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Development of Recombinant Dihydrolipoamide Dehydrogenase Subunit Vaccine against *Vibrio* Infection in Large Yellow Croaker

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Abstract: Large yellow croaker (*Larimichthys crocea*), an economically important marine fish in China, has suffered from serious vibriosis, which has resulted in great economic losses for the large yellow croaker industry. Vaccination has been considered to be a safe and effective method to prevent and control vibriosis. However, due to the complex diversity and serotypes of the *Vibrio* genus, the progress of *Vibrio* vaccine development is still slow. In this study, we prepared recombinant *Vibrio* dihydrolipoamide dehydrogenase (rDLD) protein and investigated its potential as a candidate to be a subunit vaccine against *Vibrio*. The lysozyme activity and the rDLD-specific antibody level in sera of large yellow croakers immunized with rDLD were significantly higher than those in the control group, and the transcript levels of proinflammatory cytokines (*IL-6*, *IL-8*, *IL-1β*), *MHC IIα/β*, *CD40*, *CD8α*, *IL-4/13A*, and *IL-4/13B* were significantly up-regulated in the spleen and head kidney of large yellow croakers immunized with rDLD, suggesting that rDLD could induce both specific and nonspecific immune responses in this species. In addition, rDLD protein increased the survival rate of large yellow croakers against *Vibrio alginolyticus* and *Vibrio parahaemolyticus*, with the relative percent of survival (RPS) being 74.5% and 66.9%, respectively. These results will facilitate the development of a potential subunit vaccine against *Vibrio* in large yellow croaker aquaculture.

Keywords: dihydrolipoamide dehydrogenase; subunit vaccine; *Vibrio*; relative percent survival rate; *Larimichthys crocea*

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1. Introduction

Large yellow croaker (*Larimichthys crocea*) is an economically important marine fish species in China, with the largest annual yield in Chinese farmed marine fish species [1]. However, vibriosis caused by *Vibrio alginolyticus*, *Vibrio harveyi*, and *Vibrio parahaemolyticus* has broken out frequently and resulted in tremendous economic losses for the large yellow croaker aquaculture [2–4]. Currently, antibiotics are commonly used to prevent and treat *Vibrio* infections in marine aquaculture. Nevertheless, the long-term use of antibiotics and chemotherapeutants has led to serious problems such as antibiotics residues and the drug resistance of pathogens, which prompted us to seek effective alternative means to control the *Vibrio* infection in aquaculture.

Vaccination has become an effective means to prevent and control various infectious diseases in aquaculture industry [5]. However, due to the complex diversity and serotypes of *Vibrio*, the development progress of a versatile vibriosis vaccine has been slow [6–8]. Subunit vaccines are recognized as effective tools for the prevention and control of fish diseases in aquaculture. They are typically prepared from viral capsid proteins and bacterial

glycoproteins and exhibit enhanced immune protection against pathogenic infection [9–11]. The outer membrane proteins OmpK, LamB, OmpU, and TolC have been considered as candidate subunit vaccines against *Vibrio* infections, and their cross-protective property was evaluated through challenges with heterogeneous virulent Vibrio strains in the orange-spotted grouper (*Epinephelus coioides*) [12], flounder (*Paralichthys olivaceus*) [13], zebrafish (*Danio rerio*) [14], crimson snapper (*Lutjanus erythropterus Bloch*) [15], and hybrid grouper (*E. fuscoguttatus* (♀) × *E. lanceolatus* (♂)) [16], respectively.

Dihydrolipoamide dehydrogenase (DLD), an oxidoreductase, exists in various organisms [17]. It catalyzes the NAD^+-dependent oxidation of dihydrolipoamide, and is one component of the mitochondrial-based pyruvate dehydrogenase multienzyme complex. DLD plays an important role in bacterial pathogenesis and has been confirmed to be one of the virulent determinants in *Mycoplasma gallisepticum* [18]. *Streptococcus suis* DLD mediates bacterial adhesion and contributes to the occurrence of infection [19]. In addition, DLD could be recognized by antisera of several bacteria, including *Neisseria meningitidis*, *V. alginolyticus*, and *V. harveyi* [20,21]. These studies indicated that DLD may be a common antigen and could be used as a candidate protein for developing a subunit vaccine.

In this study, we amplified the DLD gene from *V. alginolyticus* and prepared its recombinant protein with a prokaryotic expression system. Then we used rDLD as a vaccine antigen to investigate its protective effect on large yellow croaker, an economically important marine fish in China [22]. We found that the lysozyme activity and the rDLD protein-specific antibody level in sera of large yellow croakers immunized with rDLD was significantly higher than those in the control group. Challenging experiments further showed that rDLD could protect large yellow croakers against *V. alginolyticus* and *V. parahaemolyticus*, with the relative percent of survival (RPS) being 74.5% and 66.9%, respectively. Therefore, our results will facilitate the development of a potential subunit vaccine against *Vibrio* in large yellow croaker aquaculture.

2. Materials and Methods

2.1. Bacterial Strains and Animals

Four hundred large yellow croakers (weight: 100 g (mean) ± 10 g (SD), length: 15 cm (mean) ± 1.5 cm (SD)) were purchased from a marine aquaculture farm in Ningde, Fujian Province and cultured in recirculating tanks (3 tons, 25 ± 1 °C) containing filtered and oxygenated seawater for at least seven days before experiments. *V. alginolyticus* and *V. parahaemolyticus* used in the experiments were the strains preserved in our laboratory.

2.2. Cloning of V. alginolyticus DLD Gene

To obtain the *V. alginolyticus* DLD gene, the specific primers described in Table 1 were designed and used for PCR amplification. The cycling conditions were as follows: an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. The resulting PCR product was visualized via agarose gel electrophoresis and purified using OMEGA BIO-TEK Gel Extraction kit (OMEGA BIO-TEK, Norcross, GA, USA). The PCR products were cloned into pMD-18T for sequencing by Sangon Biotech (Shanghai, China).

Table 1. List of primers and their sequences used in this study.

Primers	Sequence (5′-3′)	Primers Information
DLD-F1	CGCGGATCCATGAGCAAAGAAATTAAAGC	cloning
DLD-R1	GGGAAGCTTTTACTTCTTCTTAACTGCTT	cloning
DLD-F2	CGCGGATCCCGCGGATCCATGAGCAAAGAAATTA	cloning
DLD-R2	CCGGAATTCGGGAAGCTTTTACTTCTTCTTAACTGC	cloning
IL-6-F	TGTTGTAAATAGTGGGTGTGTCG	qPCR
IL-6-R	GCTGTTCTCAAGTATGTGGCG	qPCR
IL-8-F	CTATCGTGGCACTCCTGGTT	qPCR
IL-8-R	GCAGGAATCACCTCCACTTGT	qPCR
IL-1β-F	CAGCTGTTCTCAAGTATGTGGC	qPCR
IL-1β-R	GTTGTAAATAGTGGGTGTGTCG	qPCR
IL-4/13A-F	TGGTACTGCTGGTCAATCCG	qPCR
IL-4/13A-R	TTTTGCCTTCAGCCAGATGT	qPCR
IL-4/13B-F	AGTTCTTCTGTCGCGCTGAG	qPCR
IL-4/13B-R	GCTATGTATGTGCGGTTGCTG	qPCR
MHC IIα-F	TCAGCTGCACTCGTTCC	qPCR
MHC IIα-R	CCAGCATCAACGTTCCTC	qPCR
MHC IIβ-F	TCAGTTATTGGGAACCAGAATC	qPCR
MHC IIβ-R	CCACTCTCACCTGGAGTACAC	qPCR
CD8α-F	TGCTGCTCCGATTACGGTCA	qPCR
CD8α-R	TCACTCAATCTGGTGTTAGGCCA	qPCR
CD40-F	ATAGTGCATAGGCTGGAAAATGG	qPCR
CD40-R	GCTGTGGCTCAACACAGATTTAG	qPCR
β-action-F	GACCTGACAGACTACCTCATG	qPCR
β-action-R	AGTTGAAGGTGGTCTCGTGGA	qPCR

2.3. Expression and Purification of Recombinant DLD Protein

The complete open reading frame (ORF) of DLD was amplified and cloned into the *Eco*R I/*Bam*H I sites of pET-28a vector (Novagen, Madison, WI, USA). The accuracy of recombinant expression plasmid pET-28a-DLD was confirmed by sequencing. Then recombinant expression plasmid pET-28a-DLD was transformed into the competent cells of *Escherichia coli* BL21. Expression of recombinant DLD (rDLD) protein was induced by 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) and was detected by Western blotting as described previously [23]. Briefly, *E. coli* BL21 cells transformed with recombinant plasmid pET-28a-DLD or control plasmid were collected after IPTG induction for 5 h. The cells were lysed with the lysis buffer and the supernatants of cell lysates were mixed with 5 × SDS loading buffer (250 mM Tris-HCl pH 6.8, 50% glycerin, 10%, SDS, 0.5% BPB, 5% 2-mercaptoethanol). The resulting protein samples were separated with 12% SDS-PAGE (0.125 M Tris, 1.25 M Glycine, 0.5% (*w*/*v*) SDS) and transferred to polyvinylidene fluoride membrane (PVDF). The membrane was blocked in 5% (*w*/*v*) nonfat milk in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.05% (*v*/*v*) Tween 20) at room temperature for 1 h, incubated with mouse anti-6 × His tag monoclonal antibody (Thermo Fisher Scientific, Waltham, MA, USA, 1:2000) overnight at 4 °C, washed with TBST 3 times, and then incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (H + L) polyclonal antibody (Thermo Fisher, 1:4000). After being washed with TBST 3 times, the membranes were visualized by enhanced chemiluminescence detection reagent (NCM Biotech, Suzhou, shanghai, China) and detected by azure Imager c500 (Azure Biosystems, Dublin, CA, USA).

The rDLD was purified by His Trap HP column (GE, Boston, MA, USA) under native conditions as previously described [24]. The supernatants of cell lysates above were incubated with the His Trap HP column by rocking at 4 °C overnight. The His Trap HP column was washed with washing buffer 1 (20 mM Tris-HCl, 500 mM NaCl, 10–30 mM imidazole, 0.5% Triton X-100, pH 7.4), washing buffer 2 (20 mM Tris-HCl, 500 mM NaCl, 20–40 mM imidazole, 0.5% Triton X-100, pH 7.4), and washing buffer 3 (20 mM Tris-HCl, 500 mM NaCl, 20–40 mM imidazole, pH 7.4). Then, the recombinant protein was eluted with elution buffer (20 mM Tris-HCl, 100 mM NaCl, 300 mM imidazole, pH 7.4). The eluted

recombinant protein was put into a 3 kD dialysis bag and dialyzed with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). The purified rDLD was collected by centrifugation at 10,000× *g* for 10 min, and examined by 12% SDS-PAGE and Coomassie blue staining.

2.4. Immunization of Large Yellow Croaker with Recombinant DLD Protein

Four hundred healthy large yellow croakers were divided into two groups of 200 fish each and intraperitoneally injected with 100 μL PBS and 100 μL of rDLD protein (1.5 mg/mL), respectively. After the second immunization for 7 days, the immunization was strengthened again. The serum of 5 fish in each group was collected at 7, 14, 21, 28, 35, and 42 d after the second immunization. And then filtered and frozen for later use.

2.5. Determination of Lysozyme Activity in Serum of Large Yellow Croaker

The determination of lysozyme in serum sample was based on its enzymatic activity using a classic turbidimetric assay with minor modifications. In short, *Micrococcus lysodeikticus* (0.1 mg/mL; 200 μL) in PBS (50 mM, pH = 6.4) was incubated (28 °C, 30 min) with either 5 μL of undiluted serum or lysozyme standard solution, and the decrease in absorbance at 450 nm was monitored over time. The rate of absorbance decrease at 450 nm was plotted against the lysozyme concentration to build a calibration curve. The concentration of lysozyme in serum samples was derived from the calibration curve as previously reported [25].

2.6. Determination of Antibody Titer in Serum of Large Yellow Croaker

The antibody titer in serum of large yellow croaker collected above was detected by ELISA. Briefly, 96-well plates were coated with 100 μL of rDLD protein (1 μg/mL) in ELISA Coating Buffer (Solarbio, Beijing, China) per well and incubated at 4 °C overnight. After being washed with PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.2% Tween) five times, the plates were blocked with 5% BSA (Yeasen Biotech, Shanghai, China) in PBS for 1.5 h at 37 °C. After PBST washing, the serum samples with serial dilutions were applied in blocking solution (5% skimmed milk powder) for 1 h at 37 °C. After PBS washing five times, the plates were incubated with mouse anti-large yellow croaker IgM monoclonal antibody for 1 h at 37 °C. After PBST washing, plates were incubated with HRP-conjugated sheep anti-mouse IgM antibody (Thermo Fisher; 1:4000) and then reacted with 100 μL TMB single-component substrate solution (Solarbio Science) for 10 min. Finally, the reaction was stopped by ELISA stop solution (Solarbio), and the absorbance of each well was measured in an INFINITE 200 PRO (Tecan Austria, Grödig, Austria) at 450 nm.

2.7. Expression Analysis of Immune-Related Genes by Real-Time PCR

To determine the expression change of immune-related genes based on rDLD immunization, the spleen and head kidney from large yellow croakers were collected at 1, 2, 3, and 4 w (weeks) after the second immunization. Total RNA was extracted using TRIzol Reagent (Invitrogen) and reverse transcribed into first-strand cDNA. The expression levels of proinflammatory cytokines (*IL-6*, *IL-8*, *IL-1β*), *MHC II α/β*, *CD8a*, *CD40*, *IL-4/13A*, and *IL-4/13B* genes were analyzed using real-time PCR with the gene-specific primers (Table 1). Real-time PCR was performed on a QuantStudioTM 1 Real-time PCR system (Applied Biosystems, Waltham, MA, USA) using an SYBR qPCR master mix (Vazyme, Nanjing, China). Cycling conditions were as follows: 95 °C for 5 min, followed by 40 cycles of 94 °C for 20 s, 56 °C for 20 s, and 72 °C for 1 min. The relative expression levels of genes were normalized by β-actin and expressed as fold change by comparing the normalized gene expression levels of rDLD-immunized fish with those of PBS-immunized fish (defined as 1) at the corresponding time points. All data were derived from three independent PCR analyses with three replicates in each assay.

2.8. Challenge Experiment of Large Yellow Croaker

At day 28 after the second immunization, fish immunized with rDLD or the PBS were divided into two groups, respectively. RDLD-immunized group was intraperitoneally injected with 100 µL *V. alginolyticus* or *V. parahaemolyticus* (1×10^8 CFU/mL), and PBS-immunized group also had the same treatment. Fish mortality was monitored daily for 15 days, and dead fish were removed on a daily basis. The relative percent of survival (RPS) was calculated by the following formula [26]:

$$\text{RPS} = [1 - (\%\ \text{mortality in vaccinated fish} / \%\ \text{mortality in controls})] \times 100\%$$

2.9. Statistical Analysis

All real-time PCR and hydrolyzing activity analysis were repeated three times, and all data were analyzed using SAS software (SAS Institute, Cary, NC, USA) and expressed as mean ± standard error. Two-tailed Student *t*-test was used for the significance test of the chemotactic activity between experimental and control (PBS) groups. A *p*-value of <0.05 was considered to be statistically significant.

3. Results

3.1. Expression and Purification of rDLD Protein

The recombinant plasmid pET28a-DLD was transformed into *E. coli* BL21 cells to produce rDLD fusion protein with a 6 × His tag. The lysates of *E. coli* cells harboring recombinant pET28a-DLD or empty pET28a were centrifuged at 12,000× *g* and supernatants and sediments of cell lysates were separated by SDS-PAGE. It was found that both the supernatants and sediments of lysates of cells containing pET28a-DLD generated a thick band at ~55 kDa (Figure 1, lane 3 and 4), which was consistent with the predicted size (51 kDa) of rDLD protein [27]. In contrast, cells harboring empty pET28a produced no band at the corresponding position (Figure 1, lane 1 and 2). Western blot analysis further confirmed that the thick band at ~55 kDa was rDLD protein (Figure 2). After Ni column purification, a single protein band with the same size was observed (Figure 1, lane 5). These results indicate that rDLD protein with a molecular weight of ~55 kDa was expressed in *E. coli* BL21 cells.

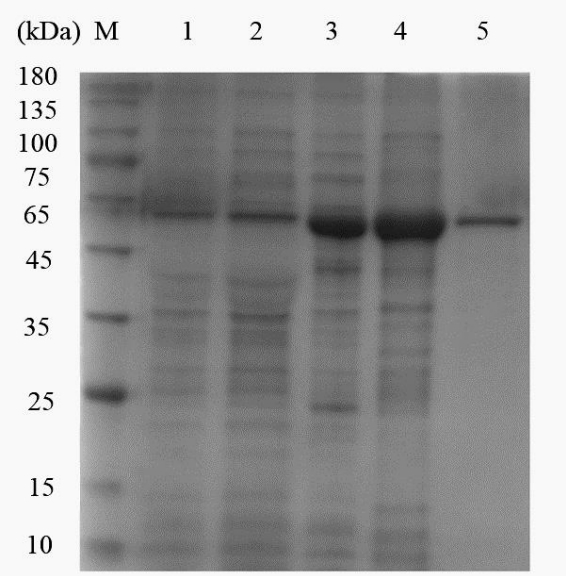

Figure 1. Expression and purification of rDLD. M: protein marker, lane 1: total lysate of *E. coli* BL21 cells harboring pET28a and induced by IPTG, lane 2: total lysate of *E. coli* BL21 cells harboring pET28a-DLD and non-induction, lane 3: lysate supernatant of *E. coli* BL21 cells harboring pET28a-DLD and induced by IPTG, lane 4: lysate sediment of *E. coli* BL21 cells harboring pET28a-DLD and induced by IPTG, lane 5: purified rDLD.

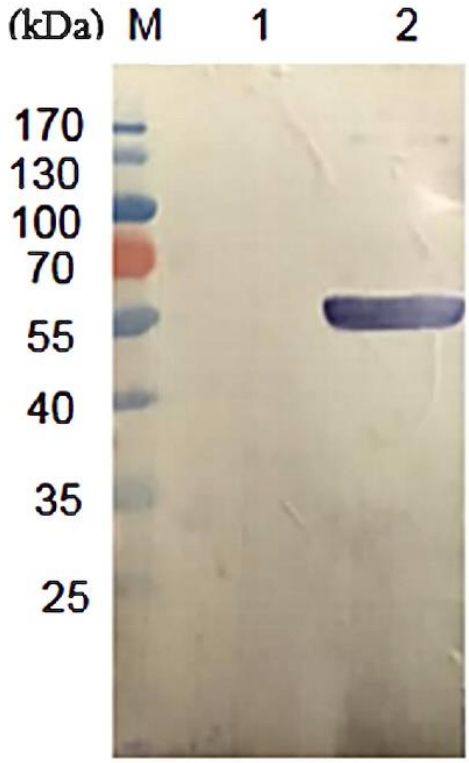

Figure 2. The expression of recombinant DLD protein was detected by Western blot. M: protein molecular weight marker; lane 1: total lysate of *E. coli* BL21 cells harboring pET28a and induced by IPTG, lane 2: lysate supernatant of *E. coli* BL21 cells harboring pET28a-DLD and induced by IPTG.

3.2. The rDLD Enhanced the Activity of Lysozyme

To study the effect of rDLD on the activity of lysozyme, a typical innate immune molecule, we analyzed the activity change of lysozyme in sera of large yellow croakers immunized with rDLD protein. The results showed that the activity of lysozyme in sera of fish immunized with rDLD protein was significantly higher than that of fish in the control group at 1, 2, 3, and 4 w, with a peak at 4 w (Figure 3). Subsequently, the activity of lysozyme in the rDLD-immunized fish gradually decreased and recovered to normal levels at 5 and 6 w.

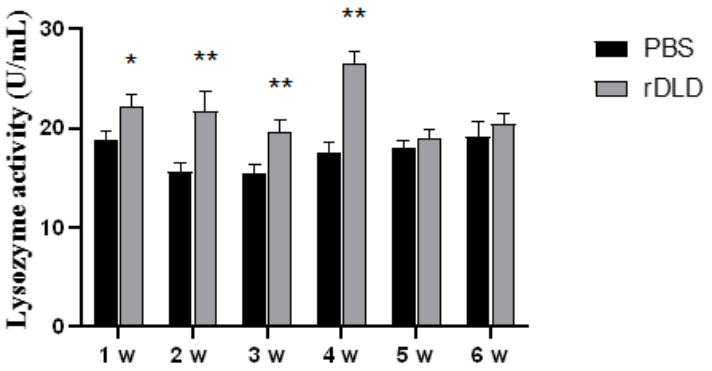

Figure 3. Determination of lysozyme activity in sera of large yellow croakers after immunization. Sera of the fish immunized with rDLD or PBS (control) were collected at 1~6 weeks. The lysozyme activity was detected by using the lysozyme detection kit. * above a bar denotes significant difference ($p < 0.05$), ** above a bar denotes highly significant difference ($p < 0.01$).

3.3. The rDLD-Induced Expression of Immune-Related Genes In Vivo

To understand whether rDLD protein induced the expression of the genes involved in innate and adaptive immune responses (*IL-6*, *IL-8*, *IL-1β*, *MHC IIα/β*, *CD40*, *CD8α*, *IL-4/13A*, and *IL-4/13B*) in vivo, we detected their expression changes after rDLD injection by real-time PCR. As shown in Figure 4, after rDLD stimulation, the mRNA levels of proinflammatory cytokines *IL-6*, *IL-8*, and *IL-1β* were significantly up-regulated in both the spleen and head kidney.

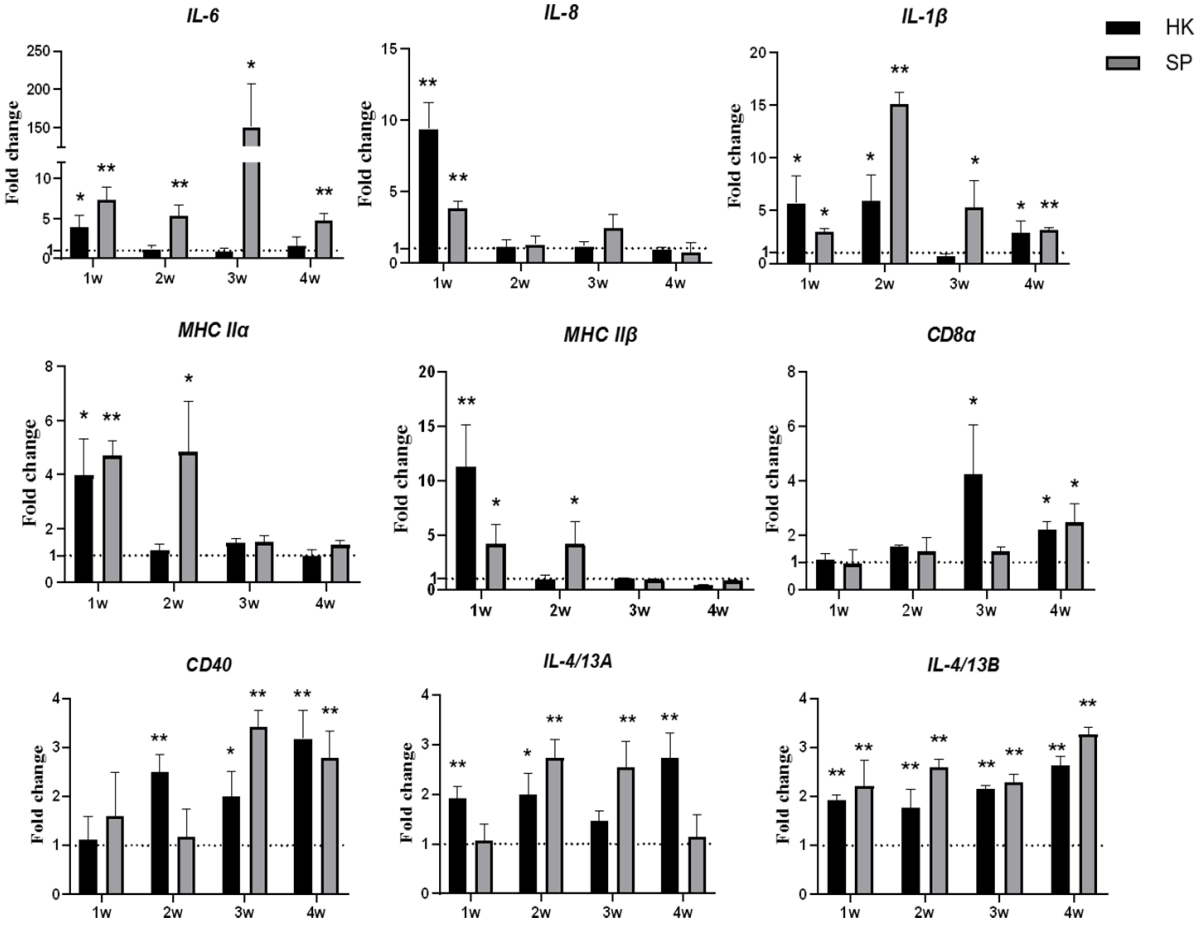

Figure 4. Expression analysis of immune-related genes in head kidney and spleen of large yellow croakers injected with rDLD. The relative expression levels of *IL-6*, *IL-8*, *IL-1β*, *MHC IIα/IIβ*, *CD40*, *CD8α*, *IL-4/13A*, and *IL-4/13B* genes were normalized by β-actin and expressed as fold changes compared with the control at each time point. All data were obtained from three independent experiments with three replicates in each experiment. Error bars represent SEM of three independent experiments. * represents $p < 0.05$, ** represents $p < 0.01$.

The expression levels of antigen-presenting molecules *MHC II α/β* in the spleen were significantly increased at 1 w and 2 w, while their expression levels in the head kidney were up-regulated at 1 w. T cell- and B cell-related molecules CD8α and CD40 were up-regulated by rDLD protein from 3 w or 2 w to 4 w. In addition, the expression levels of *IL-4/13A* and *IL-4/13B* were increased after rDLD stimulation.

3.4. The rDLD-Increased Antibody Levels in Serum

To evaluate the ability of rDLD to induce systemic humoral immune response, sera from large yellow croakers immunized with rDLD were used to test rDLD-specific antibody levels by ELISA. As shown in Figure 5, compared to the control group, an rDLD-specific

antibody in the sera of rDLD-immunized fish was detected at week 1 after the second immunization, peaked at week 3, and was maintained until week 6.

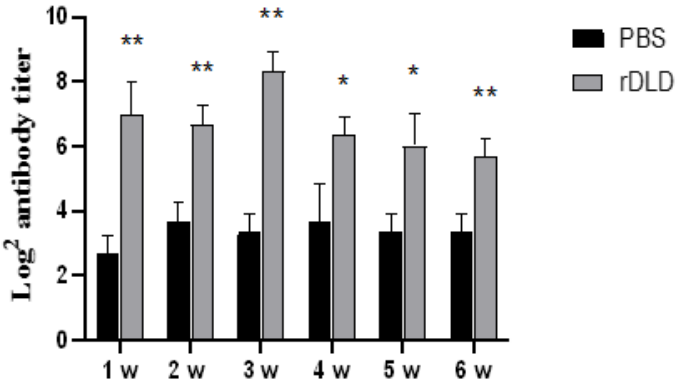

Figure 5. Determination of antibody titer in sera of large yellow croakers. Sera of the fish immunized with rDLD or PBS (control) were collected at 1~6 week. Specific antibody in sera was detected by ELISA. * above a bar denotes significant difference ($p < 0.05$), ** above a bar denotes highly significant difference ($p < 0.01$).

3.5. Vaccine Efficacy

Four weeks after the second immunization, large yellow croakers in the vaccinated group or control group were challenged by *V. alginolyticus* and *V. parahaemolyticus*. Cumulative survival rates were 17.4% for the PBS/*V. alginolyticus* group, 78.9% for the rDLD/*V. alginolyticus* group, 22.9% for the PBS/*V. parahaemolyticus* group, and 74.5% for the rDLD/*V. parahaemolyticus* group (Figure 6). Hence, the RPS of rDLD against *V. alginolyticus* and *V. parahaemolyticus* were 74.5% and 66.9%, respectively. These results showed that rDLD could protect large yellow croakers against *V. alginolyticus* and *V. parahaemolyticus* challenges to a certain extent.

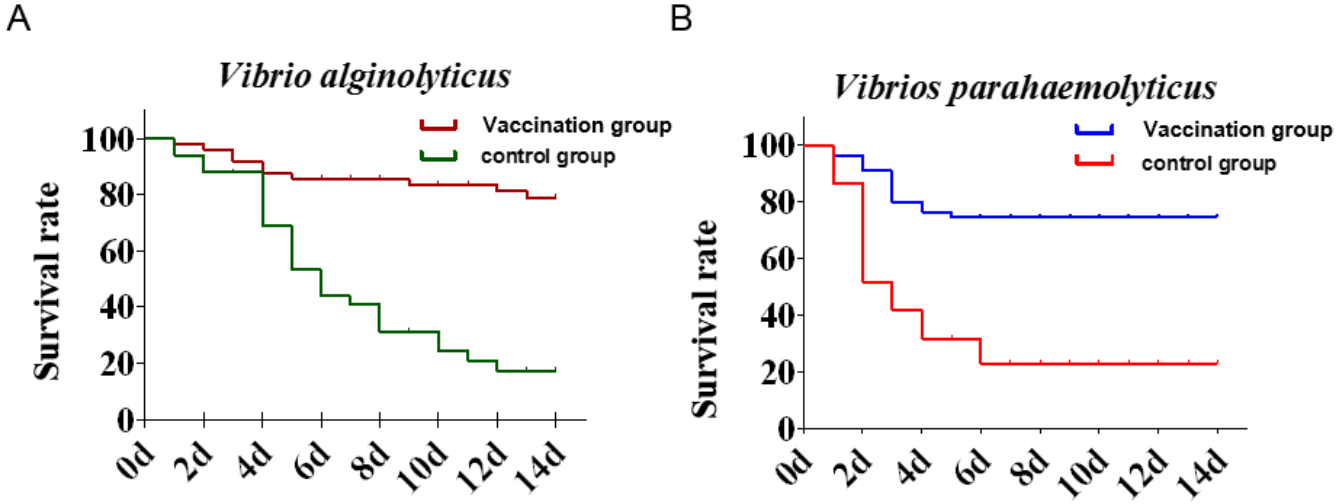

Figure 6. Relative percent of survival (RPS) of large yellow croakers vaccinated with rDLD and PBS (as the control) following challenge with *V. parahaemolyticus* and *V. alginolyticus*. (**A**) Percent survival of large yellow croakers challenged with *V. alginolyticus*. (**B**) Percent survival of large yellow croakers challenged with *V. parahaemolyticus*.

4. Discussion

Vibriosis is one of the most prevalent bacterial diseases affecting a variety of marine fishes, including large yellow croaker. The development of a *Vibrio* vaccine has been

considered to be a safe and efficacious measure to prevent and control this disease in the cultured fish. Silver bream (*Blicca bjoerkna*) immunized with formalin-killed *V. alginolyticus* showed an enhanced lymphocyte count, macrophage phagocytic activity, and agglutinating antibody titer [28]. The outer membrane proteins OmpK, OmpT, LamB, OmpU, and TolC of *Vibrio* have been regularly used as antigens for vaccine development [12,14]. In addition, other proteins of *Vibrio* such as glyceraldehyde-3-phosphatedehydrogenase (GAPDH) and protease chaperon DegQ in *V. harveyi* were also reported as suitable candidate antigens for a vaccine [29,30]. However, due to the broad host range, the complex diversity and serotypes of the *Vibrio* genus, and wide geographical distribution of *Vibrio*, a vaccine specific for a certain *Vibrio* species or strain may not be able to prevent the infection by other *Vibrio* species or strains. For example, a commercial vaccine, AquaVac® *Vibrio* (Intervet/Schering-Plough, formerly USDA, ARS), could only prevent vibriosis caused by *V. anguillarum* Types I and II, and *V. ordalii* infections in European sea bass (*Dicentrarchus labrax*) and rainbow trout (*Oncorhynchus mykiss*) [31]. A previous study reported that DLD was a high abundant cross immunogenic protein among *Vibrio* species, and orange-spotted grouper immunized with DLD had a significantly high RPS against *V. alginolyticus*, *V. harveyi*, and *V. parahaemolyticus*, suggesting that DLD may be an effective cross-protective immunogen and a protective vaccine candidate against *Vibrio* species in orange-spotted grouper [32]. However, whether DLD could be used as a protective vaccine candidate against *Vibrio* species in large yellow croaker still needs to be investigated. In this study, we prepared rDLD protein of *V. alginolyticus* separated from large yellow croaker and explored its potential as a candidate subunit vaccine against *V. alginolyticus* and *V. parahaemolyticus* in large yellow croaker. We found that the rDLD protein could activate both the innate immune system and acquired immune system of large yellow croaker. The innate immune system is the first line of host defense, in which lysozyme is an important factor and participates in the elimination of pathogenic bacteria by hydrolyzing β-1,4-glycosidic bonds of bacterial cell wall peptidoglycan [33]. The previous study showed that the goose-type lysozyme in large yellow croaker was involved in the immune response induced by bacterial vaccine as an acute-phase protein [34]. Here, the activity of lysozyme in sera of large yellow croakers immunized with rDLD protein increased quickly during the early stage. These observations suggested that the lysozyme may be a marker molecule induced by a bacterial vaccine in large yellow croaker. In addition, the expression of proinflammatory cytokines *IL-1β*, *IL-6*, and *IL-8* was significantly up-regulated in the rDLD-immunized large yellow croakers. These results indicated that the rDLD protein could activate innate immunity in large yellow croaker.

The specific antibody level is an important indicator of a humoral immune response induced by vaccine. In this study, a high titer of specific anti-DLD IgM antibody was detected in the sera of large yellow croakers immunized with rDLD protein and maintained for a long time, indicating that rDLD can induce a specific humoral immune response in large yellow croaker. Meanwhile, *MHC IIα* and *MHC IIβ*, involved in exogenous antigen presentation [35], were highly expressed in the head kidney and spleen of immunized fish, especially at an early stage (1 w), suggesting that the antigen presentation process may be activated in the early stages of immunization. T cell- and B cell-related molecules *CD8α* and *CD40* were also found to be up-regulated. *IL-4* and *IL-13* are secreted predominantly by activated T helper cell type 2 (Th2) [36–39] and participate in adaptive immune responses by promoting Th2 cell differentiation, B cell proliferation and activation, Ig production, and Ig class conversion [36,39]. In this study, the expression of *IL-4/13A* and *IL-4/13B* was up-regulated in the head kidney and spleen of large yellow croakers immunized with rDLD protein, suggesting that rDLD may induce the Th2 cell differentiation and B cell proliferation. Challenge assays further showed that large yellow croakers vaccinated with rDLD were highly protected against *V. alginolyticus* and *V. parahaemolyticus* infections, indicating that DLD may be used as a potential cross-protective vaccine candidate against *Vibrio* species in large yellow croaker. However, to achieve the best immune protection effects, the immunization strategy needs to be further optimized.

In conclusion, we investigated the immune protection effects of recombinant *V. alginolyticus* DLD (rDLD) on large yellow croaker. The rDLD could induce both innate and acquired immune responses in large yellow croaker and protect fish against *V. alginolyticus* and *V. parahaemolyticus* infections. Our results, therefore, will contribute to the development of a potential subunit vaccine against *Vibrio* species in aquaculture.

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