The protection of CpG ODNs and Yarrowia lipolytica harboring VP28 for shrimp Litopenaeus vannamei against White spot syndrome virus infection

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RESEARCH REPORT

The protection of CpG ODNs and *Yarrowia lipolytica* harboring VP28 for shrimp *Litopenaeus vannamei* against White spot syndrome virus infection

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Abstract

The white spot syndrome is one of the most serious disease which has caused high mortalities and huge economic losses to shrimp culture. In the present study, the oral administrations with CpG ODNs and *Yarrowia lipolytica* harboring VP28 (rVP28-yl) as dietary supplement for shrimp *Litopenaeus vannamei* were conducted to evaluate their protective effects against WSSV. After feeding for 15 days, the cumulative mortality and the copy number of WSSV in CpG and rVP28-yl feeding shrimps were significantly lower when they were challenged by WSSV, compared with those in control shrimps ($p < 0.05$). The caspase-3 activity was suppressed in rVP28-yl feeding shrimps but ascended in CpG feeding shrimps after WSSV challenge. Besides, the PO activity in CpG feeding shrimps was significantly increased after feeding trial, and kept increasing post WSSV challenge ($p < 0.05$). While the increased NO production was observed both in CpG and rVP28-yl feeding shrimps after feeding trial and WSSV challenge. In addition, increased mRNA expression levels of STAT and Dicer were observed in CpG group post WSSV challenge. These results together indicated that oral feeding of CpG ODNs and rVP28-yl could enhance the innate non-specific immune responses especially antiviral immunity of shrimps in varying degrees, and increase their resistance against WSSV infection.

Key Words: CpG ODNs; *Yarrowia lipolytica* surface-display VP28; White spot syndrome virus; *Litopenaeus vannamei*; disease resistance; antiviral immunity

Introduction

White spot syndrome virus (WSSV) is one of the most hampered pathogens in shrimp culture, which has caused severe disease, leading to significant economic losses (Johnson *et al.*, 2008; Haq *et al.*, 2012). Owing to the potential deteriorative environmental effects, some traditional medication such as the antibiotic and prophylactic chemicals have been gradually abandoned in application, and the enhancement of immunity of shrimp has been becoming the most promising strategy for disease control (Li and Xiang, 2013a). The performances of immunostimulants and vaccines have gained momentum by virtue of their potential use in inducing the immune response and reducing the disease impact on crustaceans (Hauton, 2012).

CpG oligodeoxynucleotides (CpG ODNs), also called bacterial DNA or synthetic oligodeoxynucleotides, have been proven to trigger innate immune responses in many animal species, and they are always employed as the well-known vaccine adjuvant in mammals (Krieg, 2002). In mammals, CpG can be recognized by Toll-like receptor 9 (TLR9) to trigger the signaling pathways, and in turn activate several transcription factors including stress kinase and NF-κB (Sparwasser *et al.*, 1998; Choudhury *et al.*, 2002). Meanwhile, the proliferation of B lymphocytes and immune responses are subsequently induced, and the immunological events occur after CpG activation of immune system include increased antiviral immunity (Krieg, 2002). In shrimps, it has been reported that CpG induce various innate immune responses and can be used in the control of virus disease for its immunostimulating properties

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Table 1 Sequences of primers used in the present study

Primer	Sequence(5'-3')	Sequence information
WSSV-F(forward)	CGCCTACCCTGTTGAATCTG	WSSVvirus detection
WSSV-R(reverse)	TTTAGTGTGTGGTCTCCGTCTC	WSSVvirus detection
WSSV-RT-F (forward)	CCAGTTCAGAATCGGACGTT	Real-time PCR for WSSV copies
WSSV-RT-R(reverse)	AAAGACGCCTACCCTGTTGA	Real-time PCR for WSSV copies
WSSV-Taqman (probe)	TCCATAGTTCCTGGTTTGTAATGTGCCG	Real-time PCR for WSSV copies
Oligo(dT)-adaptor	GGCCACGCGTCGACTAGTAC(G)17	The first strand cDNA synthesis
VP28-1F (forward)	ACCACCATGGATCTTTCTTTC	Surface display of VP28
VP28-1R (reverse)	TTACTCGGTCTCAGTGCCA	Surface display of VP28
VP28-2F (forward)	ACCACCATGGATCTTTCTTTC	Prokaryotic recombinant of VP28
VP28-2R (reverse)	TTACTCGGTCTCAGTGCCA	Prokaryotic recombinant of VP28
STAT-F (forward)	AGCCCCTGTCTGAGCGAAA	Real-time PCR for STAT gene
STAT-R (reverse)	GGTGTTCTCTTGTAACCTTCATCA	Real-time PCR for STAT gene
Dicer-F (forward)	CCGGAGATAGAACGGTTCAGTG	Real-time PCR for Dicer gene
Dicer-R (reverse)	CGATAATTCCTCCCAACACCTG	Real-time PCR for Dicer gene
LGBP-F(forward)	GGTAACCAGTACGGAGGAACGA	Real-time PCR for LGBP gene
LGBP-R (reverse)	TACTCGACGTGGGTCTTCTCGA	Real-time PCR for LGBP gene
EF-α(forward)	CATCAAGGAGAAACTGTGCT	Real-time PCR of internal control
EF-α(reverse)	GATGGAGTTGTAGGTGGTCT	Real-time PCR of internal control

(Chang *et al.*, 2003; Zhang *et al.*, 2010). For instance, the ROS production, apoptosis and phagocytosis level of shrimp hemocytes increased after they were incubated with CpG ODNs (Sun *et al.*, 2013a). The respiratory burst level and phenoloxidase activity of *Macrobrachium rosenbergii* hemocytes were enhanced after CpG ODNs treatment (Chuo *et al.*, 2005; Sung *et al.*, 2009). And it was also reported that stimulation of CpG ODNs could induce the expression of antiviral associated genes in *L. vannamei.* The copy number of WSSV in those shrimps pre-injected with CpG ODNs was lower than that in untreated group after WSSV challenge, and the survival rate in CpG pre-injected shrimps was significantly higher than that of the control (Zhang *et al.*, 2010). The accumulating evidences suggested that CpG ODN might be one eligible candidate immunostimulant to endow shrimps resistance via enhancing the non-specific immune response against virus infection. However, the underlying mechanism of dietary CpG ODNs on antiviral immune responses and disease resistance of shrimp is still not well understood.

Recently, there are accumulating reports that the application of inactivated pathogens or protein compounds derived from pathogen by means of "vaccination" was effective for disease control in crustaceans, which provided promising strategies (Kurtz and Armitage, 2006; Cong *et al.*, 2008). VP28 is a main envelope protein of WSSV acting as an important viral attachment protein for the virus to enter into the cell of shrimp (Yi *et al.*, 2004). It can interact with some host cellular receptors, such as the small GTPase (Rab7), heat-shock cognate protein 70 (Hsc70) and signal transducers and activators of transcription protein (STAT) to initiate the virus infection (Sritunyalucksana *et al.*, 2006; Liu *et al.*, 2007; Xu *et al.*, 2009). It has also been documented that VP28 is one major vaccine candidate to exert immune protective effects against WSSV infection in shrimp (Witteveldt *et al.*, 2004; Fu *et al.*, 2008; Syed and Kwang, 2011). For instance, the shrimp and crayfish vaccinated with VP28 protein showed significantly lower mortality after WSSV challenge (Witteveldt *et al.*, 2004). Because of the notable vaccine effect of VP28 against WSSV infection in shrimp, several routes and vehicles have been developed, such as the direct injection of VP28 protein, oral delivery of VP28 DNA vaccine , and prokaryotes carrying with VP28 recombinant protein(Witteveldt *et al.*, 2004; Syed and Kwang, 2011; Du *et al.*, 2013). The yeast *Yarrowia lipolytica* is one of the most attractive microorganisms for the expression of foreign genes (Madzak *et al.*, 2004), which has excellent properties compared with bacterial expression system, such as naturally secretion of high amount of proteins on the surface, lack of pathogenicity and immunological properties served as a probiotic candidate (Yue *et al.*, 2008).

In the present study, a 15 days oral administration was implemented in shrimp *L. vannamei*,

Table 2 Ingredient formulation of feeding diets. (Units: g contained in 1 kg diet)

Ingredients	CpG supplemental diet	rVP28-yl supplemental diet	Basal diet
Fish meal	300	300	300
Shrimp meal	150	150	150
Soybean meal	150	150	150
Peanut meal	50	50	50
Dextrin	20	20	20
Wheat flour	150	150	150
Oil mixture	50	50	50
Vitamin mixture	10	10	10
Lecithin	20	20	20
Chitosan	50	50	50
Sodium alginate	50	50	50
CpG powder	0.04	-	-
rVP28-yl powder	-	10^{11}cfu	-

which fed with CpG ODNs and *Y. lipolytica* surface-display VP28 supplemental diets. Some immune parameters, the expression of antiviral associated genes were measured at the end of feeding trial in shrimps as well as post WSSV challenge. WSSV copy number and the mortality were determined after the shrimps challenged by WSSV. They were contributed to evaluate the immunostimulatory effects of CpG ODNs and *Y. lipolytica*-VP28 on the innate immune response and disease resistance of *L. vannamei*, as well as to provide valuable references for their further application in shrimp aquaculture industry.

Materials and Methods

Shrimp rearing

Healthy shrimp L. vannamei, approximately 15 cm in length and 20 g in weight, were collected from a local farm in Tianjin, China, and acclimated at 20 ± 2 °C for 7 days before process. During the culture period, every 20 individuals were kept in one container, and the seawater was changed 60 % daily. From the experimental animals, gills of five shrimps in each group were randomly sampled to examine the presence WSSV in vivo by PCR with specific WSSV primers WSSV-F and WSSV-R (Table 1) (Yoganandhan *et al.*, 2003). Only healthy individuals were used in the following experiment.

WSSV and in vivo titration

WSSV virus stocks were purified from gill tissues of WSSV infected shrimps via the method of differential centrifugation described by Xu et al (Xu *et al.*, 2007). The copy number of WSSV stock was quantified by the Real-time PCR, and the stock solutions were diluted with PBS (0.1 M, pH 7.4) to a final concentration of 108 copies mL-1 and stored at −80 °C. To obtain the desired challenge pressure of WSSV (LD50), in vivo titration experiment with serial dilutions of WSSV stock was conducted according to the procedure described by previous report (Fu *et al.*, 2008). Shrimps were challenged by an injection with different WSSV dilutions, and cultured for addition 10 days. The dead shrimps were recorded and tested by PCR reaction for the presence of WSSV. For the determination of desired challenge pressure, the cumulative dead shrimp were recorded to calculate the relationship between WSSV dose and shrimp mortality. The median lethal dose was used in the following challenge experiments.

CpG ODN large-scale preparation

Five ODNs which were proved to be effective in mammals and aquatic animals were constructed in series into pUC57 vector in our laboratory (Zhang *et al.*, 2010). The pUC57-CpG was transformed into *Escherichia coli* for following fermentation in LB medium (Tryptone 10 g L^{-1}, Yeast extract 5 g L^{-1}, NaCl 10 g L^{-1}) under the ampicillin selective pressure. The large-scale plasmid extraction was performed following the previous report (Holmes and Quigley, 1981). The plasmids were dissolved in 0.1 M phosphate buffer saline buffer (PBS, pH = 7.4), heated at 100 °C for 10 min and immediately cooled in ice-water mixture. The linear CpG ODNs was quantitated and stored in PBS buffer until use in -20 °C.

Generation of recombinant VP28

VP28 was expressed in *E. coli* system following the method described by previous reports (Witteveldt *et al.*, 2004). The strain *Y. lipolytica* Po1h and the vector pINA 1317 were kindly supplied by CBAI, AgroParisTech, 78850 Thiverval-Grignon, France. VP28 was ligated into the vector pINA 1317 and then transformed into *Y. lipolytica* Po1h by lithium acetate

Table 3 The scheme sampling experiments

Samplingtime points	Group set	CpG ODNs	rVP28-yl	Basal diet (control)
	No. of shrimp	20(×6) 20(×6) 20(×6)	20(×6) 20(×6) 20(×6)	20(×6) 20(×6) 20(×6)
Before oral administration	No challenge			10
Post oral administration	WSSV challenge	20(×3)	20(×3)	20(×3)
	PBS challenge	20(×3)	20(×3)	20(×3)
On the first daypost-stimulation	WSSV challenge	20(×3)	20(×3)	20(×3)
	PBS challenge	20(×3)	20(×3)	20(×3)
On the third daypost-stimulation	WSSV challenge	20(×3)	20(×3)	20(×3)
	PBS challenge	20(×3)	20(×3)	20(×3)

method (Xuan *et al.*, 1988). VP28 displayed on *Y. lipolytica* and expressed in *E. coli* were designated as rVP28-yl and rVP28-ec, respectively. The primers for amplification of the full length ORF of VP28 were presented in Table 1. The rVP28-yl was induced in the optimized culture medium (50 mM Sucrose, 1.32 g L^{-1} Yeast extract, 25 mM NH_4Cl, 2 mM K_2HPO_4, 1 mM $MgSO_4$, 1 μM Vitamin B_1) at 28 °C overnight for an enlarge cultivation. The recombinant plasmid (pET-30a-VP28) was transformed into the strains *E. coli* Rosseta-Gami (DE3), which was cultured in the LB medium (Tryptone 10 g L^{-1}, Yeast extract 5 g L^{-1}, NaCl 10 g L^{-1}) at 37 °C and then the rVP28 was induced with the addition of IPTG at the concentration of 1 mM. The concentrations of these two strains were calculated as CFU mL^{-1}.

Preparation of feeding diet

The experimental diets were prepared following the manual procedure. The adding amount of CpG ODNs and *Y. lipolytica* was 40 mg kG^{-1} and 10^{11} CFU kG^{-1} diet respectively (Syed and Kwang, 2011; Sun *et al.*, 2013b). The diets were sufficiently mixed and pressed into strips to obtain pellets, and stored at 4 °C until use. There were three kinds of diets prepared, CpG supplemental diets, rVP28-yl supplemental diets, and basal diets respectively. The ingredients of basal diet were listed in Table 2.

Oral administration and WSSV challenge

Five hundred and fifty shrimps were divided into three sets, each with three groups. In each group, there were twenty shrimps in triplicate as subgroups. Three sets were fed by CpG, rVP28-yl and basal diets for 15 days trial, which were designated as CpG group, rVP28-yl group and the control group, respectively. The daily feeding diets were about 5 % of body weight per shrimp and nursed with five times. According to the actual intake response, the adjustment was conducted at any time. Based on the result of in vivo titration, the Lethal Dose 50 (LD50) of WSSV was calculated as $1×10^6$ copies. At the end of 15 days feeding experiment of different supplemental diet, one group in each set was randomly selected for injection of WSSV stock ($5×10^7$ copies mL^{-1}, 100 μL for each shrimp) to calculate the survival rate. The other two groups received an injection of WSSV stock ($5×10^6$ copies mL^{-1}, 100 μL) or PBS (pH = 7.4, 100 μL), and every 20 shrimps in each group were regarded as one subgroup. Six shrimps in each subgroup with 15 days' feeding trial were randomly sampled after the 1^{st} and 3^{rd} days post WSSV challenge, and the untreated shrimps in the basal diet feeding set were employed as blank group. Mortality was recorded every day for 10 days post-challenge. The scheme of feeding and challenge experiments is shown in Table 3.

Polyclonal VP28 antibody preparation and West blotting of rVP28

The purified VP28 recombinant protein was obtained and quantified, according to the previous description (Zhou *et al.*, 2011). Six weeks old healthy adult rats were immunized by four times injection of VP28 protein in one and a half month to acquire the polyclonal VP28 antibody (anti-VP28). The serum was separated and then stored at -80 °C until used. The concentrations of two strains *Y. lipolytica*-VP28 and *E. coli*-VP28 were adjusted to 10^8 CFU mL^{-1} after centrifuge at 10000×g for 10 min, the supernatant *Y. lipolytica*-VP28 and both two strain precipitates were heated at 100 °C for 10 min, and then used in western blotting. The quantitative assay of VP28 in these two recombinants was analyzed by the Quantity-One software (Bio-Rad

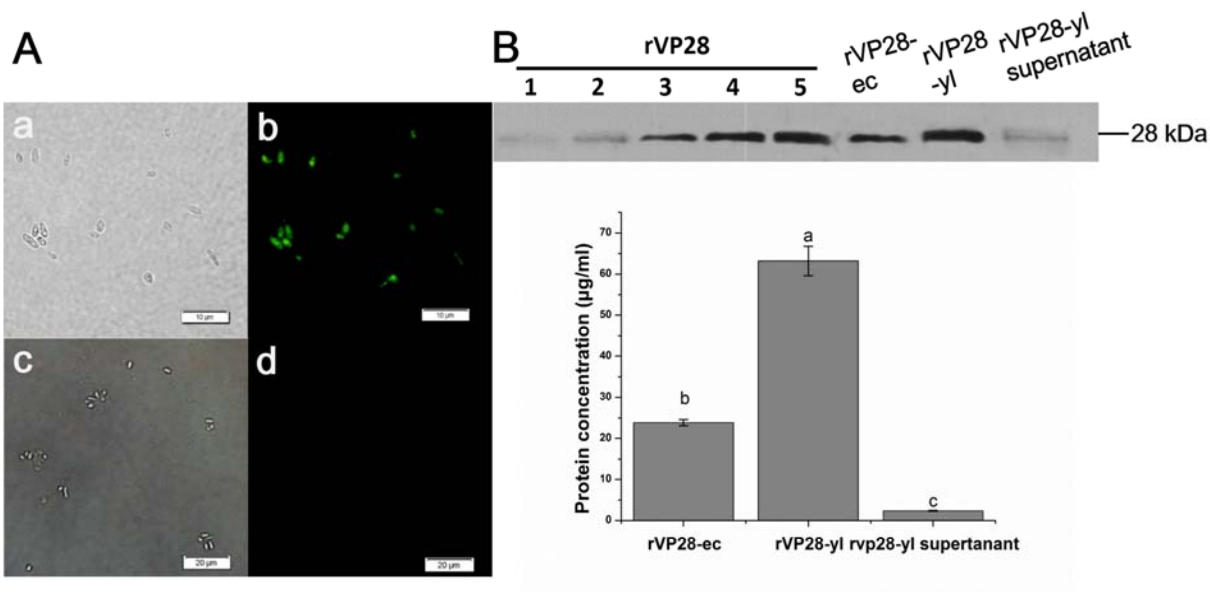


Fig. 1 Immunofluorescence and quantitative western blotting analysis. (A) The surface-display VP28 in *Y. lipolytica* was presented in the green color (b), the control group was no signal (green) detected (d), Bar = 10 μM. (B) Quantitative western blot analysis of VP28 expressed in *Y. lipolytica* system compared with VP28 expressed in bacterial system hold the same strain concentration. The purified VP28 protein as a standard. Lane 1 to Lane 5 was the purified PrVP28 with the concentration of 0.5μgmL^{-1}, 1μgmL^{-1}, 5 μgmL^{-1}, 10 μgmL^{-1}, 50 μgmL^{-1}. The amounts of Lane VP28-ec, VP28-yl and VP28-yl supernatant were analysed by Quanlity One software. Data (mean ± SD) in each column with different letters are significant (p < 0.05) from each other.

Laboratories). After SDS-PAGE, the gel was transferred to the 0.45 μM nitrocellulose membrane, and then the western blotting was carried out following the methods by Zhou *et al.* (2013) with slight modifications. The anti-VP28 at dilution 1:1,000 in 5 % BSA and Goat anti-rat IgG-HRP conjugate (Sangon Biotech, China) diluted at 1:4,000 in 5 % BSA were used as the primary antibody and the secondary antibody in the assay, respectively. The enhanced chemiluminescence staining method (ECL) was performed using luminol and hydrogen peroxide as substrates to detect the VP28 expression. Meanwhile, the VP28 protein which expressed in *E. coli* system was purified as the standard.

Hemocytes collection

Three hundred microlitres precooled anticoagulant (115 mM glucose, 336 mM NaCl, 27 mM sodium citrate, 9 mM EDTA $Na_2 \cdot 2H_2O$, pH 7.4) was preloaded into 1 mL hypodermic gauge needle and syringe, and the hemolmyph was collected from the pericardial cavity of each shrimp. Hemolmyph was immediately centrifuged at 800×*g*, 4 ℃ to harvest the hemocytes and the plasma supernatants. At each sample collection point, six shrimps were prepared from each group, and the hemolymphs collected from every two shrimps in the same treated group were pooled together as one single sample. Triplicate parallels were set for the following experiment.

The measurements of immune parameters

The PO activity was measured according to the methods reported by Zhou *et al*. (2013) with slight modifications. L-3, 4-dihydroxyphenylalanine (L-DOPA) (Sigma Aldrich, USA) was used as substrate, and the formation of dopachrome was recorded spectrophotometrically at 490 nm. Briefly, 100 μL hemolymphs samples from different groups were added in the 96-well microplate (Costar, USA), and incubated with trypsin (Solarbio, China) (1 mg mL^{-1}) at room temperature for 15 min. Then, 50 μL L-DOPA (4 mg mL^{-1} in potassium phosphate buffer) was added into each well, and the optical density at 490 nm was immediately measured every two minutes by using a microplate spectrophotometer (BioTek, PowerWave XS2) for a period of 30 min. One phenoloxidase activity unit was defined as an increase of 0.001 absorbance value at 490 nm per min, and the maximum increase between the two adjacent time points was selected and regarded as one PO activity unit. The ratio of enzyme activity unit to the total protein concentration was defined as the relative phenoloxidase activity, which was expressed as U mg^{-1} protein. The concentration of total protein was determined via the BCA protein determination Kit (Beyotime, China). The NO production of plasma samples was measured by the kit (Nanjing Jiangcheng, China) according to the previous report by Shi et al (Shi *et al.*, 2012). The caspase-3 activity was detected by a Caspase 3 Activity Assay Kit

(KeyGEN, China) according to the manufacturer's instructions. The relative caspase 3 activity was expressed by $OD_{experiment}/OD_{control}$.

Quantitative real-time PCR analysis of WSSV viral numbers and STAT, Dicer genes

The absolute TaqMan real-time assay was performed to determine the WSSV viral numbers according to the previous report (Zhang *et al.*, 2010). Total genomic DNA extraction was followed by the manuscript of DNA extraction Kit (TaKaRa, Dalian, China), and the DNA concentrations were determined by using Nanodrop 2000 (USA). The real-time PCR was carried out to quantify the mRNA expression of STAT, Dicer. Total RNA of hemocytes collected from each experimental group was extracted using Trizol reagent (TaKaRa, Japan). The first-strand cDNA was obtained according to M-MLV RT Usage manual protocol (Promega, USA). The qPCR and the equation of $2^{-\Delta\Delta Ct}$ to calculate the relative expression level of genes were performed as described by Wang et al (Wang *et al.*, 2013). Shrimp EF-1α (elongation factor) was selected as the internal control gene described by Roux et al (Roux *et al.*, 2002). The primers for the synthesis of cDNA template and the real-time PCR assays were presented in Table 1.

Statistical analysis

The data were analyzed by SPSS17.0 software using one-way ANOVA and Duncan test. All experiments were implemented in triplicate, and the values were given as Means ± SD. Differences were considered as significant at $p < 0.05$.

Results

Generation of recombinant Y. lipolytica and assessment

The VP28 protein displayed on the surface of *Y. lipolytica* was detected by immunofluorescence assay. The positive signal was observed in green, while there was no fluorescence signal detected in the control (Fig.1A). The recombinant *Y. lipolytica* and *E. coli* were designated as rVP28-yl and rVP28-ec respectively. Western blotting was used to determine and compare the concentration of recombinant VP28 protein in two strains with anti-VP28 polyclonal antibody (Fig. 1B) by Quality One software. The VP28 presented on the surface of *Y. lipolytica* was about average of 63.2 μg mL^{-1} (10^8 CFU), and only 3.4 μg mL^{-1} in the supernatant of yeast. The VP28 productin in *E. coli* was about 23.8 μg mL^{-1} (10^8 CFU) (Fig. 1B). At the same CFU, the VP28 protein generated by *Y. lipolytica* was 2.7 fold higher ($p < 0.05$) than that generated from *E. coli*. And the VP28 protein in the supernatant of yeast culture was significantly lower, which confirmed that the *Y. lipolytica* strain was more suitable for the preparation of feeding diet.

The cumulative mortality

WSSV challenge experiment was performed following oral administration, and the mortality of shrimps was recorded (Fig. 2). Shrimps began to die after 2^{nd} day in all groups. On the 6^{th} day, the cumulative mortalities in the CpG and rVP28-yl group was 39.4 ± 3.0 % and 36.4 ± 2.1 %,

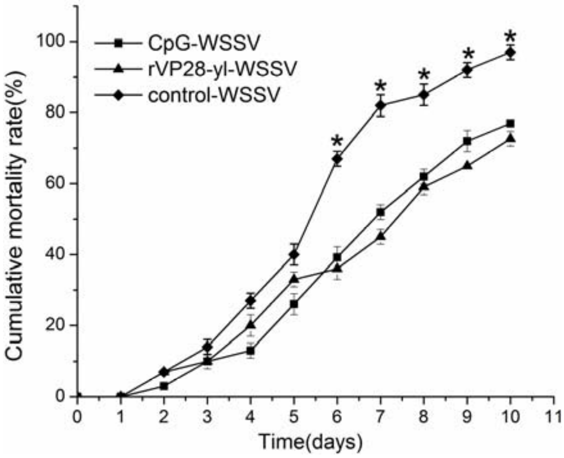


Fig. 2 Comparative protection effects of CpG ODNs, rVP28-yl and basal diet feeding shrimps after WSSV challenge, cumulative mortality rates from CpG group(■), rVP28-yl group(▲) and the control group (♦)were presented. Significant differences of the control group and supplemental diet feeding groups were indicated with asterisk at $p < 0.05$.

respectively, which was significantly lower than that in the the control group (67.0 ± 3.0 %) ($p < 0.05$). From the 7^{th} day to the 9^{th} day post WSSV challenge, the cumulative mortalities in the control group were significantly higher than that in CpG and rVP28-yl group ($p < 0.05$). On 10^{th} day, the cumulative mortality nearly reached 100 % in the the control group, whereas it was only 77.4 ± 2.1 % and 71.2 ± 2.1 % in CpG and rVP28-yl group ($p < 0.05$).

WSSV quantification

After the feeding trial, WSSV copy numbers were measured in the hemocytes of all shrimps, which were 23.41, 12.53 and 19.23 copies ng^{-1} DNA in CpG, rVP28-yl, and the control group, respectively. After WSSV challenge, the virus copy numbers in CpG and rVP28-yl group were significantly lower than that of the control group. On the 1^{st} day after WSSV challenge, the copy number increased in all shrimpswith no significant difference observed among the groups. On the 3^{rd} day post challenge, the mean copy number in the control group increased to 1.30×10^6 copies ng^{-1} DNA, while 5.18×10^5 and 3.65×10^5 copies ng^{-1} DNA were detected in CpG and rVP28-yl feeding shrimps, respectively. The virus number in the control group was 2.5 and 3.5 fold higher ($P<0.05$) than that in the CpG and rVP28-yl groups.

The caspase-3 activity in shrimp hemocytes

The caspase-3 activity was recorded as relative ratio ($OD_{experiment}/OD_{control}$). It ascended in CpG group after feeding trial, which was 2.3 and 1.9 fold ($p < 0.05$) higher than that of rVP28-yl and the control group, respectively. The caspase-3 activity in CpG feeding shrimps increased significantly on the 1^{st} day post WSSV challenge ($p < 0.05$) and dropped to normal level on the 3^{rd} day. As time progressed during experimental trials, there was no significant

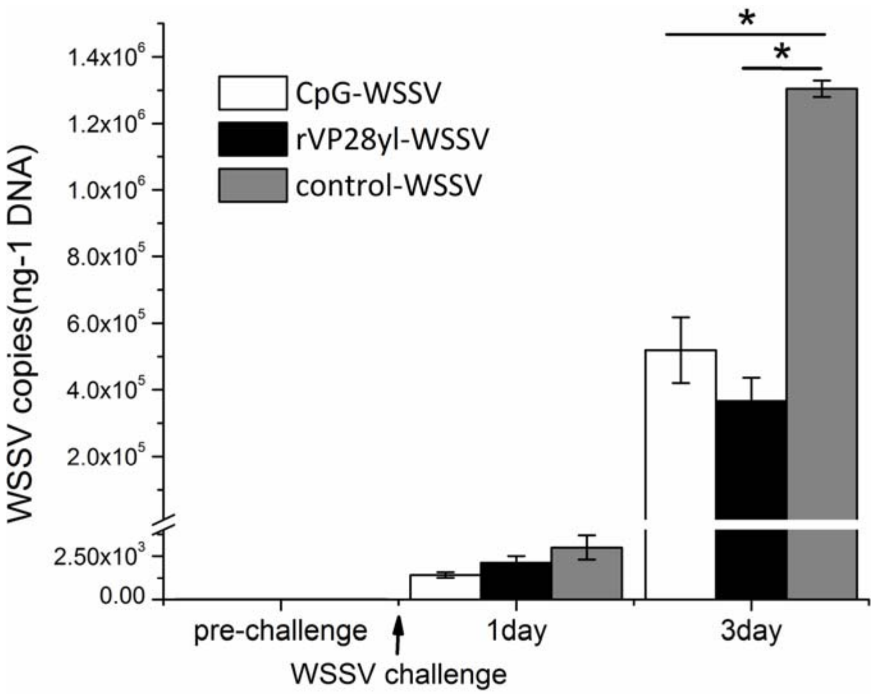


Fig. 3 Quantification of WSSV viral copies number in CpG, rVP28-yl and control feeding shrimps pre-challenge and post WSSV challenge on the 1st and 3rd day. Each symbol and vertical bars represented the means of triplicate assays with standard deviation (SD). Significant differences of WSSV copies number between the two supplemental feeding shrimps and the control feeding ones were indicated with asterisk at $p < 0.05$.

alteration of the caspase-3 activity in rVP28-yl group. In addition, the WSSV challenge resulted in significantly higher caspase-3 activity in the control group on both the 1st day and 3rd day post challenge, and the activity was 3.3 and 3.7 fold higher than that in CpG and rVP28-yl group on the 3rd day (Fig. 4).

The PO activity and NO production in shrimp hemocytes

CpG feeding shrimps resulted in a significant increase ($p < 0.05$) in PO activity throughout the experimental trial. After feeding trial, PO activity in CpG group was significantly increased to 2.1 and 2.3 fold of that in rVP28-yl and the control group ($p < 0.05$). After WSSV challenge, it was significantly higher ($p < 0.05$) than that in all other groups on the the 1st and 3rd day, respectively. Furthermore, after PBS stimulation, the PO activity also exhibited significantly higher level ($p < 0.05$) in CpG group. However, the values of PO activity in rVP28-yl group displayed no significant alteration post feeding trial and WSSV challenge. The PO activity in the control group was significantly decreased on the the 1st and 3rd day post WSSV challenge ($p < 0.05$) (Fig. 5A).

The NO productions in CpG and rVP28-yl group were higher than that of the control group ($p < 0.05$) at the end of feeding trial. After WSSV challenge, the production of NO in all three groups increased on the the 1st day. It was 23.3 μM in CpG group which was significantly higher than that in rVP28-yl (13.0 μM) and and the control group (13.6 μM), respectively ($p < 0.05$). And on the 3rd day, it decreased in CpG group (15.1 μM), while increased to 17.23 μM in rVP28-yl group, which were both significantly higher than that in the control group (11.2 μM) ($p < 0.05$) (Fig. 5B).

The mRNA expression of immune-related genes

The mRNA expression of STAT and Dicer exhibited different variation tendency post feeding and WSSV challenge. The expression level of STAT mRNA exhibited no significant variation in each group post feeding trial. But it increased significantly in hemocytes of CpG fed shrimps on the the 1st and 3rd day post WSSV challenge ($p < 0.05$), which was 15.5 fold higher than that of blank on the 3rd day post challenge (Fig. 6A). The expression level of STAT in the control group decreased significantly on the 3rd day compared with other groups ($p < 0.05$).

The mRNA expression levels of Dicer in CpG feeding shrimps were up-regulated both after feeding trial and WSSV challenge, which were significantly increased than that in other group ($p < 0.05$) (Fig. 6B). There was no significant difference of Dicer mRNA expression in rVP28-yl group between WSSV and PBS injected subgroups throughout the experiment.

Discussion

In shrimp culture industry, immunological approaches including the use of immunostimulants and vaccination have been validated with beneficial effects on the prevention and control of WSSV

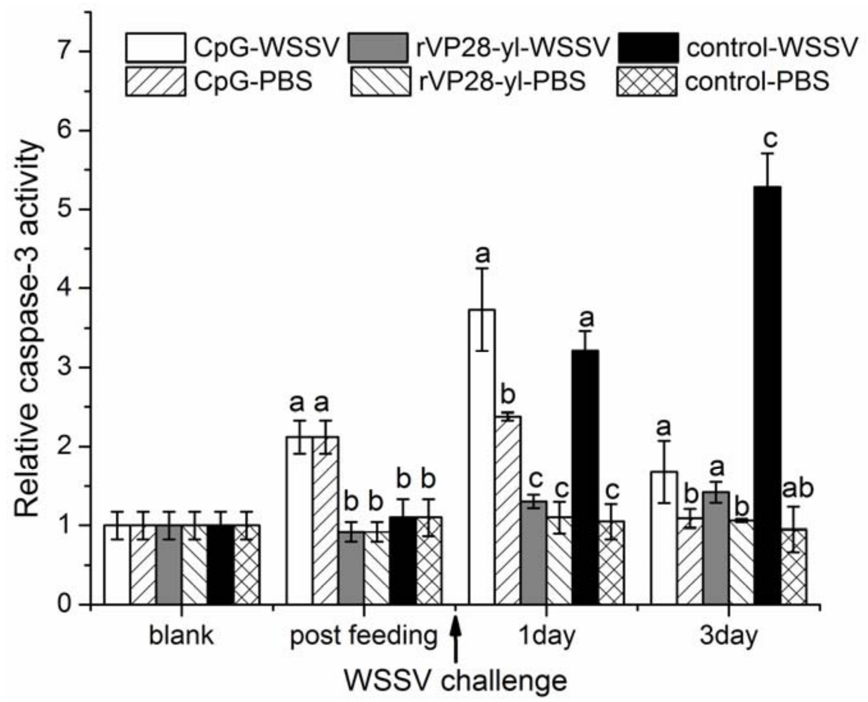


Fig. 4 Caspsase-3 activity in CpG ODNs, rVP28-yl and control feeding shrimps, including post feeding and post WSSV and PBS challenge on the 1st and 3rd day. Each symbol and vertical bars represented the means of triplicate assays with standard deviation(SD). Bars with different letters are statistically significant from each other in the same sampling point (p < 0.05).

diseases (Xu *et al.*, 2011; Haq *et al.*, 2012). It has been demonstrated that CpG ODNs can trigger various immune responses to enhance the immune capability, and it could be used as an immunostimulant candidate (Carrington and Secombes, 2006). VP28 is one of main structural proteins of WSSV participating in the virus entry into cells, and it has been considered as a potential "vaccine" candidate for crustaceans against WSSV infection (Johnson *et al.*, 2008). In the present study, CpG ODNs and VP28 displayed on *Y. lipolytica* surface (rVP28-yl) were employed by oral routes to investigate their protective effects of shrimps against WSSV.

Since oral feeding is the basic approach for the intake of nutriments in all stages of shrimp life, it is generally applicable in shrimp aquaculture (Syed and Kwang, 2011). The yeast *Y. lipolytica* has always been employed as a probiotic candidate for its immunological properties. In the present study, VP28 was highly expressed on the surface of *Y. lipolytica*, and the new constructed *Y. lipolytica* strain was employed as the vehicle of VP28. Meanwhile, CpG ODNs were large-scale prepared via plasmid extraction with alkali method. After fed with CpG and rVP28-yl for 15 days, the shrimps were challenged with WSSV, and the mortality was recorded for ten days. From 6th day to 10th day post WSSV challenge, the mortality rates of shrimps in CpG and rVP28-yl group were significantly lower than that in the the control group. On the 10th day, almost all the shrimps died in the control group (100 % mortality rate), while the survival rate in CpG and rVP28-yl group were 22.6 ± 2.1 % and 28.8 ± 2.1 %, respectively. Meanwhile, the WSSV copy numbers were also significantly lower in CpG and rVP28-yl group than that in the control group on the 3rd day post WSSV challenge. It is generally accepted that there is a relationship between the high survival rate of culture shrimp and the low virus load (Jang *et al.*, 2009). These results indicated that the replication and proliferation of WSSV could be partially inhibited by CpG and rVP28-yl, and the oral administration of CpG and rVP28-yl enhanced antiviral immunity of shrimp against WSSV infection.

In invertebrates, hemocytes are generally regarded as the main component of immune defense system, which participate in the immune responses against pathogen, such as apoptosis, encapsulation, melanization, oxidation and so on (Bachere *et al.*, 2004). In the present study, shrimps fed with CpG and rVP28-yl displayed the obvious enhanced capability to reduce the mortality rate caused by WSSV. Some relative immune parameters and mRNA expression of antiviral genes in hemocytes were then analyzed to address the innate immune responses of *L. vannamei* after oral administration. Apoptosis, as one of vital cellular defense mechanisms, can eliminate the pathogen infected cells to avoid their delivering into surrounding cells, and it has been reported to exert comparably obvious function against WSSV infection in shrimp (Leu *et al.*, 2013; Wang and Zhang, 2008). In the intricate apoptotic course,

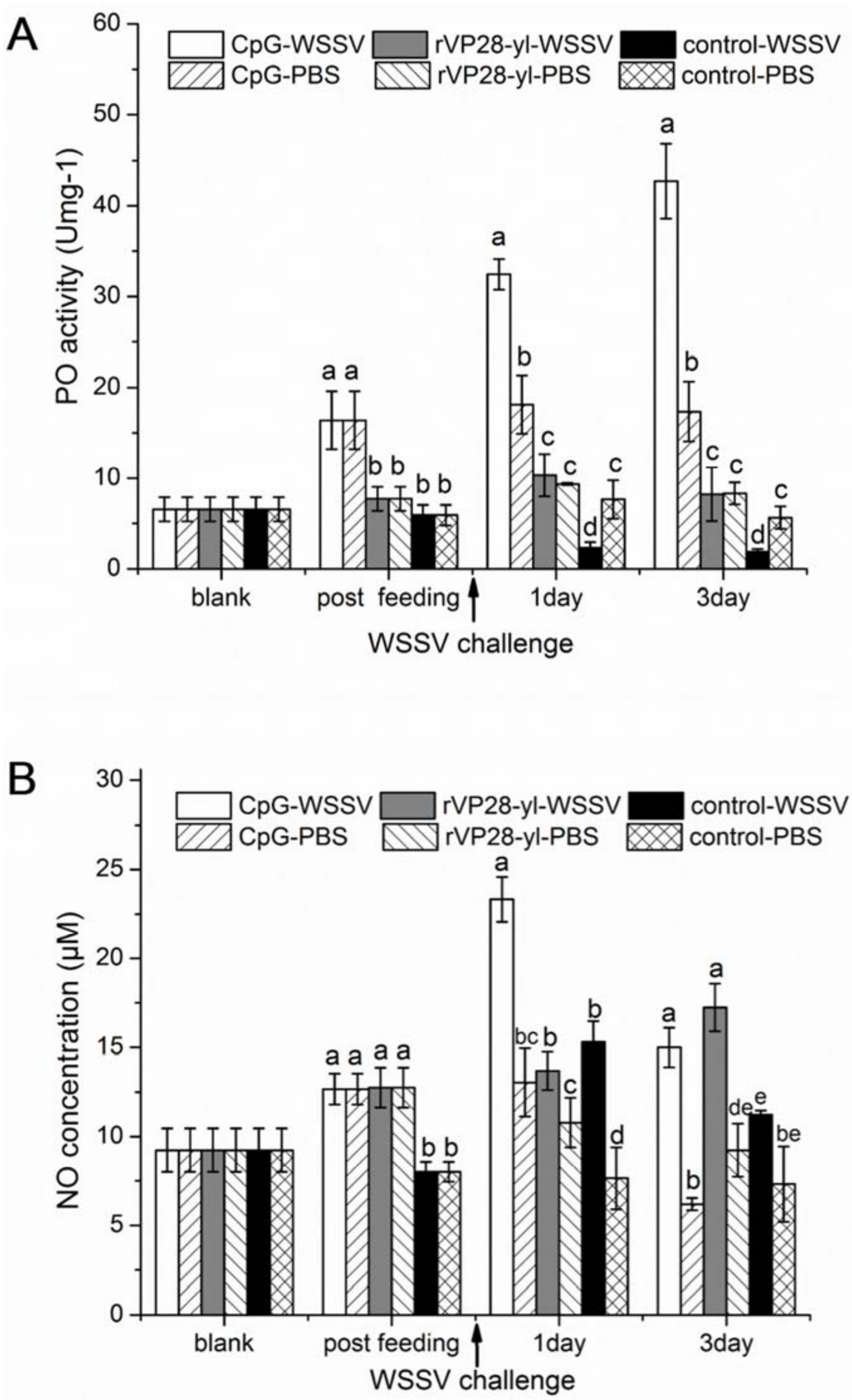


Fig. 5 Phenoloxidase (PO) activity(A) and NO production(B) in CpG ODNs, rVP28-yl and control feeding shrimps, including post feeding and post WSSV and PBS challenge on the 1st and 3rd day. Each symbol and vertical bars represented the means of triplicate assays with standard deviation (SD). Bars with different letters are statistically significant from each other in the same sampling point ($p < 0.05$).

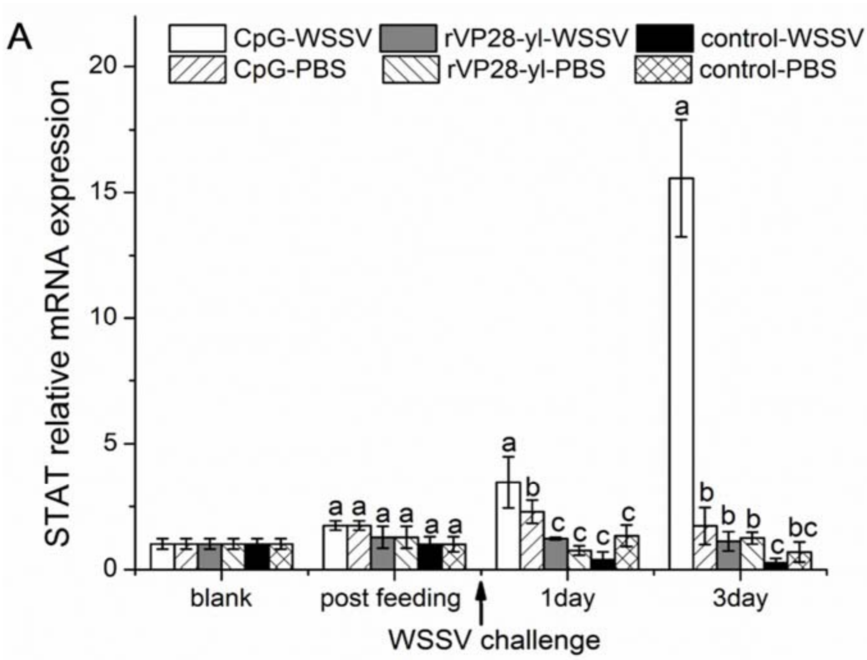
caspase protein family members are the central effectors, among which Caspase-3 has been confirmed to be the crucial one and also the indicator used to mirror the level of apoptosis (Fu *et al.*, 2010). In our previous study, CpG ODNs have be confirmed to boost apoptosis in shrimp hemocytes (Sun *et al.*, 2013a). In the present study, a significant increase of caspase-3 activity was observed after the shrimps were fed with CpG ODNs, and it ascended further at early stage of WSSV infection, followed by a decrease on the 3rd day post WSSV challenge. The lower mortality rate and WSSV copies, and the enhanced caspase-3 activity suggested that CpG ODNs could induce apoptosis which might contribute to the effective protection for shrimps to eliminate WSSV at the early stage of infection. In the control group, caspase-3 activity kept rapid increase after WSSV challenge, which indicated that the higher apoptosis level induced by WSSV could generate damage effects for shrimps. However, there was no obvious alteration of caspase-3 activity in the rVP28-yl group before and post WSSV challenge. It indicated that the apoptosis induced by WSSV could be suppressed in rVP28-yl feeding shrimps. It has been reported that VP28 could bind with the host cell receptor to reduce the possibility of WSSV entry into cells (Sritunyalucksana *et al.*, 2012). Therefore, CpG and rVP28-yl might contribute to the protective effect against WSSV via inducing adequate apoptosis activity to eliminate WSSV and inhibiting the cell infection.

PO is one of the significant components of proPO system in crustaceans, and it is also the key enzyme to control melanism cascade (Li and Xiang, 2013b). It was documented that CpG ODN could activate the proPO system and enhance the phenoloxidase activity effectively in giant freshwater prawn (Chuo *et al.*, 2005). In the present study, PO activity increased significantly after feeding trial in CpG group, indicating that PO system was provoked by CpG ODNs stimulation. The enzyme activity in CpG ODNs feeding shrimps kept increasing after WSSV challenge, which confirmed that the effect of CpG ODNs on the activation of PO system was long-lasting. Meanwhile, there was no significant difference of PO activity in rVP28-yl group post feeding and WSSV challenge, which was in agreement with the result obtained in oral administration of rVP28 in *F. chinensis* (Fu *et al.*, 2010). However, the PO activity was significantly decreased after WSSV infection in the control group, suggesting that WSSV could inhibit PO activity and immune response induced by proPO system, then continue to infect into host cells. It was interesting that the PO activity in rVP28-yl group was significantly higher than that in the control group, but it did not increase post WSSV challenge, suggesting that rVP28-yl was functional in the defense against WSSV. These results together suggested that CpG ODNs and rVP28-yl could partially neutralize the inhibitory effect of WSSV on proPO system, and enhance the protective immunity of shrimps.

NO is considered as an important signal molecule playing versatile roles in many physiological processes including immune defense. The production of NO in cells is one important immune response, mediating an oxidative progress with reactive oxygen species such as superoxide anions to enhance the non-specific immunity against pathogenic invasion (Colasanti and Venturini, 1998; Bogdan, 2001). Haiqi *et al.* (2003) reported that CpG ODN significantly stimulated NO production in avian. In the present study, the NO production in CpG group was significantly higher than that in the control group after feeding trial, and also significantly increased on the 1st and 3rd day post WSSV challenge, suggested that the CpG ODNs induced NO production contributed to the resistance against WSSV infection. It was also observed that the NO production in rVP28-yl group increased after feeding trial and on the 3rd day post WSSV challenge. Similar results were also reported in *F. chinensis* that rVP28 could significantly heighten the iNOS activity (Fu *et al.*, 2010). Because iNOS regulated the production of NO and it was involved in innate response against WSSV in shrimp (Jiang *et al.*, 2006).

It has been demonstrated that some potent inducers contribute to the antiviral immune response, such as antiviral immunoregularory factors and double-stranded RNA (Tassanakajon *et al.*, 2013). STAT is one of transcription factors that regulate antiviral pathways (Darnell, 1997; Li and Xiang, 2013b), and the previous reports have documented that several virus replication and proliferation could be inhibited by the regulation of JAK/STAT pathway (Darnell, 1997; Decker *et al.*, 2002; Tassanakajon *et al.*, 2013). STAT deficient mice were more susceptible than wild ones when they were undergone the RNA virus infection (Durbin *et al.*, 1996). STAT in invertebrates was also confirmed to play roles in antiviral process which was similar to that in mammals. For instance, STAT from shrimps could be activated when they were infected with WSSV (Dostert *et al.*, 2005; Chen *et al.*, 2008), and the mRNA expression of STAT was down-regulated as WSSV infection progressed (Syed and Kwang, 2011). In the present study, the expression level of STAT mRNA continued decreasing in the control group post WSSV challenge, indicating that WSSV might inhibit immune response induced by STAT in the early stage of infection. Furthermore, there were significant difference of STAT expression in CpG group and rVP28-yl group compared to that in the control group post WSSV stimulation, suggesting that CpG ODNs and rVP28-yl could remove the inhibition generated by WSSV to activate STAT expression. It indicated that CpG ODNs and rVP28-yl could induce the STAT mediated antiviral response in *L. vannamei*. And the significant higher STAT mRNA expression on the 3rd day in CpG group after WSSV challenge suggested that CpG ODNs was a more effective inducer for the STAT-mediated antiviral immunity in shrimp.

RNAi has been accepted to be one of the most promising strategies to combat both DNA and RNA virus in invertebrate (Robalino *et al.*, 2004). Dicer is a key enzyme involved in RNA interference, which recognizes a viral RNA and splices it to small RNA to exerts a protective role for host against RNA and DNA virus (Lee *et al.*, 2004 (Lee *et al.*, 2004; Kemp and Imler, 2009) . Dicer could inhibit the replication of HIV-1 virus, and the knockdown of Dicer gene yielded the increased virus production (Haase *et al.*, 2005). In the present study, the mRNA expression of Dicer in CpG group was significantly higher than that in rVP28-yl and the control group after feeding trial, and a delayed up-regulation was observed in CpG group on 3rd day post WSSV challenge, suggesting that CpG ODNs might induce the Dicer expression to render the corresponding inhibitory effect on WSSV replication. Interestingly, the feeding of rVP28-yl didn't lead to significant alteration of Dicer mRNA expression compared to control. After WSSV challenge, there was also no significant difference of Dicer expression between rVP28-yl and the control



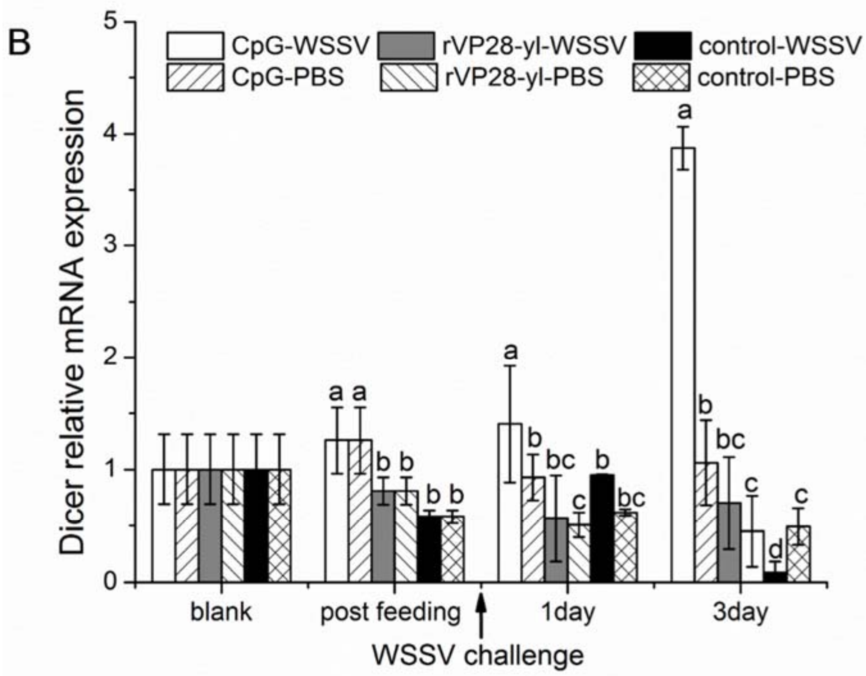


Fig. 6 The mRNA expreesion level of STAT (A) and Dicer (B) genes relative to EF-α gene in CpG ODNs, rVP28-yl and control feeding shrimps, including post feeding and post WSSV and PBS challenge on the 1st and 3rd day. Each symbol and vertical bars represented the means of triplicate assays with standard deviation (SD). Bars with different letters are statistically significant from each other in the same sampling point ($p < 0.05$).

group, indicating that Dicer was not involved in the immune defense caused by rVP28-yl. In contrast, CpG ODNs might contribute to the enhanced antiviral immunity of shrimps for the induction of Dicer gene to interfere the replication of WSSV.

In summary, the oral administration with CpG ODNs and rVP28-yl in shrimps significantly induced the immune responses including PO activity, NO concentration, caspase-3 activity, and expressions of STAT and Dicer, which might endow shrimps with enhanced protective capability under WSSV challenge. CpG and rVP28-yl exhibited almost similar efficacy in terms of protective effect for *L. vannamei* against WSSV infection through different mechanisms. The present results provided insights into the immunological prevention management in shrimp culture industry.

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