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Synergistic effect of a combined live *Vibrio anguillarum* and *Edwardsiella piscicida* vaccine in turbot

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ABSTRACT

In aquaculture, more than one pathogen usually be isolated from the sick fish, creating an urgent need for developing combined vaccines to control fish disease caused by multiple pathogens simultaneously. In our previous work, two live attenuated vaccines against *Vibrio anguillarum* and *Edwardsiella piscicida* were vaccinated in turbot, exhibiting an efficient protection. However, some immunological processes such as antigenic competition, antigenic cross-reaction and antigen induced suppression during combined vaccination are unknown. In this study, we evaluated the effectiveness of the combined live vaccines and explored the immunological processes after vaccination. We found that the combined two live attenuated vaccines for *V. anguillarum* and *E. piscicida* induced a stronger immune response without existing antigen competition. Instead, a synergistic effect was observed not only for triggering innate immune response but for stimulation of adaptive immunity. Our study suggested that the two combined live vaccines against *V. anguillarum* and *E. piscicida* could be used simultaneously in the future.

1. Introduction

Turbot (*Scophthalmus maximus* L.) is an important farmed fish in China, especially in Bohai Rim area. However, bacterial disease becomes a thorny problem of turbot farming, resulting in serious economic losses [1]. Among these, vibriosis and edwardsienosis are two main diseases respectively caused by *Vibrio anguillarum* and *Edwardsiella piscicida* (formerly *Edwardsiella tarda*) [2] in turbot industry. Worse still, more than one pathogen often be isolated from the sick fish, which is considered as multiple pathogens infection. Therefore, the rise in occurrence of these bacterial diseases creates an urgent need for efficient measures to combat currently notorious pathogens.

Vaccination is one of the most effective disease control strategies that has contributed to a significant reduction of disease outbreaks and antibiotics use in aquaculture [3]. To date, different kinds of vaccines have been developed in aquaculture. Live attenuated vaccine is a popular form due to its merits. For example, it can express a full range of protective antigens without influencing the physico-chemical characteristics of surface antigens [4]. Meanwhile, it can mimic a natural portal of entry for pathogens thus might be conveniently used for immersion inoculation for fish. Moreover, it can be used as presenter of heterogenous antigens and as polyvalent vaccines.

Two live attenuated vaccines have been constructed in our laboratory with high relative percent survival rate (RPS). MVAV6203 is a live vaccine by curing the virulence plasmid pEIB1 and deletion of the *aroC* gene from the virulent *V. anguillarum* [5]. A highly specific antibody level is found in the peripheral blood of zebrafish after MVAV6203 bath-vaccination [6]. Moreover, it can induce a Th17-like immune response in turbot [7]. While, WED is an *E. piscicida* mutant with deletions in the T3SS genes, along with the *aroC* gene for the biosynthesis of chorismic acid, as well as the curing of endogenous plasmid pEIB202 [8]. The immunoprotection mechanism of WED has also been studied that cytotoxic T lymphocyte responses play a major role in the protection against *E. piscicida* infection in zebrafish [9] and low specific antibody but up-regulation of some immune-related genes contribute to the protection in turbot [8]. Considering that *V. anguillarum* and *E. piscicida* are often detected simultaneously from one deathly fish, we wonder whether these two vaccines could be used together to prevent vibriosis and edwardsienosis.

Several combined vaccines containing more than one inactivated pathogen have been reported. A combined three inactivated bacterins showed the RPS of more than 80% after challenge with *Vibrio alginolyticus*, *Vibrio parahaemolyticus* and *Photobacterium damsela* subsp. *Piscicida* in cobia (*Rachycentron canadum*), respectively [10]. Recently,

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a combined inactivated bacteria-virus vaccine consisted of *V. alginolyticus*, *Vibrio harveyi*, *Vibrio vulnificus* and infectious spleen and kidney necrosis virus has been suggested as an effective candidate to protect groupers against multiple bacterial and viral pathogens [11]. Still, there are some drawbacks such as short duration [12] and low efficiency of immunoprotection [13] of inactivated vaccines. In addition, some polyvalent vaccines targeting more than one species of pathogens have been developed in order to control multiple pathogens infection. An OmpA from *V. alginolyticus* is reported as a polyvalent immunogen against infections caused by different genus and species of bacteria *V. parahaemolyticus*, *Aeromonas hydrophila*, and *Pseudomonas fluorescens* [14]. Similarly, two recombinant proteins from *V. parahaemolyticus* showed effective immune protection against at least two genera of bacteria, *Vibrio* (*V. parahaemolyticus* and *V. alginolyticus*), *Pseudomonas* (*P. fluorescens*) or/and *Aeromonas* (*A. hydrophila*) [15]. However, whether combined live vaccines could induce a stronger immune response with a prolonged immune protection is a valuable issue to study.

Generally, a series of immune responses such as antigen recognition, uptake, processing and presentation are happened following the vaccination. However, there might exist some immunological processes such as antigenic competition, antigenic cross-reaction and antigen induced suppression. Herein, we evaluated the effectiveness of the combined live vaccines and explored the immunological processes after vaccination.

2. Materials and methods

2.1. Fish maintenance

Turbots weighing 35.0 ± 5.0 g were obtained from a commercial fish farm (Tianyuan, Shandong, China) and acclimatized in our laboratory for two weeks before experimental manipulation. Fish were reared in aerated tanks supplied with a continuous flow of sand-filtered seawater at 15.0 ± 1.0 °C. They were fed twice daily with commercial feed and were sampled for examination of bacterial recovery from liver, kidney and spleen on thiosulfate citrate bile salts sucrose agar (TCBS, Shengsi, Shanghai, China) and deoxycholate hydrogen sulfide lactose agar (DHL, Shengsi, Shanghai, China) plates to confirm that the turbot were not infected by *V. anguillarum* and *E. piscicida*. All fish experiments were carried out according to the guidelines and approval of the Animal Research and Ethics Committees of East China University of Science and Technology.

2.2. Vaccination, challenge and sampling

240 turbots were randomly divided into vaccinated and control groups: Va, 50 fish vaccinated with the live attenuated vaccine *V. anguillarum* MVAV6203; Ed, 50 fish vaccinated with the live attenuated vaccine *E. piscicida* WED; Va + Ed, 70 fish combined-vaccinated with MVAV6203 and WED; C, 70 fish mock-vaccinated with sterile physiological seawater (PSW, 20 g NaCl, 4.8 g MgCl₂·6 H₂O, 3.5 g MgSO₄·7 H₂O, 0.7 g KCl, 0.11 g NaHCO₃, 1.21 g CaCl₂·2H₂O per liter of deionized water).

For vaccination, the two live attenuated vaccines were cultured and conducted routinely as in our laboratory [8,16]. Turbots were intraperitoneally (i.p.) vaccinated with 100 µl/fish and the inoculation dosage of MVAV6203 and WED was both 1×10^6 CFU/fish. The combined vaccination group of fish were vaccinated with a dosage of 2×10^6 CFU/fish which contained the mixture of MVAV6203 and WED in a ratio of 1:1. Fish in control group was injected with 100 µl of PSW. After 4 weeks, 20 fish in Va group, in Va + Ed group and in control group were intramuscularly (i.m.) challenged with 1×10^7 CFU/fish of *V. anguillarum* MVM425. Meanwhile, 20 fish in Ea group, in Va + Ed group and in control group were i.m. challenged with 2×10^3 CFU/fish of *E. piscicida* EIB202. RPS was calculated according to the following formula. Both vaccination and challenge were conducted in triplicate.

$$RPS = \left(1 - \frac{\% \text{ mortality of vaccinated fish}}{\% \text{ mortality of control fish}}\right) \times 100\%.$$

For sampling, spleen and kidney from three vaccinated fish and three control fish were isolated at 1, 2, 5, 7, 14, 21 and 28 days post vaccination (d.p.v.), respectively, for the analysis of gene expression. As well, blood was extracted at the same time and serum was collected after centrifugation at 3000 rpm for 10 min and stored at -80 °C until use.

2.3. Specific antibody detection

Antibody titers in turbot sera against *V. anguillarum* and *E. piscicida* were determined using a modified ELISA method. Briefly, microplate was coated with 1.0×10^8 CFU/ml wild type *V. anguillarum* MVM425 or *E. piscicida* EIB202 in 100 µl/well coating buffer (50 mM carbonate buffer, pH 9.6) at 4 °C overnight. Wells were washed in PBS with 0.05% Tween-20 (PBST) and blocked in PBST with 1% BSA (PBSTB) at 22 °C for 2 h. After blocking, sera from vaccinated fish and control fish were diluted with a 1:1 dilution and added into wells (100 µl/well) in duplicate, respectively. After incubation at room temperature (RT) for 3 h, microplate was washed three times with 300 µl/well PBST, and 100 µl/well of mouse-anti-turbot IgM (Aquatic Diagnostics Ltd, Stirling, UK, 1:33 dilution in PBSTB) was added to each well. Then, microplate was washed three times with 300 µl/well PBST after incubation at RT for 1 h, followed by incubation with 100 µl/well goat-anti-mouse IgG conjugated to HRP (Abgent, San Diego, CA, USA, 1:200 dilution in PBSTB) for 1 h. Finally, microplate was washed five times with 300 µl/well PBST, and 100 µl/well TMB was added. After incubation for 10 min at RT, 50 µl/well of H₂SO₄ (2 M), which was used as a stop solution, was added to each well. Finally, the absorbance of the solution was determined at OD₄₅₀ using a microplate reader. Each sample was assessed in duplicate.

2.4. Total serum protein

Total serum protein was determined by Biuret reaction using the total protein quantification kit (Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instruction. Briefly, 250 µl of biuret reagent was added to serum sample and protein standard or distilled water, respectively. Then the mixture was incubated at 37 °C for 30 min. Total serum protein was calculated at 562 nm following the formula. Total serum protein (mg/ml) = $\frac{OD_{\text{serum}} - OD_{\text{water}}}{OD_{\text{standard}} - OD_{\text{water}}} \times \text{standard concentration of protein}$.

2.5. Lysozyme activity

Lysozyme activity in sera of turbot was evaluated using a lysozyme assay kit (Jiancheng Bioengineering Institute, Nanjing, China) according to Zhu et al. with slight changes [17]. Briefly, 200 µl of serum sample, standard solution, and distilled water were placed on ice, added with 2 ml of bacterial suspension (*Micrococcus lysodeikticus*), respectively, and incubated at 37 °C for 15 min. Double distilled water was used as a blank. Lysozyme activity was evaluated at 530 nm and reported as mg/ml.

2.6. Total RNA isolation, cDNA synthesis and RT-qPCR

Total RNA was extracted from samples using Trizol (Invitrogen, CA, USA) according to the manufacturer's instruction. To remove residual genomic DNA, RNA samples were digested with RNase-free DNase I (Tiangen, Shanghai, China). Immediately, 1 mg total RNA was amplified in cDNA synthesis reactions by using PrimeScript RT reagent kit (TaKaRa, Shiga, Japan). The reaction solution was mixed and incubated at 37 °C for 15 min, followed by heat inactivation at 85 °C for 5 s. Negative controls lacking reverse transcriptase or RNA were included for each group. The final cDNA reaction mixtures (20 ml) were diluted

Table 1
Primers used in RT-qPCR.

Gene	Primer sequence (5'–3')
Hepcidin	CGAGTCACATCAGGCAGAAG TCCTCAGAACTTGACGACAGA
C3	GGTACAACCTTCAACAACAACAACAA AGCGTAGTACAGCGACACCATT
CD83	AGTACTACGTGGCTTGGAC CTGTACAGTGGAGGAGACC
IL-1β	GAGAGCATCGTGAAGAACA GTTTCGGACCAGAACGAAGT
IL-8R	GGCTCAGCAAAGACTCGCA CCCGTTGATGACAAACCTCC
IFN1	TGCTCTGCCACAGTCAAAGGT GGTCTTCAGGACGGAGAGG
TLR2	AGGAGCCAAAGGAGACCCGAT GGCGCTCATGATGTTGTGC
TLR5	CGGCCTCAGTATAAGCTCCA GGGGAGGCTAGGAAGTTGTT
TLR21	CAGCTGTCTATCCTATCACCG TTGTGATTTGCCCTGCGTAG
MHC I	CATGGCTGCCATTGGAGTCT CCCTGCCTGTTTACAGGAGAT
MHC II	TGTCCTCAGTGTCTCTGCTGAAG GTATGTCTCTCTCCACCAGTGTCT
TCR	GTGGAGCAAACCAAATCAACA CCGGCTTACAGCACAGTAGTA
β-actin	TGAACCCCAAAGCCAAACAGG AGAGGCATACAGGGACAGCAC

with 80 ml of water and stored at 20 °C until use.

RT-qPCR was carried out following the manufacturer's instruction of SYBR green real-time PCR mix (Tiangen, Shanghai, China) using ABI 7500 Real-time Detection System (Applied Biosystems, Foster City, USA). All samples were analyzed by RT-qPCR in triplicate for technical replicate. A melting curve analysis was performed for all PCR products to confirm the occurrence of specific amplification peaks and the absence of primer-dimer formation. Primers for each gene were listed in Table 1. Each primer pair was specificity. The relative expression of each immune-related gene was determined by the comparative threshold cycle method ($2^{-\Delta\Delta Ct}$ method) with β-actin as the reference gene.

2.7. Statistical analysis

Independent-sample *t*-tests were performed for statistical significance of gene expression using SPSS software as well. Significant differences were considered present at **P* < 0.05 and ***P* < 0.01.

3. Results

3.1. Protection of the single-vaccine and the combined-vaccine

The protection of the single-vaccine MVAV6203 and WED and the combined-vaccine MVAV6203 plus WED were determined firstly. As a result, the RPS of MVAV6203 (Va) and WED (Ed) group against *V. anguillarum* MVM425 and *E. piscicida* EIB202 were 77.65% and 70% at 28 d.p.v., respectively. While, combined-vaccine group (Va + Ed) exhibited higher RPS of 83.3% against *V. anguillarum* MVM425 and of 70% against *E. piscicida* EIB202. These results showed that the combined-vaccine produced stronger immunoprotection against *V. anguillarum* compared with the single vaccine.

3.2. Antibody levels in sera from turbot

Then, specific antibody production in sera of vaccinated turbot was determined. As shown in Fig. 1A, from 14 d.p.v., specific antibody production against *V. anguillarum* MVM425 were induced in Va and

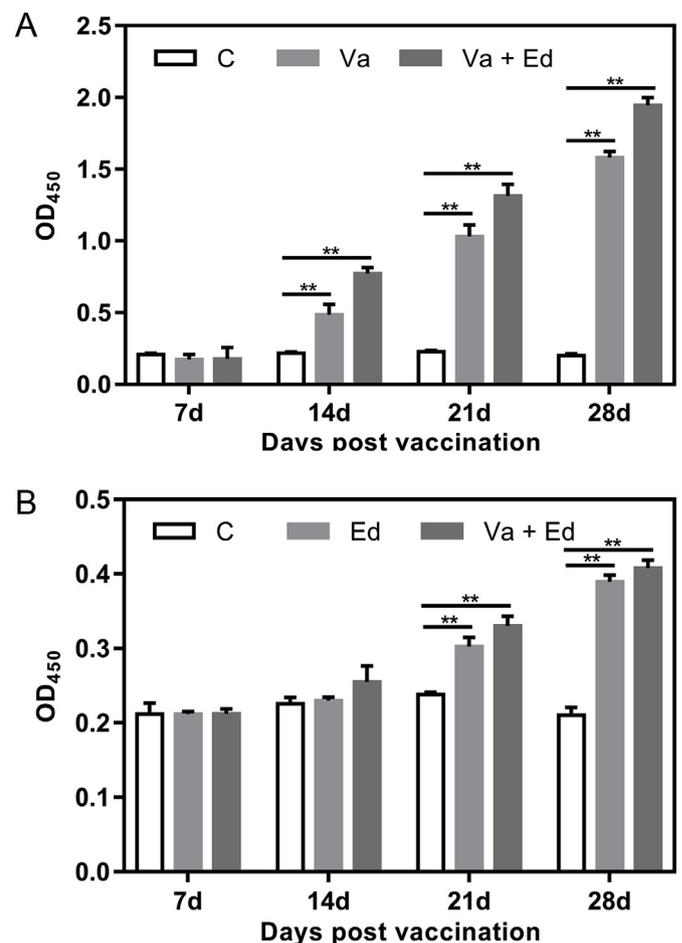


Fig. 1. Specific antibody titers of *V. anguillarum* (A) and *E. piscicida* (B) in sera of vaccinated turbot. Briefly, fish were vaccinated intraperitoneally with 100 μl of vaccines. Va, 50 fish vaccinated with the live attenuated vaccine *V. anguillarum* MVAV6203; Ed, 50 fish vaccinated with the live attenuated vaccine *E. piscicida* WED; Va + Ed, 70 fish combined-vaccinated with MVAV6203 and WED; C, 70 fish mock-vaccinated with PSW. At 7, 14, 21 and 28 d.p.v., sera were collected from vaccinated fish and control fish. Specific antibody titers were determined by ELISA. Statistical significance was analyzed between vaccinated and control turbot (**P* < 0.05, ***P* < 0.01).

Va + Ed groups, and the later group produced more than the former. However, though significant difference was observed, the absolute value of antibody titer in Va + Ed group was as low as the Ed group as shown in Fig. 1B. These results suggested that the combined-vaccine could enhance the production of specific antibody against *V. anguillarum* but failed to enhance a powerful specific antibody against *E. piscicida*.

3.3. Total protein and lysozyme activity in sera from turbot

Total serum protein and lysozyme activity in sera of vaccinated turbot were tested. As shown in Fig. 2A, notable differences were obtained between combined Va + Ed group and control group at each time points. Meanwhile, a similar trend of elevation was observed for lysozyme activity in Fig. 2B. Besides, significant increase was also observed in Va and Ed groups at 21 and 28 d.p.v., respectively. These results suggested that more total protein and higher lysozyme activity were induced after the combined vaccination.

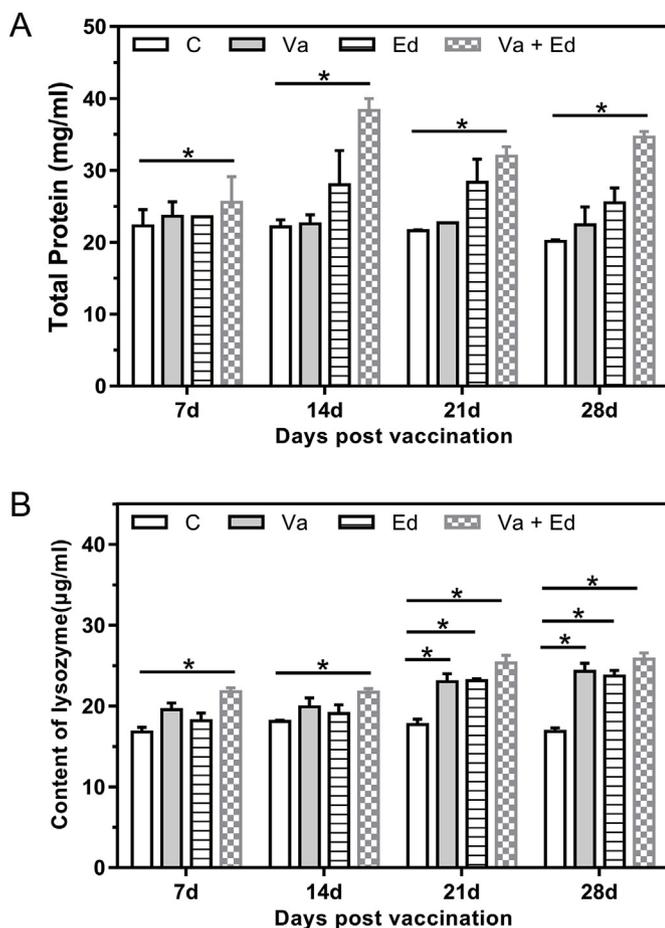


Fig. 2. Total serum protein (A) and lysozyme activity (B) in sera of vaccinated turbot. The experimental procedure was as described in the legend for Fig. 1. Total serum protein and lysozyme activity were determined using total protein quantification kit and lysozyme assay kit. Statistical significance was analyzed between vaccinated and control turbot (* $P < 0.05$, ** $P < 0.01$).

3.4. Gene expression profiles in single-vaccinated and combined-vaccinated turbot

Finally, the expressions of some immune related genes in spleen and kidney were evaluated. As shown in Fig. 3, genes involved in innate immune resistance including Hepcidin, C3 and CD83 notably increased in spleen at 14 d.p.v with receiving 9.06, 57.91, 3.6-fold changes in combined vaccinated group. However, genes involved in antigen recognition including TLR2, TLR5 and TLR21 were observed simultaneously upregulated in kidney at 7 d.p.v. with receiving 4.59, 6.91, 4.10-fold changes. Comparatively, adaptive immune response related genes such as MHC I, MHC II and TCR were firstly increased at 1 d.p.v., and a second elevation of TCR were obtained at 14 d.p.v. Meanwhile some inflammatory cytokines including IL-1 β , IL-8R and IFN1 were also significantly upregulated in both spleen and kidney in combined vaccination group. These results suggested that an enhanced immune response was induced by the combined vaccine.

4. Discussion

The rise in occurrence of multiple-pathogens infection creates an urgent need for efficient measures to combat currently notorious pathogens. Combined vaccination is welcomed for controlling diseases caused by multiple pathogens simultaneously. Previously, two live attenuated vaccines against *V. anguillarum* and *E. piscicida* were vaccinated in turbot, exhibiting an efficient protection. In this study, we

further investigated the immunological processes induced by the combined vaccines in turbot.

Though antigen competition may occur when fish are vaccinated with more than one antigen, the composition of vaccine has a marked effect during the vaccination. Swain et al. found that there was no antigenic competition among *A. hydrophila*, *E. piscicida* and *P. fluorescens*, which did not further jeopardize the specific immune response to the vaccine components [18]. A suppressive antibody response was observed when rainbow trout was vaccinated with *Aeromonas salmonicida*, *Listonella anguillarum* and both Th and Fd serotypes of *Flavobacterium psychrophilum* antigens compared with that with *A. salmonicida*, *L. anguillarum* and only Fd serotype of *F. psychrophilum* [19]. Herein, we believed that there was no antigen competition but a synergistic effect induced by the combined vaccine in our study according to the RPS of combined vaccine group and MVAV6203 vaccine group. One possible reason was that *V. anguillarum* MVAV6203 was an extracellular bacterium while *E. piscicida* WED was an intracellular bacterium. Both these two live vaccines may utilize different antigenic epitope to induce immune response instead of using the same antigenic epitope for antigen recognition. Coincidentally, a humoral immunity-based mechanism of protection was induced by inactivated *E. piscicida* and *V. anguillarum* in flounder, existing a mutual and specific immunostimulatory effect between these two vaccines [20]. The different result in our study that the combined vaccine group showed a similar RPS with WED vaccine group of 70% might owing to the different antigenic epitope of different *E. piscicida* strain we use, since WED mainly induce cell mediated immunity rather than humoral mediated immunity [9].

Innate immune response is immediately triggered after antigens enter the host. Several immunological parameters such as lysozyme, antimicrobial peptide (hepcidin) and complement (C3) were determined. Lysozyme can activate complement system and phagocytes, thereby controlling disease by bacteriolysis [21]. Hepcidin, a widely studied AMP, is considered to exert its antimicrobial properties on cellular pathogens by penetrating the pathogen's plasma membrane to increase permeability and lead to its death [22]. C3 is another important and conventional terminal index of fish innate immunity that is responsible for various immune effector functions [23]. In our study, notable increase of lysozyme activity in sera of turbot vaccinated with the combined vaccine as well as hepcidin and C3 were obtained, indicating that a stronger innate immune response was induced by the combined vaccine.

Pathogen pattern recognition receptors (PRRs) is identified to sense particular structures of the microorganisms (pathogen-associated molecular patterns, PAMPs) and initiate a well orchestrated immune response [24]. Herein, expressions of three PRRs including TLR2, TLR5 and TLR21 were determined. TLR2 of turbot was proved to be induced by LPS, PGN and poly (I:C) in immune and non-immune tissues [25], while TLR21 was induced by poly (I:C), turbot reddish body iridovirus and CpG-ODN in immune tissues [26], and TLR5 was considered as a sensor to bacterial flagellum. Previously, antigenic cross-reactivity of different bacterial whole cells, cell lysates, LPS and outer membrane proteins had been reported in several species of fish bacterial pathogens. For instance, LPS is a typical antigenic structure of Gram-negative bacteria and fish appear to often generate antibodies against LPS [19]. However, different bacterial strains show similarities in LPS structure that have led to cross-reaction of antibodies [27]. Another example as mentioned above, two outer membrane proteins of *V. parahaemolyticus* were proved as polyvalent vaccine candidates against more than one bacteria based on cross-immune protection owing to stimulation of innate immune response involved in humoral immunity such as lysozyme and C3b, and cellular immunity, instead of generation of antibodies of cross-immune protection [15]. In our previous study, a recombinant glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from EIB202 also induced a cross-reaction with *V. anguillarum* MVM425 [28]. It was speculated that a similar cross-reaction might be produced of the

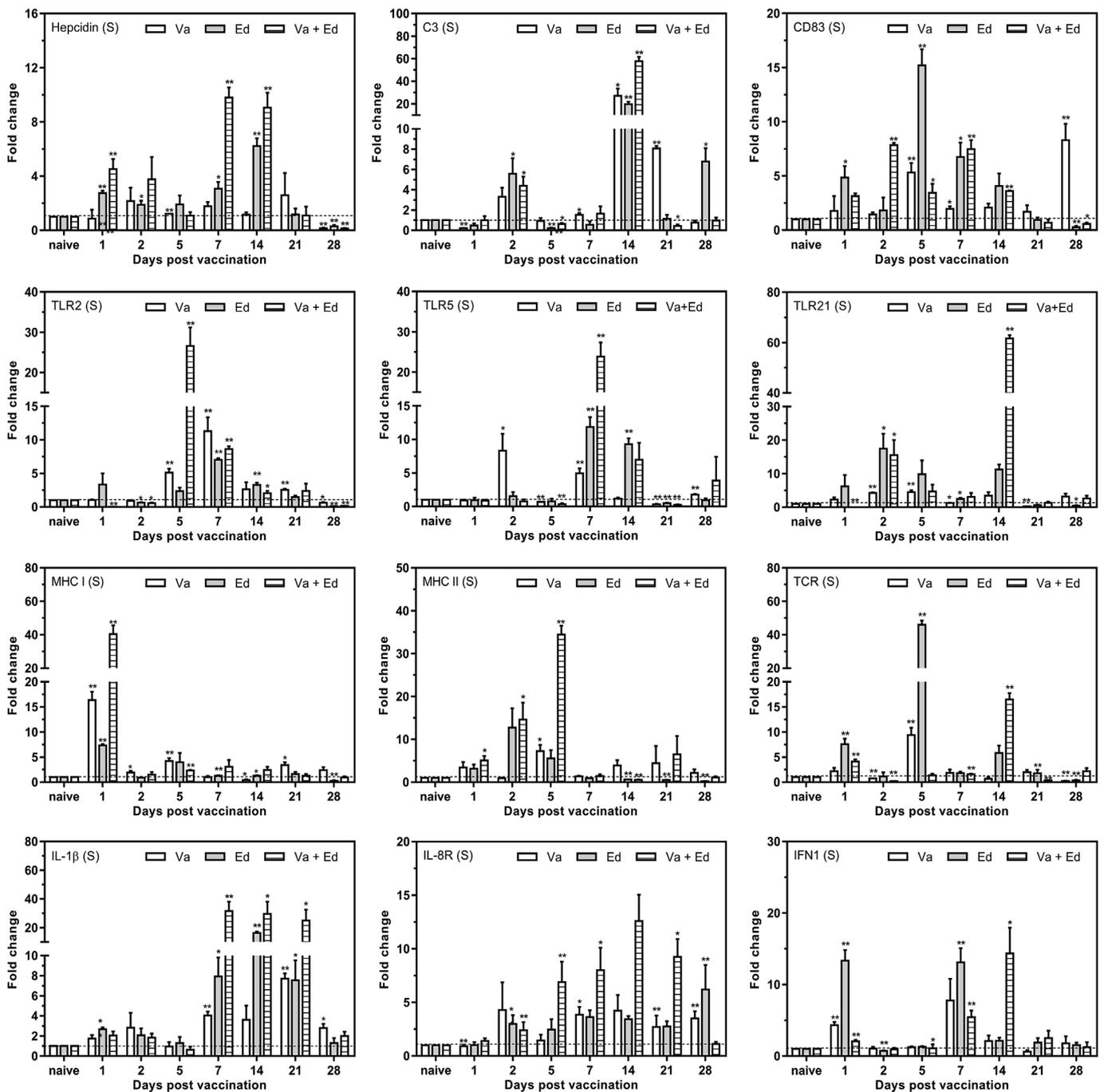


Fig. 3. Gene expressions in vaccinated fish at 1, 2, 5, 7, 14, 21 and 28 d.p.v. in spleen (A) and kidney (B) RNA from spleen and kidney were extracted. mRNA level of each gene was normalized to that of β -actin and relative expression was calculated by dividing values of the vaccinated tissues by those of the controls. Bars represented the mean relative expression of three biological replicates and error bars represented standard deviation. Statistical significance was analyzed between vaccinated fish and naive fish (* $P < 0.05$, ** $P < 0.01$).

wild type or even of the derived vaccine. Nevertheless, significant up-regulations of TLR2, TLR5 and TLR21 at 7 d.p.v. in combined vaccination group indirectly indicated an enhanced antigenic cross-reactivity was induced after the combined vaccination.

Immune response are activated initially by PRRs followed by an inflammatory response including recruitment of leukocytes, activation of antimicrobial effector systems and stimulation of adaptive immunity [29]. A DC-like antigen-presenting cell marker, CD83, was selected as a marker for activated macrophages in turbot [30]. In addition, some inflammatory cytokines including IL-1 β , IL-8R and IFN1 were also evaluated. As expected, the expression of these genes increased

significantly in combined vaccine group. Subsequently, genes related to antigen presentation (MHC I and MHC II) and the T cell marker were determined. The result was consistent with our previous study that both MHC I and MHC II pathway was triggered after the combined vaccination [31].

Antibody titer is a vital factor for judging the efficiency of a vaccine. Interestingly, only higher production of specific antibody against *V. anguillarum* was produced in combined vaccination group. Due to an intracellular bacterium, humoral immune response might play weak role in defending *E. piscicida*. The low titer of specific antibody of *E. piscicida* may be one of strategies of host immune system to save

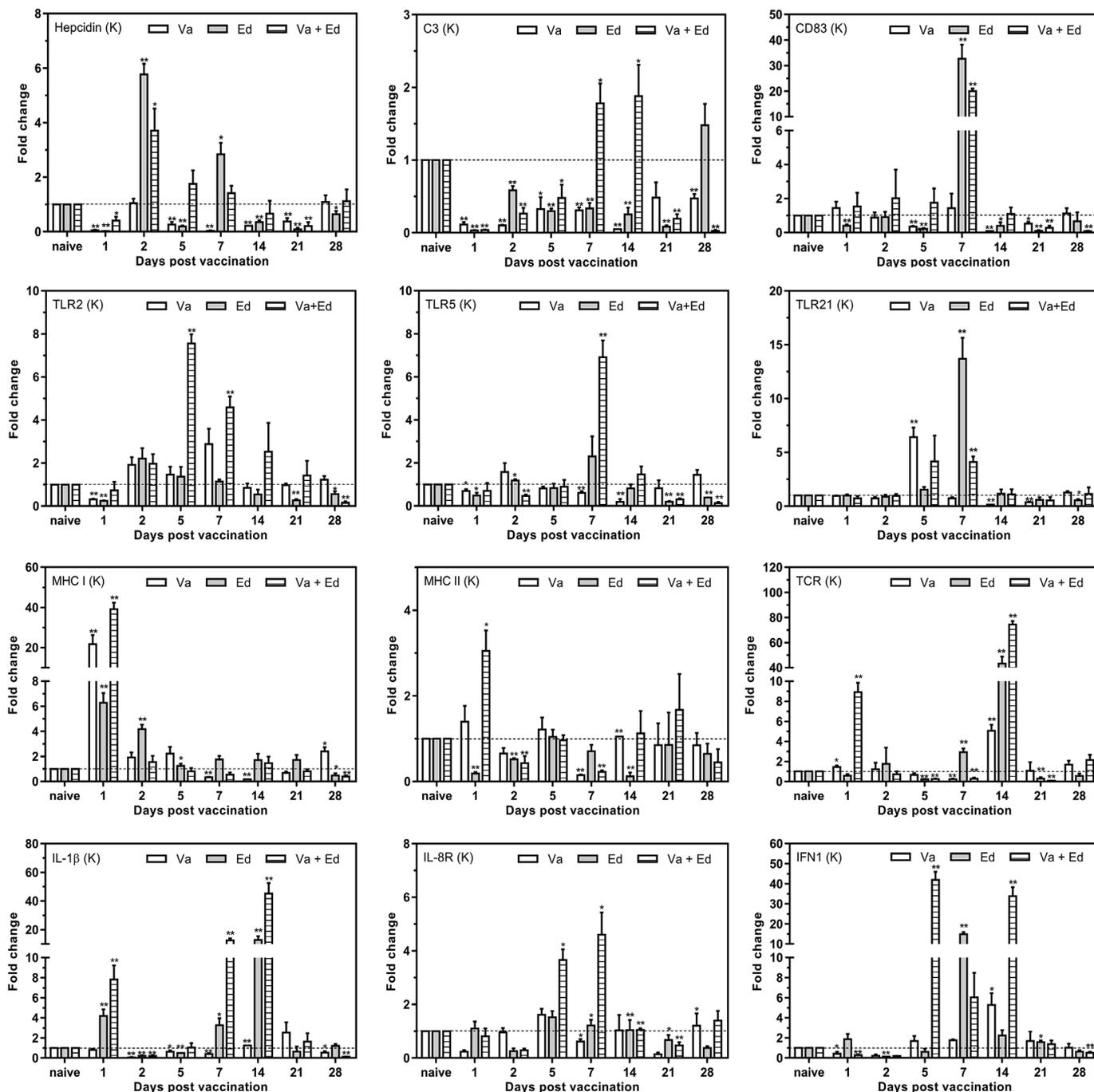


Fig. 3. (continued)

unnecessary energy. It is reported that the protective antibodies can fix complement to the microbial surface thus enhancing the phagocytosis and killing of microbes and enhance the activation of antigen-specific B cells in mammals [32]. However, some researchers found excess of specific antibodies can inhibit the phagocytosis and complement activities [33] and moreover, immunoglobulin has the ability to enhance or suppress the antibody response in human and mouse [34]. Whether antibodies induced by the combined vaccines in our study enhance or suppress the antibody dependent complement activation and phagocytosis remains to be elucidated.

In conclusion, the combined two live attenuated vaccines of *V. anguillarum* and *E. piscicida* only produced stronger immunoprotection and highly specific antibody against *V. anguillarum*, but with a stable immunoprotection against *E. piscicida* and failed to enhance a powerful

specific antibody. However, a synergistic effect was observed after combined vaccination owing to the increased total protein and lysozyme activity in sera of vaccinated fish and upregulations of some immune related genes involved in innate immune resistance, antigen recognition, adaptive immune response and inflammatory cytokines. Totally, we suggested that the combined two live attenuated vaccines played a synergistic role not only in triggering innate immune response but in stimulation of adaptive immunity.

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