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# Identification and functional characterization of a c-type lysozyme from *Fenneropenaeus penicillatus*

Shuanghu Cai[a,*], Yilin Zhang[a], Fan Wu[a], Rimin Wu[a], Shiping Yang[a], Ya Li[b], Youhou Xu[c,**]

[a] Fisheries College of Guangdong Ocean University, Guangdong Provincial Key Laboratory of Pathogenic Biology and Epidemiology for Aquatic Economic Animals & Key Laboratory of Control for Diseases of Aquatic Economic Animals of Guangdong Higher Education Institutes, Zhanjiang, China
[b] Agricultural College of Guangdong Ocean University, Zhanjiang, China
[c] Guangxi Key Laboratory of Beibu Gulf Marine Biodiversity Conservation, Beibu Gulf University, Qinzhou, China

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ABSTRACT

Lysozyme is an important defense molecule of the innate immune system and possess high antimicrobial activities. In this study, a full-length c-type lysozyme cDNA (*Fplysc*) was cloned and characterized from *Fenneropenaeus penicillatus*. The cDNA contains an open reading frame of 477 bp encoding 158 amino acids, with 53–94% identity with those of other crustaceans. The recombinant Fplysc had antibacterial activities against Gram-positive bacteria *(Streptococcus agalactiae* and *Micrococcus luteus*) and Gram-negative bacteria (*Vibrio alginolyticus* and *Escherichia coli*), and showed antiviral activity against WSSV and IHHNV. The qRT-PCR analysis showed that *Fplysc* expression levels were most abundant in hemocytes and less in eyestalk. The expression levels of *Fplysc* were significantly upregulated in gill, intestine and hemocytes when challenged with WSSV and *V. alginolyticus*. *Fplysc*-silencling suppressed *Fplysc* expression in cephalothoraxes and increased mortality caused by WSSV and *V. alginolyticus*, and exogenous rFplysc led to a significant decrease of shrimp mortality by injecting rFplysc into *Fplysc* silenced shrimp, suggesting Fplysc is the important molecule in shrimp antimicrobial and antiviral response. In conclusion, the results provide some insights into the function of Fplysc in shrimp against bacterial and viral infection.

## 1. Introduction

*Fenneropenaeus penicillatus*, generally called red tail shrimp by the Food and Agriculture Organization, is an important aquaculture species, and widely distributed in the India and Indonesia coastal waters, especially in the East China Sea and South China Sea [1]. It used to be an important breeding species in Guangdong, Fujian and Taiwan of China because of its delicious taste and rapid growth. However, in recent decades, the cultured capacity and wild resource of red tail shrimp sharply declined. In 2005, *F. penicillatus* was included in the Red List by the Chinese government as an endangered species [2]. Currently, several factors such as excess exploitation, unreasonable utilization, environmental deterioration and frequent disease already affected reproduction of red tail shrimp [3,4]. In order to preserve red tail shrimp wild stocks, it is important and urgent to study how to promote the immune resistance against the disadvantage conditions.

The innate immune system plays a crucial role in defense against invading of pathogen microorganisms. Lysozymes are key immune factors in the innate immune system of animals, plants and microorganisms, and participate in some different types of body innate immune responses [5]. In animals, lysozymes can be divided into four types including c-type (chicken or conventional type), g-type (goose type), i-type (invertebrate type), and ch-type (chalaropsis type) [6]. Some studies show only c-type and g-type can be found in vertebrates, and all four types have been identified in invertebrates [6,7]. Moreover, lysozymes are multi-functional enzymes responsible for immune defense and foods digestion in animals [8].

Several c-type lysozymes had been identified and characterized in crustaceans, and these lysozymes exhibit variable expression patterns and antibacterial spectrums. A c-type lysozyme from kuruma shrimp *Marsupenaeus japonicus* was identified and found it could inhibit the proliferation of hemocytes microbiota [9]. In addition, c-type lysozyme was released around the hemocytes extracellular trap complex (ET) after pathogen-stimulation, suggesting the enzyme could be the

* Corresponding author. Fisheries College of Guangdong Ocean University, No. 1 Haida Road, Mazhang District, Zhanjiang, Guangdong, China.
** Corresponding author. Guangxi Key Laboratory of Beibu Gulf Marine Biodiversity Conservation, Beibu Gulf University, No. 89 West Street of Nanzhu, Qinzhou, Guangxi, China.
*E-mail addresses:* shuanghucai@gmail.com (S. Cai), xuyouhou@gmail.com (Y. Xu).

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component of ET [10]. The c-type lysozyme showed a broad spectrum of antimicrobial activity against both Gram-negative pathogenic bacterium *Salmonella typhimurium* and two fungus *Pichia pastoris* and *Saccharomyces cerevisiae* in *Fenneropenaeus indicus*. Furthermore, c-type lysozyme expression levels correlated with WSSV (White Spot Syndrome Virus) load suggested the enzyme was involved in shrimp antiviral response [11]. The c-type lysozymes were also identified in other Penaeidae species including *Litopenaeus vannamei*, *Penaeus monodon* and *F. mergiurensis*, and challenge with pathogen associated molecular patterns (PAMPs) can significantly promote c-type lysozyme mRNA expression levels [12–14]. Furthermore, Knockdown of lysozyme gene by dsRNA could lead to high mortality without any artificial bacterial infection in *M. japonicas* [15]. All these findings suggest c-type lysozyme play a crucial role in crustacean immunity.

In this study, we investigated the structural and functional features of c-type lysozyme in *F. penicillatus*. Based on transcriptome data constructed by our research group, the c-type lysozyme gene (*Fplysc*) was identified and its expression level was analyzed after virus and bacteria challenge from *F. penicillatus*. The function of Fplysc was also studied in recombinant Fplysc protein and *Fplysc*-silenced shrimps. Overall, this study may provide better understand of the further mechanism on c-type lysozyme participant in the shrimp innate immunity.

## 2. Materials and methods

### 2.1. Experimental animals

Healthy red tail shrimp *F. penicillatus* with an average body weight 12 ± 2.42 g were obtained from Sanniang Bay in Guangxi, China. Before the experiments, all of shrimp were detected using the methods of loop-mediated isothermal amplification according to the previous reports [16,17] for WSSV and *Vibrio alginolyticus*. Only shrimps of WSSV and *V. alginolyticus*-free were randomly selected as experimental animals. The shrimp were fed with artificial diet for 7 days and acclimated to laboratory conditions.

### 2.2. Molecular cloning of Fplysc cDNA in red tail shrimp

Total RNA was extracted from the hemocytes of red tail shrimp using Trizol reagents (Invitrogen, USA) following the manufacturer's instructions, and the residual genomic DNA was removed using Rnase-free Dnase (RQ1; Promega, Madison, WI). Complementary DNA (cDNA) was prepared using PrimeScript RT reagent kit (TaKaRa, Dalian, China). *Fplysc* primers were designed (Table 1) based on a partial sequence from the transcriptome data. Rapid amplification cDNA ends (RACE) were performed using the SMARTer™ RACE cDNA Amplification kit (Clontech, Japan) according to the manufacturer's protocol. The PCR products were cloned into pMD-18T Cloning Vector (Takara) and then sequenced by Shanghai Sangon Biologic Engineering & Technology and Service Co. Ltd. To reconfirm the sequence of full-length cDNA, we amplify the complete fragment of *Fplysc* by PCR technique using the primers Lysc-fF and Lysc-fR. The amplification conditions were performed as following: 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, followed by 72 °C for 10 min. The PCR product was ligated with the pMD18-T plasmid and sequenced.

### 2.3. Bioinformatics analysis

The cDNA Sequence was analyzed with the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The protein characteristic of Fplysc was described using ExPASy software (https://www.expasy.org/). The functional domains were presumed using CDD software (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Signal peptide was predicted with SignalP (http://www.cbs.dtu.dk/services/SignalP/). Multiple sequence alignments were performed using the ClustalX 2.0 program. Phylogenetic analyses were performed using the Neighbor-Joining method in the MEGA 5.0. A bootstrap analysis was performed based on 1000 bootstrap replications.

### 2.4. Recombinant expression and purification of rFplysc

The pair of primers FplyscF and FplyscR (Table 1) was designed to amplify the sequence encoding Fplysc mature peptide. The PCR fragment was digested and inserted into a pET-28a expression vector. The constructed plasmid was transformed into competent *Escherichia coli* BL21 (DE3) cells for recombinant expression. After induction with a final concentration of 0.5 mM IPTG at 28 °C for 12 h and the fusion protein was purified from the inclusion bodies. Briefly, the bacteria were pelleted through centrifugation and then re-suspended in PBS containing 0.1% Triton X-100 for probe sonication lysis. The recombinant protein (rFplysc) was purified by Ni-NTA Sefinose™ Resin (Sangon Biotech, China) and eluted with imidazole according to the manual.

### 2.5. Antibacterial and antiviral activity of rFplysc in vitro

Gram-positive bacteria (*Streptococcus agalactiae* and *Micrococcus luteus*) and Gram-negative bacteria (*Vibrio alginolyticus* and *E. coli*) were used to analyze the antibacterial activity of rFplysc in bacterial inhibition assay. Cultured bacteria in mid-logarithmic phase were collected and washed with sterile PBS and diluted to approximately $1 \times 10^6$ cells/ml. Bacteria suspension (10 ml) were incubated with rFplysc (2 mg) for 2 h at 28 °C, while the bacteria incubated with PBS was used as control. Bacteria-protein mixture was added to the 96-well plate containing 180 μl medium per well and were cultured for 24 h at 28 °C. The $OD_{600}$ was measured in order to monitor the bacterial growth with Bio-tekELx800 microplate reader (Biotek, Winooski, USA). The bacterial growth of control was defined as 100%. All samples were tested in triplicate.

WSSV and IHHNV (Infectious Hypodermal and Hematopoietic Necrosis Virus) were prepared according to a previously described method [1]. The titers of WSSV and IHHNV suspension were determined by qRT-PCR assay using the primers Wssv270 and Wssv345 for WSSV, Ihhnv24 and Ihhnv100 for IHHNV [18]. And then the suspensions were diluted with PBS and copy numbers adjusted to $1 \times 10^6$ copies/ml, respectively. Viral suspensions (10 ml) were incubated with rFplysc (2 mg) for 2 h at 28 °C as experimental group, while with PBS as control. Finally, the mixtures of experimental group and control were analyzed by qRT-PCR assay. The viral titer of control was defined as 100%. All samples were tested in triplicate.

### 2.6. Tissue distribution of Fplysc mRNA

Transcriptional tissue distributions of *Fplysc* mRNA in red tail shrimp were examined by using real-time quantitative RT-PCR (qRT-PCR) in selected tissues. The total RNA was extracted from brain, gill, heart, hepatopancreas, intestine, hemocytes, eyestalk, stomach and muscle from healthy shrimp, respectively. RNA was extracted and cDNA was synthesized following the above protocols. The obtained cDNA samples were used as templates in qRT-PCR with the specific primers (Table 1) designed based on the sequence of *Fplysc* cDNA. The *elongation factor-1* (*FpEF1α*) gene was used as internal control in all qRT-PCR experiments according to the study of Dhar et al. [19]. The qRT-PCR analysis were performed and PCR conditions were as follows: 94 °C 2 min, followed by 30 cycles of 94 °C 20 s, 60 °C 20 s, and 72 °C 20 s. The PCR products were analyzed on 1.2% agarose gel stained with ethidium bromide. Expression level of *Fplysc* was calculated using $2^{-\Delta\Delta Ct}$ method after normalization to *FpEF1α* [20]. All samples were tested in triplicate.

**Table 1**
Primers used for PCR amplification in this study.

| Primers | Primer sequence (5′→3′) |
|---|---|
| RACE | |
| Lysc-5R | CGAATTCGCGAGTCTGTTGG |
| Lysc-5NR | CAAGAACTGGGTGTGCATCG |
| Lysc-3F | TCGCTGCCACACCAGTATTT |
| Lysc-3NF | ATACCGTAGTCTGTGCTGCG |
| Primers for full-length cDNA of *Fplysc* | |
| Lysc-fF | GTAGGGAACATTCGCTGGGT |
| Lysc-fR | CTCCAGTATCTGCCGTGCC |
| Primers for recombinant expression | |
| FplyscF | CATGCCATGGGCGTGCTTCCTCTG (*Nco* I) |
| FplyscR | CCGCTCGAGAGAATGGGTATATCCCG (*Xho* I) |
| Primers for qRT-PCR analysis | |
| Lysc-F | TCGAGTCGTCCTTCAACACG |
| Lysc-R | CTTGCCATAATCGCTGCCAC |
| FpEF1α-F | CTACTCACCTGTGCTTGATTGC |
| FpEF1α-R | TTGCTGGGAACCATCTTTACGA |
| Ihhnv24 | AAACTGAACACTGGCCTAGTAACAA |
| Ihhnv100 | TAGGACTTTCCGATGAGGTTTTG |
| Wssv270 | ACCATGGAGAAGATATGTACAAGCA |
| Wssv345 | GGCATGGACAGTCAGGTCTTT |
| Primers for gene silencing | |
| dsFplysc-T7-F | GGATCCTAATACGACTCACTATAGGGTTGGGCTTCTGGCCGTTTC |
| dsFplysc-T7-R | GGATCCTAATACGACTCACTATAGGCGGCCATGTACTGATCGAGG |
| dsFplysc-F | TTGGGCTTCTGGCCGTTTC |
| dsFplysc-R | CGGCCATGTACTGATCGAGG |
| dsGFPT7-F | GGATCCTAATACGACTCACTATAGGATGGTGAGCAAGGGCGAGGA |
| dsGFPT7-R | GGATCCTAATACGACTCACTATAGGTTACTTGTACAGCTCGTCCA |
| dsGFPF | ATGGTGAGCAAGGGCGAGGA |
| dsGFPR | TTACTTGTACAGCTCGTCCA |

### 2.7. The expression pattern of Fplysc during microbial infection

WSSV suspension was prepared and diluted with PBS and copy numbers adjusted to $1 \times 10^6$ copies/ml. *V. alginolyticus* were cultured in Luria broth (LB) medium and the concentration was adjusted to $1 \times 10^8$ CFUs/ml. For challenge experiments, healthy red tail shrimps were divided into 3 groups, in which each red tail shrimp was injected intramuscularly at the third abdominal segment of 50 μl WSSV suspension ($5 \times 10^4$ copies), 50 μl V. *alginolyticus* suspension ($5 \times 10^6$ CFUs) or 50 μl PBS, respectively. Five shrimps from each group were selected randomly and brain, gill, heart, hepatopancreas, intestine, hemocytes, eyestalk, stomach and muscle were collected at 0, 1, 2, 4, 8, 12, 24, 48 and 72 h post-injection. Three samples were collected at each time point, and the total RNA was extracted from each individual sample. The mRNA levels of *Fplysc* were detected by qRT-PCR as the above method.

### 2.8. Gene silencing of Fplysc in F. penicillatus

Specific double-stranded RNAs (dsRNA) of *Fplysc* and GFP (as a control) were generated *in vitro* using a T7 RiboMAX™ Express RNAi System (Promega, USA) according to the manufacturer's instructions. The primers were designed to produce sense and antisense strands separately (Table 1). Two separate PCRs were performed, one with ds*Fplysc*-T7-F and ds*Fplysc*-R for the sense strand template, the other with ds*Fplysc*-F and ds*Fplysc*-T7-R for the anti-sense strand template. The *GFP* gene as a negative control was amplified with pEGFP-1 vector by using GFP-T7-F and GFP-R for the sense strand template, and GFP-F and GFP-T7-R for the anti-sense strand template. The T7 RiboMAX™ Express RNAi System was used to generate single stranded RNA. Equal amounts of the single stranded RNA were annealed to generate dsRNA. Finally, two groups of shrimp were injected with either ds*Fplysc*, ds*GFP* (10 μg dsRNA/shrimp) or PBS. To confirm the efficiency of the dsRNA-mediated RNA interference, the cephalothoraxes samples from at least three shrimp per treatment group were collected for total RNA isolation and cDNA preparation at 0, 1, 2, 4, 8, 12, 24, 48 and 72 h post-dsRNA injection by qRT-PCR assay.

### 2.9. Microbial infection experiments in dsRNA injected shrimp

At 24 h post-injection of ds*Fplysc* or ds*GFP*, shrimps were received the second injection of 50 μl WSSV suspension ($5 \times 10^4$ copies) or 50 μl V. *alginolyticus* suspension ($5 \times 10^6$ CFUs), and that of PBS (50 μl) as a control, respectively. For the rescue, rFplysc (5 μg) was injected into *Fplysc* pre-silencing shrimp, and WSSV or *V. alginolyticus* was injected another 1 h later. Shrimps were maintained in a separate tank in tanks with air-pumped seawater for 10 days following infection. Cumulative mortality of each group was recorded each day. For each group, 40 individuals were used.

### 2.10. Statistical analysis

All statistical analyses were performed using Student's t-test or analysis of variance (ANOVA) followed by Duncan's new multiple range test with SPSS 19.0 software, and statistical significance was defined as $P < 0.05$. Results were expressed as the mean ± SE.

## 3. Results

### 3.1. cDNA cloning and sequence analysis of Fplysc

The complete cDNA sequence of *Fplysc* was obtained by RACE and reconfirmed by PCR. The full-length cDNA of *Fplysc* is 759 bp long with an open reading frame (ORF) of 477 bp encoding a putative protein of 158 amino acids, a 5′-untranslated region of 72 bp, and a long 3′-untranslated region of 210 bp including putative polyadenylation consensus signal (AATAAA) and a poly (A) tail (GenBank accession no. MK192267). The calculated molecular mass of the deduced mature Fplysc was 18.07 kDa and the protein had a theoretical isoelectric point of 8.47. Sequence analysis showed that Fplysc protein contained a signal peptide of 18 residues and a highly conserved lysozyme domain. Sequence comparison showed the protein sequence of Fplysc shared

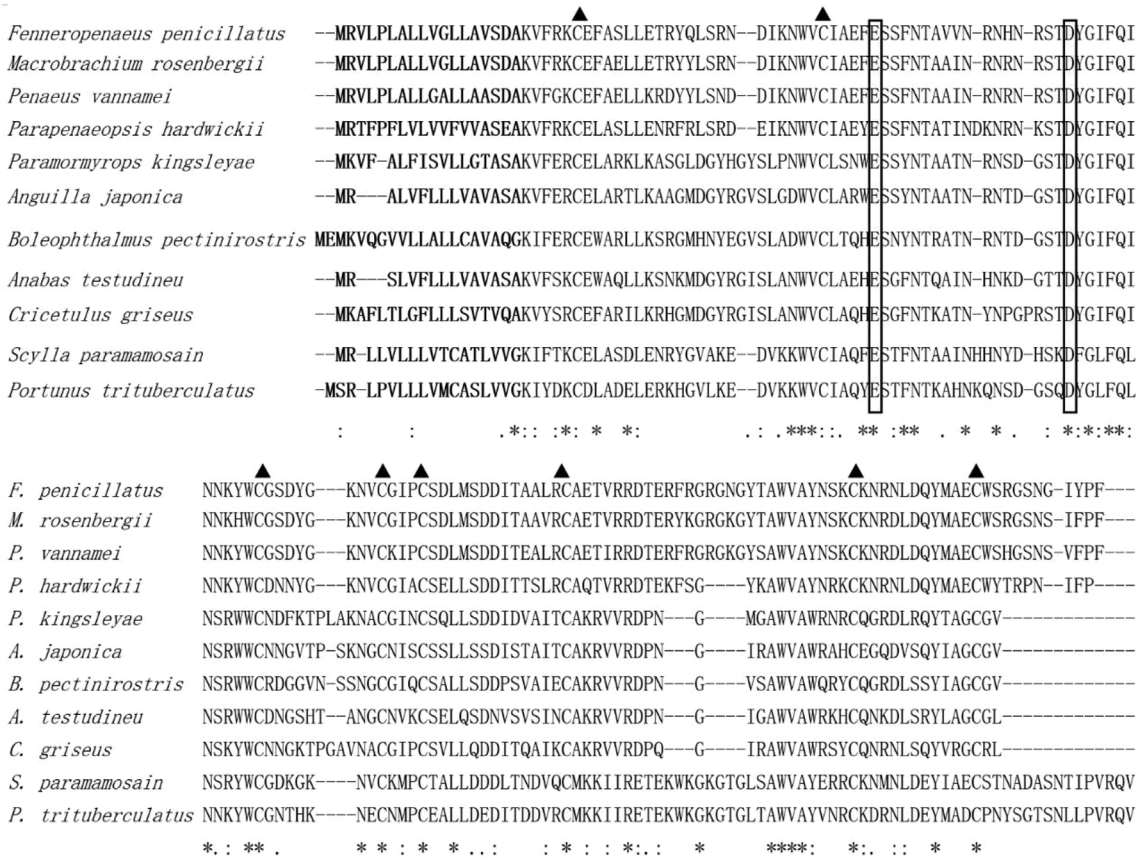


**Fig. 1.** Alignment of the deduced amino acid sequences of Fplysc homologues. The predicted signal peptide is in bold. The conserved catalytic residues ($E^{51}$ and $D^{68}$) and eight Cys residues are indicated with box (□) and triangle (▲), respectively. Accession number for each sequence is *Fenneropenaeus penicillatus* (MK192267), *Macrobrachium rosenbergii* (AAP13577.2), *Penaeus vannamei* (AAL23948.1), *Parapenaeopsis hardwickii* (AEV91334.1), *Paramormyrops kingsleyae* (XP_023662412.1), *Anguilla japonica* (AIZ00421.1), *Boleophthalmus pectinirostris* (XP_020780154.1), *Anabas testudineu* (XP_026200227.1), *Cricetulus griseus* (XP_027248149.1), *Scylla paramamosain* (ADM33942.1) and *Portunus trituberculatus* (ACM24796.2).

53–94% identity with those of other crustaceans (Fig. 1). Phylogenetic relationship analysis revealed that Fplysc protein was clustered with the other c-type lysozymes, including seven from crustaceans (*F. merguiensis*, *F. chinensis*, *P. vannamei*, *P. monodon*, *Macrobrachium rosenbergii*, *Portunus trituberculatus* and *Scylla paramamosain*), three from arachnoidea (*Haemaphysalis longicornis*, *Dermacentor variabilis* and *D. andersoni*), seven from fish (*Hucho taimen*, *Oncorhynchus mykiss*, *Paramormyrops kingsleyae*, *Scophthalmus rhombus*, *Solea senegalensis*, *Gambusia affinis* and *Poecilia mexicana*) and three from mammals (*Cricetulus griseus*, *Mus caroli* and *Mesocricetus auratus*) (Fig. 2).

### 3.2. Antimicrobial and antiviral activity of rFplysc

To study antimicrobial and antiviral activity of rFplysc *in vitro*, recombinant protein was successfully expressed in *E. coli* BL21 (DE3) cells under 0.5 mM IPTG induction at 28 °C and purified by Ni-NTA chromatography. The recombinant vector would add 6 × His tag to the recombinant protein, and the molecular mass of rFplysc fusion protein (19 kDa) observed from the SDS-PAGE was in agreement with prediction (Fig. 3A). The antimicrobial and antiviral activity of rFplysc was examined, and the results exhibited rFplysc significantly inhibited the growth of all tested bacteria and virus (Fig. 3B).

### 3.3. Expression levels of Fplysc in different tissues

The qRT-PCR was performed to quantify the mRNA expression levels of *Fplysc* in different tissues. The results showed *Fplysc* expression could be detected in all tested tissues including brain, gill, heart, hepatopancreas, intestine, hemocytes, eyestalk, stomach and muscle when normalized to the mRNA expression level of *FpEF1α* (Fig. 4). The highest level was detected in the hemocytes (5.24-fold) and the lowest level in the eyestalk (1.69-fold).

### 3.4. Expression pattern of Fplysc after microbial infection

The temporal expression pattern of *Fplysc* in different tissues after microbial infection was shown in Fig. 5. After WSSV injection, *Fplysc* expression levels in gill, hepatopancreas, intestine and hemocytes increased gradually during the first 4 h post-injection, reached the highest level and then decreased gradually till the end of the experiment. Moreover, *Fplysc* expression levels in heart, hepatopancreas, eyestalk, stomach and muscle maintained at a low level at all of the tested time points. *Fplysc* expression levels showed significantly changes in gill, intestine and hemocytes at 2–8 h post-injection, and in hepatopancreas at 4–8 h. After *V. alginolyticus* injection, *Fplysc* expression levels was significantly upregulated in intestine and hemocytes at 4–72 h post-injection, in gill at 4–48 h post-injection, and in muscle at 12–48 h. In contrast, no changes in *Fplysc* expression levels in the brain, heart, hepatopancreas, eyestalk and stomach were observed when compared to the PBS injection group.

### 3.5. dsRNA-mediated silencing of Fplysc

To further explore the function of Fplysc *in vivo*, we successfully suppressed *Fplysc* expression in the cephalothoraxes by dsRNA-mediated gene silencing. The qRT-PCR results showed that *Fplysc* expression

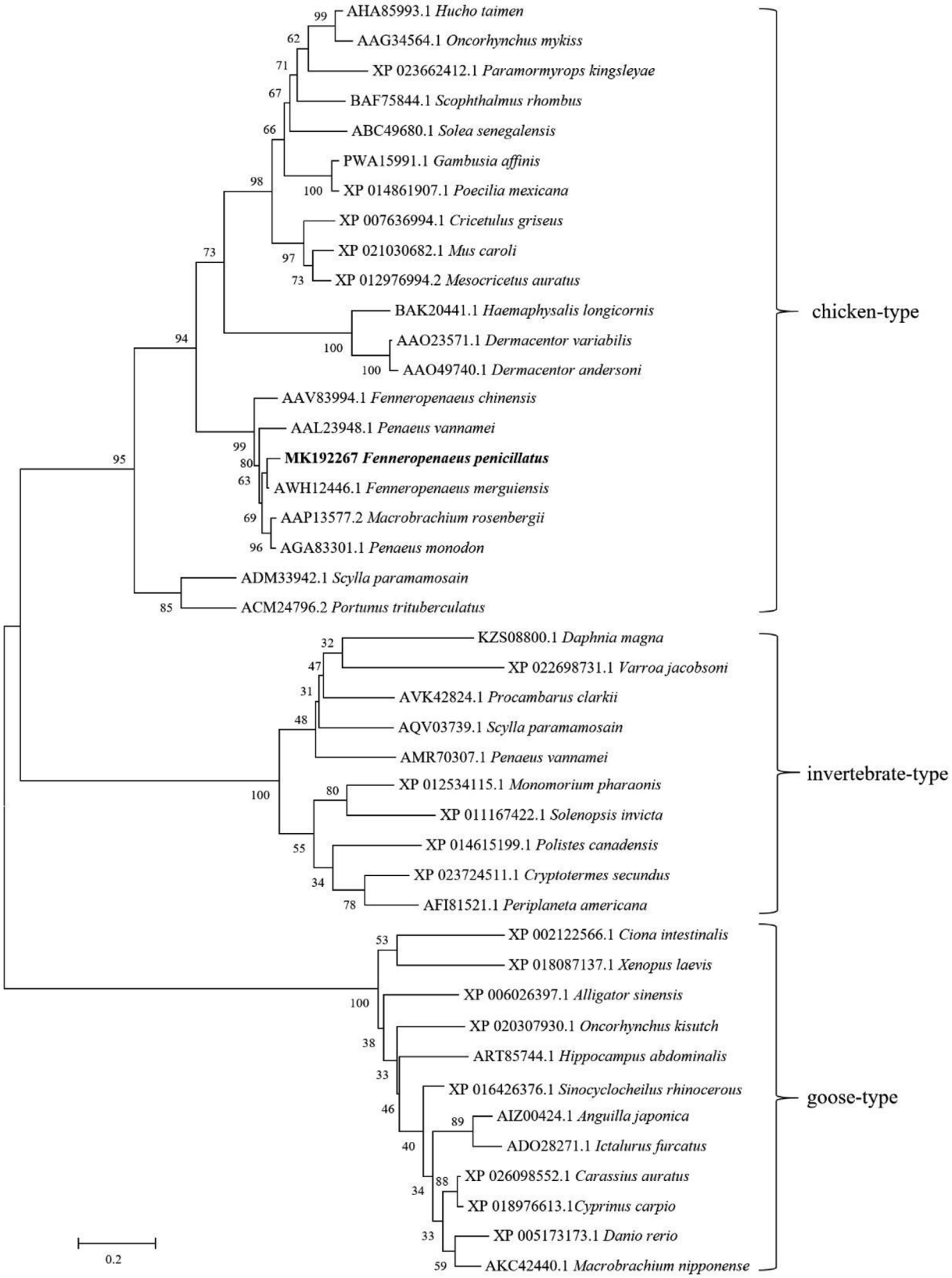


**Fig. 2.** Neighbor-joining phylogenetic analysis of Fplysc with other members of lysozyme family. The tree was built with MEGA 5.0 with bootstrap of 1000. The numbers marked on the tree branches represent the bootstrap values.

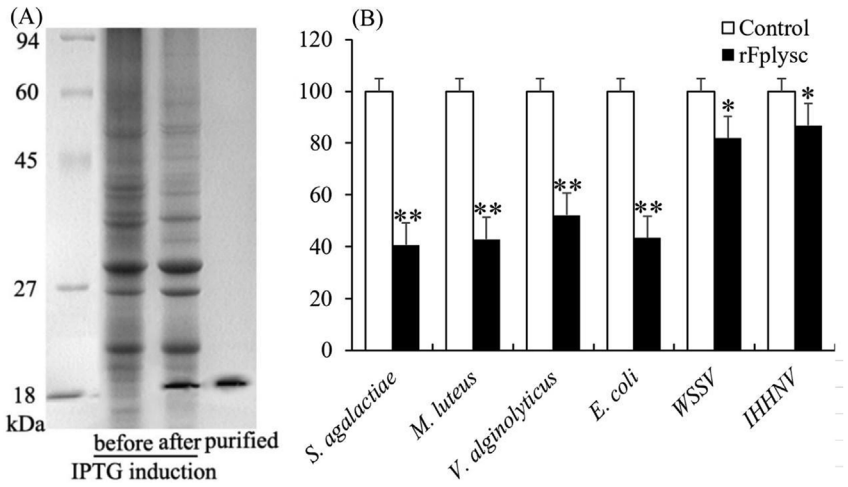


**Fig. 3.** Recombinant expression and antimicrobial and antiviral activity of rFplysc in *vitro*. (A) Recombinant expression and purification of rFplysc. Lane1, protein marker; lane 2, total protein of recombinant *E. coli* before IPTG induction; lane 3, total protein of recombinant *E. coli* after IPTG induction; lane 4, purified protein. (B) Antimicrobial and antiviral activity of rFplysc. Data showed the mean ± SE from three independent experiments. **, $P < 0.01$; *, $P < 0.05$, calculated by Student's t-test compared the control sample.

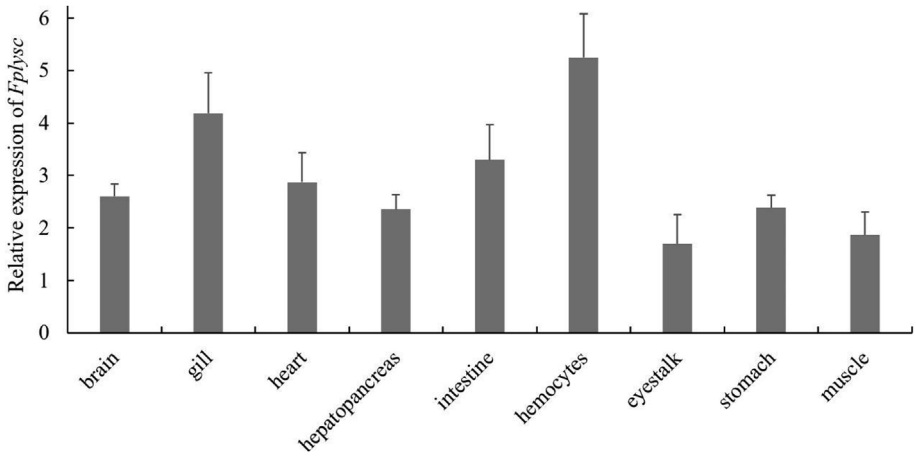


**Fig. 4.** Tissue distribution of *Fplysc* in healthy shrimp. The expression of shrimp *Fplysc* was based on the ratio of *FpEF1α* expression.

was significantly reduced in the cephalothoraxes at 8–72 h post-dsRNA injection when compared to the control groups of dsGFP or PBS injection (Fig. 6). The expression levels of *Fplysc* in the cephalothoraxes of ds*Fplysc*-injected shrimp were only 25.7%, 9.5%, 6.3%, and 6.7% of those in the dsGFP-injected shrimp at 8, 12, 24, 48 and 72 h post-injection, respectively.

### 3.6. Antimicrobial and antiviral activity of Fplysc in vivo and protection of shrimp after microbial infection

To reveal the function of Fplysc after microbial infection, we performed microbial infection experiments in Fplysc-silenced shrimp. At 24 h after dsRNA-injection, red tail shrimp were infected with WSSV or *V. alginolyticus*. We found that the cumulative mortality of Fplysc-silenced shrimp injected with WSSV or *V. alginolyticus* was remarkably increased, and the final mortality rates reached 100%, 100% and 47.5% for WSSV, *V. alginolyticus* and PBS groups, respectively ($P < 0.01$). Nevertheless, the cumulative mortality rates of dsGFP-injection shrimp were 50%, 45% and 0 after WSSV, *V. alginolyticus* or PBS injection, respectively. In rescue experiments, rFplysc introduced into the Fplysc-silenced shrimp, and the cumulative mortality injected with WSSV, *V. alginolyticus* or PBS was remarkably decreased compared with those of Fplysc-silenced shrimp without injecting rFplysc ($P < 0.05$). The final mortality rates only reached 70%, 67.5% and 17.5% for WSSV, *V. alginolyticus* and PBS injection, respectively (Fig. 7).

## 4. Discussion

Because of lacking an adaptive immune system, shrimps rely mainly on their innate immune system to resist invasion of microbial pathogens [21]. Lysozymes are considered as a forceful barrier to resist surrounding bacterial pathogen invasion as a widespread hydrolase in innate immunity. Most lysozymes have antibacterial activities by exhibiting muramidase activity through the hydrolysis of the glycosidic bond between hydrolyzing the β-1, 4-glycosidic bond between *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG) of peptidoglycan to trigger the lysis of the bacterial cell wall [22]. Although many c-type lysozymes have been cloned in invertebrates [23], the knowledge of c-type lysozymes in crustaceans is still limited. In this study, a c-type lysozyme *Fplysc* gene was identified from the red tail shrimp *F. penicillatus*. The deduced amino acid sequence of *Fplysc* shared a similarity with those from other species. Multiple alignments revealed eight conserved cysteine residues that form four intra-molecular disulfide bonds, two conserved catalytic sites $Glu^{51}$ and $Asp^{68}$ for muramidase activity and two conserved calcium binding sites $Ser^{98}$ and $Ala^{103, 104}$ [24]. In addition, Fplysc possesses a 17 aa N-terminal signal peptide sequence, which is similar with that in most known crustaceans, and was predicted to be localized extracellularly. Phylogenetic analysis showed that Fplysc was clustered with crustaceans, arachnoidea, fish and mammals c-type lysozymes. We can clearly see that Fplysc belongs to the crustaceans group and has the highest similarity with *F. merguiensis* c-type lysozyme. The showed several duplication events could occur in different periods of evolution in fish, mammals, arachnoidea and crustaceans and resulted in c-type lysozyme

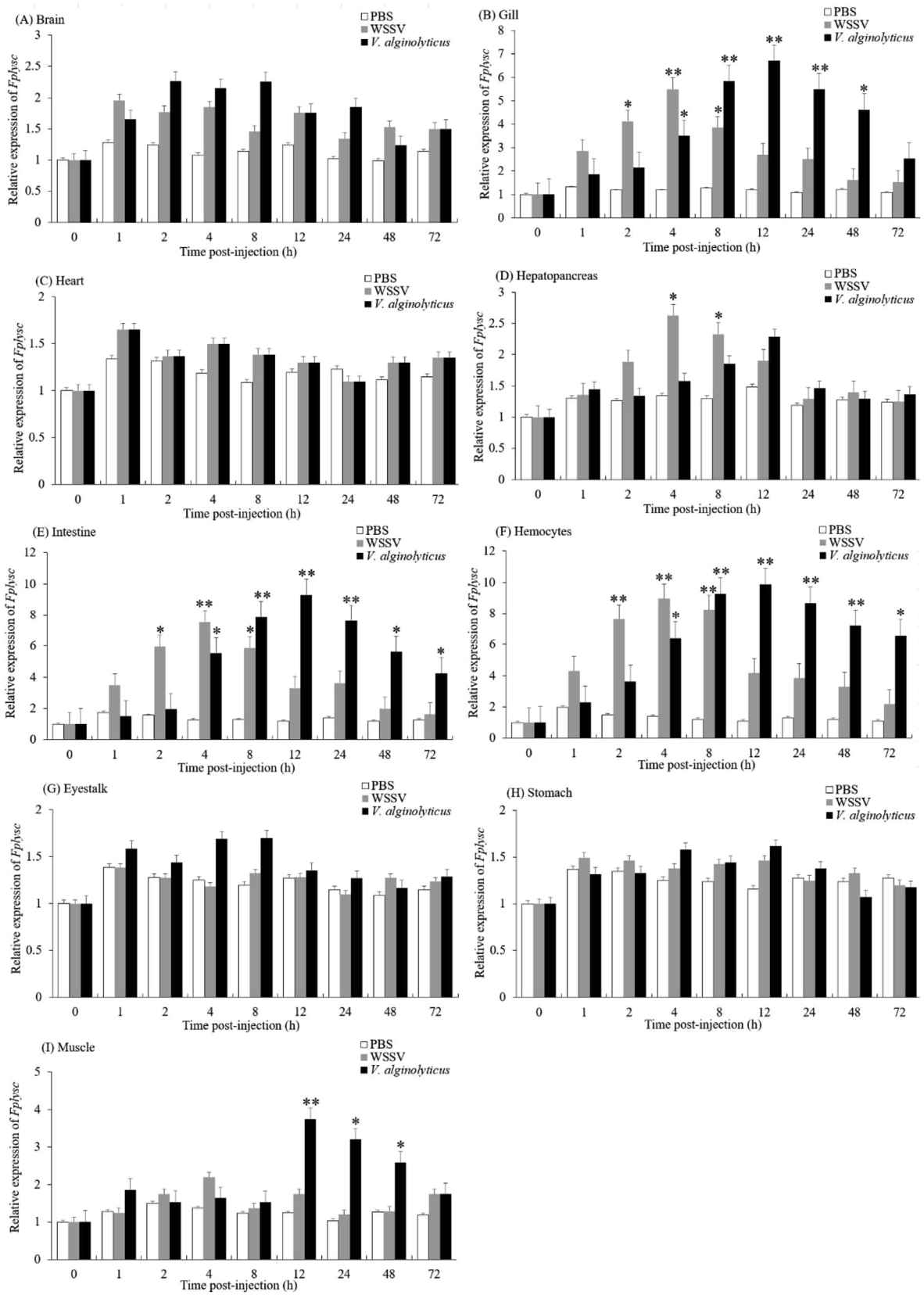


**Fig. 5.** The expression pattern of *Fplysc* in the different tissues after WSSV and *V. alginolyticus* challenge. Data are expressed as the mean fold change relative to the ratio of *FpEF1α* expression. Significant difference was indicated by asterisks, *, $P < 0.05$, **, $P < 0.01$.

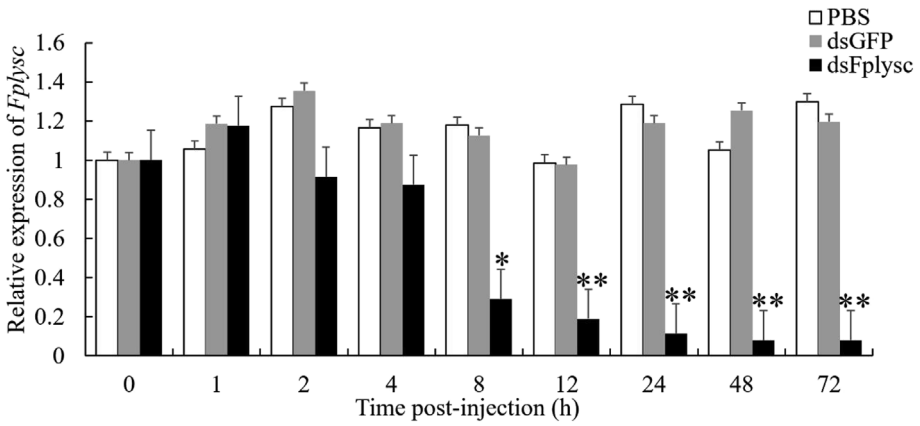


**Fig. 6.** The temporal expression levels of *Fplysc* in the cephalothoraxes of *Fplysc*-silencing shrimp by dsRNA-mediated RNAi. Data are expressed as the mean fold change relative to the ratio of *FpEF1α* expression. Significant difference was indicated by asterisks, **, $P < 0.01$; *, $P < 0.05$.

differentiation in different species.

Some c-type lysozymes from mollusks and shrimp showed high activity only against Gram-negative bacteria, and some displayed activity only against Gram-positive bacteria [25–27]. In the study, recombinant Fplysc exhibited strong antibacterial activity against both of Gram-positive and Gram-negative bacteria. The wide spectrum antibacterial activity of rFplysc suggested that it could be involved in host defense against kinds of bacteria, and highlighted its significance to resist bacterial infection as an effector [28]. Similarly, rFplysc showed moderate antiviral activity against WSSV and IHHNV, suggesting Fplysc could be effective at blocking virus infection in *F. penicillatus* and Fplysc modulates the cellular and humoral defense mechanisms to suppress virus [29].

The qRT-PCR analysis suggested that Fplysc was expressed in all tested tissues, indicating a constitutive and ubiquitous expression of Fplysc. The broad tissue distribution of Fplysc was also been detected in many of aquatic animals, such as *Hucho taimen*, *Procambarus clarkia*, *Apostichopus japonicas* [30–32]. A relative higher expression levels of Fplysc were detected in hemocytes and gill. Fplysc was mainly synthesized and stored in hemocytes, and then released to the hemolymh against pathogen invasion. The gill was easy subjected to invasion of pathogenic bacteria in shrimp, and lysozyme is a first line defensive peptide in innate immune systems against invading pathogens. Therefore, the high expression of Fplysc indicated and proved c-type lysozome in these first line defensive tissues was important as the first defensive peptide in shrimp immune system.

In order to explore the immune-related functions of Fplysc, a challenge experiment was performed using WSSV and *V. alginolyticus*. Comparing with no significant change in the brain, heart, eyestalk and stomach after WSSV or *V. alginolyticus* challenge, Fplysc was strongly upregulated in gill, intestine, hemocytes during challenge of the pathogens, suggesting Fplysc could be activated during defense against pathogens. Interestingly, the expression levels of *Fplysc* in responses to WSSV and *V. alginolyticus* were significantly upregulated, suggesting its being involved in the bacterial and viral defense activities. The previous studies showed c-type lysozyme had exerted mainly the function of bacterial degradation as a bacteriolytic enzyme [25,30–32]. However, it is unknown whether c-type lysozyme have some important functions in viral defense as an important innate immune molecule. In our study, rFplysc showed antiviral activity against WSSV and IHHNV *in vitro* and the expression levels of *Fplysc* significantly increased *in vivo* post WSSV-injection, indicating that Fplysc could be an important defense factor during blocking WSSV infection. The entire mode of antiviral action of the lysozyme is not yet fully understood, although some studies have shown that lysozyme causes specific and nonspecific humoral responses in fish and shrimp [29,33,34]. In crustaceans, innate cellular and humoral immunes are the major lines defense resisting pathogens invasion and eliminating the invading microbes from the blood and tissues. After WSSV and *V. alginolyticus* infection, Fplysc expression patterns show different trends with time in different tissues suggesting that Fplysc was

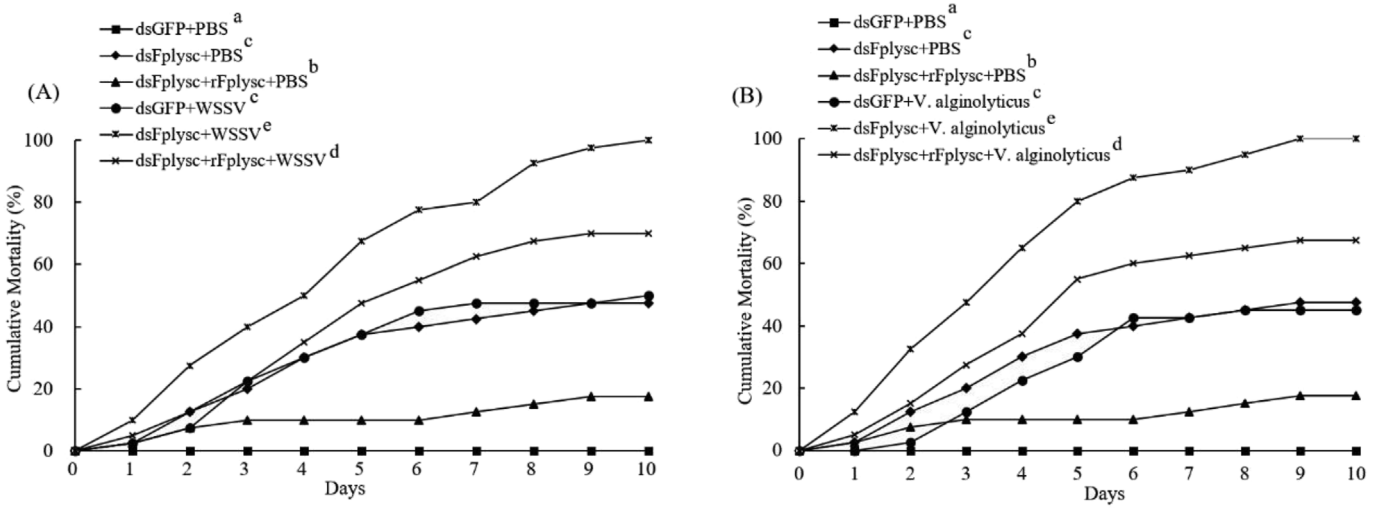


**Fig. 7.** Cumulative mortality rates of *Fplysc*-silencing shrimp after WSSV (A) or *V. alginolyticus* (B) infection. Significant difference was indicated by lowercase a, b, c, d and e.

established to attend the immune response towards pathogens challenge, and may play a different function in various tissues.

To verify the protecting role of Fplysc, RNAi-mediated silencing was performed to determine the survival post WSSV and *V. alginolyticus* injection. The dsRNA injection could suppress *Fplysc* expression in cephalothoraxes, suggesting that *Fplysc* was successfully knockdown. *Fplysc* pre-silencing led to increasing mortality caused by WSSV, *V. alginolyticus*, and even PBS injection, indicating that Fplysc play very important role in pathogenesis and shrimp immune responses [35]. Then a rescue experiment was performed by injecting rFplysc into *Fplysc* silenced shrimp, and exogenous rFplysc led to a significant decrease of shrimp mortality post WSSV, *V. alginolyticus*, and PBS injection. These results together suggested that Fplysc have the important functions in shrimp antimicrobial and antiviral response.

In conclusion, the identification and characterization of c-type lysozyme were performed in *F. penicillatus*. *Fplysc* expression levels could be up-regulated by WSSV and *V. alginolyticus* challenge in some tissues. Fplysc-silencing significantly caused high cumulative mortality post WSSV and *V. alginolyticus* challenge, and exogenous rFplysc led to a significant decrease of shrimp mortality by injecting rFplysc into *Fplysc* silenced shrimp. The results may help to elucidate the function of Fplysc in shrimp against bacterial and viral infection.

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