. In conclusion, the bivalent inactivated vaccine against *A. salmonicida* and *V. vulnificus* was immunogenic, efficiently preventing turbot from infection, which has the potential to be applied in aquaculture.

Keywords: *Aeromonas salmonicida*; *Vibrio vulnificus*; bivalent inactivated vaccine; RPS; turbot

Key Contribution: The formalin-inactivated bivalent vaccine can increase the serum antibody titer, ACP activity, and LZM activity of turbot; induce the expression of relevant immune genes; and effectively prevent the infection of *A. salmonicida* and *V. vulnificus* in turbot.

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1. Introduction

Turbot (*Scophthalmus maximus*) is a cold-water fish popular within the aquaculture industry around the world because of its refined flavor and white flesh. It not only provides abundant protein, vitamin B3, and B12 but also serves as a source of minerals such as selenium, magnesium, and phosphorus. After being imported from Europe, it is cultured in quantity in China. One major threat to turbot farming is the outbreak of infectious diseases, particularly those caused by various bacteria. These diseases dramatically influence turbot farming in Europe and China. *Vibrio vulnificus* [1], *Edwardsiella tarda* [2], and *Aeromonas salmonicida* [3] are the most common bacterial pathogen in turbot, causing significant economic losses. Currently, effective methods to control these diseases are needed urgently.

A. salmonicida has caused significant economic losses in the global aquaculture industry as a primary bacterial pathogen, particularly in salmonid culture systems, ever since its first documentation in the 19th century [4]. Previously, *A. salmonicida* was only considered a major pathogen in fish; however, recent reports have indicated its potential to cause

zoonotic diseases [5–7]. This bacterium can be found almost worldwide in both marine and freshwater environments. *A. salmonicida* is the pathogen of furunculosis, which can lead to muscle necrosis, hemorrhagic septicemia, and death in turbot [8]. Clinical symptoms include redness of the skin, inflammation, and ulcers [9].

In bacterial diseases of aquaculture animals, *V. vulnificus* is one of the most common pathogens that inhabit marine environments [10]. Furthermore, *Vibrio* infections can lead to severe mortality in fish, shellfish, crustaceans, and other farmed populations [11], and are also pathogenic to humans [12,13]. What is more, infections with *V. vulnificus* can easily result in massive mortality of economically important animals. Especially in turbot, *V. vulnificus* can cause skin and fin hemorrhage as well as ulcerative necrosis, even leading to extensive mortality in turbot cultures [14].

In conclusion, *A. salmonicida* and *V. vulnificus* are two Gram-negative bacteria that can cause diseases in turbot, leading to high morbidity and mortality [4]. However, there are no studies of bivalent inactivated vaccines against *A. salmonicida* and *V. vulnificus* infections. In this research, a bivalent inactivated vaccine supplemented with Montanide™ ISA 763 AVG adjuvant was developed to protect turbot against infections caused by *A. salmonicida* and *V. vulnificus*. We recorded the survival rate in the vaccinated group and control group to evaluate the RPS of the bivalent inactivated vaccine. Additionally, in the vaccinated group and control group, we assessed the acid phosphatase activity, lysozyme activity, serum antibody titer, and expression levels of immunity genes (*TLR5*, *CD4*, *MHCI*, and *MHCII*).

2. Materials and Methods

2.1. Fish Rearing

The healthy turbot, with an average weight of 30 ± 5 g and an average length of 8 ± 2 cm, were procured from a commercial aquaculture farm located in Haiyang City, Shandong Province, China. These fish were reared in tanks filled with 18 °C aerated seawater, which was changed twice a day. The oxygen content in seawater was greater than 7 mg/L, and the salinity was 28 ppt. Throughout the duration of the experiment, turbots were fed with commercial feed (the main components of the feeds were fish meal, soybean meal, fish oil, etc.) twice daily. Before experimentation, samples were taken from the liver, kidney, and spleen for examination to verify that turbots were free from *A. salmonicida* and *V. vulnificus*.

2.2. Preparation of Inactivated Vaccine

Highly pathogenic *A. salmonicida* and *V. vulnificus* isolated from diseased turbot were used for vaccine production. The bacteria were cultured with Trypticase Soy Broth (TSB, Hopebio, Qingdao, China) and incubated in an incubator shaker at 200 rpm at 28 °C for 24 h. Bacteria were centrifuged at 4 °C at 6000 rpm for 15 min, after which the supernatant medium was abandoned. Bacteria were washed with sterile phosphate-buffered saline (PBS; Welgene, Gyeongsan, Republic of Korea) and then diluted to a concentration of 2×10^8 CFU/mL in sterile PBS. The concentration of bacteria was determined by optical density measurement. The resuspended bacteria were subjected to inactivation by adding 0.5% formaldehyde and then incubated at 4 °C for 24 h. A combined vaccine was prepared by combining equivalent quantities of inactivated *A. salmonicida* and *V. vulnificus*. The inactivation of bacteria was checked by plating on TSB agar plates after incubating for five days. The bivalent inactivated vaccine was ultimately a mixture of inactivated vaccine and Montanide™ ISA 763 AVG adjuvant (Seppic, Shanghai, China) in a ratio of 3:7.

2.3. Fish Immunization and Challenge

After acclimating for 7 d, 300 randomly selected turbot specimens were divided into 2 groups, including a vaccinated group and a control group, with 150 specimens in each group. The vaccinated group was administered with an intraperitoneal injection of 0.1 mL bivalent inactivated vaccine (2×10^8 CFU/mL) using a disposable sterile syringe, while the control group was injected with 0.1 mL PBS using the same method. The blood

from nine vaccinated and control fish was isolated, respectively, at 0, 1, 2, 3, and 4 weeks post-vaccination (wpv), with three biological replicates per time point and three fish per biological replicate. After centrifugation at 3000 rpm for 15 min, the serum was collected and stored at −80 °C until further use. Meanwhile, head kidneys from nine vaccinated and control fish were isolated at 0, 1, 2, 3, and 4 wpv. The kidneys were collected under aseptic conditions and immediately frozen in liquid nitrogen. Subsequently, they were preserved at −80 °C for the purpose of future RNA extraction. Before any operation, fish were anesthetized with Ethyl 3-aminobenzoate methanesulfonate (Aladdin, Shanghai, China, 60 mg/L).

According to the previous experimental results, *A. salmonicida* and *V. vulnificus* were cultured [3,15]. After centrifugation, the bacterial suspensions were separately resuspended in sterile PBS to achieve concentrations of 2.6×10^6 CFU/mL and 1.8×10^8 CFU/mL, respectively. They were then mixed in a 1:1 ratio to form a mixture of the two bacteria. On day 29 after vaccine immunization, 22 turbots were used for bacterial infection, and each fish was injected intraperitoneally with 200 μL of mixed bacterial suspension and monitored daily. Each fish in the control group was injected with 200 μL of PBS in the same manner. The cumulative mortality rates were recorded over a span of 15 d, and the following formula was used to calculate the relative percent survival (RPS): RPS = {1 − (% mortality in vaccinated fish/% mortality in control fish)} × 100%.

2.4. Analysis of Serum Enzyme Activity

2.4.1. ACP Activity

Acid phosphatase (ACP) can be hydrolyzed to hydrolase under acidic conditions, and hydrolase can destroy the cell walls of bacteria and make them inactive. The activity of ACP is related to the immune response of fish and can be measured to respond to the strength of the immune response of fish. The ACP activity of turbot serum was assessed using a commercially available assay kit (Jiancheng Bioengineering Institute, Nanjing, China). Acid phosphatase can decompose disodium phenylphosphate to produce free phenols. Phenols react with specific reagents in alkaline solutions to form red compounds, which are used to determine the activity of the enzyme. Following the specified protocol, 50 μL of the test serum, double distilled water, and standard solution were added to seven centrifugal tubes. Then, 500 μL of the matrix solution and buffer solution was added into each centrifugal tube, thoroughly mixed, and left at 37 °C for 30 min. Subsequently, 1500 μL of color development solution and 1000 μL of alkaline solution were added and left out for 10 min. A spectrophotometer was used to measure the absorbance at a wavelength of 520 nm. The previous serum sample was diluted twice and assessed three parallels. OD values were presented as the means ± SE (N = 3).

2.4.2. LZM Activity

Lysozyme (LZM) lyses bacteria in the body. When bacteria invade the fish, it is activated and lyses the cell wall of the bacteria, resulting in bacterial death. Therefore, the activity of LZM can reflect the activation of the immune system. The LZM activity of turbot serum was assessed using a commercially available assay kit (Jiancheng Bioengineering Institute, Nanjing, China) [16]. LZM can dissolve bacteria and release their contents, so the activity of LZM can be evaluated by measuring the OD value. According to the protocol, 200 μL of the test serum, double distilled water, and standard solution were placed in different tubes on ice, after which 2 mL of Micrococcus luteus was added, and the mixture was kept at 37 °C for 15 min under incubation. A blank control was conducted using double distilled water. The absorbance was measured at 530 nm using a spectrophotometer. The previous serum sample was diluted twice and assessed three parallels. OD values were presented as the means ± SE (N = 3).

2.5. Specific Antibody Levels in Serum

The procedure for the enzyme-linked immunosorbent assay (ELISA) was as follows. In brief, microtiter plates (Nunc, MaxiSorp, San Diego, CA, USA) were coated overnight at 4 °C with 100 μL of formalin-killed 1.0×10^8 CFU/mL *A. salmonicida* and *V. vulnificus* in carbonate–bicarbonate coating buffer (pH 9.6), respectively. Subsequently, the microtiter plate was washed three times with PBST (PBS containing 0.3% Tween-20) and then blocked with PBST containing 1% BSA for a duration of 3 h at 37 °C. After being washed three times, the turbot serum was diluted in PBST at a 1:100 ratio, with 100 μL per well in triplicate, and incubated for 3 h at 37 °C. Subsequently, the microplates were washed as described previously and added to 100 μL/well mouse anti-turbot IgM mAb (1:100 Diluted by PBSTB), incubating for 1.5 h at 37 °C. Then, following the previous washing steps, the microplates were subjected to incubation with 100 μL/well goat anti-mouse IgG conjugated to HRP (TransGen, Beijing, China, 1:2000 Diluted by PBSTB) for 1.5 h. Lastly, the microplates were washed five times according to the previous steps, and 100 μL/well TMB solution (Solarbio, Beijing, China) was added. Following a 5 min incubation period at room temperature, each well received 100 μL H_2SO_4 (1 M) to stop the reaction, and the OD_{450} was measured using a microplate reader.

2.6. Quantitative Real-Time Reverse Transcription-PCR (qRT-PCR) Analysis of Immune-Related Genes

TLR5 recognizes bacterial flagellin and increases phagocytic activity. MHCI and MHCII are related genes involved in antigen presentation to T cells. CD4 is a T-cell surface molecule that can be involved in T-cell proliferation and differentiation and is also a receptor for MHCII molecules. All these genes are closely related to the immune response after bacterial invasion, so they can reflect the level of immune response of the fish. We designed qRT-PCR primers with specific sequences for amplification of TLR5, CD4, MHCI, and MHCII using NCBI Primer (Table 1). In the extraction of RNA, frozen kidney tissue from the vaccinated group and control group were homogenized in liquid nitrogen in a mortar, and total RNA was extracted from the tissue using a SteadyPure Universal RNA Extraction Kit (Accurate Biology, Changsha, China) according to the manufacturer's instructions. Agarose gel electrophoresis was utilized to assess the quality of the total RNA that was extracted. The genomic DNA in the extracted RNA was removed by using one-step gDNA removal, and the total RNA was immediately synthesized into cDNA using the cDNA Synthesis SuperMix kit (TransGen, Beijing, China), in accordance with the guidelines provided by the manufacturer. Finally, we stored the synthesized cDNA at −20 °C for future use. The expression levels of immune genes were confirmed by qRT-PCR. Briefly, qRT-PCR was conducted utilizing the SYBR® Green qPCR superMix kit (TransGen, Beijing, China). The reaction was performed in a total volume of 10 μL, which includes 5 μL of 2× SYBR Green qPCR SuperMix, 0.4 μL each for forward and reverse primers (10 μM), 0.8 μL of cDNA, and 3.4 μL of ddH2O. The amplification protocol consisted of an initial denaturation step at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, and extension at 60 °C for 34 s. The expression level was determined using the comparative threshold cycle method ($2^{-\Delta\Delta Ct}$) with β-actin as the housekeeping gene. The samples were performed in triplicate and the values were presented as the means ± SE. The experimental data were correlated and processed using the one-way ANOVA method. Statistical significance was determined by considering the $p < 0.05$ to indicate significant differences.

Table 1. List of primers used for qRT-PCR.

Gene	Primer Name	Nucleotide Sequence of Primer (5′–3′)
β-actin	*β-actin*-F	AATGAGCTGAGAGTTGCCCC
	β-actin-R	AGCTTGGATGGCAACGTACA
TLR 5	*TLR 5*-F	GATCCCGGGCTTTAACACCA
	TLR 5-R	GGGGAGGCTAGGAAGTTGTT
CD 4	*CD 4*-F	ACATACCAATCCGTGGCGAG
	CD 4-R	GAAATCGCGTCGGACGATCA
MHC I	*MHC I*-F	TGCTGAGAAAGCTCGACTCAC
	MHC I-R	CTCGCCCCAAAGTTCACGTA
MHC II	*MHC II*-F	ACTGGACTTCACCCCACAGT
	MHC II-R	CATCAACCAATCAGCTGCACTC

2.7. Statistical Analyses

All statistical analyses were performed using the SPSS 19.0 package for one-way analysis of variance, and $p < 0.05$ was considered a statistically significant difference.

3. Results

3.1. Immune Protective Ability of Vaccine

To examine the protective effect of the bivalent inactivated vaccine against *A. salmonicida* and *V. vulnificus* immunization on turbot, the cumulative mortality was recorded for 15 d, after which the RPS rates were calculated. As shown in Figure 1, the cumulative mortalities of the vaccinated group and control group were 13.6% and 59.1%. There was a notable decrease in mortality among vaccinated fish compared to the control group. The bivalent inactivated vaccine was calculated to have an RPS rate of 77% against the combined challenge of *A. salmonicida* and *V. vulnificus*. These data indicate that the bivalent inactivated vaccine against *A. salmonicida* and *V. vulnificus* can effectively protect turbot from *A. salmonicida* and *V. vulnificus* infections and can be used as an effective vaccine for turbot.

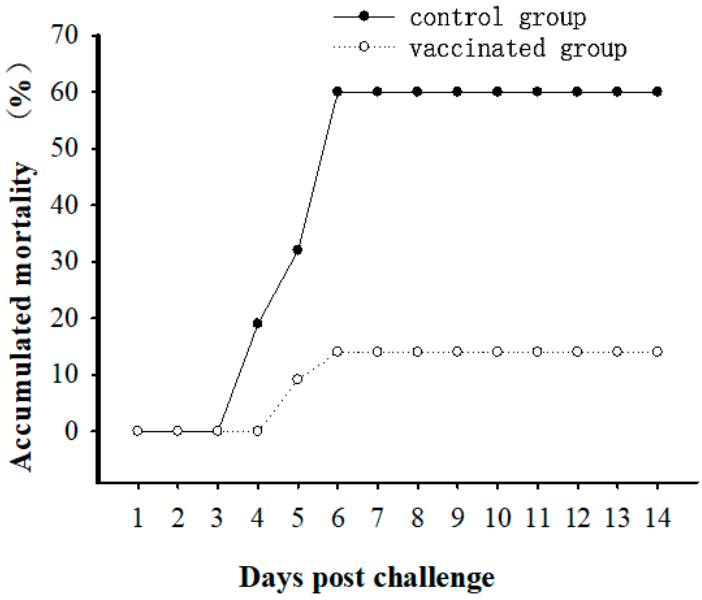

Figure 1. Cumulative mortality of vaccinated and control group after challenge with *A. salmonicida* and *V. vulnificus*. The *x*-axis represents the days post-vaccination and the *y*-axis represents the cumulative mortality rate.

3.2. Analysis of ACP Activity

As shown in Figure 2, the ACP activity in the vaccinated group was significantly higher than that in the control group. At 1 wpv, the results revealed that the ACP activity of the vaccinated group significantly exceeded the control group. At 2 wpv, the ACP activity of the vaccinated group reached the highest activity, which was significantly higher than that of the control group, and the peak ACP activity reached 32.7 U/mL. Until 4 wpv, the ACP activity remained elevated compared to the control group.

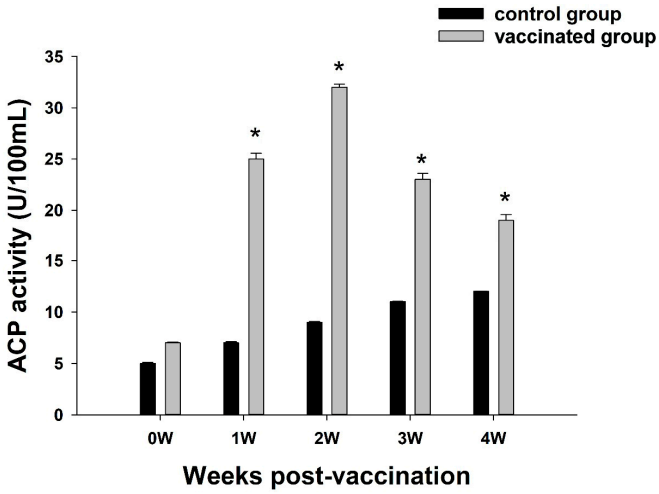

Figure 2. ACP activity in serum of turbot after vaccination. The *x*-axis represents the weeks post-vaccination and the *y*-axis represents the ACP activity. Data are presented as the means ± SE (N = 3). The asterisk (*) indicates the statistical significance ($p < 0.05$) between the vaccinated and control groups.

3.3. Analysis of LZM Activity

As shown in Figure 3, the LZM activity in the vaccinated group was significantly higher than in the control group. At 2 wpv, the LZM activity of the vaccinated group reached the highest activity, which was significantly higher than the control group, and the maximum activity of LZM was 241.7 μg/mL. These results indicate that the bivalent inactivated vaccine induced higher LZM activity compared with the control group.

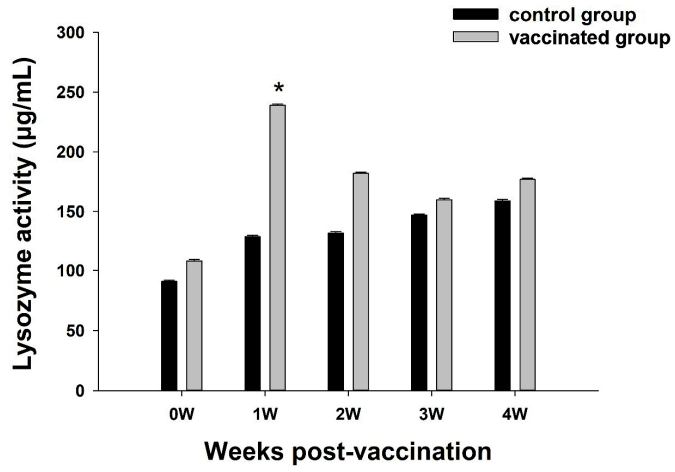

Figure 3. Lysozyme activity in serum of turbot after vaccination. The *x*-axis represents the weeks post-vaccination and the *y*-axis represents the LZM activity. Data are presented as the means ± SE (N = 3). The asterisk (*) indicates the statistical significance ($p < 0.05$) between the vaccinated and control groups.

3.4. The Analysis of Antibody Titers

The titer of specific antibodies in serum after immunization was measured with ELISA. As shown in Figure 4, the antibody titers against *A. salmonicida* and *V. vulnificus* showed slight fluctuations from 0 to 2 wpv, after which a significant increasing trend was observed from 3 to 4 wpv. The vaccinated group showed a higher antibody response than the control group from 0 to 4 wpv (Figure 4A). At 3 wpv, the titer of the antibody against *A. salmonicida* reached a peak, which was significantly higher than the control group ($p < 0.05$). As shown in Figure 4B, the levels of antibodies against *V. vulnificus* demonstrated a significant increase trend at 3 wpv, with a peak at this time point. Compared to the control group, this level of antibodies was significantly higher ($p < 0.05$).

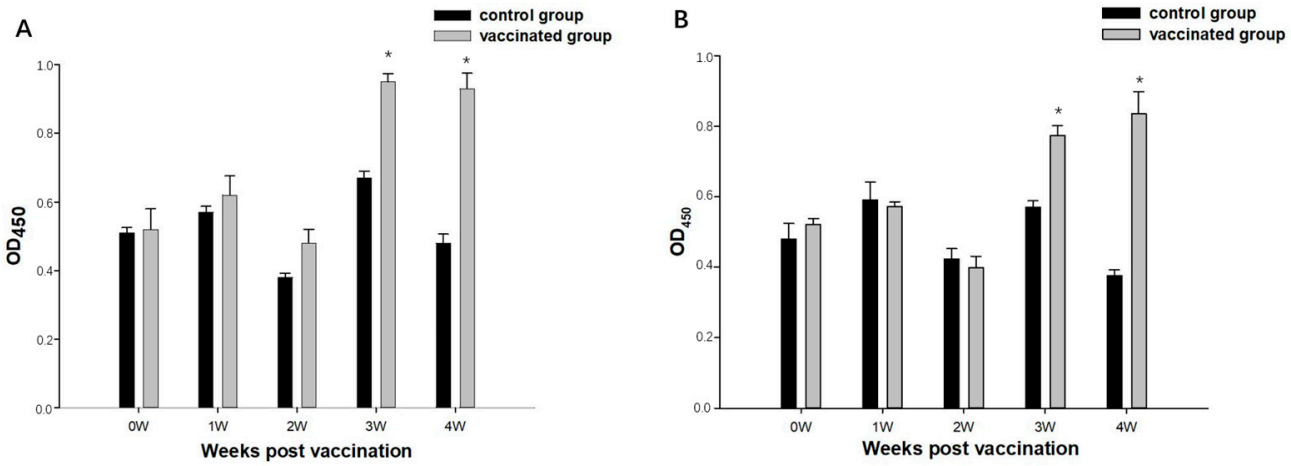

Figure 4. Variation trend of serum antibody titer against *A. salmonicida* (**A**) and *V. vulnificus* (**B**). The *x*-axis represents the weeks post-vaccination and the *y*-axis represents the serum antibody titers. The asterisk (*) indicates the statistical significance ($p < 0.05$) between the vaccinated and control groups.

3.5. Expression Pattern of Immune-Related Genes during Immunization

The expression of some immune-related genes (*TLR5*, *CD4*, *MHCI* and *MHCII*) in the kidney tissue was determined by qRT-PCR during the post-vaccination period. To reflect the changes in gene expression before and after immunization, the immune genes of vaccinated groups 0, 1, 2, 3 and 4 wpv were detected. As shown in Figure 5, the expression of *TLR5*, *CD4*, *MHCI* and *MHCII* was significantly increased in the kidney. The *TLR5* transcript in the kidney was significantly increased to the maximum expression levels at 1 w; after, it decreased gradually but was still higher than 0 w until 3 w. The *CD4* in the kidney was significantly increased to the highest expression levels at 3 wpv; then, it decreased at 4 wpv but was still higher than at 0 wpv. The *MHCI* transcript in the kidney showed a significant increase at 2 wpv, reaching its highest expression level. Subsequently, it gradually decreased but remained higher than at 0 wpv. The *MHCII* transcript was significantly up-regulated in a time-dependent manner and to the highest expression levels at 4 wpv.

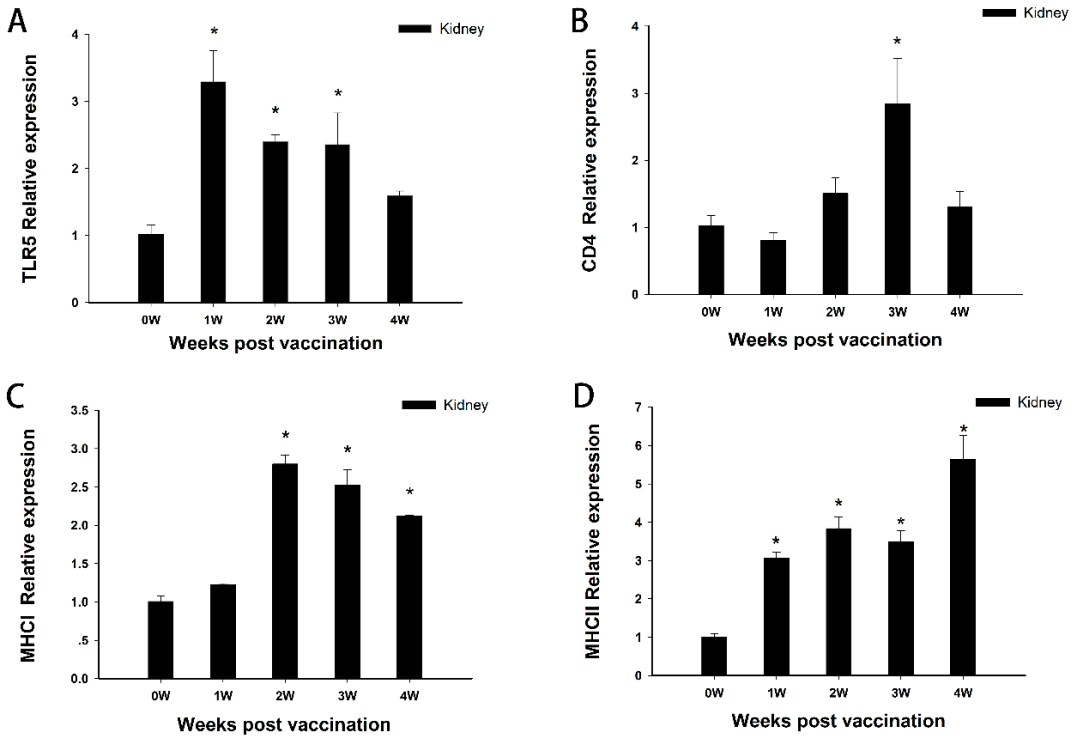

Figure 5. The expression levels of immune-related genes were analyzed by qRT-PCR. (**A**): *TLR5* relative expression. (**B**): *CD4* relative expression. (**C**): *MHCI* relative expression. (**D**): *MHCII* relative expression. The kidney was sampled at 0, 1, 2, 3 and 4 weeks post-vaccination. Each bar represents the mean of three biological replicates. The asterisk (*) indicates the statistical significance ($p < 0.05$).

4. Discussion

In recent years, the cultivation scale of turbot has been continuously expanded after its introduction into China. However, due to intensive cultivation, turbot frequently becomes infected with various pathogens, including bacteria, viruses, and parasites [17]. Vaccination has become an effective and popular measure in aquaculture, as we place increasing emphasis on food safety and environmental protection.

Vaccination is commonly utilized in finfish aquaculture, particularly for Atlantic salmon (*Salmo salar*). However, the vaccination capabilities for many other fish species are limited or nonexistent due to factors such as limited availability, poor performance, or high costs [18]. It is known that various adjuvants are frequently used in fish vaccines to enhance the protective effect of vaccines [19]. Oil-adjuvant vaccines show better efficacy in terms of protective effects compared to aqueous vaccines, and the Montanide™ ISA 763 AVG was used as an adjuvant in our bivalent inactivated vaccine against *A. salmonicida* and *V. vulnificus*. The RPS has always been considered a primary indicator to evaluate the effectiveness of vaccines. In previous reports, the RPS values of *A. salmonicida* formalin-killed with the same adjuvant and *V. vulnificus* formalin-killed vaccine without an adjuvant were 83% and 60% [20,21]. The addition of the Montanide™ ISA 763 AVG adjuvant improved protection against *Vibrio vulnificus* infection compared to our vaccine. The inactivated *A. salmonicida* vaccine with the same adjuvant provided better protection against *A. salmonicida* infection, whereas our bivalent inactivated vaccine provided protection against both bacteria. In addition, the RPS of the bivalent inactivated vaccine against *A. salmonicida* and *E. tarda* with the Montanide™ ISA 763 AVG adjuvant was 77.1% [22]. In this study, our bivalent inactivated vaccine also had a high protective effect, with an RPS of 77%, indicating the vaccine is promising for practical use.

The first line of defense against pathogens in fish is constructed by the non-specific immune system, in which ACP and LZM play crucial roles in the humoral immunity response [23–25]. LZM is a crucial protein in the defense against bacteria as it can lyse

bacteria in vivo and activate the complement system and phagocytic cells to provide protection [26]. Therefore, LZM activity is a quantitative measure of innate immune response in aquatic animals. ACP is a hydrolase that hydrolyzes various orthophosphate monoesters under acidic conditions and is a marker enzyme for macrophage lysosomes. ACP is one of the quantitative indicators of the non-specific immune system in aquatic animals. The current results indicate that ACP and LZM activities in the vaccinated group significantly exceed that of the control group, which implies that the bivalent inactivated vaccine can effectively increase ACP and LZM activity and improve the non-specific immunity of the immunized fish.

Immunoglobulins are the most important mediators of specific immune responses in fish humoral immunity, which are mainly present in the internal environment in the form of soluble antibodies and participate in humoral immune responses. Therefore, specific antibody titers are an important factor in determining the effectiveness of vaccines. The positive correlation between antibody titers and protection levels in immunized fish also suggests that antibody titers can reflect vaccine efficacy [27]. In our research, antibody levels were significantly increased in the vaccinated group at 3 w and 4 w after immunization. Therefore, we conclude that the combined vaccine of *A. salmonicida* and *V. vulnificus* can effectively activate specific immunity in immunized turbot.

A qRT-PCR analysis was used to examine the effect of the bivalent inactivated vaccine against *A. salmonicida* and *V. vulnificus* on the expression of immune genes. The results showed that the majority of the evaluated genes exhibited increased expression. In particular, the significantly elevated expression levels of *TLR5*, *MHCI*, and *MHCII* imply the initiation of the innate immune response. The *MHC* gene cluster can encode the histocompatibility system that participates in immune responses, which is involved in antigen presentation. The main role of the *MHCI* is to deliver newly synthesized antigens in antigen-presenting cells to $CD8^+$ T cells [28]. Similar to the role of *MHCI*, *MHCII* plays a role in the immune response by presenting exogenous proteins to T cell receptors, mainly in cellular immunity. $CD4^+$ T cell function as a helper T cell, which can assist in cellular immunity. *CD4* factor is a marker of helper T lymphocytes; $CD4^+$ T cell function assists in cellular immunity and is also a receptor for *MHCII* molecules. According to the results of this experiment, *CD4* and *MHCII* have an inseparable role in exerting immune responses, and the combined vaccine of *A. salmonicida* and *V. vulnificus* contributes to stimulating the activation of $CD4^+$ T cells and improving the autoimmunity of fish. It can be hypothesized that the *MHCI* and *MHCII* molecules would bind with the antigen and thus present to $CD8^+$ T cells and $CD4^+$ T cells, which means the combined vaccine of *A. salmonicida* and *V. vulnificus* helps to stimulate the antigen presentation pathway. In addition, toll-like receptors (TLRs) have a crucial function in identifying microbial components [29,30]. In this experiment, *TLR5* was up-regulated in kidney tissue after vaccination, reaching a peak after 1 wpv. Subsequently, *TLR5* showed a downward trend, but the relative expression was still higher than at the 0 wpv level. *TLR5* can improve the phagocytic activity of phagocytes, enhance the defense ability against bacteria, fungi, etc., and play an important role in the resistance to infection in immunized individuals. It can be hypothesized that the bivalent inactivated vaccine can promote the expression of *TLR5* and induce immune effects in fish against pathogenic bacteria.

5. Conclusions

In this study, we prepared a formalin-inactivated combined vaccine for *A. salmonicida* and *V. vulnificus* and demonstrated that it exhibits good immune protection effects in turbot. The bivalent inactivated vaccine was used to immunize turbot by intraperitoneal injection, and the relevant immune indexes were detected. It was found that the serum antibody titer, LZM activity, and acid ACP were greatly increased after immunization, which was significantly different from the control group. In the kidney tissue, relevant immune genes (*TLR5*, *CD4*, *MHCI*, *MHCII*) were also up-regulated, which further confirmed the immunization effect of the bivalent inactivated vaccine against *A. salmonicida* and

V. vulnificus. The findings from related experiments indicate that the bivalent inactivated vaccine for *A. salmonicida* and *V. vulnificus* can be used as an effective treatment to facilitate turbot aquaculture development.

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Data Availability Statement: Data are contained within the article.

Conflicts of Interest: The authors declare no conflicts of interest.

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