Adjuvant effects of β-defensin on DNA vaccine OmpC against edwardsiellosis in flounder (*Paralichthys olivaceus*)

Heng Chi[1,2*], Qingqing Qin[1], Xiaokai Hao[1], Roy Ambli Dalmo[3], Xiaoqian Tang[1,2], Jing Xing[1,2], Xiuzhen Sheng[1,2], Wenbin Zhan[1,2*]

[1] Laboratory of Pathology and Immunology of Aquatic Animals, KLMME, Ocean University of China, Qingdao 266003, China;

[2] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266071, China.

[3] Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries and Economics, University of Tromsø, The Arctic University of Norway, Tromsø N-9037, Norway.

*To whom correspondence should be addressed

Mailing address:

5 Yushan Road

Qingdao, 266003, China

Tel: +86 53282032284

E-mail: chiheng@ouc.edu.cn (Heng Chi); wbzhan@ouc.edu.cn (Wenbin Zhan)

Abstract

β-defensin of flounder plays an important role in immunomodulation by recruiting immune cells and has a potential vaccine adjuvant effect in addition to its bactericidal activity. In this study, adjuvant effects of β-defensin on DNA vaccine OmpC against edwardsiellosis in flounder (*Paralichthys olivaceus*) were investigated. The bicistronic eukaryotic expression plasmid pBudCE4.1 plasmid vector with two independent coding regions was selected to construct DNA vaccine of p-OmpC which express only the gene for the outer membrane protein of *Edwardsiella tarda* and the vaccine of p-OmpC-βdefensin which express both the outer membrane protein of the bacterium and β-defensin of flounder. *In vitro* and *in vivo* studies have shown that the constructed plasmids can be expressed in flounder embryonic cell lines and injection sites of muscles. After vaccination by intramuscular injection, both p-OmpC and p-OmpC-βdefensin groups showed significant upregulation of immune-response. Compared to the pBbudCE4.1 and the p-OmpC vaccinated groups, the p-OmpC-βdefensin vaccinated group showed significantly more cell aggregation at the injection site and intense immune response. The proportion of $sIgM^+$ cells, as well as the $CD4\text{-}1^+$ and $CD4\text{-}2^+$ cells in both spleen and kidney was significantly higher in the p-OmpC-βdefensin vaccinated group at peak time point than in the control groups. The relative survival rate of the p-OmpC-βdefensin vaccine was 74.17%, which was significantly higher than that of the p-OmpC vaccinated group 48.33%. The results in this study determined that β-defensin enhances the responses in cellular and humoral immunity and evokes a high degree of protection against *E. tarda*, which is a promising candidate for vaccine adjuvant.

Keywords: β-Defensin; DNA Vaccine; Adjuvant; Immunomodulation; Fish

1. Introduction

DNA vaccines are so-called third generation vaccines, which in many instances mimic live virus infection and responses [1]. Compared to a traditional vaccine such as inactivated and subunit vaccine, a DNA vaccine has the advantages of safety, ease of production, low cost, and stability which give them a commercial value as they often show high vaccine efficacy against hard-to-combat diseases [2, 3]. *Edwardsiella tarda* (*E. tarda*) is an intracellular parasitic Gram-negative bacterium which infects various aquatic animals, spreads rapidly in water, and causes significant compromised animal welfare and economic loss to the aquaculture industry [4, 5]. The often-encountered problem of antibiotic drug resistance often caused by antibiotic spillovers from treatment for *E. tarda* have led to a search for other ways to control edwardsiellosis [4].

In mammals, DNA vaccines have been shown have applications for several diseases, including infectious diseases, cancer, autoimmune diseases, and allergies [6]. In recent years, the technology on DNA vaccine of fish has developed rapidly. Puente-Marin et al. detected high specific antibodies induced by a DNA vaccine constructed with glycoprotein G of viral hemorrhagic septicemia virus in rainbow trout, suggesting that the DNA vaccine elicits humoral immunity [7]. In flounder, Zhang et al. showed that DNA vaccines can induce cellular immunity and changes in the T/B lymphocyte ratios [8]. A DNA vaccine mixed with chemokines (CCL9) showed rise in the proportion of $sIgM^+$ cells, up-regulation of MHCII, IFN, and MHCI expression, and increased the relative survival rate (RPS) 74.1% [9]. As such, a selection of appropriate functional vaccine adjuvants may enhance the potency and efficacy of DNA vaccines.

Antimicrobial peptides (AMPs) are a class of small molecule peptides that play a key role in the host immunity [10]. They can be considered as a first line of defense against pathogenic microorganisms and can be rapidly produced and transported to the site of infection [11]. Furthermore, AMPs have broad-spectrum anti- microbial properties, including inhibition of viruses, bacteria, fungi, and parasites [12]. It has been shown in cattle, that a neutrophil beta-defensin 3 and glycoprotein D of bovine herpesvirus fused together in a DNA vaccine increased the induction of glycoprotein D-specific cytotoxic T lymphocytes (CTLs) [13]. In zebrafish, β-defensin 2 has been shown to possess antiviral and immunomodulatory properties and has thus been suggested to act as

an adjuvant for viral DNA vaccines [14].

Our previous studies showed that β-defensin of flounder (*Paralichthys olivaceus*) had broad-spectrum antimicrobial activity and the ability to attract leukocytes [15]. However, the efficacy of β-defensin as vaccine adjuvant was not addressed. In the present study, the gene encoding flounder β-defensin was co-inserted with a gene encoding membrane C (OmpC) of *E. tarda* in a DNA vaccine plasmid, in a bicistronic fashion. Following immunization, the regulation of immune response, and the efficacy of the vaccine were evaluated.

2. Materials and Methods

2.1 Fish, cell line, bacteria, and antibodies

Clinically healthy flounders, weighing 200 *g* ± 50 *g* and 35 ± 5 *g* were purchased from a marine farm in Rizhao City of Shandong Province, China. Fish were cultured in aerated seawater tanks at 20 ± 0.5 °C and fed commercial dry feed for one week before immunization. The flounder embryonic cell lines (HINAE) were kindly provided by Ikuo Hirono, University of Tokyo of Marine Science and Technology, Japan and cultured in L15 medium (Thermo, Waltham, MA, USA) [16]. *Edwardsiella tarda* strain (HC01090721) was previously isolated from diseased fish and stored in our laboratory [17]. *E. tarda* was cultured using Luria-Bertani (LB) liquid medium at 28 ℃ for 12 hours. Afterward, the bacterial suspension was adjusted to 1×10^7 cfu/ml with PBS, which was used for the later experiments. The antibodies of OmpC, β-defensin, IgM, CD4-1, and CD4-2 involved in the experiment were previously produced and stored in our laboratory [15, 18, 19].

2.2 Gene cloning and plasmid construction

Total RNA of flounder head kidney was extracted by using the TRIZOL method reported previously [20]. The purity and quality of RNA were assayed by the NanodropND-8000 spectrophotometer (Thermo, Waltham, MA, USA). Later, RNA was reverse transcribed to cDNA according to the instructions of the Hiscript III RT Super Mix kit (Vazyme, Nanjing, China). Then, the cDNA as template was used to amplify β-defensin (GenBank No. OL631146) by using primers fBD-F/fBD-R. Based on the genome sequence of *E. tarda* EIB202 (GenBank No. CP001135.1), the gene sequence encoding OmpC was obtained and the accession number was ETAE3470. The OmpC was amplified by PCR using *E. tarda* suspension as template and primers OmpC-F/OmpC-R. The

sequence of primers used in the experiment are listed in Table 1.

In order to construct recombinant plasmids p-OmpC, the OmpC gene was inserted into the pBudCE4.1 vector at *Xho*I site with Trelief SoSoo Cloning Kit (Tsingke, Qingdao, China). Then, the OmpC gene and the β-defensin gene were inserted into the pBudCE4.1 vector at *Xho*I site and *Bam*HI sequentially to produce the recombinant plasmids p-OmpC-βdefensin. The plasmids p-OmpC, p-OmpC-βdefensin and pBudCE4.1were transformed into *E. coli* DH5α for a large quantity production, then the plasmids were extracted by using the Plasmid Extraction Kit (TIANGEN, Beijing, China) and stored in a 4°C refrigerator for later use.

2.3 Plasmid transfection *in vitro*

The plasmids p-OmpC, p-OmpC-βdefensin and pBudCE4.1 were separately transfected into HINAE cells by using Lipofectamine® 3000 (Thermo Fisher, MA, USA) to investigate expression of the plasmids. The HINAE cells were seeded in 6-well cell culture plates and cultured with L15 medium with 5% fetal bovine serum (FBS) at 22 ºC. The cells were transfected using DNA-liposomes when the cells grew to cover 70%-80% of the culture well area, according to the instruction of the manufacturer. Briefly, 125 μl Opti-MEM was added to tube A and tube B respectively, then 5 μl p3000 and 5000 ng plasmids were added to tube A and 5 μl lipo3000 was added to tube B. After incubation at RT (15 min) , the mixture in tube A was gently mixed with the solution in B. Then, the final solution was transferred to the corresponding 6-well plate (250 μl per well). After incubation for 6 hours at RT, the transfection cocktail was replaced by L15 medium with 5% FBS and incubated further for 48 hours. Finally, the cells were washed three times and fixed with 4% (w/v) paraformaldehyde for subsequent indirect immunofluorescence experiments.

2.3 Vaccination and sampling

Three hundred and sixty flounders weighing 35 ± 5*g* were randomly divided into three groups (120 fish / group). After two weeks of acclimation, the fish was injected intramuscularly with 100 μl （200 ng/μl）pBudCE4.1, p-OmpC, or p-OmpC-βdefensin plasmids, respectively. The muscle tissue at the injection site, spleen, and head kidney samples were obtained at day 7 after immunization for RNA extraction and for histology to image the expression of the plasmids. In addition, the injection sites from three fish per treatment groups was sampled on day 3, 5, 7, 21, and 28 for RNA extraction by using the TRIZOL method for semiquantitative RT-PCR. Head kidney and spleen were sampled

at 0 h, 12 h, 24 h, 3 d, 9 d, and 28 d post-immunization for qRT-PCR and flow cytometry.

2.4 Immunofluorescence

Indirect immunofluorescence was done to detect the expression of OmpC and β-defensin by HINAE cells *in vitro* according to the previous report [21]. In brief, the fixed HINAE cells were washed three times with PBS and added the rabbit anti-OmpC (1:1000) or mouse anti-β-defensin (1:1000) polyclonal antibody. After incubation at 37 ℃ for 1.5 h, the cells were washed three times and incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG or Fluor 648-conjugated goat anti-mouse IgG (1:1000, Sigma, Nawa Prefecture, Japan) as a secondary antibody at 37 ℃ for 1 hour. After that, 4,6-diamidino-2-phenylindole (DAPI, Bio-Legend, Santiago, Chile) was added to the cells and incubated at room temperature for 15 mins. After washing three times with PBS, the cells were assessed using a Zeiss fluorescence microscope (Zeiss, Jena, Germany).

2.5 Histochemical staining

The muscle samples from the injection site obtained from each treatment group 5 days after injection were fixed in 4% (w/v) paraformaldehyde. After 24 hours, the samples were immersed in ethanol solution with concentrations of 50%, 75%, 95%, and 100% for one hour respectively – to remove water, followed by immersion in a 1:1 solution of ethanol and xylene concentration. The samples were finally dehydrated by immersing the specimens in a pure xylene solution. The samples were embedded in paraffin wax and sliced with a rotary microtome (Leica, Wetzlar, Germany). After remove the paraffin by xylene, the sections were rehydrated by sequential immersion in ethanol solutions at concentrations of 100%, 85%, 75%, 65%, and 50% for 5 min, respectively. The sections were immersed in hematoxylin solution for 18 minutes and soaked with acid alcohol for 50 s for color separation. The color-separated sections were sequentially immersed in ethanol solutions at concentrations of 50%, 65%, 75%, 85%, and 95%, respectively, for 5 min and stained for 10 s using an eosin solution. After staining, the sections were transferred to 95% ethanol solution for 45 s and immersed in ethanol and xylene solution for dehydration and transparency. Finally, the sections stained by hematoxylin and eosin (H&E) were sealed and observed by a light microscope (Zeiss, Jena, Germany).

2.6 Real time PCR

Semiquantitative RT-PCR was performed to detect of β-defensin and OmpC gene transcripts at the

injection site using the primersOmpC-F/OmpC-R and fBD-F/fBD-F on day 3, 4, 5, 7, 14, 21, 28 after immunization. The expression of IFN-γ, GATA-3, CD83, MHC I, and MHC II in muscle tissue at the injection site were assessed on day 5 post immunization. The expression of IL6, TNFα, CD4-1, and MHC II were also analyzed in the head kidney and spleen at 0 h, 12 h, 24 h, 3 d, 5 d, 9 d, and 28 d after immunization. Briefly, the total RNA was extracted from the muscle tissue at the injection site, head kidney, and spleen from three fish per treatment groups and reverse-transcribed to cDNA. The qRT-PCR system was configured according to instructions of manufacture (Abclonal, Qingdao, China) with three replicates per fish. 18S rRNA transcripts served as the internal reference. The real-time PCR results were analyzed using the $2^{-\Delta\Delta Ct}$ method. The following is the qRT-PCR reaction steps: 95 °C, 3 min, (95 °C, 5 s → 60 °C, 30 s) × 40 cycles. The primers used in this experiment are shown in Table 1.

2.7 Flow Cytometry

The leukocytes of spleen and head kidney were isolated at week 0, 1, 2, 3, 4, 5, and 6 after immunization following our previous experiment [21]. The mouse monoclonal antibodies against flounder CD4-1, CD4-2, and IgM were added to the leukocytes respectively, and incubated at 22 ℃ for 1.5 hours. After washing three times with PBS, the leukocytes were incubated with Alexa Fluor 488/649-labeled goat-anti-mouse IgG antibody at a ratio of 1:1000 at 22 ℃ for 50 min. After washing three times with PBS, the concentration of cells was adjusted to 1×10^6 for the detection of the CD4-1^+, CD4-2^+, and IgM^+ cells by using flow cytometry (Accuri C6, BD, NJ, USA).

2.8 Challenge

Six weeks after vaccination, 90 flounders were randomly selected from each group (3 replicates, 30 fish in each tank). The solution containing *E. tarda* was adjusted to 1×10^7 cfu/ml in PBS and injected intraperitoneally to fish (100 μL/ fish). Mortality was recorded over 14 days after the challenge and the relative percentage survival (RPS) was calculated.

2.9 Statistical Analysis

Statistical analyses were performed by using Statistical Product and Service Solution (SPSS) 20.0 software (IBM, Armonk, NY, USA) with one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. All data are expressed as the mean ± standard deviation (SD). Statistical parameters and details are provided in the figure legends, and exact *p*-values are shown

in the figures. GraphPad Prism software version 5.01 (GraphPad 265 Software, San Diego, CA, USA) was used for plotting graphs.

3. Results

3.1 *In vitro* expression of OmpC and ß-defensin from the bicistronic plasmid

The OmpC from *E. tarda* and β-defensin from flounder were successfully cloned (Figure S1). The constructed p-OmpC, p-OmpC-βdefensin, and control plasmids were transfected into HINAE cells and analyzed by indirect immunofluorescence. Microscopic examination showed that only green signals (GFP) were detected in the p-OmpC transfection group, but both green and red signals (RFP) were detected in the p-OmpC-βdefensin transfection group. No fluorescence signals were seen in cells transfected by the pBudCE4.1 empty plasmid (Figure 1). The green and red colors represented expression of OmpC and β-defensin proteins, respectively. The above results showed that the plasmid expressed recombinant OmpC and β-defensin successfully in the cells as expected.

3.2 Expression of bicistronic DNA vaccines in flounder muscle

After total RNA extracted from muscle tissues at the injection site on day 3, 4, 5, 7, 14, 21, and 28 after immunization, the transcription of target genes was examined by RT-PCR using the 18s gene as an internal reference. The results showed that transcripts of both OmpC and β-defensin genes were detected in the p-OmpC-βdefensin plasmid-injected group within two weeks after immunization, and transcripts of the OmpC gene were detected in the p-OmpC plasmid-injected group three weeks after immunization. In contrast, the pBudCE4.1 empty plasmid injection group did not detect transcripts of OmpC and β-defensin genes (Figure 2).

3.3 Infiltration of inflammatory cells and expression of immune genes at the injection sites

The results of H&E staining of muscle tissues showed that the injection site of the p-OmpC-βdefensin plasmid injected fish had significant infiltration of inflammatory cells. The number of inflammatory cells in the injection sites of the p-OmpC plasmid injected fish, in general, were less compared to fish injected with the p-OmpC-βdefensin plasmid. After injection of pBudCE4.1 (empty plasmid), there was minor infiltration of inflammatory cells (Figure 3).

The results of qRT-PCR showed that the expression of GATA-3, CD83, and MHC I genes in fish from the p-OmpC-βdefensin group was significantly higher compared to the expression levels in

the fish that received the p-OmpC plasmid and the pBudCE4.1 plasmid. The expression of IFN-γ and MHC II were not different between the p-OmpC-βdefensin and the p-OmpC treatments, but both were slightly higher than the corresponding expressions in the pBudCE4.1 group (Figure 4).

3.4 The dynamics of immune gene expression in the spleen and head kidney after immunization

To compare the immune response in fish belonging to the three treatment groups, assessment of IL6, TNFα, CD4-1, and MHC II expression in spleen and head kidney were examined by qRT-PCR at different time points after vaccination. In spleen, the expression of the TNFα, IL-6, MHC II, CD4-1, peaked at 12 h, 24 h, 5 d, 9 d post-immunization, respectively (Figure 5). Compared to the p-OmpC plasmid treatment group, the expression levels of all tested genes in p-OmpC-βdefensin vaccine groups were increased at different levels at various time points (p<0.05). Similarly, the relative expression of IL6 and MHC II genes in head kidney reached their highest level at 24 h and 5 d after immunization, respectively, and the peak level in the p-OmpC-βdefensin group were higher than corresponding gene expression levels of the p-OmpC group (Figure 5). The highest TNFα and CD4-1 expressions were found in fish at 12 h and on day 9 in fish from the p-OmpC-βdefensin and p-OmpC plasmid groups. The expression levels of TNFα and CD4-1 in p-OmpC-βdefensin vaccine groups were higher than in control fish at all time points.

3.5 The proportions of T and B lymphocyte subsets changes in spleen and head kidney

Changes in the proportion of T and B lymphocyte subsets were analyzed by flow cytometry. The result showed that the proportion of CD4-1$^+$ and CD4-2$^+$ T lymphocytes from spleen in the p-OmpC-βdefensin plasmid and p-OmpC plasmid injected groups gradually increased from one week after immunization, peaked at the second week and leveled off after four weeks (Figure 6). The proportion of CD4-1$^+$ and CD4-2$^+$ T lymphocytes in fish from the p-OmpC-βdefensin group (20.3% and 22.2%) was significantly higher than those in the p-OmpC group (15.2% and 16.9%) and pBudCE4.1 group (8.4% and 11.4%) at week 2. The percentage of splenic IgM$^+$ B lymphocytes in the p-OmpC-βdefensin and p-OmpC groups gradually increased one week after immunization and peaked on week 6 and thereafter declined. The percentage of IgM$^+$ B lymphocytes at week 6 was significantly higher in spleens from fish in the p-OmpC-βdefensin group (24.9%) compared to spleen in fish from the p-OmpC (18.4%) and the pBudCE4.1 group (10.8%).

Such change with respect to the T and B lymphocytes ratio in the head kidney was similar to the spleen. The proportions of CD4-1$^+$ and CD4-2$^+$ T lymphocytes and IgM$^+$ B lymphocytes from head kidney cells in the p-OmpC-βdefensin and the p-OmpC group all showed an increasing trend followed by a decrease. Meanwhile, the percentage of CD4-1$^+$ and CD4-2$^+$ T head kidney lymphocytes and IgM$^+$ B lymphocytes in fish from the p-OmpC-βdefensin injected group at the peak time points was significantly higher than the p-OmpC and pBudCE4.1 empty plasmid injection group (Figure 7).

3.6 Vaccine efficacy following experimental challenge with *E. tarda*

The mortality rates in pBudCE4.1, p-OmpC and p-OmpC-βdefensin treatment groups within two weeks after the challenge were 100%, 52.67% and 25.83%, respectively. Fish in the pBudCE4.1 injection group all died on day 9 after the challenge. The RPS in the p-OmpC injection group was 47.33%, and in the p-OmpC-β-defensin group was 74.17% (Figure 8).

4. Discussion

DNA vaccines are effective to induce both antibacterial and antiviral response and protection, by the induction of humoral and cellular immunity [22]. Outer membrane proteins of bacteria are essential components for functions such as nutrient uptake, cell adhesion, and cell signaling. OMPs from *Vibrio anguillarum*, *Vibrio harveyi*, and *Edwardsiella tabaci* have already been shown to have potential as DNA vaccine or subunit candidates [23-25]. OmpC of *E. tarda* is a surface protein involved in bacterial invasion and infection. Our previous studies have demonstrated that immunization of flounder with recombinantly expressed OmpC protein from *E. tarda* induced humoral immunity [17].

A targeted approach to find an optimal molecular adjuvant is needed to increase DNA vaccine efficacy, especially if the DNA vaccine itself shows poor efficacy [26]. Antimicrobial peptides, such as defensins, may harbor attractive features to be included as a molecular adjuvant in DNA vaccines targeting bacterial diseases. Defensins are one of the antimicrobial peptides well conserved during evolution, as proposed by Ganz et al. who first isolated defensin from human neutrophils [12]. As antimicrobial and immunomodulatory peptides, defensins have previously been shown to adjuvant vaccines. Zhang et al. showed that β-defensin-1 cloned from chickens possessed adjuvant activity

as co-inserted in the plasmid vaccine together with the IBDV VP2 gene. It was shown that, following immunization, that the proportion of immune cells in peripheral blood increased [27]. In the present study, we constructed a p-OmpC-βdefensin plasmid containing both OmpC and β-defensin genes at two independent coding regions of pBudCE4.1. After being transfected by the plasmid, HINAE cells expressed both the OmpC and β-defensin proteins. Similar results were encountered after intramuscular injection of the plasmid, where the muscle cells expressed both the OmpC and β-defensin. The vaccine resulted in significant recruitment of cells to the injection site and increased immune gene expression. The vaccination strategy from OmpC and β-defensin genes as co-inserted in a DNA plasmid backbone yielded a higher relative protection rate compared to the DNA vaccine that expressed only OmpC. These findings strongly suggested that β-defensin as adjuvant enhanced the vaccine efficacy of the present DNA against *E. tarda*.

Defensins play important roles in a variety of immune processes. It is reported that defensins secreted by human neutrophils have the ability of increase chemotaxis of monocytes [28]. Our previous findings also confirmed that flounder β-defensins were indeed capable to recruit inflammatory cells [15]. In this study, the inflammatory cell infiltration in the injection site of fish given the p-OmpC-βdefensin, was notable on day 5 after immunization. The samples of p-OmpC or pBudCE4.1 plasmid injected group showed a lower number of inflammatory cells in injection area. The extra recruitment of immune cells was likely due to the chemotactic properties by the defensin. A prerequisite of DNA vaccine efficacy, caused by both humoral and cellular responses, is that target genes in the plasmid of DNA vaccine are transcribed into proteins/peptides in host cells [29, 30]. In the present study where gene expression was assessed in the injection site, the high expression of the gene involved in T cell differentiation (GATA-3) and markers of antigen presenting cells, such as CD83 and MHC I suggested an increased expression and/or increased presence of cells. H&E stain sections of the muscle tissue at the injection site strengthened this suggestion. TNFα is an inflammatory cytokine produced by macrophages/monocytes during inflammation and is involved in many immune pathways including regulation of CD4 T cells, and MHC class I and II expression [31-33]. The high expression of TNFα, CD4-1, MHC I and MHC II in fish from the p-OmpC-βdefensin group suggested a robust immune response. Furthermore, the spleen and head kidney expression of IL-6 was consistently and significantly higher in the p-OmpC-βdefensin group than

in the p-OmpC group. It is reported that IL-6 in mammals, is promptly and transiently produced in response to infections and vaccination and contributes to host defense through the stimulation of acute phase responses and immune reactions [34]. In mammals, it has been shown that β-defensin can adjuvant Hepatitis B vaccine by enhancing the expression of IL-6 [36]. In grouper, β-defensin increased the expression of MHC II in the head kidney as an adjuvant [35]. Similarly, a significant co-increase of IL-6 and MHC II was observed in head kidney and spleen of flounder after vaccination, and an overexpression of β-defensin induced up-regulated the expression of IL-6 and MHC II.

In contrast to higher animals, some fish species express two CD4 subtypes, namely CD4-1 and CD4-2 [37]. In this study, an increased expression of CD4-1, CD4-2 and IgM suggested the presence of a higher number of transcripts (or higher number of T and B cells) which in turn may be translated to increased adaptive immune capacity. The ratios of $CD4\text{-}1^{+}$, $CD4\text{-}2^{+}$, and IgM^{+} in the spleen and head kidney of the p-OmpC-βdefensin group were significantly higher compared to fish given p-OmpC group. This strongly suggests that β-defensin as vaccine adjuvant is efficient to induce both T-cell and B-cell response, which likely increased the vaccine efficacy. Furthermore, the RPS for vaccine was a visual indicator of efficacy and the gap between the p-OmpC-βdefensin and p-OmpC groups also showed adjuvant function of β-defensin.

5. Conclusion

β-defensin, being both an antimicrobial peptide and a chemokine, has also a function of chemoattractant for inflammatory cells, is an attractive candidate as a vaccine adjuvant. In this study, we confirmed the adjuvant property of β-defensin possibly by activating both cellular and humoral defense mechanisms. In a future study, the contribution of the antibody response will be included, to unveil the importance of antibody-mediated protection compared to protection caused by cellular immune response.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CRediT authorship contribution statement

Heng Chi: Conceptualization, Statistical analyses, Writing - original draft, Funding acquisition. Qingqing Qin: Methodology, Statistical analyses, Writing - original draft. Xiaokai Hao: Methodology, Statistical analyses. Roy Ambli Dalmo: Writing - review & editing to the final version. Xiaoqian Tang: Writing - review & editing, Supervision. Jing Xing: Writing - review & editing, Supervision. Xiuzhen Sheng: Writing - review & editing, Supervision. Wenbin Zhan: Writing - review & editing, Supervision.

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Figure legends

Table 1. List of primers used in this study.

Figure 1. Detection of β-defensin and OmpC expression in transfected HINAE cells. Indirect immunofluorescence of HINAE cells after plasmid transfected by p-OmpC, p-OmpC-βdefensin and pBudCE4.1. Blue indicates cell nuclei, green indicates OmpC, and red indicates β-defensin. Bar = 20 μm.

Figure 2. RT-PCR detection of β-defensin and OmpC gene transcripts in muscle tissue. (A) p-OmpC-βdefensin immune group; (B) p-OmpC immune group; (C) pBudCE4.1 immune group. Lane M, markers; lane 1, 2, 3, 4, 5, 6, 7, muscle tissue samples on day 3, 4, 5, 7, 14, 21, 28 after immunization. 18S rRNA transcripts as an internal reference.

Figure 3. Injection of recombinant plasmids leads to local recruitment of inflammatory cells by using histology analysis. Injection site muscle sections were obtained on day 5 after immunization of flounder with p-OmpC-βdefensin, p-OmpC, and pBudCE4.1, respectively, and were stained with hematoxylin and eosin. Bar = 40 μm.

Figure 4. Expression of immune-related genes at the injection site. Expression of immune-related genes at the injection site on the 5 days after immunization. The vertical bar represents the mean ± SD (n=3). Different letters (a, b, and c) above the bars represent the statistical significance ($p < 0.05$) among different groups of the same gene.

Figure 5. The expression of immune-related genes induced by recombinant plasmids in spleen and head kidney. Expression of immune-related genes in the spleen and head kidney at 0, 12, and 24 hours, 3, 5, 9, and 28 days after immunization were tested by qPCR. The mRNA level of each gene was normalized to that of 18S rRNA. The y-axis represents the mRNA abundance relative to the expression in fish injected with the pBudCE4.1 at 0 h. Different letters (a, b, and c) on the bars indicate statistically significant differences ($p < 0.05$) compared to the same gene in each group at same time, and the vertical bar represents the mean ± SD (n=3).

Figure 6. Dynamic changes in the ratio of T and B lymphocyte subpopulations in the spleen. Flow cytometry results of CD4-1$^+$, CD4-2$^+$ T lymphocytes, and IgM$^+$ B lymphocytes of the spleen in the 5 weeks after immunization. As well as, the proportion of CD4-1$^+$, CD4-2$^+$ T lymphocytes, and IgM$^+$ B lymphocytes in the spleen at 0, 1, 2, 3, 4, 5, and 6 weeks after immunization. The vertical bar represents the mean ± SD (n=3)and different letters (a, b, and c) above the bars represent the statistical significance ($p < 0.05$) among different groups at the same time point.

Figure 7. Dynamic changes in the ratio of T and B lymphocyte subpopulations in the head kidney. Flow cytometry results of CD4-1$^+$, CD4-2$^+$ T lymphocytes, and IgM$^+$ B lymphocytes in the 5 weeks of the head kidney after immunization. As well as, the proportion of CD4-1$^+$, CD4-2$^+$ T lymphocytes, and IgM$^+$ B lymphocytes in the head kidney at 0, 1, 2, 3, 4, 5, and 6 weeks after immunization. The vertical bar represents the mean ± SD (n=3) and different letters (a, b, and c) above the bars represent the statistical significance ($p < 0.05$).

Figure 8. Immune protection rate of each group post-challenge with live *E. tarda*. The mortality of each injection group of flounder within two weeks after the challenge was counted and the immune protection rate of each injection group was calculated. RPS represents the relative protection rate. The bar represents the mean ± SD (n=3). *** indicates levels of significance of $p<0.001$.

491

492 **Table 1**

Primer name	Primer sequence	use
OmpC-F	CGAGCTCATGATGAATAAAATCC(XhoI)	Plasmid construction
OmpC-R	CCGCTCGAGTTAGAACTTATAGTTCA(Xho I)	Plasmid construction
fBD-F	CGGGATCCATGTCTCGTTATCGTGTGGCT(BamH I)	Plasmid construction
fBD-R	CCCTCGAGTTATGGTTTGGTTACGCAACATA(BamH I)	Plasmid construction
18S-F	GGTCTGTGATGCCCTTAGATGTC	qRT-PCR
18S-R	AGTGGGGTTCAGCGGGTTAC	qRT-PCR
IFN-γ-F	TGTCAGGTCAGAGGATCACACAT	qRT-PCR
IFN-γ-R	GCAGGAGGTTCTGGATGGATGGTTT	qRT-PCR
GATA-3-F	CAGGAGGACAAAGAGTGCATAAAGT	qRT-PCR
GATA-3R	GAAGATGACCCACCTATCAGGCTAC	qRT-PCR
CD83-F	CCCAACGGCACGACGCTACAC	qRT-PCR
CD83-R	CCCAAAGGTGCTGCCAGGTGA	qRT-PCR
MHCI-F	AGACCACAGGCTGTTATCACCA	qRT-PCR
MHCI-R	TCTTCCCATGCTCCACGAA	qRT-PCR
MHCII-F	ACAGGGACGGAACTTATCAACG	qRT-PCR
MHCII-R	TCATCGGACTGGAGGGAGG	qRT-PCR
IL6-F	CTCCGCAATGGGAAGGTG	qRT-PCR
PIL6-R	AGTGGATGGGTGGAATAA	qRT-PCR
TNFα-F	GTCCTGGCGTTTTCTTGGTA	qRT-PCR
TNFα-R	CTTGGCTCTGCTGCTGATTT	qRT-PCR
CD4-1-F	CCAGTGGTCCCCACCTAAAA	qRT-PCR
CD4-1-R	CACTTCTGGGACGGTGAGATG	qRT-PCR

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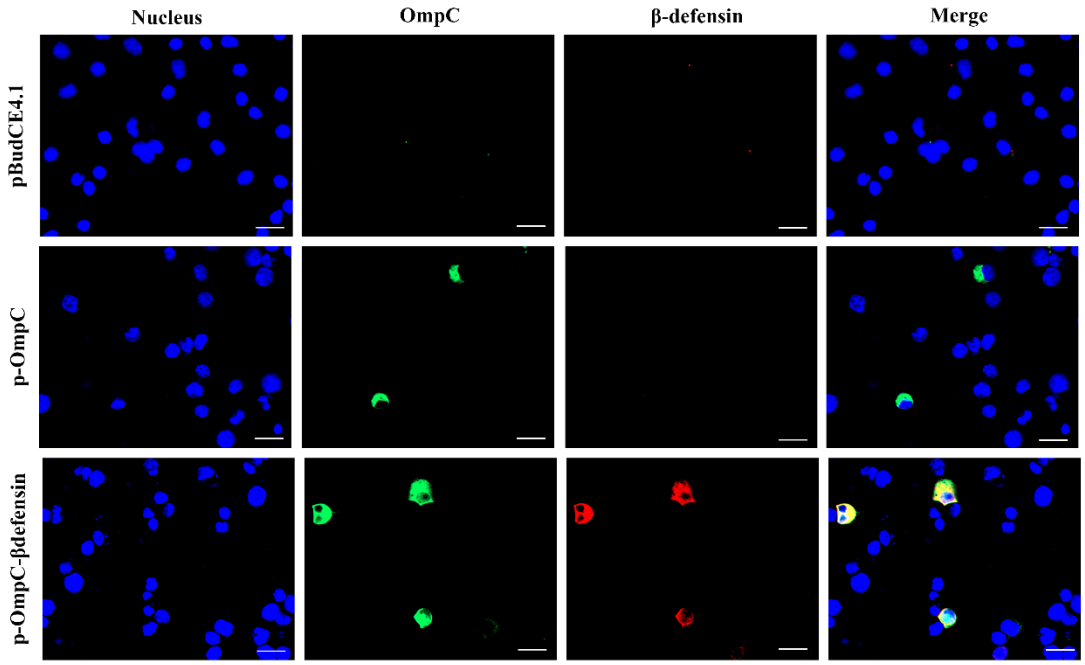
Figure 1


Nucleus
OmpC
β-defensin
Merge
pBudCE4.1
p-OmpC
p-OmpC-βdefensin

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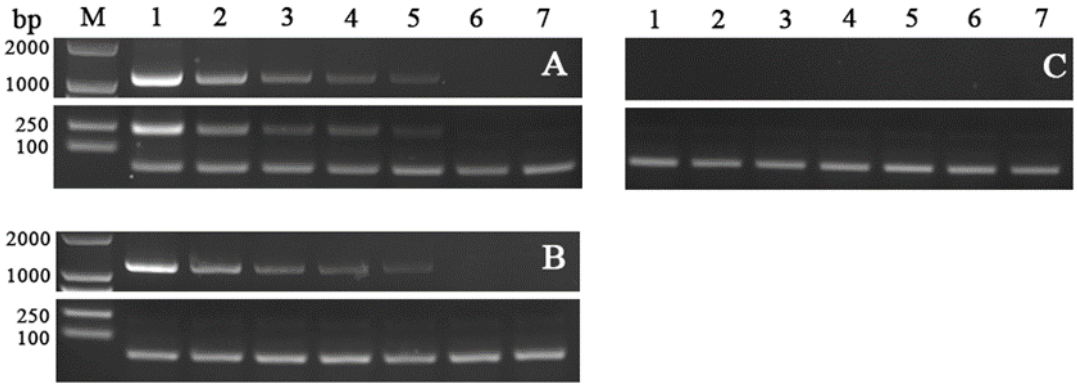
499 Figure 2



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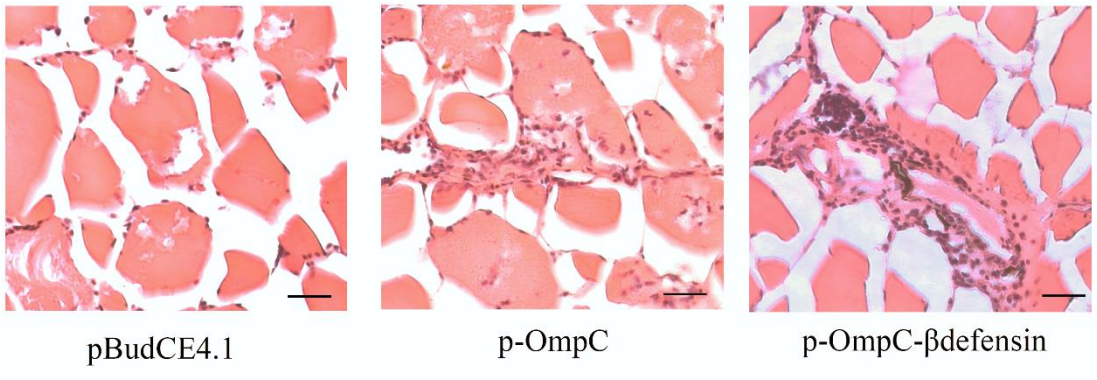

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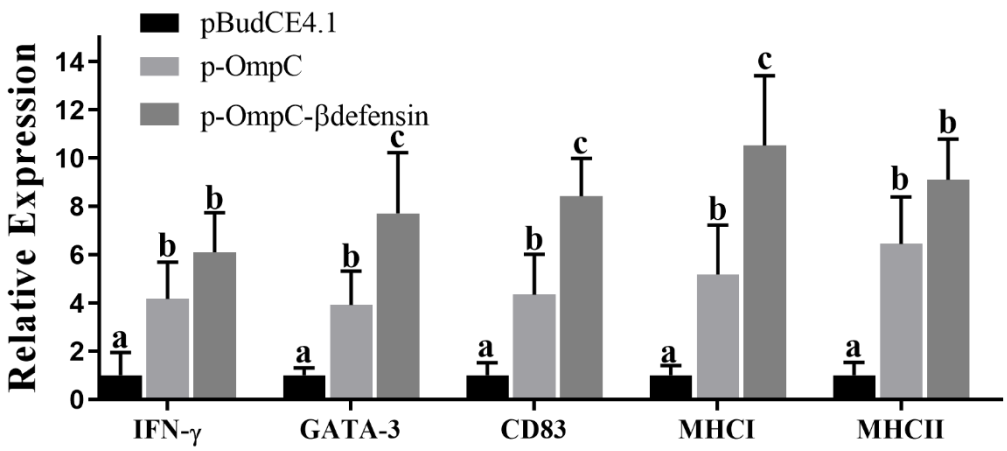
Figure 4


pBudCE4.1
p-OmpC
p-OmpC-βdefensin
Relative Expression
0
2
4
6
8
10
12
14
a
b
b
a
b
c
a
b
c
a
b
c
a
b
b
IFN-γ
GATA-3
CD83
MHCI
MHCII

515 Figure 5

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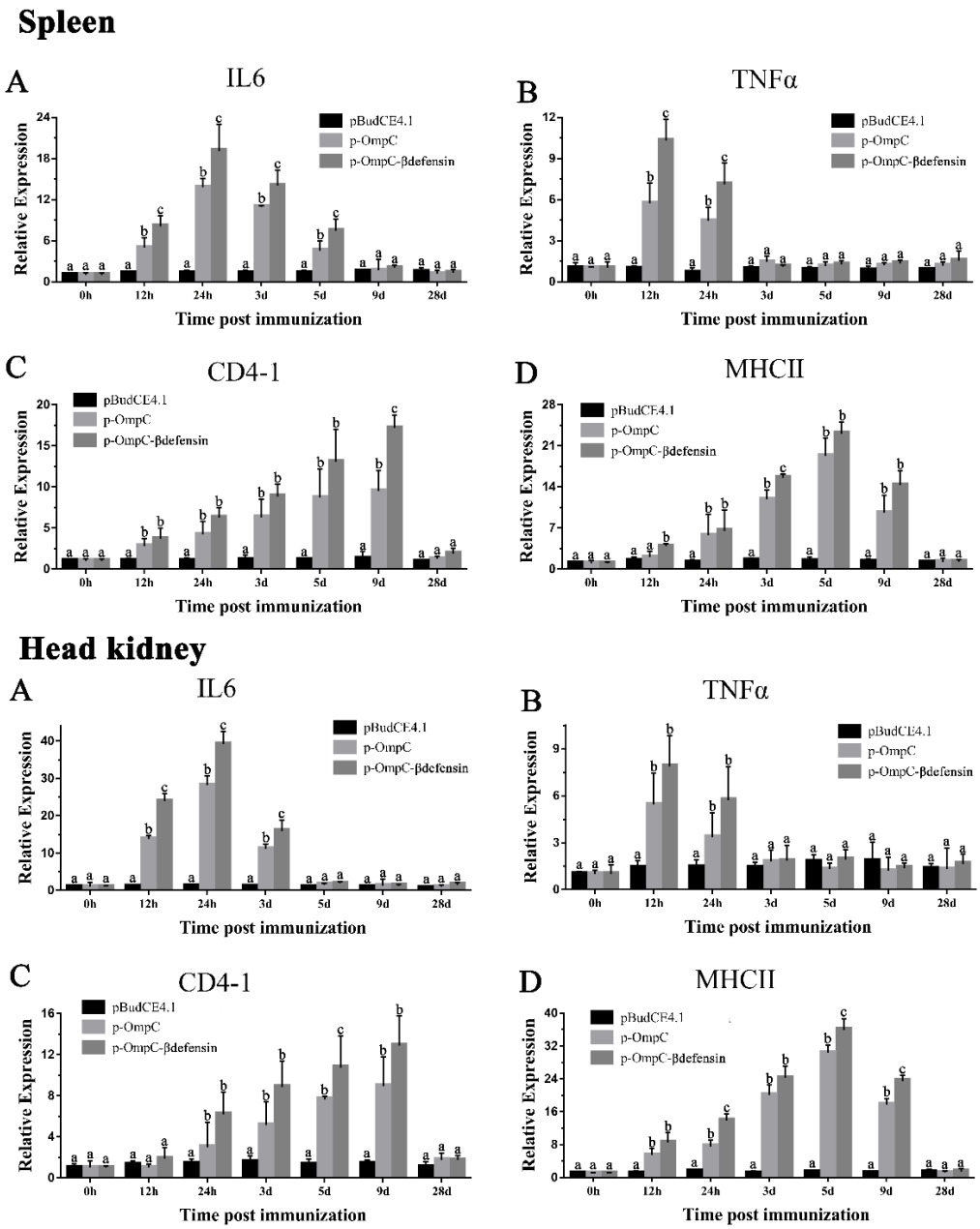
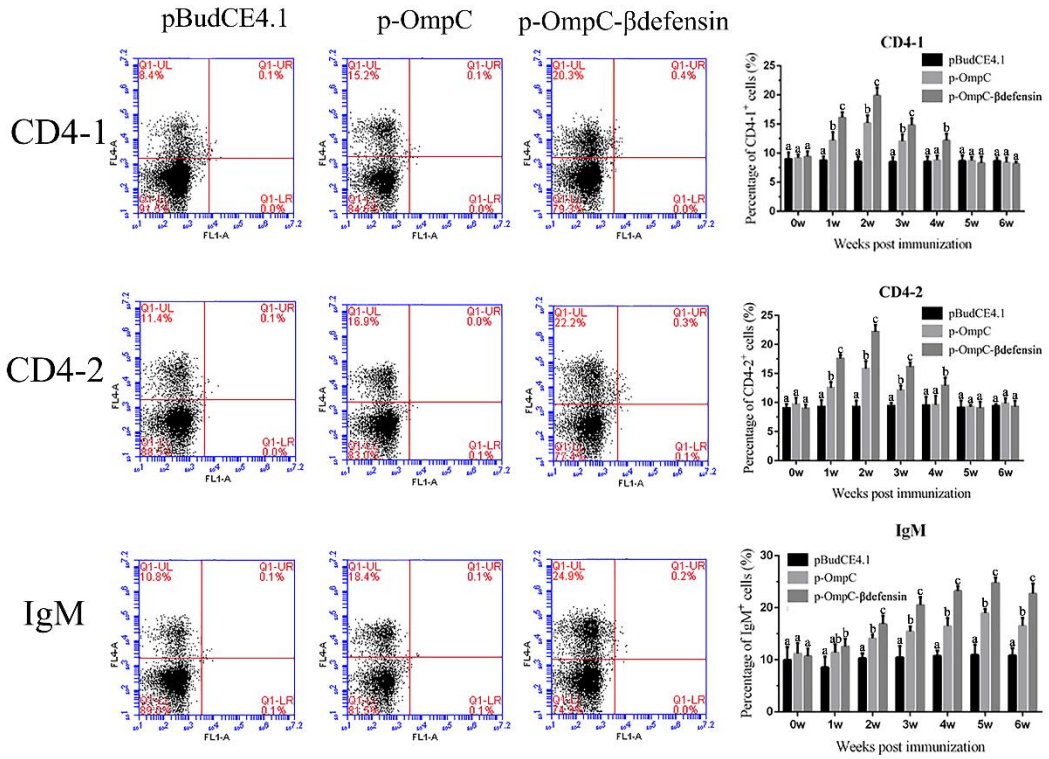
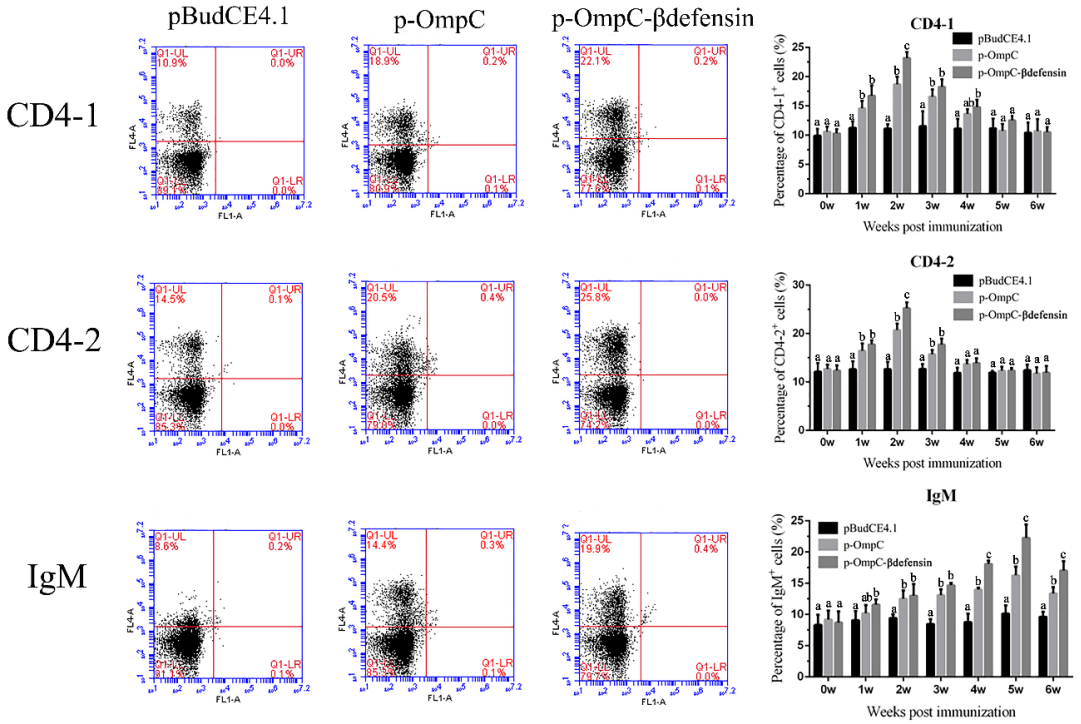


Figure 6


pBudCE4.1
p-OmpC
p-OmpC-βdefensin
CD4-1
CD4-2
IgM
Q1-UL
Q1-UR
Q1-LR
FL4-A
FL1-A
CD4-1
CD4-2
IgM
pBudCE4.1
p-OmpC
p-OmpC-βdefensin
Percentage of CD4-1+ cells (%)
Percentage of CD4-2+ cells (%)
Percentage of IgM+ cells (%)
0w
1w
2w
3w
4w
5w
6w
Weeks post immunization

532

533 Figure 7



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537 Figure 8

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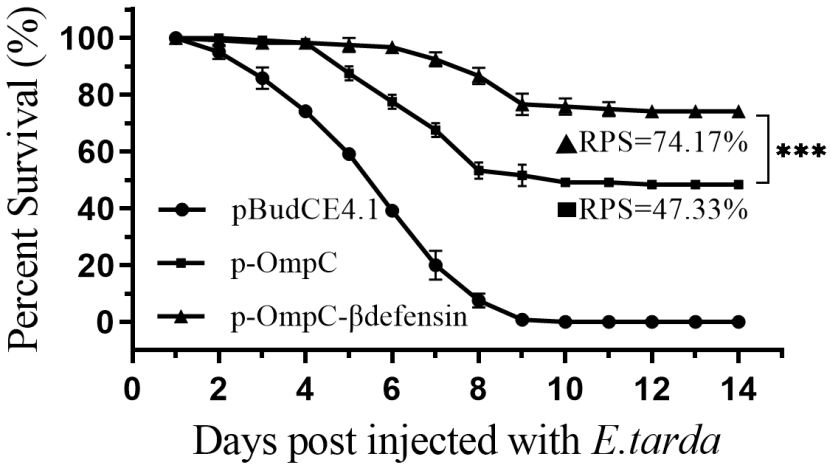

Highlights:

1. β -defensin is an attractive candidate as a DNA vaccine adjuvant.
2. β-defensin enhanced local immunity at the DNA vaccine injected site.
3. β-defensin assisted the DNA vaccine to stimulate more intense adaptive immunity.

Figure S1

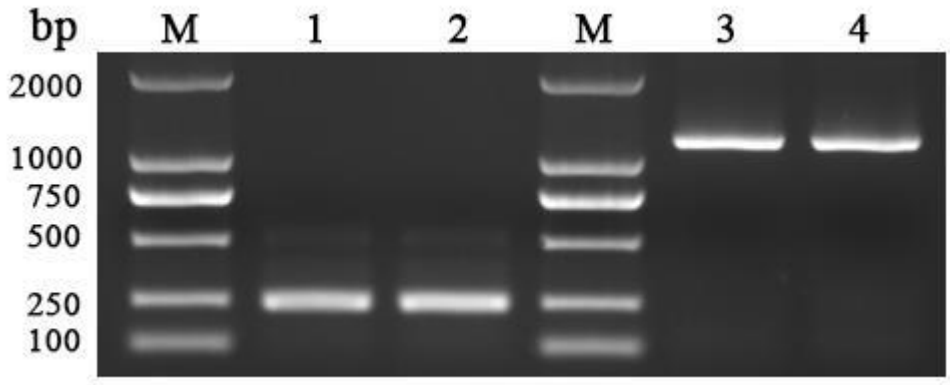

Figure S1. Agarose electrophoresis analysis of PCR products of β-defensin in flounder and OmpC of *Edwardsiella tarda*. Lane M, DNA marker; lane 1and 2, PCR products of β-defensin; lane 3 and 4, PCR products of OmpC.