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An attenuated *Vibrio harveyi* surface display of envelope protein VP28 to be protective against WSSV and vibriosis as an immunoactivator for *Litopenaeus vannamei*

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ABSTRACT

Surface display can expose foreign antigenic protein on the surface of the vaccine vector, which is promising choice to elicit better immune responses. In this study, we apply this strategy to develop an immunoactivator by using a live attenuated *Vibrio harveyi* as an antigenic protein carrier with surface displayed VP28, a major envelope protein of white spot syndrome virus (WSSV), for two major pathogens of *Litopenaeus vannamei*. As a result, the immunoactivator showed self-limited growth and attenuation of virulence in shrimp via different inoculation routes either with single-repetitive dose or high dose. Moreover, either intramuscular injection or oral administration of the immunoactivator did not affect growth of shrimp body weight or cause pathologic changes. Additionally, the rapid immunoprotection was induced by the immunoactivator after administration for one week with highly relative percent survival (RPS) more than 90% against both *V. harveyi* and WSSV. Until 4 weeks post administration, the immunoactivator still possessed efficient immune effect with no less than 60% RPS for both pathogens. Totally, the attenuated *V. harveyi* surface displaying VP28 could be a potential immunoactivator for WSSV and vibriosis control in *L. vannamei*.

1. Introduction

Litopenaeus vannamei is the dominant economic cultivated shrimp species globally. Currently, China's production of this species has reached more than 1 million tons. However, the threat of various diseases is one of the biggest risk factors for healthy and sustainable development in shrimp aquaculture worldwide. Among these diseases, approximately 60% are viral diseases and 20% are bacterial diseases [1]. White spot disease (WSD) caused by white spot syndrome virus (WSSV) is the most serious viral disease threat to penaeid shrimp, especially for *L. vannamei* in Asia, leading to a cumulative mortality up to 100% within 3–10 days [2]. In addition, vibriosis mainly caused by *Vibrio harveyi*, *Vibrio parahamelyticus* and *Vibrio alginolyticus*, is the most prevalent bacterial disease in global shrimp farming [3–5].

Though shrimps lack adaptive immunity as that existed in vertebrates and depend on innate immunity to combat various pathogenic infections, the development of such a vaccination regimen based on stimulating the shrimp immune response is still considered a promising strategy against viral and bacterial diseases. Much approaches such as inactivated vaccine, immunostimulant, DNA vaccine and dsRNA treatment has been investigated to prevent WSSV and *Vibrio* respectively [6–11]. However, prior to this work, there has been no report on developing polyvalent vaccines against both WSSV and *Vibrio* in shrimp.

As a major protein of WSSV, envelope protein VP28 (28 kDa) is involved in the systemic infection of shrimp, of which acts as a viral attachment protein to the shrimp cells and interacts with host cellular proteins [12]. Heterologous recombinant VP28 prepared through various expression systems has been confirmed to be effective against WSSV in *L. vannamei* and *Litopenaeus monodon* [13,14]. We wonder whether a live attenuated vibrio strain can act as a vector to express the recombinant protein VP28 and obtain immunoprotection against both vibriosis and WSSV.

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Table 1
Strains and plasmids in this study.

Strain or plasmid	Genotype and characteristics	Source or Reference
***V. harveyi* strains**		
SDW715	Wild-type strain, shrimp pathogen	This study
HTVhs-5306 (Δ*aroA*::*Livp28*)	Amp^r, SDW715 in-frame deletion in *aroA and vhsp*, with fusion fragment *LacZ-inp-vp28* insertion in Δ*aroA*.	This study
***E. coli* strains**		
INPVP28	harboring pLivp28	This study
CC118 λ*pir*	λ*pir* lysogen of CC118, Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1*	Sheng et al. (2013)
CC118-λ*pir*Δ*aroA*::*Livp28*	CC118/λ*pir* harboring pDM4Δ*aroA*::*Livp28*	This study
CC118-λ*pir*Δ*vhsp*	CC118/λ*pir* harboring pDM4Δ*vhsp*	This study
SM10 λ*pir*	Donor for bacterial conjugation; *thi thr leu tonA lacY supE recA*::*RP4-2-Tc*::*Mu*, *pirR6K*, Km^r	Sheng et al. (2013)
SM10λ*pir*Δ*aroA*::*Livp28*	SM10/λ*pir* harboring pDM4Δ*aroA*::*Livp28*	This study
SM10λ*pir*Δ*vhsp*	SM10/λ*pir* harboring pDM4Δ*vhsp*	This study
Plasmids		
pDM4	*SacBR*, suicide vector that contains an R6K origin of replication (*pir* requiring), Cm^r	Wang et al. (2003)
pDM4Δ*aroA*::*Livp28*	pDM4 containing truncated *aroA* region, Cm^r	This study
pDM4Δ*vhsp*	pDM4 containing truncated *vhsp* region, Cm^r	This study
pLivp28	Amp^r, pUC18 derivative; containing *lacZ-inp-vp28* fusion gene	This study

Bacterial cell surface display, as a widely developed antigen delivery system, is an attractive approach for recombinant vaccines. So far, bacteria and yeast have been successfully used for displaying a variety of antigen proteins via fusion expression with various carrier proteins (anchoring motifs) for vaccine development [15–17]. Ice nucleation protein (INP), a successful carrier protein derived from *Pseudomonas syringae*, is considered to be a very efficient carrier, particularly suitable for developing recombinant vaccines for human, animal and aquatic products [18,19]. In this study, we have constructed an antigen surface expression vector using INP. The VP28 gene was inserted into the surface display system and displayed on the surface of a live attenuated *V. harveyi* strain. Then safety and efficacy of this immunoactivator for *L. vannamei* was evaluated to see whether it could be an immunoactivator against both vibrio and WSSV through oral administration.

2. Materials and methods

2.1. Bacteria strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise specified, both wild type *V. harveyi* SDW715 and HTVhs-5306 were grown on Luria Bertani (LB) mediums supplemented with 2% sodium chloride (LB20). *Escherichia coli* strains were grown in LB. For all strains used in this study, ampicillin (100 mg/ml) and chloramphenicol (20 mg/ml) were added as required. *V. harveyi* and *E. coli* strains were routinely cultured at 30 °C and 37 °C respectively.

2.2. Construction of the live attenuated V. harveyi surface displaying INP-VP28 protein

To obtain a live attenuated vector stain, the *aroA* (encoding the 5-enolpyruvylshikimate-3-phosphate synthase) and *vhsp* (encoding serine protease) genes of *V. harveyi* SDW715 were inactivated by in-frame double deletion of chromosomal DNA, while a surface expression system containing a INP-VP28 fusion element (a fusion fragment of *LacZ* promoter, INP gene fragment *inpV* containing N terminus with most of the central repeating domain (CRD) deleted, and *vp28* gene, ϕ (*LacZ-inp-vp28*)) was inserted into the mutant-site of *aroA* by allelic exchange. Overlap PCR was used to obtain the fragments for constructing the in-frame deletion and substitution mutants of selected genes. The PCR products containing in-frame deletion or substitution fragments were separately cloned into the suicide vector pDM4, which carried R6K ori, *sacB* sucrose-sensitivity gene, and Cm resistance [20]. The resulting plasmids with the deletion fragments in the MCS were mated from *E. coli* SM10 λ*pir* into *V. harveyi* SDW715 by conjugation. To create the in-frame deletion mutants, a double-crossover recombination event was counter-selected on LB20 agar containing 10% sucrose [21]. The targeted in-frame deletion and substitution mutants were confirmed by sequencing of the deleted and fused region on chromosome. The SDW715 derived mutants harboring the targeted deletion and substitution was designated as Δ*aroA*:*livp28*Δ*vhsp*, namely HTVhs-5306. Nucleotide sequences were determined by the dideoxynucleotide chain termination method with double-stranded templates by Applied Biosystems 3730xl (GENWIZ, China). All primers used in this study were listed in Table 2.

2.3. Preparation of polyclonal antibody against VP28 protein and Western blot analysis

Preparation of polyclonal antibody against VP28 protein was carried our as previously described [22]. Roughly, vp28 gene was firstly ligated into pET28a (+) expression vector for expression. After purification, the VP28 protein was injected into New Zealand white rabbits and the antisera was raised. Finally, the IgG fractions of antisera were then purified by affinity chromatography. Antibodies against recombinant VP28 were stored at −80 °C for further use. For Western blot, the recombinant VP28 were electrophoresed by SDS-PAGE and then were electrically transferred to nitrocellulose paper. The VP28 polyclonal antibodies at a dilution of 1:4000 were used as primary antibody and goat anti-rabbit IgG (Abbkine, America) at a dilution of 1:10000 were used as secondary antibody to detect the expression of VP28.

2.4. WSSV and in vivo titration

For viral propagation, *L. vannamei* were injected intramuscularly between second and third abdominal segments with WSSV obtained from infected *P. vannmei* collected in Tianjin, China in 2012. Infected tissues were collected and homogenized in 10 × volume (w/v) of TN buffer (20 mM Tris-HCL and 0.4 M NaCl, pH7.4), centrifuged twice at 4 °C with 12,000 × *g* for 5 min. The final supernatant was filtered through 0.22-μm filter and virus stocks were stored in aliquots at −80 °C. Virus stock was titrated *in vivo* to determine the amount of virus required for WSSV challenge via injection above 90% mortality in 10 days. The desired mortality was induced using 30 μl of the virus sample in shrimps from negative control group.

2.5. Shrimp maintenance

L. vannamei, average body weight 5 g each for juvenile respectively, were obtained from a commercial farm in Yangjiabo, Tianjin, China. Shrimps were temporarily raised in tanks containing disinfected

Table 2
Primer pairs of target genes used in this study.

Primer	Sequence
aroA-P1	GAGCTCAGGTTACCCGCATGCAAGATCTATATTAAGCCAACGTTTGCACT
aroA-P2	GTGCTCTAGATAAAAGTTGATAATCCTTTG
aroA-P3	GTAACTGCAG GCTGGTAGCGCTAAGCGACA
aroA-P4	CCCTCGAGTACGCGTCACTAGTGGGGCCCTATACTGCGCTCACTTAAGTT
aroA-in-F	TCACTTAGTGACAGCCTTAA
aroA-in-R	CCTTGATGGCAGCTGCGGCC
aroA-out-F	CAACAAACTGATCAACGTCA
aroA-out-R	TAGCCACTGCACGTATATAC
vhsp-P1	GAGCTCAGGTTACCCGCATGCAAGATCTATGGTGGCCAGCGTCCTGGTGG
vhsp-P2	TCAAACGCTC CGTTTTCATCTTTTGTCAGC
vhsp-P3	GATGAAAACG GAGCGTTTGAAGAAAGAAGC
vhsp-P4	CCCTCGAGTACGCGTCACTAGTGGGCCCT TGCGAACGCTCAGATTCCAA
vhsp-in-F	TAGTAACAGTAGCAATGGAC
vhsp-in-R	TGCACGACCTGTTGCTTTCG
vhsp-out-F	ATGGCGTGGAATGAGCCTGG
vhsp-out-R	TTAGTATCTCCCTTGACGTG
inp-F	GGATGCTGTAATGAATCTCGACAAGGCGTT
inp-R	TCACAGTGTTGGATCCCCGGGTACCATGAT
vp28-F	CCGGGGATCCAACACTGTGACCAAGACCAT
vp28-R	CGCTACCAGCCTGCAGTTACTCGGTCTCAG
LacZ-F	TCAACTTTTA TCTAGAGCACGACAGGTTTC
LacZ-R	CGAGATTCAT TACAGCATCCTCAGAATTCG

seawater with aeration at 28 °C for 5 days. Before experiments, shrimps were randomly selected for health examination and tested by PCR to ensure they were WSSV-free. As well, whether shrimps were infected with *V. harveyi* was analyzed by plating tissues on thiosulfate citrate bile salts sucrose agar (TCBS, Difco, USA). Then shrimps were divided into the control groups and experiment groups according to the experimental requirements.

2.6. Immunoactivator administration

After growth in LB20 for 24 h at 30 °C, the live cells were harvested by centrifugation at 3000 × *g* and resuspended with sterile saline. The suspension was diluted with sterile saline to 10^6–10^9 CFU/ml according to experiments. Shrimp was inoculated by intramuscular injection (i.m.) between second and third abdominal segments with 50 μl the immunoactivator. For oral route, the suspension was mixed with sodium alginate as binder and applied uniformly on the commercial shrimp feed (equivalent to 2×10^6 CFU per gram feed). The control feed was prepared using only the coated solution. The immunoactivator coated or binder coated control feed were air dried under shade for 2 h before feeding. Shrimps feeded with the immunoactivator preparation in a proportion of 5% of weight for 5 days continuously.

2.7. Immunoactivator safety evaluation

To evaluate safety of the immunoactivator, shrimps were randomly divided into two groups for safety evaluation on single and repetitive dose and large dose inoculation respectively. The two groups were subdevided into several groups (50 shrimps/group) and the detailed grouping situation was as shown in Fig. 1. Cumulative survival rate was recorded after administration. Meanwhile, wild type strain SWD715 was also manipulated comparably of large dose inoculation group. The experiment was performed in triplicate.

2.8. Immunoactivator effectiveness evaluation

To evaluate the effectiveness of the immunoactivator, shrimps were randomly divided into three groups: V_{or}, shrimps received immunization via oral route with 2×10^6 CFU/shrimp and challenged through i.m. route with *V. harveyi* and WSSV respectively; C_n, shrimps raised normally without any manipulation; C_p,shrimps mock-immunized with sterile saline and challenged via i.m. with *V. harveyi* or WSSV. At 1, 2, 3 and 4 week post administration (p.a.), 50 shrimps from each group were challenged with 1.2×10^8 CFU of SDW715 in 0.1 ml and equal shrimps from each group were challenged with 30 μl of WSSV sample by i.m. Cumulative survival rate was recorded for lasting 14 days and relative percent survival (RPS) was calculated according to the following formula devised by Amend (1981). $RPS = \left(1 - \frac{\%\ \text{mortality of administrated shrimp}}{\%\ \text{mortality of control shrimp}}\right) \times 100\%$. Moreover, the survival curve of group that challenged at 4 w p.v. was drew. Both administration and challenge were conducted in triplicate.

2.9. Histopathologic examination of shrimp tissues for the immunoactivator

For histopathology, tissues including hepatopancreas, intestine, gills and myocardium were dissected from administrated shrimps of each experimental group and excised samples were placed immediately into Bouin's solution fixative for histology analysis. Fixed samples were sequentially dehydrated by passage through a gradient of ethanol solutions and embedded in paraffin wax using standard protocols. 4 μm thick sections were cut and were mounted onto glass slides before staining with hematoxylin-eosin (H&E) for microscopic examination. Stained sections were analyzed by light microscopy (OLYMPUS BX53) and images were taken using the Image-Pro Plus™ analysis software (OLYMPUS).

2.10. The impact on shrimp growth of the immunoactivator

To determine the impact on shrimp production performance, juvenile shrimp weighing approximately 5 g were randomly divided into three groups (100 shrimp/group): V_{im}, shrimp administrated with 2×10^5 CFU/shrimp of the immunoactivator via i.m.; V_{or}, shrimp inoculated with 2×10^6 CFU/shrimp of immunoactivator via oral route; C, shrimp mock-inoculated with sterile saline. At 0, 2, 4, 6, 8 and 10 w p.v., average weight of f shrimp from each group were calculated. The experiment was performed in triplicate.

2.11. Persistent carrier state of the immunoactivator in tissues

For the determine persistent carrier state of the immunoactivator in tissues, shrimps were randomly divided into three groups (50 shrimp/group): V, shrimp administrated with 1×10^7 CFU of HTVhs-5306 by i.m. or oral route; C, shrimps injected with 1×10^7 CFU/shrimp of WT. Hepatopancreas, intestine and gills from three shrimps in each group

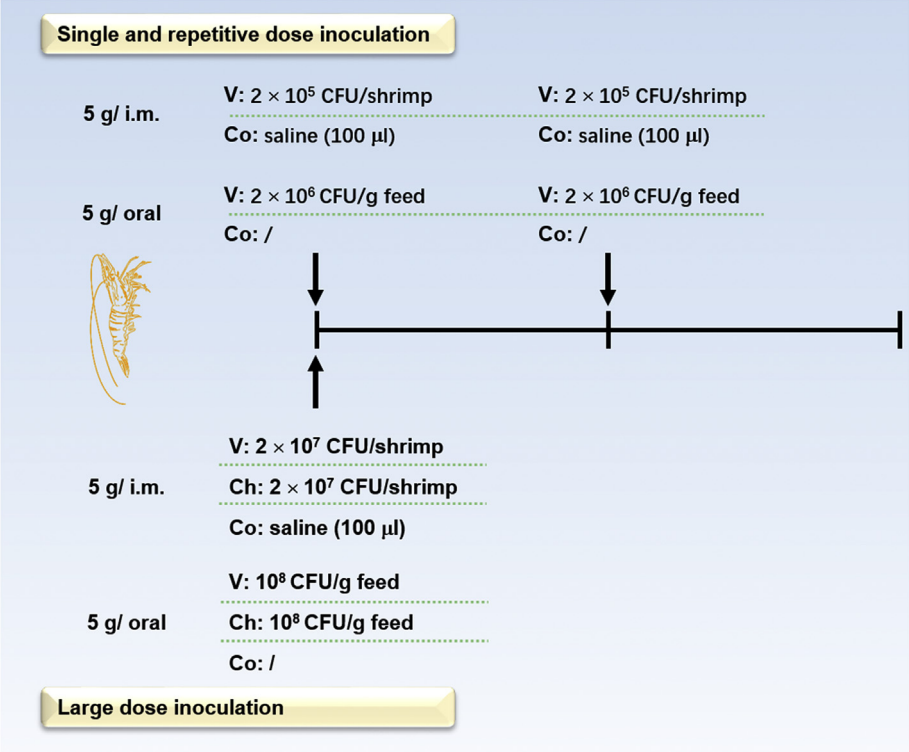

Fig. 1. The scheme of determination for safety evaluation of the immunoactivator. shrimps were randomly divided into two groups for safety evaluation on single and repetitive dose and large dose inoculation respectively. The two groups were sub-devided into ten groups (50 shrimps/group) with different treatment.

were sampled, weighed, and homogenized in 1 ml sterile saline. Homogenates were serially diluted and plated on TCBS at 28 °C for 48 h. Colonies that featured with yellow centers were counted. Bacteria counts were calculated by dividing the weights of the tissues and from the mean of three samples in two parallel experiments.

2.12. Statistical analysis

Independent-sample t-tests were performed with SPSS software (Version 11.5, SPSS Inc.) to determine statistical significance. Significant differences were considered at $*P < 0.05$ and $**P < 0.01$.

3. Results

3.1. Construction of live attenuated V. harveyi vector strain surface displayed VP28

Firstly, the fusion fragment of *LacZ*, *inpV* and *vp28* gene was obtained by overlap PCR, which was then inserted into the mutant-site of *aroA* (size in 1473 bp, Fig. 2A). Moreover, the vhsp-deleted strain was further constructed based on the *aroA* mutant, with an expected fragment of 415 bp (Fig. 2B). Furthermore, the expression of VP28 protein was determined by Western blot, and a clear band at the expected position was observed as shown in Fig. 2C. These results indicated that the live attenuated *V. harveyi* candicate was successfully constructed and the antigenic protein VP28 could be expressed.

3.2. Safety profiles of the live attenuated strain as an immunoactivator

3.2.1. Safety evaluation of the live attenuated vaccine

Firstly, safety of the live attenuated vaccine HTVhs-5306 to juvenile shrimp was tested both on single-repetitive dose and large dose via i.m. injection or oral route. As a result shown in Table 3, no shrimp died in the administrated and control groups for single-repetitive dose inoculation. Only 5% of shrimp died after injected with 2×10^7 CFU of the immunoactivator, compared with a highly mortality of 100% observed when injected with 2×0^7 CFU of the wild type strain. Besides, 1% of juvenile shrimps were died after giving the immunoactivator via oral route. As well, the safety of the immunoactivator to shrimp larva was also tested via immersion inoculation owing to its small length

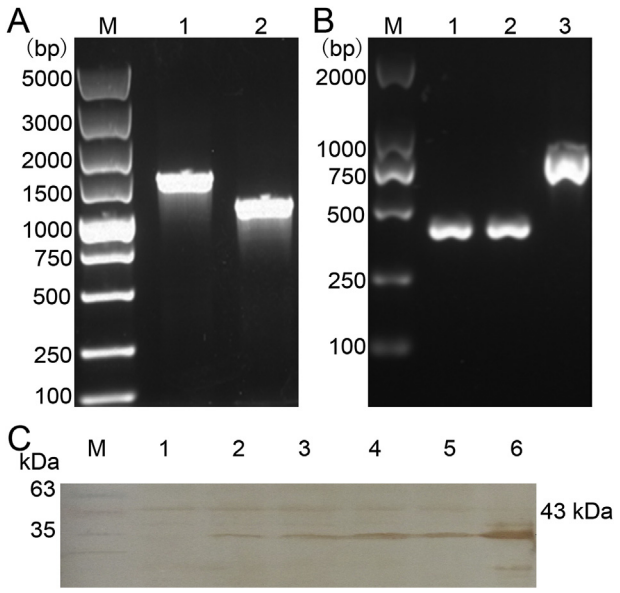

Fig. 2. Identification of strain *V. harveyi* HTVhs-5306 construction. A, PCR amplification of Δ*aroA*:*Livp28*: lane M, DNA marker; lane 1, Δ*aroA*:*Livp28* (1473 bp); lane 2, wild-type *aroA* (1112 bp). B, PCR amplification of *vhsp*: lane M, DNA marker; lane1 and lane2, Δ*vhsp* (415 bp); lane 3, wild-type *vhsp* (798 bp). C, Western blot analysis of expressed Δ*aroA*:*Livp28*. Lane M, protein marker; lane 1 *V. harveyi* HTVhs-5306 un-induced by IPTG; lane 2 and 3, strain cultured in LB20 induced by IPTG; lane 4 and 5, strain cultured in LB20 with 40 mg/ml aromatic compounds induced by IPTG; lane 6, purified Livp28 protein.

Table 3
Safety evaluation of the immunoactivator via i.m. and oral for juvenile shrimp.

Group	Strain	Animal size	Route	Administration dose	Readministration dose	Mortality (%)
Single and repetitive dose	HTVhs-5306	Juvenile (5 g)	i.m.	2×10^5 CFU/shrimp	2×10^5 CFU/shrimp	0
		Juvenile (5 g)	oral	2×10^6 CFU/g feed[a]	2×10^6 CFU/g feed	0
	Control	Juvenile (5 g)	i.m.	saline (100 μl)	saline (100 μl)	0
		Juvenile (5 g)	oral	Normal feed	Normal feed	0
High dose	HTVhs-5306	Juvenile (5 g)	i.m.	2×10^7 CFU/shrimp		5.0
		Juvenile (5 g)	oral	10^8 CFU/g feed[a]		1.0
	SDW715	Juvenile (5 g)	i.m.	2×10^7 CFU/shrimp		100
		Juvenile (5 g)	oral	10^8 CFU/g feed[a]		98
	Control	Juvenile (5 g)	i.m.	saline (100 μl)		0
		Juvenile (5 g)	oral	Normal feed		0

[a] Feed shrimp by artificial diet (2×10^6 CFU/g feed or 10^8 CFU/g feed) according to 5% of shrimp weight for 5 days continuously.

(Table S1). For single-repetitive dose inoculation, about 1% of larva died in both administrated group and control group, which might be due to nonspecific death. For large dose inoculation, 3% of larva shrimp were died after immersion with 10^8 CFU/ml of the live attenuated for 15 min, comparably, 100% of larva shrimp died after immersion with the same dose of the wild type strain. These results suggested that the immunoactivator has a good safety to shrimp via different inoculation route including immersion, i.m. injection and oral immunization either for single-repetitive dose or large dose.

3.2.2. The impact on shrimp growth and carrier state of the immunoactivator

Then we determined the impact on growth and carrier state of the live attenuated vaccine via i.m. injection and oral immunization in juvenile shrimp (weighing approximately 5g). After inoculation, shrimp were weighted twice a week. As the results shown in Fig. 3, live vaccine did not bring negative impact on shrimp weight gain, as no differences were observed between administrated and control fish during the first eight weeks. However at 10 w p.v. even though there was no significant growth, shrimps inoculated with the immunoactivator via different route exhibited increase at all. As a safety property of an attenuation, persistent carrier state of live vaccine should not be allowed in the host. In our study, hepatopancreas, intestine and gill from shrimps injected with the immunoactivator or injected with *V. harveyi* wild type were sampled for bacterial persistence. The immunoactivator was eliminated from hepatopancreas, intestine and gills within one week (Fig. 4A). However, wild type strain presented a significant multiplying in the host and all shrimps died within 3 days. Moreover, the carrier state of the immunoactivator also was proved by *in vivo* expression of VP28 from collected sample in vaccinated shrimp (Fig. 4B). These results support that live attenuated strain present good safety profiles for shrimp via different administrations.

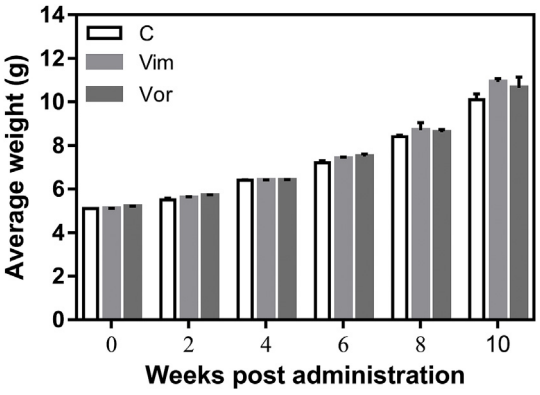

Fig. 3. The impact on shrimp growth. Juvenile shrimp weighing approximately 5 g were randomly divided into three groups (100 shrimp/group): V_{im}, shrimp administrated with 2×10^5 CFU/shrimp of the immunoactivator via i.m.; V_{or}, shrimp inoculated with 2×10^6 CFU/shrimp of immunoactivator via oral route; C, shrimp mock-inoculated with sterile saline. Shrimp were weighted at 0, 2, 4, 6, 8 and 10 w p.v.

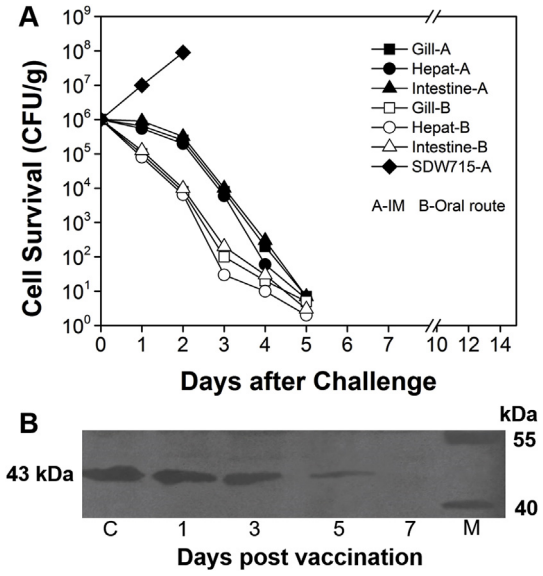

Fig. 4. The persistant carrier state of the immunoactivator. A, The elimination of immunoactivator from shrimp organs (hepatopancreas, gill and intestine) after inoculation within one week, whereas the shrimp injected WT succumbed to lethal infection with a significant multiplication after 3 days. The pooled data set from two independent experiments. B, Western blot showed that the expressed VP28 *in vivo* could not be detected after 5 days p.a. in shrimp. C, positive control for INP/VP28.

3.2.3. Pathologic change induced by the immunoactivator

Then, sections of tissues including hepatopancreas, intestine, gill and myocardium from administrated shrimps via i.m. injection and oral route were stained using H&E to examine histopathological changes induced by the vaccine. Also, shrimps challenged with *V. harveyi* WT and WSSV were tested. No significant pathologic change was observed in administrated group via oral route (data not shown). Herein, we showed the results of that in i.m. injection group in Fig. 5. In the hepatopancreas, no significant pathological changes were observed in shrimps injected with the immunoactivator or in control group and acinus maintained a normal astroid shape or siphonate shape. Comparably, gap between gland cells grew bigger and cells showed partly falling and vacuolating in *V. harveyi* infected group, and in WSSV infected group, cells shrinked gradually and the astroid shape was disappeared. Meanwhile, the structure of intestinal lumen was consecutive with villus in administrated group while in *V. harveyi* infected group and WSSV infected group, the wall became thin or scattered in the lumen with destroyed villus. The structure of the gills in vaccinated

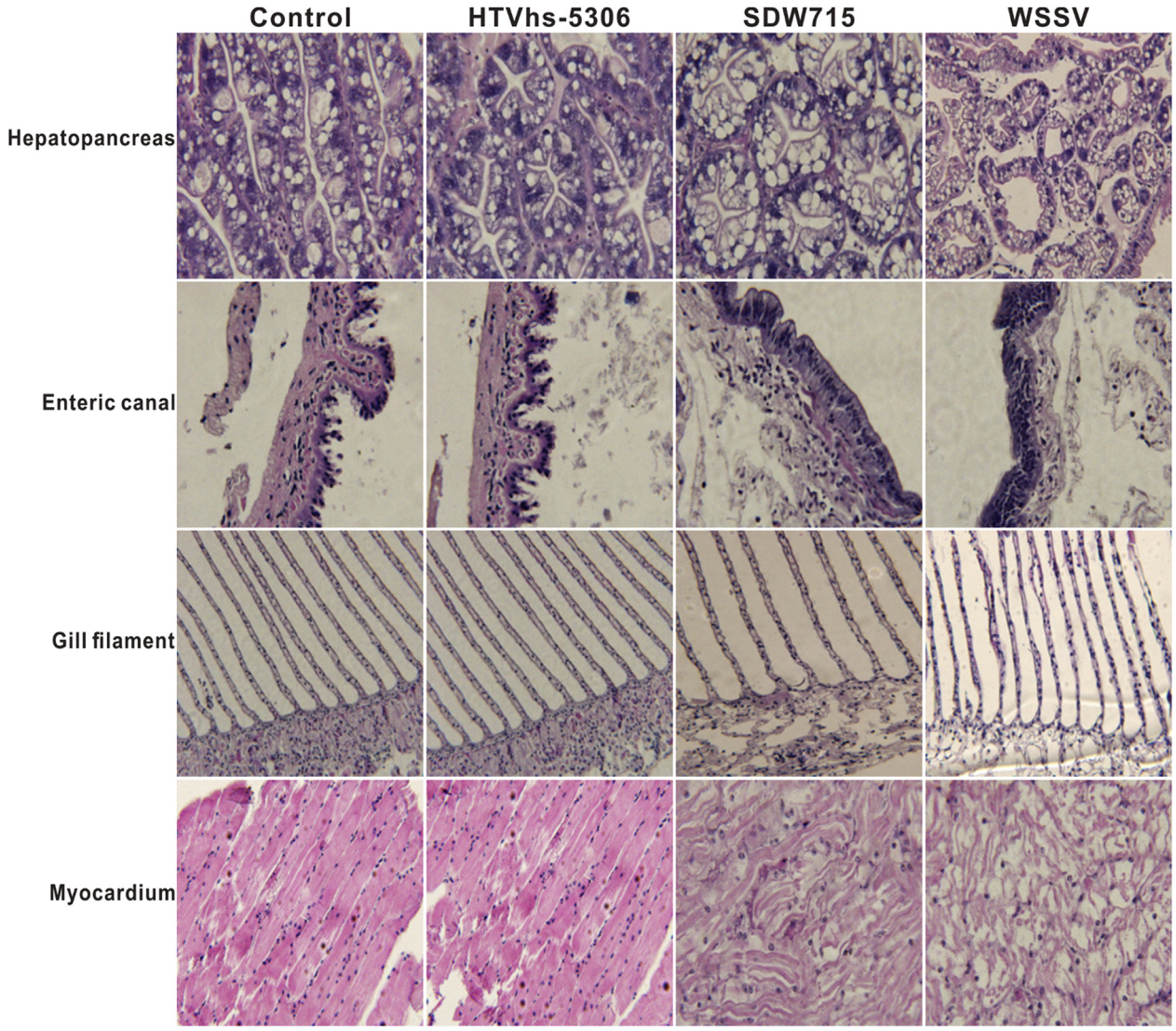

Fig. 5. Micrographs of the hepatopancreas, intestine, gill and myocardium tissues of *L. vannamei*. All samples were placed immediately into Bouin's solution fixative for histology analysis. 4 μm thick sections were cut and were mounted onto glass slides before staining with hematoxylin-eosin (H&E) for microscopic examination. Magnification 400×, scale bars = 50 μm.

group was well-ranged but with epithelial cells necrosis and abscission in the lamellae in infected groups. Meanwhile, the structure of myocardium in administrated group was normal but disordered in infected groups. Totally, these results suggested that the immunoactivator was safety without destroying tissues structure of shrimps.

3.3. Protective immunity of the immunoactivator against WSSV and vibrio infection

Secondly, the efficacy of the immunoactivator against vibriosis and WSSV was determined in juvenile shrimps via oral route later. As a result shown in Fig. 6A, a survival rate more than 60% was observed when shrimps were giving the immunoactivator via oral route and challenged with WSSV. In contrast, after the challenge, 98% of shrimps died once they were mock-immunized with sterile saline. During the experiment, no death was obtained of shrimps raised normally without any manipulation. Meanwhile, shrimps were also challenged with *V. harveyi* to investigate its protective efficacy against *Vibrio* infection. As a result shown in Fig. 6B, significant protective efficacy was observed when shrimps received inoculation via oral route and 71% shrimps were survived after challenged with *V. harveyi*. However, 95% of shrimps died once they were mock-immunized with sterile saline. In addition, the immune effect induced by the immunoactivator after administration at 1, 2, 3 and 4 w p.v. was also determined. As shown in Fig. 6C, after administrated for one week, highly RPS more than 90% were obtained in both groups that challenged with *V. harveyi* and WSSV, then a decreasing trend was observed. Still, RPS more than 60% were obtained in these groups after administrated for four week and challenged with *V. harveyi* and WSSV. These results suggested that the immunoactivator possessed an excellent protective capability against both WSSV and vibriosis through oral route.

4. Discussion

The rise in occurrence of diseases becomes a theat to aquaculture industry, leading to serious economic losses. To control this situation, vaccines are considered as one of the most effective strategies, which had been confirmed absolutely in teleosts. Though crustaceans are considered lacking a true adaptive immune system, researchers found that quasi-immune response play a role in defensing against WSSV, and the conception of ‘administration’ or ‘vaccines’ was proposed and different approaches have been attempted [23,24], thus, wo named the strains by us as “immunoactivator”. Inactivated WSSV and recombinant proteins against WSSV have been proved to protect disease caused by

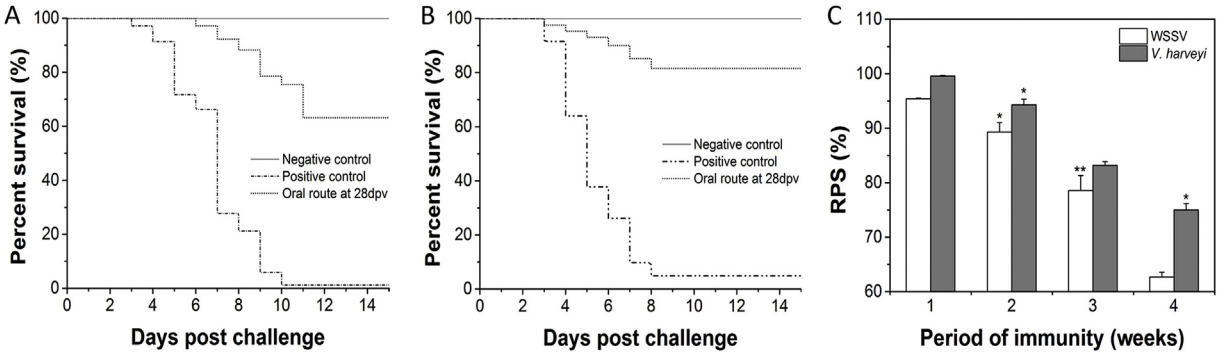

Fig. 6. Protective effect against WSSV and *Vibrio* infection. Survival rate of *L. vannamei* administrated with immunoactivator after challenge by WSSV (A), and *V. harveyi* WT (B). Briefly, shrimp were administrated by feeding artificial diet (2×10^6 CFU/g feed) according to 5% of shrimp weight for 5 days continuously, and challenged with SDW715 or WSSV at 1, 2, 3 and 4 w p.v. respectively. Cumulative survival rate was recorded for lasting 14 days until there were no dead shrimp. C, RPS of the period of immunity for immunoactivator via oral route. Error bars represented standard deviations.

WSSV, with inducing memory responses [25,26]. Surface display is a useful method to express heterologous proteins on the exterior of phages or cells [27]. *Bacillus subtilis* spores and yeast are common tools for surface display technology. In this study, we developed a live attenuated vibrio immunoactivator by deletion of *vhsp* and *aroA* gene and introduced surface display system with expressing VP28, a major envelope proteins of WSSV. We wondered whether the immunoactivator could be a bivalent "vaccine" against both vibrio and WSSV.

The immunoactivator was constructed initially and determined for VP28 expression. Expected band was obtained with indicating that the immunoactivator was *aroA and vhsp* mutant strain of *V. harveyi* and expressing VP28 of WSSV (Fig. 2). To further evaluate whether the antigen protein could be expressed *in vivo*, viscera from administrated shrimps were sampled for analysis. Until 5 d p.v., expression of VP28 was proved by Western blot (Fig. 4B). Thus, we believed that the live vibrio strain with expressing VP28 was constructed successfully, that prompted us to evaluate the safety and efficacy of the immunoactivator.

Safety is a crucial factor in vaccine development. An ideal candidate strain should be non-pathogenic or avirulent to the target animal species [28]. Herein, the immunoactivator developed in this study was proved safety to juvenile shrimp via different inoculation route either with single-repetitive dose or large dose. As mentioned above, *B. subtilis* spores and yeast are non-pathogenetic and usually used for expressing heterologous proteins. There are truly several reports on expressing VP28 against WSSV infection. Nguyen et al. used spores of *B. subtilis* to display VP28 on the spore surface and found it have potential as a prophylactic treatment of WSS [29]. Besides, an engineered *Pichia pastoris* displaying biologically active mPmRab7 and pVP28 is efficacy in protecting shrimp against WSSV by oral administration [30]. However, there is no report on that using common bacterium in aquaculture as an antigenic protein carrier, even harmful to breeding industry. Considering that *L. vannamei* is often suffered from vibriosis caused by *V. harveyi*, we attempted to use the attenuated *V. harveyi* as the carrier. As a matter of fact, the immunoactivator is attenuated significantly compared with the wild type (Table 3). Moreover, this immunoactivator did not affect the growth of shrimp according to the weight after administration within 10 weeks (Fig. 3) and have limited ability to proliferate in shrimp with survival duration within one week (Fig. 4A), which was in accordance with VP28 expression *in vivo* (Fig. 4B). Additionally, the immunoactivator did not induce unhealth pathologic changes at all (Fig. 5). All of these suggested that the immunoactivator we developed is safety to shrimp. Recently, the immune stimulatory effect of vibrio bacterin in commercial tiger shrimp was reported under oral administration and be proved to improve the immunity, reduce anatomical deformities and enhance the production in commercial shrimp culture operations [31]. Similarly, in our study, shrimps inoculated with the immunoactivator via immersion or oral administration exhibited more growth than control group. One of the reason might be that the immunoactivator enhance the disease resistance of the shrimp. Therefore, then, we evaluated the efficacy of the immunoactivator.

The immunoactivator we developed possess an efficient immunoprotection against both *V. harveyi* and WSSV, of which are desired by us. Thus, it is convenient to use the immunoactivator to control both vibriosis and WSS. Nowadays, multiple-pathogens infection creates an urgent need for efficient measures to combat current pathogens. However, most researches are focus on developing vaccines against one pathogen. If one vaccine could play roles in defensing one more pathogens, it is not only beneficial for breedings but for saving operation cost. Among different inoculation route, oral administration is a simple method. Under this situation, the RPS of our immunoactivator against *V. harveyi* and WSSV are 70% and 63% at 4 w p.v., respectively. Interestingly, Fu et al. previously reported that the RPS value of VP28 using *B. subtilis* as vehicles fed shrimp was 83.3% when challenged on the 14th day p.a [23]. The difference between us was the challenge time point we selected, which prompted us to see the protective efficacy of the vaccine in a shorter period of time. As a result, a decreasing trend of RPS was observed after administration at 1, 2, 3 and 4 w p.v. This result was quite different from that in teleost. In our previous study, the efficacy of a vaccine in teleost increased gradually, and reached a highly RPS at 4 w p.v. However, in this study, excellent RPS were obtained at 1 w p.v. and then decreased. This might due to the different immune system between teletost and shrimp. Invertebrates lack true adaptive immunity and it solely depends on the primitive innate immunity [32]. Maybe this is the reason that high protective efficacy is acquired immediately after administration. Our result supply evidence for immune stimulation of shrimp. Recently Lin et al. conferred that shrimp may have specific memory [33]. Nevertheless, the RPS of the immunoactivator we developed against *V. harveyi* and WSSV are more than 60% at 4 w p.v.

In conclusion, we developed an immunoactivator by using *V. harveyi* as an antigenic protein carrier with VP28 expression for shrimp. The immunoactivator was safety via different inoculation routes either for single-repetitive dose or large dose. Moreover, the immunoactivator was not harmful for shrimp in suppressing growth or inducing pathologic changes. Additionally, an immediate immunoprotection was induced by the immunoactivator after administration for one week with highly RPS more than 90% against both *V. harveyi* and WSSV. Until 4 w p.v., the immunoactivator still possessed efficient immune effect. Totally, the attenuated *V. harveyi* strain displaying VP28 is a potential immunoactivator for *L. vannamei*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.10.016.

References

[1] T.W. Flegel, Historic emergence, impact and current status of shrimp pathogens in Asia, J. Invertebr. Pathol. 110 (2012) 166–173.
[2] S. Thitamadee, A. Prachumwat, J. Srisala, P. Jaroenlak, P.V. Salachan, K. Sritunyalucksana, et al., Review of current disease threats for cultivated penaeid shrimp in Asia, Aquaculture 452 (2016) 69–87.
[3] G. Qiao, D.H. Xu, Z. Wang, I.K. Jang, Z. Qi, M. Zhang, et al., Comparison of immune response of Pacific white shrimp, *Litopenaeus vannamei*, after multiple and single infections with WSSV and *Vibrio anguillarum*, Fish Shellfish Immunol. 44 (2015) 257–264.
[4] Z. Wang, F. Zhu, Different roles of a novel shrimp microRNA in white spot syndrome virus (WSSV) and *Vibrio alginolyticus* infection, Dev. Comp. Immunol. 79 (2018) 21–30.
[5] Z. Zheng, F. Wang, J.J. Aweya, R. Li, D. Yao, M. Zhong, et al., Comparative transcriptomic analysis of shrimp hemocytes in response to acute hepatopancreas necrosis disease (AHPND) causing *Vibrio parahemolyticus* infection, Fish Shellfish Immunol. 74 (2018) 10–18.
[6] H. Cho, N.H. Park, Y. Jang, Y.D. Gwon, Y. Cho, Y.K. Heo, et al., Fusion of flagellin 2 with bivalent white spot syndrome virus vaccine increases survival in freshwater shrimp, J. Invertebr. Pathol. 144 (2017) 97–105.
[7] P. Nilsen, M. Karlsen, K. Sritunyalucksana, S. Thitamadee, White spot syndrome virus VP28 specific double-stranded RNA provides protection through a highly focused siRNA population, Sci. Rep. 7 (2017) 1028.
[8] P. Palanikumar, D.J.D. Benitta, C. Lelin, E. Thirumalaikumar, M. Michaelbabu, T. Citarasu, Effect of *Argemone mexicana* active principles on inhibiting viral multiplication and stimulating immune system in Pacific white leg shrimp *Litopenaeus vannamei* against white spot syndrome virus, Fish Shellfish Immunol. 75 (2018) 43–52.
[9] K.C. Pham, H.T.T. Tran, C. Van Doan, P.H. Le, A.T. Van Nguyen, H.A. Nguyen, et al., Protection of *Penaeus monodon* against white spot syndrome by continuous oral administration of a low concentration of *Bacillus subtilis* spores expressing the VP28 antigen, Lett. Appl. Microbiol. 64 (2017) 184–191.
[10] A.K. Ray, C. Gopal, H.G. Solanki, T. Ravisankar, P.K. Patil, Effect of orally administered vibrio bacterin on immunity, survival and growth in tiger shrimp (*Penaeus monodon*) grow-out culture ponds, Lett. Appl. Microbiol. 65 (2017) 475–481.
[11] N.S. Sudheer, G. Poulose, A. Thomas, K. Viswanath, A. Kulkarni, R.B. Narayanan, et al., Expression profile of bio-defense genes in *Penaeus monodon* gills in response to formalin inactivated white spot syndrome virus vaccine, Antivir. Res. 117 (2015) 60–68.
[12] G. Solis-Lucero, K. Manoutcharian, J. Hernandez-Lopez, F. Ascencio, Injected phage-displayed-VP28 vaccine reduces shrimp *Litopenaeus vannamei* mortality by white spot syndrome virus infection, Fish Shellfish Immunol. 55 (2016) 401–406.
[13] L.H. Chen, S.W. Lin, K.F. Liu, C.I. Chang, J.R. Hseu, J.M. Tsai, Comparative proteomic analysis of *Litopenaeus vannamei* gills after administration with two WSSV structural proteins, Fish Shellfish Immunol. 49 (2016) 306–314.
[14] S. Taengchaiyaphum, H. Nakayama, J. Srisala, R. Khiev, D.J. Aldama-Cano, S. Thitamadee, et al., Administration with multimeric recombinant VP28 induces high protection against white spot syndrome virus in shrimp, Dev. Comp. Immunol. 76 (2017) 56–64.
[15] J.S. Lee, K.S. Shin, J.G. Pan, C.J. Kim, Surface-displayed viral antigens on Salmonella carrier vaccine, Nat. Biotechnol. 18 (2000) 645–648.
[16] J.L. Wasilenko, L. Sarmento, S. Spatz, M. Pantin-Jackwood, Cell surface display of highly pathogenic avian influenza virus hemagglutinin on the surface of *Pichia pastoris* cells using alpha-agglutinin for production of oral vaccines, Biotechnol. Prog. 26 (2010) 542–547.
[17] J. Bal, H.Y. Jung, L.N. Nguyen, J. Park, Y.S. Jang, D.H. Kim, Evaluation of cell-surface displayed synthetic consensus dengue EDIII cells as a potent oral vaccine candidate, Microb. Cell Factories 17 (2018) 146.
[18] S. Gao, D. Li, Y. Liu, E. Zha, T. Zhou, X. Yue, Oral immunization with recombinant hepatitis E virus antigen displayed on the *Lactococcus lactis* surface enhances ORF2-specific mucosal and systemic immune responses in mice, Int. Immunopharmacol. 24 (2015) 140–145.
[19] K. Hao, X.H. Chen, X.Z. Qi, B. Zhu, G.X. Wang, F. Ling, Display of GCRV vp7 protein on the surface of *Escherichia coli* and its immunoprotective effects in grass carp (*Ctenopharyngodon idella*), Fish Shellfish Immunol. 72 (2018) 199–209.
[20] S.Y. Wang, J. Lauritz, J. Jass, D.L. Milton, A ToxR homolog from *Vibrio anguillarum* serotype O1 regulates its own production, bile resistance, and biofilm formation, J. Bacteriol. 184 (2002) 1630–1639.
[21] L.L. Sheng, Y.Z. Lv, Q. Liu, Q.Y. Wang, Y.X. Zhang, Connecting type VI secretion, quorum sensing, and c-di-GMP production in fish pathogen *Vibrio alginolyticus* through phosphatase PppA, Vet. Microbiol. 162 (2013) 652–662.
[22] X. Li, H. Wu, M. Zhang, S. Liang, J. Xiao, Q. Wang, et al., Secreted glyceraldehyde-3-phosphate dehydrogenase as a broad spectrum vaccine candidate against microbial infection in aquaculture, Lett. Appl. Microbiol. 54 (2012) 1–9.
[23] L.L. Fu, J.B. Shuai, Z.R. Xu, J.R. Li, W.F. Li, Immune responses of *Fenneropenaeus chinensis* against white spot syndrome virus after oral delivery of VP28 using *Bacillus subtilis* as vehicles, Fish Shellfish Immunol. 28 (2010) 49–55.
[24] D. Ning, X. Leng, Q. Li, W. Xu, Surface-displayed VP28 on *Bacillus subtilis* spores induce protection against white spot syndrome virus in crayfish by oral administration, J. Appl. Microbiol. 111 (2011) 1327–1336.
[25] R.K. Jha, Z.R. Xu, S.J. Bai, J.Y. Sun, W.F. Li, J. Shen, Protection of *Procambarus clarkii* against white spot syndrome virus using recombinant oral vaccine expressed in *Pichia pastoris*, Fish Shellfish Immunol. 22 (2007) 295–307.
[26] F. Zhu, H. Du, Z.G. Miao, H.Z. Quan, Z.R. Xu, Protection of *Procambarus clarkii* against white spot syndrome virus using inactivated WSSV, Fish Shellfish Immunol. 26 (2009) 685–690.
[27] J. Kim, W. Schumann, Display of proteins on *Bacillus subtilis* endospores, Cell. Mol. Life Sci. 66 (2009) 3127–3136.
[28] X. Cai, B. Wang, Y. Peng, Y. Li, Y. Lu, Y. Huang, et al., Construction of a *Streptococcus agalactiae phoB* mutant and evaluation of its potential as an attenuated modified live vaccine in golden pompano, *Trachinotus ovatus*, Fish Shellfish Immunol. 63 (2017) 405–416.
[29] A.T. Nguyen, C.K. Pham, H.T. Pham, H.L. Pham, A.H. Nguyen, L.T. Dang, et al., *Bacillus subtilis* spores expressing the VP28 antigen: a potential oral treatment to protect *Litopenaeus vannamei* against white spot syndrome, FEMS Microbiol. Lett. 358 (2014) 202–208.
[30] V. Ananphongmanee, J. Srisala, K. Sritunyalucksana, C. Boonchird, Yeast surface display of two proteins previously shown to be protective against white spot syndrome virus (WSSV) in Shrimp, PLoS One 10 (2015) e0128764.
[31] A.K. Ray, C. Gopal, H.G. Solanki, T. Ravisankar, P.K. Patil, Effect of orally administered vibrio bacterin on immunity, survival and growth in tiger shrimp (*Penaeus monodon*) grow-out culture ponds, Lett. Appl. Microbiol. 65 (2017) 475–481.
[32] S.K. Syed Musthaq, J. Kwang, Evolution of specific immunity in shrimp a administration perspective against white spot syndrome virus, Dev. Comp. Immunol. 46 (2014) 279–290.
[33] Y.C. Lin, J.C. Chen, W.Z. Morni, D.F. Putra, C.L. Huang, C.C. Li, et al., Administration enhances early immune responses in white shrimp *Litopenaeus vannamei* after secondary exposure to *Vibrio alginolyticus*, PLoS One 8 (2013) e69722.