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Characterization of a new homologous anti-lipopolysaccharide factor SpALF7 in mud crab *Scylla paramamosain*

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ABSTRACT

Invertebrates, such as mud crab *Scylla paramamosain*, rely only on innate immunity. Anti-lipopolysaccharide factors (ALFs), are one of the typical antimicrobial peptides (AMPs) commonly found in crustaceans, playing a vital role in the immune defense against pathogens. In this study, a new homologous ALF, named SpALF7, was identified from *S. paramamosain*. Its full-length cDNA sequence consists of 640 bp, including 121 deduced amino acid residues. The homology between SpALF7 and the other six reported ALFs in *S. paramamosain* is about 24.69%–43.59%. Absolute quantitative PCR (qPCR) analysis showed that SpALF7 was widely distributed throughout the embryonic and larval developmental stages and the tissues of adult crabs, with the highest transcription level in the seminal vesicles of adult male crabs. Not surprisingly, SpALF7 gene could be significantly upregulated in hepatopancreas under LPS and *Vibrio alginolyticus* challenge analyzed by relative qPCR. Through the *Pichia pastoris* eukaryotic expression system, the SpALF7 recombinant protein (rSpALF7) was successfully obtained and a truncated peptide containing the putative LPS binding domain (sSpALF7) was chemically synthesized. Both of them exhibited potent antibacterial activity against several Gram-positive and Gram-negative bacteria. The bactericidal kinetic curves produced by rSpALF7 showed that it could kill more than 90% *Pseudomonas stutzeri* and *Shigella flexneri* within 5 min and 120 min at a concentration of 1 μM and 6 μM, respectively. After rSpALF7 treatment, the disruption of cell membrane integrity in *S. flexneri* and *P. stutzeri* was observed by scanning electron microscope, which was further confirmed by the flow cytometry assay with SYTO9 and propidium iodide (PI) stainning. In addition, it was worth noting that although rSpALF7 had no bactericidal effect on the crab pathogen *V. alginolyticus*, it could significantly reduce the bacterial endotoxin levels. Furthermore, the *in vivo* studies showed that rSpALF7 could obviously improve the survival of crabs infected by *V. alginolyticus*. Taken together, these results suggested that SpALF7 might play an important role in the innate immunity of *S. paramamosain*, and has potential applications in aquaculture.

1. Introduction

Anti-lipopolysaccharide factors (ALFs), a class of typical antimicrobial peptides (AMPs), play vital roles in the innate immunity of crustaceans lacking adaptive immunity (Tassanakajon et al., 2015). The first ALF was obtained from the hemocytes of the horseshoe crab *Limulus polyphemus*, and was named according to its ability to inhibit lipopolysaccharide (LPS)-mediated activation of the Limulus coagulation system (Tanaka et al., 1982). It was later confirmed that this ALF had strong antibacterial activity against Gram-positive and Gram-negative bacteria (Patrulea et al., 2020). Since then, many ALFs had been found in crustaceans.

In shrimp and crab species, the reported full-length transcripts of ALFs encode a precursor composed of a signal peptide (22 to 28 residues), followed by a mature peptide (10.74 to 12.23 kDa), containing two conserved cysteine residues, of which the isoelectric points (pI) varies between 5.0 and 11.0. The three-dimensional structure of both crab and shrimp ALFs consist of three α-helices packed with four β-sheets (Guani-Guerra et al., 2010). In this structure, the two cysteines are flanked by a 20 residues central β-hairpin stabilized by a single disulfide bond. This central β-hairpin structure (also known as "LPS-binding domain (LBD)" or LPS-BD, pI 4.37–10.00) is the functional domain of ALFs and contains key amino acids involved in the recognition and binding of microbial cell wall components (such as LPS from Gram-

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negative bacteria, lipoteichoic acid from Gram-positive bacteria and β-glucans from fungi) (Schmitt et al., 2016a). Notably, LBD is an active center and plays a key role in resistance to Gram-positive and Gram-negative bacteria, fungi, and viruses (Hoeger and Harris, 2020). For example, the LBD of FcALF2 (Yang et al., 2016) and FcALF8 (Li et al., 2019) from *Fenneropenaeus chinensis,* MnALF1-5 (Wang et al., 2015) from *Macrobrchium nipponense,* ALF*Pm*3 (Somboonwiwat et al., 2005) and ALF*Pm* 11(Zhou et al., 2019b) from *Penaeus monodon,* EcALF1 (Lv et al., 2017), EcALF3 (Lv et al., 2018) and EcALF4 (Lv et al., 2018) from *Exopalaemon carinicauda,* RspALF1 (Gu et al., 2018) from *Rimicaris exoculata*, ALFsp (Imjongjirak et al., 2007) and ALFsp2 (Imjongjirak et al., 2011) from *Scylla paramamosain* showed a broad-spectrum of antimicrobial activities, including Gram-positive and Gram-negative bacteria. In addition, the LBDs of FcALF2, FcALF5, FcALF7 from *F. chinensis* (Li et al., 2015), the EcALF2–5 (Lv et al., 2018) from *E. carinicauda* had anti-WSSV activity. Moreover, the LBDs of ALF*Pm*3 (Somboonwiwat et al., 2005) from *P. monodon*, Sp-ALF1 and Sp-ALF2 (Liu et al., 2012a) from *S. paramamosain* have antibacterial, antifungal and anti-WSSV activities.

Most reported shrimp ALFs are highly expressed in hemocytes, and could be significantly induced under bacterial challenge (Hou et al., 2017; Li et al., 2019; Supungul et al., 2004; Wang et al., 2015; Zhou et al., 2019a). Furthermore, the recombinant products of shrimp ALFs could significantly inhibit the growth of several aquaculture pathogens (Gu et al., 2018; Li et al., 2014; Li et al., 2019; Somboonwiwat et al., 2005; Tang et al., 2020; Yang et al., 2015; Zhu et al., 2019). For crab ALFs, seven ALFs were isolated from the swimming crab *Portunus trituberculatus* (Liu et al., 2013a; Liu et al., 2012b, 2012c; Liu et al., 2013b; Liu et al., 2011), and most of them (PtALF1, PtALF2, PtALF3, PtALF5, PtALF7) were also highly detected in hemocytes, while PtALF4 mainly distributed in eyetalks and PtALF6 in gills. PtALF1–7 could be upregulated after being challenged with *Vibrio alginolyticus*, except PtALF2. Similarly, the recombinant PtALFs had antibacterial activity against certain Gram-negative bacteria, Gram-positive bacteria, and fungi. In the mud crab *S. paramamosain*, six ALFs had been identified, including ALFSp (Imjongjirak et al., 2007), ALFSp2 (Imjongjirak et al., 2011), Sp-ALF1 and Sp-ALF2 (Liu et al., 2012a), SpALF4 (Zhu et al., 2014), SpALF5 (Sun et al., 2015), SpALF6 and SpALF6-V (Hou et al., 2017). Except for SpALF5, which was highly expressed in brain, other SpALFs were mainy distributed in hemocytes. After being challenged with *Vibrio harveyi*, the transcripts of SpALF4 and SpALF5 in the hemocytes were upregulated (Sun et al., 2015; Zhu et al., 2014). In addition, the recombinant products of ALFSp2, Sp-ALF1, Sp-ALF2, SpALF4, SpALF5, SpALF6 and SpALF6-V also have antibacterial activity against several Gram-positive and Gram-negative bacteria. Among them, Sp-ALF1 and Sp-ALF2 could inhibit the replication of WSSV; SpALF4, SpALF5 and SpALF6 have antifungal activity. Taken together, these data suggested that ALFs are essential effectors in the innate immune system of crustaceans.

In addition to the activity of ALFs on bacteria and even viruses *in vitro*, some studies on the immunoprotective effect of ALFs on crustaceans have also been reported. Such as the chemically synthesized peptides LBD1, LBD2, LBD5 and LBD7 derived from FcALF1, FcALF, FcALF5, ALFFc, respectively in *F. chinensis*, although these synthetic peptides had no anti-WSSV activity *in vitro*, when they were pre-incubated with WSSV and then injected into *E. carinicauda*, it was found that the copy number of WSSV in the treatment group was obviously lower than that of the control group (Li et al., 2015). In the freshwater prawn *Marsupenaeus japonicus*, both rMjALF-E2 and rMnALF4 could significantly increased the bacterial clearance rate in the hemolymph, thereby protecting the prawns against infection (Jiang et al., 2015; Tang et al., 2020). Similarly, rRspALF1 and its synthetic LBD ALF1P1 from the hydrothermal vent shrimp *R. exoculata* could effectively combat *V. harveyi* infection in Pacific white shrimp *Litopenaeus vannamei* (Gu et al., 2018). However, the underlying mechanism is still unclear.

In the present study, a new homologous ALF gene, named SpALF7 from mud crab *S. paramamosain* was identified. Absolute quantitative PCR (qPCR) was used to determine the transcription level of SpALF7 in the embryonic and larval developmental stages and the tissues of adult crabs. And the mRNA expresstion patten of SpALF7 in the hepatopancreas of crabs challenged by *V. alginolyticus*, *Staphylococcus aureus* or LPS was analyzed by relative qPCR. The recombinant protein (rSpALF7) was obtained by *Pichia pastoris* eukaryotic expression system and a truncated peptide containing the putative LPS binding domain (sSpALF7) was chemically synthesized. The antimicrobial activity assay of rSpALF7 and sSpALF7 were performed, and the bactericidal kinetic curves of rSpALF7 against *Shigella flexneri* and *Pseudomonas stutzeri* were conducted. The antibactierial mechanism of rSpALF7 against *S. flexneri* and *P. stutzeri* was preliminarily elucidated by scanning electron microscope (SEM) and flow cytometry assay. Additionally, the endotoxin level of *V. alginolyticus* after rSpALF7 treatment was detected, and the mortality of the crabs was calculated by first challenging the crabs with *V. alginolyticus* and then injecting rSpALF7 to investigate its immunoprotective function *in vivo*.

2. Materials and methods

2.1. Animals, strains, vectors, and reagents

S. paramamosain with a weight of about 300 ± 30 g were purchased from a crab farm in Xiamen, which were used for tissue sample collection, LPS, *V. alginolyticus* and *S. aureus* challenge assays. Crabs weighing about 30 ± 10 g were used for immunoprotection experiments. *Escherichia coli* DH5α was used for gene subcloning. *P. pastoris* GS115 and pPIC9K (Invitrogen, USA) were used as recombinant expression host and vector. The standard bacterial strains used for testing were purchased from Institute of Microbiology, Chinese Academy of Sciences, including *Listeria monocytogenes* (CGMCC:1.10753), *Shigella flexneri* (CGMCC: 1.1868), *Staphylococcus epidermidis* (CGMCC: 1.4260), *Pseudomonas stutzeri* (CGMCC: 1.1868), *Micrococcus lysodeikticus* (CGMCC: 1.0634), *Micrococcus luteus* (CGMCC:1.634), *Bacillus subtilis* (CGMCC: 1.108), *V. alginolyticus* (CGMCC: 1.1615) and *S. aureus* (CGMCC:1.363), *Candida albicans* (CGMCC: 2.2411), and *Cryptococcus neoformans* (CGMCC: 2.1563). The restriction enzymes *EcoR* I, *Not* I, *Sac* I were purchased from Thermo Fisher Scientific. Lipopolysaccharides(LPS) from *E. coli* 055: B4, lipoteichoic acid (LTA) from *B. subtilis* were purchased from Merck, Germany. Mouse anti-His antibody, goat anti-rabbit IgG antibody conjugated with horseradish peroxidase were purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., China.

2.2. Full-length cDNA and genomic DNA cloning of SpALF7

Total RNA of normal crab testes was prepared using Trizol reagent (Invitrogen) following the manufacturer's instructions. According to the partial cDNA sequence of SpALF7 from the transcriptome database established by our laboratory, primers (5RACE1, 3RACE1; 5RACE2, 3RACE2) were designed through PrimerPrimer5 software. The cDNA was synthesized following the instructions of SMARTer® RACE 5′/3′Kit User Manual (Takara) and RACE PCR was performed as follows: 95 °C, 5 min; 29 cycles of 95 °C, 30 s, 69 °C -0.5/cycle, 30s, 72 °C, 3 min; 9 cycles of 95 °C, 30 s, 54 °C, 30 s, 72 °C, 3 min; 72 °C, 10 min; 16 °C 10 min. The expected fragments were then purified from 1% agarose gel by Tiangen Universal Purification Kit (Tiangen Biotech), ligated into pMD18-T vector (Vazyme), and then recombinant clones were screened by bacterial colony PCR. The selected positive clones were sequenced at Sangon Biotech. The resulting sequences were then performed bioinformatic analysis for verification. TIANamp Marine Animal Tissue Genomic DNA Extraction Kit was used to obtain genomic DNA from crab muscle. The PCR was performed using primers GenF and GenR (Table 1) as follows: 95 °C for 5 min; 35 cycles of 94 °C for 15 s, 60 °C for 15 s, and 72 °C for 2 min; finally extended at 72 °C for 10 min. The target fragment was then purified and sequenced as previously described.

Table 1
Primers used in the present study.

Primer name	Nucleotide sequence (5′-3′)
LUPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
SUPM	CTAATACGACTCACTATAGGGC
5RACE1	GCTTCCTCCTCCGTAATCAG
3RACE1	GGCTCTGGAGGAACGAAGAG
5RACE2	CTTGATCTTGGCTTGACG
3RACE2	TTTGGGTCGCAAGTGTCA
M13F	GTAAAACGACGGCCAGT
M13R	CAGGAAACAGCTATGAC
Gen-F	CAGGACTGGGAATCACTACTGGTTT
Gen-R	TCAGGCTTCTAACCACACCTTGGCT
RT-F	GGGCTGGACTCCCATCGT
RT-R	TGGCTTCCTCCTCCGTAA
GAPDH-F	CTCCACTGGTGCCGCTAAGGCTGTA
GAPDH-R	CAAGTCAGGTCAACCACGGACACAT
ZHF	GGAATTCCAGGACTGGGAATCACTACTGGTTT
ZHR	ATAAGAATGCGGCCGCTCAATGGTGATGGTGATGATGGGCTTCTAACCACACCTTGGCT
5′AOX	GACTGGTTCCAATTGACAAGC
3′AOX	GCAAATGGCATTCTGACATCC

Fast digesting site are marked with underlined solid lines, and his-tagged proteins are marked with underlined dotted lines.

2.3. Sequence and phylogenetic analysis

Homology analysis of nucleotides and deduced amino acids was performed using BLASTn and BLASTp algorithm of the NCBI (http://www.ncbi.nlm.nih.gov/blast). Signal peptide (SP) was identified using SignalP5.0 program (http://www.cbs.dtu.dk/services/SignalP). Domain and motif analysis was performed using the conserved domain search program of NCBI(https://www.ncbi.nlm.nih.gov/)and SMART database (http://smart.embl-heidelberg.de/smart/). The theoretical molecular mass and isoelectric point were predicted using ExPASy (http://web.expasy.org/protparam). PSIPRED Workbench (http://bioinf.cs.ucl.ac.uk/psipred/) was used to predict secondary structure. The tertiary structure was predicted by PHYRE2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/). Phylogenetic tree was constructed using neighbour-joining method with MEGA software version 7.0, and the reliability of the obtained tree was evaluated by bootstrap method using 1000 replications.

2.4. The expressinon pattern of SpALF7 in various tissues and different developmental stages of S. paramamosain

The hemolymph of adult normal mud crabs, including males (300 ± 30 g, *n*=5) and females (200 ± 30 g, n=5) were collected as previously described (Qiao et al., 2016). Other tissues, including eyestalk, gills, heart, mid-gut gland, hepatopancreas, muscle, stomach, subcuticular epidermis, nerves, tissues from reproductive system of female crabs (spermatheca, reproductive duct and ovaries) and male crabs (testes, anterior vas deferens, seminal vesicle, posterior vas deferens, ejaculation ducts, posterior ejaculation ducts) were also sampled and ready for total RNA isolation and cDNA synthesis as previously described. In order to determine the SpALF7 mRNA transcripts at different developmental stages of crabs, the entire embryonic stages (Em1-Em9), zoea larval stages (Z1-Z5), megalopa larval stage and juvenile were sampled. Absolute quantitative real-time PCR (qPCR) was performed in a 20 μL reaction mixture containing 1 μL of the pMD18-T/SpALF7 plasmid cDNA with eight independent serial dilutions (10^{-8}–10^{-2} copies/μL), 0.8 μL of each gene-specific primers (as listed in Table 1: RT-F, RT-R) and 10 μL of Power SYBR Green PCR Master Mix (Roche). The standard PCR cycling conditions were: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. The SYBR Green RT-PCR assay was carried out in Qtower 2.2 (Analytik Jena, Germany). A standard curve was generated by plotting the Ct values, with 95% confidence intervals, relative to the logarithm of the initial copy numbers. The copy numbers of experimental RNAs can be calculated after real-time PCR amplification according to the linear regression of the standard curve. qPCR soft 3.4 was used to calculate the original relative quantitative data.

2.5. The immune challenges of LPS, V. alginolyticus and S. aureus to crabs

For the immune challenge experiments, healthy male *S. paramamosain* (300 ± 30 g) were purchased from a commercial crab farm in Xiamen, China, and acclimated at 25 ± 1 °C for 3–4 days before processing. During the experiment, 96 crabs were randomly divided into two groups, including the experimental group and the control group, with 48 crabs in each group. For the experimental group,100 μL of *V. parahaemolyticus* (3×10^7 CFU mL^{-1}) or 100 μL of *S. aureus* (1×10^7 CFU mL^{-1}) or 0.5 mg/kg·crab LPS (2 mg/mL) were injected into the base of the fourth leg of each crab. For the control group, 100 μL of crab saline (4.96 mM NaCl; 9.52 mM KCl; 0.8 mM $MgSO_4$; 16.2 mM $CaCl_2$; 0.84 mM $MgCl_2$; 5.95 mM $NaHCO_3$; 20 mM HEPES; pH 7.4) was injected. The experiments were conducted under laboratory conditions (salinity: 3‰, temperature: 24–28 °C). Hepatopancreas were collected from five crabs in each group at 0, 3, 6, 12, 24, 48 and 72 h post-injection (hpi). Tissues were packed by sterilized tin foil and then stored in −80 °C for later use. Total RNA was extracted using Trizol as previously described. Relative qPCR was performed in a 20 μL reaction mixture containing 1 μL of total transcribed cDNA, 0.8 μL of each gene-specific primers (as listed in Table 1: RT-F, RT-R; GAPDH-F, GAPDH-R;) and 10 μL of Power SYBR Green PCR Master Mix (Roche). The PCR cycling conditions were described as absolute qPCR. qPCR soft 3.4 was used to calculate the original relative quantitative data and GAPDH gene was employed as an internal standard. The fold change of gene expression relative to the control was determined by the $2^{-\Delta\Delta Ct}$ method. ANOVA comparison tests were used for statistical analysis by GraphPad Prism 7. Values were considered to be significant as $P < 0.05$.

2.6. Optimization and purification of recombinant SpALF7 (rSpALF7) in P. pastoris eukaryotic expression system

The recombinant expression vector of SpALF7 mature peptide was constructed using pPIC9K plasmid, and the primers (ZHF and ZHR) used were shown in Table 1. After linearization by *Sac* I, 1 mg of the recombinant plasmid was transformed into competent *P. pastoris* cells by electroporation according to the manufacturer's instructions (Invitrogen). After 48 h of incubation on minimal dextrose medium (MD) plates at 30 °C, the positive clones were screened by PCR with primers

5'AOX and 3'AOX (Table 1) to ensure that the SpALF7 gene was integrated into the *P. pastoris* genome.

A positive clone was selected and the expression condition was optimized by detecting the expression of the recombinant protein at different time points (24 h, 48 h, 72 h, 96 h) after induction. After 24 h of induction with 0.5% methamphetamine for large-scale expression, the protein-containing supernatant was separated from the yeast precipitate and dialyzed with phosphate buffer (28.5 mM Na_2HPO_4, 21.5 mM NaH_2PO_4, 50 mM NaCl, pH 7.5). Then the target protein was purified by Ni^{2+} affinity chromatography. The purity of the protein was analyzed by SDS-PAGE combined with Coomassie brilliant blue staining, and the concentration was determined by the Bradford protein assay kit (Beyotime Institute of Biotechnology, China). The recombinant protein with a purity of more than 90% was frozen and stored at −80 °C for later use. The purified protein was processed as described previously (Liu et al., 2012a) and sequenced by the Mass Spectrometry Center of the School of Life Sciences, Xiamen University (Liu et al., 2012a).

2.7. Chemical synthesis of SpALF7 LPS-binding domain (sSpALF7)

A linearized LPS-binding domain with two amino acid residues overhang beyond the conserved cystine residues were chemically synthesized by Genscript. The synthetic peptide was derived from positions 55th to 76th of SpALF7 (CQLTVKPKIKNLQLYHEGRMWC). Both N-terminal residue of the peptide was blocked by acetylation, and the C-terminal residue was amidated. The synthetic peptides were purified by reverse phase HPLC with a purity of over 95%.

2.8. The antimicrobial and bactericidal activity assay

The antimicrobial and bactericidal activities of rSpALF7 and sSpALF7 were analyzed. The microorganisms included Gram-positive bacteria (*L. monocytogenes, S. flexneri*) and Gram-negative bacteria (*S. epidermidis, P. stutzeri, M. lysodeikticus, B. subtilis, M. luteus)*, fungi (*C. albicans, C. neoformans*). The minimum growth inhibition concentration (MIC) was determined by the liquid growth inhibition assay (Shan et al., 2016). Briefly, the bacteria were diluted to 3.6×10^5 CFU mL^{-1} in 10 mM NaPB (1.225 g $Na_2HPO_4{\cdot}12H_2O$, 0.246 g $NaH_2PO_4{\cdot}2H_2O$, dissolved in 0.5 L distilled water, and sterilized at 121 °C for 20 min). In sterile 96-well plates, the assay mixture consisted of 50 μL diluted purified rSpALF7 or sSpALF7, 30 μL of diluted bacteria suspension and 20 μL of the corresponding culture medium (Nutrient Broth for bacteria and YPD for fungi) (Liu et al., 2012a; Yang et al., 2015). All bacteria were cultured on Mueller–Hinton broth medium except for *C. albicans* and *C. neoformans* that were cultured on YPD medium (1% yeast extract, 1% peptone, 2% glucose). After 24 h of incubation at 28 °C or 37 °C, the MIC was defined as the lowest peptide concentration with no detectable bacterial growth compared with the negative control. This assay was performed in triplicate. A 2 μL aliquot of the culture was then plated on Nutrient Broth agar, and MBC was the minimum peptide concentration which killed more than 99.9% microorganisms after overnight incubation at 28 °C or 37 °C.

2.9. Bactericidal kinetic acesessment

Bactericidal kinetic acesessment was performed using *S. flexneri* and *P. stutzeri*. They were incubated with rSpALF7 at concentrations of 6 μM and 12 μM or 0.5 μM and 1 μM, respectively at 37 °C for 0 min, 2 min, 15 min, 30 min, 60 min, 120 min, 240 min, and 360 min. At each incubation time point, 6 μL of the mixture was taken, serially diluted in 10 mM NaPB, and then plated on nutrition broth agar. The plates were incubated at 37 °C for 24 h. Meanwhile, an equal volume of NaPB without rSpALF7 was added to the control tube, and the total viable count (TVC) was estimated. The assays were performed in triplicate. The killing efficiency was determined by caculating the survival rate of colony-forming units (% CFU) as follows: % CFU = recovered CFU/initial CFU × 100%, where the initial CFU refers to the TVC at 0 min, and the recovered CFU refers to the TVC at different sampling points.

2.10. Scanning electron microscope (SEM) observation of antibacterial activity of rSpALF7 against S. flexneri and P. stutzeri

S. flexneri and *P. stutzeri* cells were cultured in LB medium to the logarithmic phase (OD_{600} 0.2), and collected by centrifugation at 6000 *g* for 5 min. The medium was removed and washed twice with PBS. *S. flexneri* and *P. stutzeri* were resuspended in PBS, and then incubated with 3 μM/6 μM and 1.5 μM/3 μM rSpALF7 respectively at 37 °C for 1 h. The mixture was then harvested by centrifugation (5000 *g*, 10 min), and the bacterial pellets were fixed with a 2% glutaraldehyde solution at 4 °C for 2 h. The bacteria were dehydrated in a series of graded alcohols (30%, 50%, 70%, 80%, 95%, 100%). After that, the samples were coated with gold and observed with a FEI Quanta 650 FEG Field Emission Environment Scanning Electron Microscope (American).

2.11. Membrane integrity measurement by flow cytometry

The bacterial suspensions of *S. flexneri* and *P. stutzeri* were prepared according to the instructions of the LIVE/DEAD® BacLight™ Bacterial Viability Kits (Invitrogen). *S. flexneri* and *P. stutzeri* (10^6 cells/mL) were incubated with 3 μM/6 μM and 1.5 μM/3 μM rSpALF7 respectively. Meanwhile, an equal volume of PBS (KH_2PO_4 42 mM, Na_2HPO_4 8 mM, NaCl 136 mM, KCl 2.6 mM, pH 7.4) without rSpALF7 was added as a control group. All groups were incubated at 37 °C for 1 h, and then immediately stained with SYTO9 and PI. They were incubated in the dark at room temperature for 20 or 25 min, respectively, before subjected to be analyzed by flow cytometry CytoFLEX (BECKMAN COULTER, American).

2.12. Binding assay

To determine the binding properties of rSpALF7 with LPS and LTA, a modified enzyme-linked immunosorbent assay (ELISA) was performed following the previous method description (Yang et al., 2020). Briefly, a 96-well ELISA plate was coated with LPS, and LTA (2 μg) overnight, then blocked with 5% skim milk (prepared in PBS with 0.05% Tween20), and incubated with a serial dilutions of rSpALF7 (0 to 20 μg/mL, prepared in PBS). Bound proteins were detected by incubation with mouse anti-His antibody (1:3000, prepared in 1% skim milk) followed by goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (1:5000, prepared in 1% skim milk). After the colorimetric reaction, the absorbance at 450 nm was measured using a microplate reader (TECAN GENios). The experiment was carried out in triplicate, and the apparent dissociation constant (Kd) was generated by Scatchard plot analysis.

2.13. V. alginolyticus endotoxin assay

Toxin Sensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, NJ, U.S.A.) was used to perform *V. alginolyticus* endotoxin assay. The overnight culture of *V. alginolyticus* was centrifuged (5000 *g*, 10 min, 4 °C) and resuspended in Zobell Marine Broth 2216. Then, 50 μL of the cell suspension (OD_{600} 0.5) and rSpALF7 were added to Zobell Marine Broth 2216 to make the final protein concentrations of 0, 12, 24, 48 and 96 μM. The bacterial cells growed to logarithmic phase at 37 °C. The uninoculated 2216 was used as a control. The samples were processed following the manufacturer instructions and analyzed by a spectrophotometer (Agilent Technologies; Malaysia.).

2.14. Cytotoxicity assay

Hemocytes from the healthy male crab (300 ± 30 g) were maintained in L15 medium supplemented with 5% FBS and 1.2% NaCl. Cells were seeded at ~2.0×10^4 cells $well^{-1}$ on a 96-well cell culture plate (Thermo

Fisher), and cultured at 26 °C overnight. The cells were incubated with NaPB (solution control) or rSpALF7 (1.5, 3, 6, 12, 24, 48 and 96 μM). After 24 h of incubation, the cell viability was assessed using the Cell-Titer 96®AQueous Kit (Promega). Experiments were carried out in triplicate.

2.15. Effect of rSpALF7 on the survival rate of S. paramamosain

Mud crabs (30 ± 5 g) were acclimated for about one weeks before subjected to experiment. *V. alginolyticus* was cultured to logarithmic phase and washed three times, and then adjusted to a concentration of 1.2×10^8 CFU mL^{-1} with crab saline. 15 crabs were randomly selected, and each crab was injected with 25 μL *V. alginolyticus* with a micro-injector. One hour after the bacterial injection, 25 μL (600 μg/mL) of rSpALF7 were injected into these crabs, and equal volume of the crab saline instead of rSpALF7 was used as a negative control. The number of dead crabs at different time points (0 h, 3 h, 6 h, 9 h, 12 h, 24 h, 36 h, 48 h, 72 h, 96 h, 120 h) was recorded, and a motality curve was drawn.

2.16. Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.0 Software (GraphPad Software Inc., CA, United States), with a confidence level of 95% being considered statistically significant. Data were shown as mean ± standard deviation.

3. Results

3.1. Full lengh cDNA cloning and gene structure of SpALF7

The full-length cDNA sequence of SpALF7 (Genbank accession No. MT074682) was obtained by RACE PCR analysis. The complete sequence of 640 bp contains an open reading frame of 372 bp, encoding a 121-amino acid protein, a 5'untranslated region (UTR) of 63 bp, and a 3'UTR of 205 bp with a poly(A) tail (Fig. 1A). The deduced protein sequence has a signal peptide (residues 1st to 25th) and a putative LBD (residues 55th to 76th). The PI of the mature peptide and LBD were 8.64 and 9.31, respectively, and the molecular mass of the mature peptide is 11.6 kDa. Fig. 1B and C showed the gene structure of SpALF7 (Genbank accession No. MW119265), which is 923 bp and contains three exons and two introns.

3.2. Sequence and phylogenetic analysis of SpALF7

The amino acid sequence encoded by SpALF7 had a homology of 24.69% -43.59% with other ALFs isloated from mud crab such as SpALF1, SpALF2, ALFSp2, SpALF3, SpALF4, SpALF5, SpALF6 and SpALF6-v (Fig. 2A). Compared with ALFs in other crustaceans, includng shrimps (*Litopenaeus stylirostris*, *L. vannamei, Litopenaeus schmitti, Farfantepenaeus paulensis, Farfantepenaeus aztecus, Fenneropenaeus penicillatus, Fenneropenaeus indicus, F. chinensis*, *P. monodon*, *M. japonicus*, *Macrobrachium rosenbergii, Homarus americanus, Litopenaeus paulensis, Penaeus schmitti, Pacifastacus leniusculus, Procambarus clarkii, Macrobrachium nipponense, E. carinicauda, Rimicaris sp.*) and crabs (*Eriocheir sinensis, Scylla serrata, P. trituberculatus*, *L. polyphemus*), SpALF7 had the highest homology with MjALF-C1 isolated from *M. japonicus* (the amino acid sequence identity ratio is 73.39%), and the phylogenetic analysis also found that they clustered into the same branch (Fig. 2B). We analyzed the phylogenetic tree of ALFs (including 89 ALFs in crustaceans) and tried to classify them. According to the different branches formed by the cluster analysis, we divided the crustaceans ALFs into five categories. We found that Sp-ALF1, SpALF2, SpALF4 and SpALF7 have been clustered to Classification V, and SpALF5 and SpALF6-V were grouped in Classification III, SpALF6 and ALFSp2 in Classification II, and

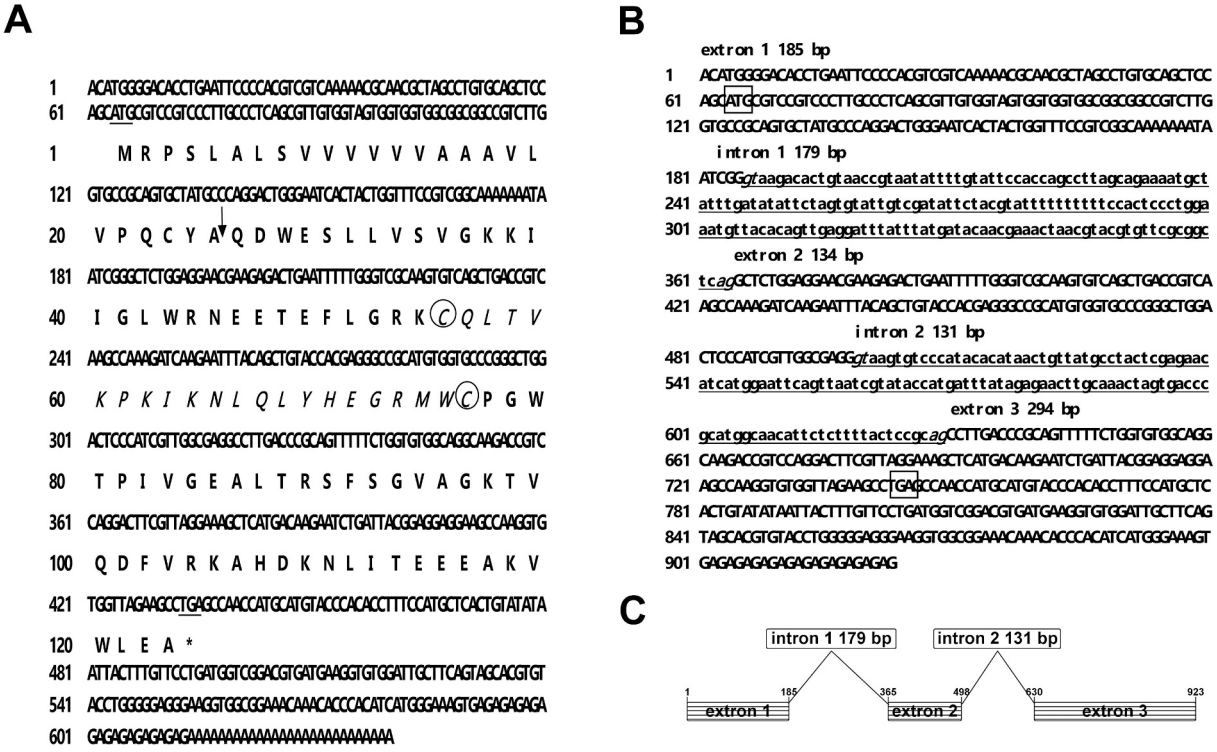

Fig. 1. The full-length cDNA sequence, genomic DNA sequence and genomic organization of SpALF7 in mud *crab S. paramamosain*. (A) The full-length cDNA sequences (above) of SpALF7 and its predicted amino acid sequences (below). The proposed start codon and stop codon were underlined. The arrow indicated the predicted cleavage site of the signal peptide. The conseved cysteine residues forming the LPS-binding domain were marked with circles andasterisks. (B) Genomic DNA sequence of SpALF7. The intron dinucleotide acceptor and donor sites (gt/ag) for RNA splicing are italics. The start codons and stop codons are boxed. (C) Genomic organization of SpALF7.

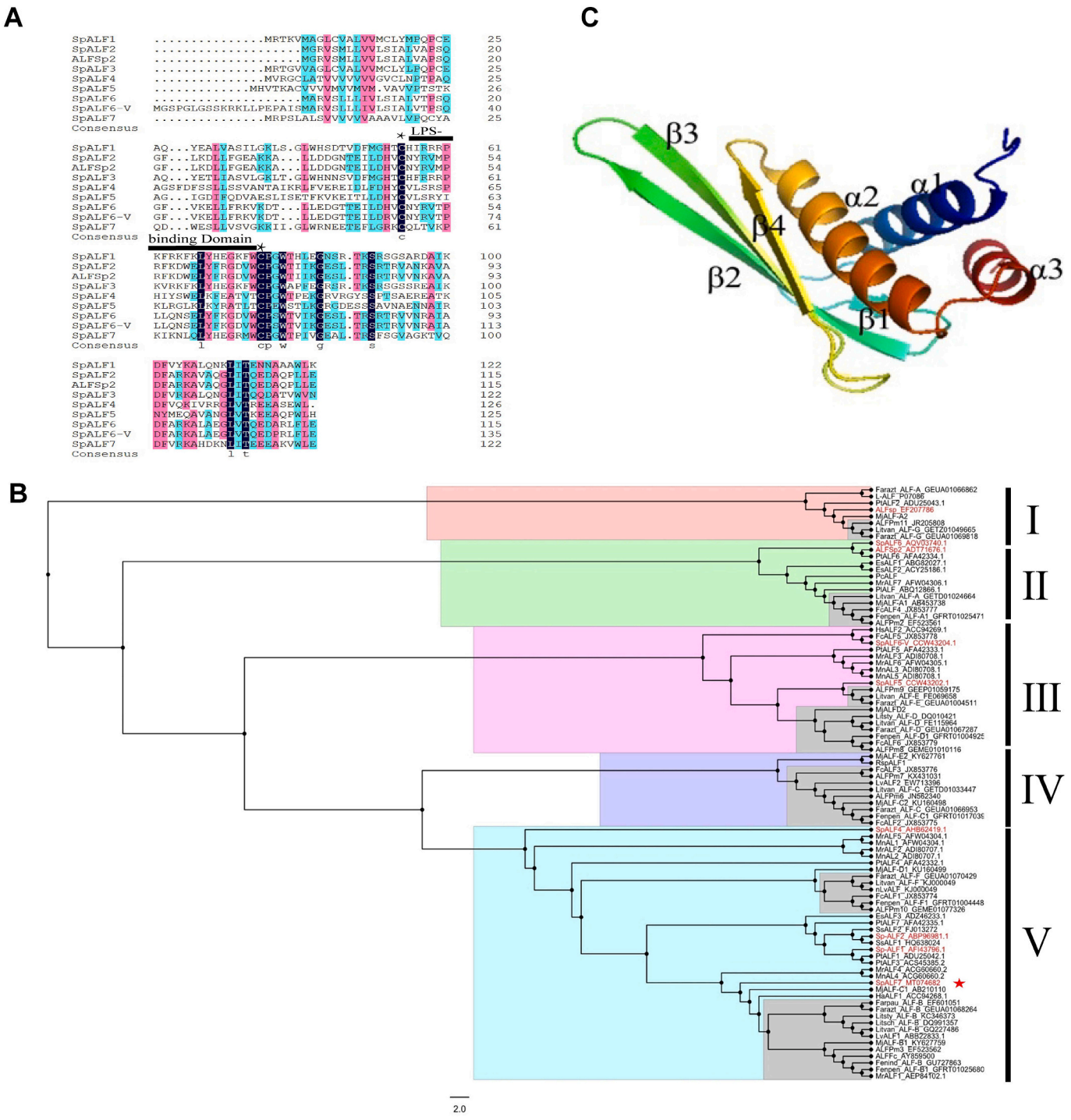

Fig. 2. Multiple alignment of SpALF7 amino acid sequences with other ALFs and prediction of its tertiary structure. (A) Multiple alignment of SpALF amino acid sequences with other known ALFs, and the LPS-binding domain is underlined. (B) Phylogenetic analysis of SpALF7 relative to other ALFs in crustaceans. 1000 bootstraps were performed on the NJ trees to check the repeatability of the results. SpALFs were shown in red, and SpALF7 was marked with an red asterisk. The gray highlights in the figure indicated different groups of ALFs identified in shrimps. Litvan, *L. vannamei*; Litsch, *L. schmitti*; Litsty, *L. stylirostris*; Farpau, *F. paulensis*; Farazt, *F. aztecus*; Mj, *M. japonicus*; Fc, *F. chinensis*; Fenpen, *F. penicillatus;* Fenind, *F. indicus*; Pt, *Portunustritu trituberculatus*; Sp, *S. paramamosain*; Pm, *P. monodon*; Fc, *F. chinensis*; Mr., *M. rosenbergii*; Ha, *H. americanus*; Es, *E. sinensis*; Fenpen, *F. paulensis*; Pl, *P. leniusculus*; L, *L. polyphemus*; Pc, *P. clarkii*; Ss, *S. serrata*; Mn, *M. nipponense*. (C) Prediction of the tertiary structure of SpALF7 by PHYRE2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ALFsp in Classification I (Fig. 2B).The tertiary structure of SpALF7 consists of three α helices and four β sheets, which is consistent with typical ALFs (Fig. 2C).

3.3. The tissue distribution and immmune response of SpALF7 mRNA in hepatopancreas stimulated by LPS, V. alginolyticus and S. aureus

Absolute quantitative PCR (qPCR) was used to detect the expression of SpALF7 mRNA in embryos, zoea larval stage, megalopa larval stage,

juvenile, and tissues of adult male and female crabs. Fig. 3A shows that the highest expression level of SpALF7 was found on the embronic stage at seven days post hatching. In adult crabs, SpALF7 was widely distributed in different tissues, with the highest expression in the seminal vesicles of male crabs (Fig. 3B–C). Relative qPCR results showed that the transcripts of SpALF7 gene was significantly upregulated at 6 h and 12 h in hepatopancreas under LPS or *V. alginolyticus* challenge (Fig. 3E–F), but there was no obvious change after infection with *S. aureus*.

3.4. Optimization and purification of recombinant SpALF7 (rSpALF7) expression in P. pastoris GS115

The clone of GS115/pPIC9K- SpALF7 was induced by 0.5% methanol at pH 6.0 for 96 h. A single protein band around 12 kDa was observed at 24 h, and its production was almost the same during subsequent induction untill 96 h. The molecular weight of this peptide was consistent with the size of the predicted one (11.6 kDa) (Fig. 4A). In addition, the expression concentration of recombinant SpALF7 did not show significant difference before and after optimization of GS115/pPIC9K- SpALF7 induction(data not shown). Therefore, 24 h (1 days) was selected as the optimal induction time for SpALF7 expression. The recombinant SpALF7 (rSpALF7) was successfully obtained from *P. pastoris* eukaryotic expression system (Fig. 4B). The mass spectrometry results confirmed that the amino acid sequence of the purified protein was the target

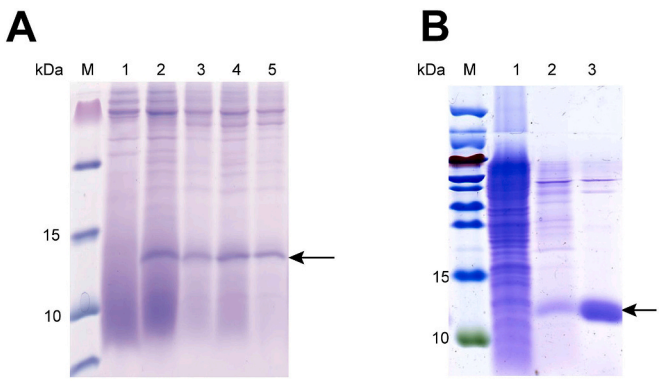

Fig. 4. Expression and purification of rSpALF7. (A) SDS-PAGE analysis of optimization of rSpALF7 expression. M, marker; 1, cells induced for 0 h; 2, cells induced for 24 h; 3, cells induced for 48 h; 4, cells induced for 72 h; 5, cells induced for 96 h. (B) SDS-PAGE analysis of purification of rSpALF7. M, marker; 1, cells induced for 0 h; 2, cells induced for 24 h; 3, purified protein.

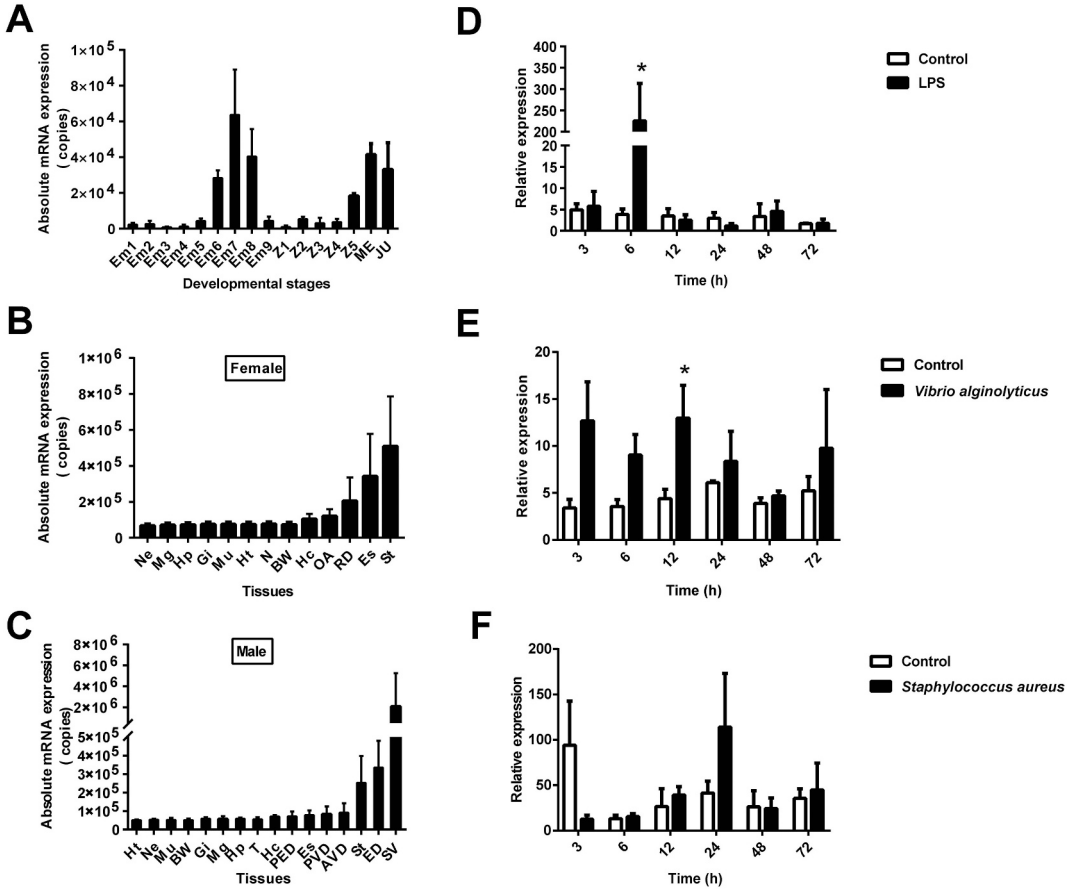

Fig. 3. The distribution of SpALF7 in different developmental stages and adult crab tissues and the expression pattern of SpALF7 in hepatopancreas after challenge with LPS, *V. alginolyticus* and *S. aureus*. (A) The expression of SpALF7 at different developmental stages of *S. paramamosain*. (B) The expression of SpALF7 in various tissues of female crabs. (C) The expression of SpALF7 in various tissues of male crab; Em1-9: the mud crab embryos from day 1 to day 9 post hatching; Z1-5: zoea larval stage 1-5; ME: megalopa larval stage; JU: juvenile; Hc: hemocytes; Es: eyestalk; Gi: gills; Hp: hepatopancreas; Mu: muscle; Ne: nerve; Ht: heart; St: stomach; Mg: midgut; N: spermatheca; OA: ovaries; BW: subcuticular epidermis; RD: reproductive tract; AVD: anterior vas deferens; PED: posterior ejaculation ducts; T: testis; ED: ejaculation duct; Sv: seminal vesicle; PVD: posterior vas deferens. The expression pattern of SpALF7 (D) after challenge with LPS; (E) after challenge with *V. alginolyticus*; (F) after challenge with *S. aureus*. The asterisks indicates a significant difference compared with the control group (*: $P < 0.05$).

protein rSpALF7 (Fig. S1).

3.5. The antibacterial activity of rSpALF7 and sSpALF7 and the preliminary antibacterial mechanism of rSpALF7

The antibacterial activity of recombinant SpALF7 (rSpALF7) and chemically synthesized LBD peptide (sSpALF7) were analyzed. The results showed that they could significantly inhibit several Gram-positive and Gram-negative bacteria (Table 2). Obviously, the antibacterial spectrum of sSpALF7 is broader than that of rSpALF7. And rSpALF7 exhibited stronger antimicrobial activities on the growth of Gram-positive bacteria *S. flexneri* with MIC and MBC values of 3–6 μM (24–48 μM for sSpALF7) and Gram-negative bacteria *P. stutzeri* with MIC and MBC value of 3–6 μM (12–24 μM for sSpALF7), but had no obvious antibacterial activity against *L. monocytogenes, S. epidermidis, M. lysodeikticus, M. luteus,* and *B. subtilis.* In contrast, sSpALF7 showed antimicrobial activity against all tested microorganisms. Common pathogens including *S. flexneri*, *P. stutzeri*, *M. lysodeikticus*, *M. luteus*, *B. subtilis* (values for MIC and MBC ranging from 3 to 48 μM) were susceptible to sSpALF7.

In the bactericidal kinetic assay, *S. flexneri* and *P. stutzeri* were selected to evaluate the bactericidal efficiency of rSpALF7. The results showed that rSpALF7 could kill *P. stutzeri* within 5 min at a concentration of 1 μM (Fig. 5A). Over 90% *S. flexneri* were killed within 50 min by rSpALF7 at a concentration of both 6 μM and 12 μM (Fig. 5B).

After co-incubating rSpALF7 with *S. flexneri* and *P. stutzeri*, the surface morphology of the cells was observed with a scanning electron microscope (SEM). The results showed that the cell surface of the control group was intact and there was no obvious desruption (Fig. 5C). In the experimental group, *S. flexneri* and *P. stutzeri* cells aggregated, the cell morphology changed significantly, the cell surface was ruptured, and the contents flowed out. Eventually all cells became shrinked, causing cell death.

SYTO9 and PI staining assay are used to evaluate the integrity of bacterial cell membranes after rSpALF7 treatment. The results showed that after incubation with 1.5 μM rSpALF7, the proportion of *P. stutzeri* stained with PI accounted for 27.78%, while for the 3 μM rSpALF7 treatment group, the PI staining rate increased to 72.88% (Fig. 5D). Similar results were observed in *S. flexneri* (Fig. 5E), that is, almost all bacterial cells were stained with PI (99.02%) after treatment with 6 μM rSpALF7. However, for the PBS control group, it showed a very small amount of red fluorescence cells (0.79% for *P. stutzeri* and 0.16% for *S. flexneri*, respectively).

Table 2
Antibacterial activity of rSpALF7 and sSpALF7.

Microorganisms	CGMCC No.[a]	rSpALF7		sSpALF7	
		MIC[b]	MBC[b]	MIC	MBC
Gram-negative bacteria					
Listeria monocytogenes	1.10753	>48	>48	48–96	>96
Shigella flexneri	1.1868	3–6	3–6	24–48	24–48
Gram-positive bacteria					
Staphylococcus epidermidis	1.4260	>48	>48	24–48	48–96
Pseudomonas stutzeri	1.1868	1.5–3	1.5–3	12–24	12–24
Micrococcus lysodeikticus	1.0634	>48	>48	3–6	3–6
Micrococcus luteus	1.634	>48	>48	6–12	6–12
Bacillus subtilis	1.108	>48	>48	12–24	24–48

[a] China general microbiological culture collection number; MIC[b] and MBC[b]: All the concentrations showed in this table were in μM. The values of MIC (minimal inhibitory concentration) and MBC (minimal bactericidal concentration) were expressed as the lowest concentration yielding no detectable microbial growth or that killed more than 99.9% of microorganisms (n=3).

3.6. rSpALF7 had a strong binding affinity to LPS and could reduce V. alginolyticus endotoxin in vitro

To further investigate the related mechanism of rSpALF7, we performed a modified ELISA assay to assess its binding properties to Gram-positive and Gram-negative bacteria surface molecules. As shown in Fig. 6A, rSpALF7 could bind to LPS and LTA in a concentration-dependent manner. Scatchard plot analysis showed that SpALF7 bound to LPS and LTA with the calculated apparent dissociation constants (Kd) of 0.007 μM and 0.426 μM, respectively. These results indicated that rSpALF7 had a strong binding affinity to LPS and a moderate binding affinity with LTA. (Fig. 6A).

V. alginolyticus is a common pathogenic bacteria in aquaculture, and rSpALF7 had no obvious activity against it. But whether rSpALF7 could reduce the endotoxin of *V. alginolyticus* is worth exploring, and we evaluated that. The original endotoxin concentration of *V. alginolyticus* was found to be 0.93 ± 0.006 EU/mL (Fig. 6C). After treatment with different concentrations of rSpALF7, a significant reduction of bacterial endotoxin was observed. The 12 μM and 24 μM rSpALF7 treatment resulted in a significant decrease ($P < 0.0001$ and $P = 0.0001$ respectively) in the endotoxin concentration of *V. alginolyticus* to 0.83 ± 0.003 EU/mL and 0.82 ± 0.012 EU/mL, respectively (Fig. 6B). Especially, when the concentrations of rSpALF7 reached 48 and 96 μM, it caused a sharp decrease in endotoxin concentration ($P < 0.0001$) to 0.04 ± 0.013 EU/mL and 0.009 ± 0.003 EU/mL, respectively (Fig. 6C).

3.7. rSpALF7 showed no cytotoxicity and had an in vivo protective effect in S. paramamosain

To determine whether rSpALF7 could be used safely in *in vivo* experiments, the cytotoxicity of rSpALF7 was analyzed using hemocytes isolated from healthy male crabs (300 ± 30 g). The results showed that rSpALF7 had no cytotoxicity, and the cell viability of hemocytes was significantly improved after treatment with 1.5 μM and 3 μM rSpALF7 (Fig. 6B).

To assess the effect of rSpALF7 *in vivo*, *S. paramamosain* was challenged with *V. alginolyticus.* One hour after the bacterial injection, rSpALF7 or crab saline was injected into the crabs. The survival rate of the control group decreased to 36.84% at 24 h post-injection, while the rSpALF7 treatment group showed a 70.83% survival rate (Fig. 6D). At 96 h after challenge, all the crabs in the control group died, and the survival rate of the experimental group was matained at about 47.22% (Fig. 6D). In general, *S. paramamosain* treated with rSpALF7 after bacterial challenge exhibited a significantly higher survival rate than that of crabs injected with crab saline until 120 h ($P < 0.01$).

4. Discussion

Currently, due to the contribution of AMPs to host defense, they have received increasing attention and are considered as potential antibiotic alternatives to solve the global antibiotic resistance (Tang et al., 2020). Since anti-lipopolysaccharide factors (ALFs) is one of the most important antimicrobial peptides (AMPs) involved in the innate immunity of mud crab, studies on ALFs will help to understand the immune defense mechanisms of *S. paramamosain* and provide new insights for diseases control in crab aquaculture. In this study, a new homologous ALF gene was identified in *S. paramamosain*, named SpALF7. SpALF7 has certain homology with the six previously reported ALFs in *S. paramamosain*. It showed a conserved sequence and structure characteristics of ALFs, and had relatively high pI value in both the LBD region and mature peptide. Functional analysis showed that both the recombinant protein rSpALF7 and chemically synthesized LBD peptide sSpALF7 could inhibit the growth of both Gram-negative and Gram-positive bacteria to varying degrees. Furthermore, rSpALF7 could significantly reduce the endotoxin level of the crab pathogen *V. alginolyticus in vitro* and improve the survival rate of *S. paramamosain in vivo*.

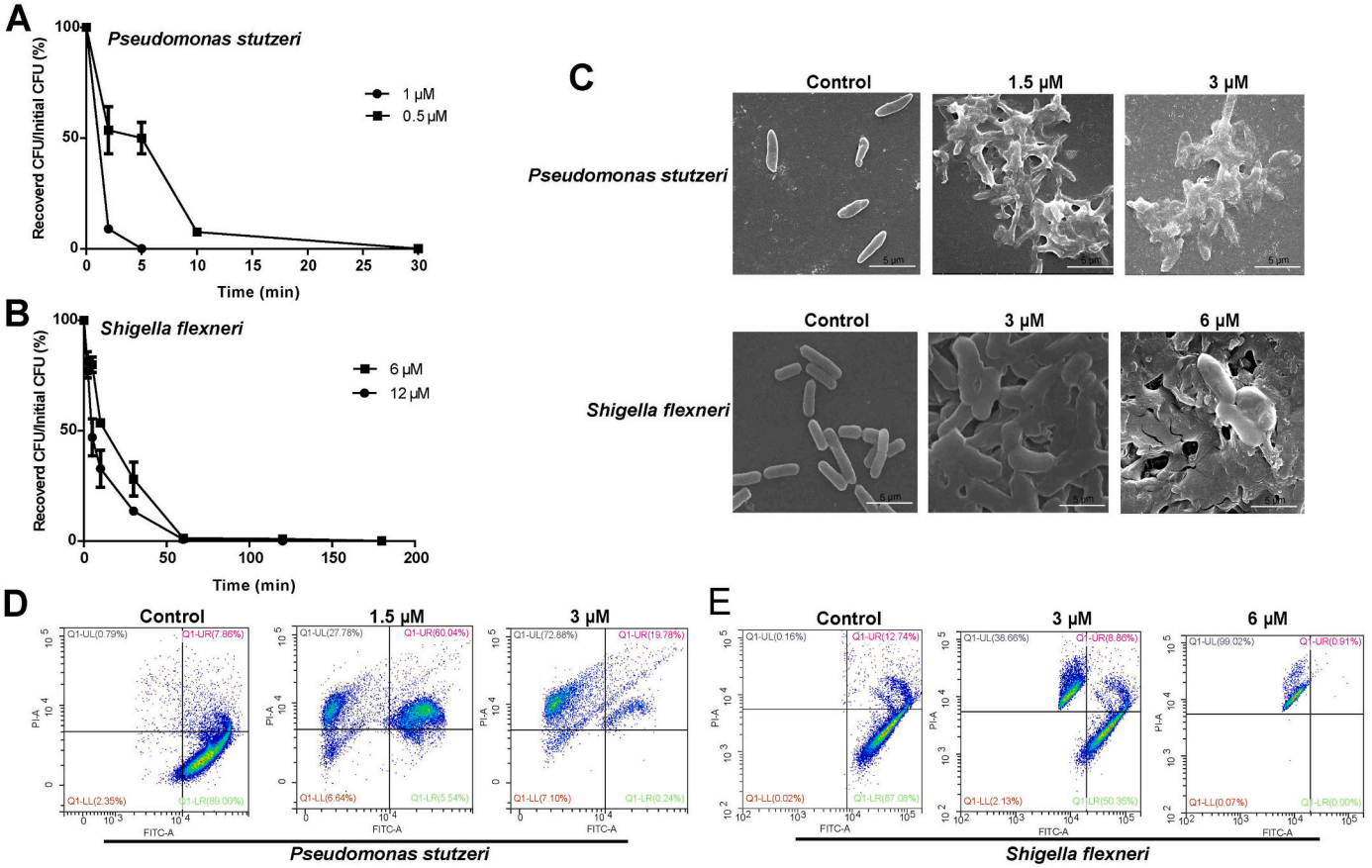

Fig. 5. The antibacterial effect of rSpALF7 on *P. stutzeri* and *S. flexneri*. The time-killing curves of *P. stutzeri* (A) and *S. flexneri* (B) treated with rSpALF7. (C) Effect of rSpALF7 on morphology of *P. stutzeri and S. flexneri*. Flow cytometry analysis of *P. stutzeri* (D) and *S. flexneri* (E) after 1 h incubation with PBS (negative control) or rSpALF7.

As reported, there are seven groups of the ALF family (Groups A to G) in shrimp, all of which are encoded by different loci with conserved gene organization (Schmitt, et al., 2016; Matos et al., 2018). The result of phylogenetic anaysis showed that SpALF7 clustered into Classification V. The PI of the ALF mature peptides and LBD from Classification V ranged from 8.18 to10.29 and 9.93–10.04, respectively, and those ALFs exhibited a broad spctrum of antimicrobial activity, such as ALF*Pm*3–5 isolated from *P. monodon* (Shown in Table. S1) (Tharntada et al., 2008). ALF*Pm*3 had strong antibacterial activity against Gram-positive and Gram-negative bacteria, fungi, and viruses (Methatham et al., 2017; Supungul et al., 2004; Somboonwiwat et al., 2005; Tharntada et al., 2008). As for SpALF7, the PI of the mature peptide and LBD were 8.64 and 9.31, and it also could significantly inhibit several Gram-positive and Gram-negative bacteria (shown in Table S1). The tertiary structure of SpALF7 is composed of three α helices and four β folds, which is commonly found in crustacean ALFs, such as PenmonALF8 from *P. monodon* (Zhou et al., 2019b), MnALF4 from freshwater prawn *M. nipponense* (Tang et al., 2020), PtALF7 from swimming crab *P. trituberculatus* (Liu et al., 2013a) and mud crab ALFs, SpALF4 (Zhu et al., 2014) and SpALF5 (Sun et al., 2015). Like other ALFs, such as PenmonALF3 (*P. monodon*) (Tharntada et al., 2008), PenmonALF5 (*P. monodon*) (Tharntada et al., 2008), PenpauALF1 (*Macrobra chiumolfersi*) (Rosa et al., 2008), PenvanALFAA-K, PenvanALFAV-K, PenvanALFAV-R, Penva nALFVV-R (*P. vannamei*) (Tinwongger et al., 2019), PenchiALF (*Penaeus chinensis*) (Tang et al., 2014), SpALF7 also contains a typical LBD with six net positive charges, resulting in a high pI value (9.31). These domains are considered to be capable of interacting with the negatively charged LPS and essential for the biological activities of ALFs (Sun et al., 2015). Some ALFs with high pI values for LBD are proven to first interact with LPS and further inhibit the growth of different Gram-negative bacteria (Liu et al., 2013a; Liu et al., 2012b, 2012c; Liu et al., 2013b; Liu et al., 2011; Lv et al., 2018; Methatham et al., 2017). The synthetic ALFSp LBD peptide (54th to 77th) was highly active in inhibiting the growth of Gram-positive, Gram-negative bacteria and *V. harveyi* with MIC values ranging from 0.1 to 50 μM (Imjongjirak et al., 2007). The ALFSp2 LBD peptide (47th to 70th) showed antimicrobial activity against certain tested bacteria, with MIC values of 3.13 to 6.25 μM for *Aerococcus viridans* and *M. luteus*, and 12.5 to 25 μM for *V. harveyi* and *Vibrio anguillarum* (Imjongjirak et al., 2011). Besides, both synthetic LBD peptides sALF1 and sALF2 (53th to 78th) exhibited strong or similar activities against all the tested Gram-positive bacteria (*B. subtilis, C. glutamicum, M. luteus, M. lysodeikticus, S. epidermidis*) and one Gram-negative bacterium (*P. fluorescens*) tested (Liu et al., 2012a). In this study, the synthesized LBD peptide of SpALF7 also had strong antimicrobial activity against almostly all tested bacteria (*M. luteus*, *M. lysodeikticus*, *P. stutzeri*, and *B. subtilis*), indicating that the LBD of SpALF7 might play a improtant role in the immune defense process of *S. paramamosain*.

Most mud crab ALFs (SpALFs) are predominantly distributed in hemocytes (details shown in Table S1) (Hou et al., 2017; Imjongjirak et al., 2011; Imjongjirak et al., 2007; Liu et al., 2012a; Rosa et al., 2008; Zhu et al., 2014), except for SpALF5, whose highest expression level has been reported in the brain(Lv et al., 2017; Sun et al., 2015). Among other crustaceans, the Group G ALF (ALF*Pm*11) from *P. monodon* was expressed abundantly in the hepatopancreas (Zhou et al., 2019b), and EcALF2 and EcALF4 (Lv et al., 2017) from ridgetail prawn *E. carinicauda* were mainly distributed in gills, epidermis, and stomach, while EcALF1 (Lv et al., 2017), EcALF3 and EcALF5 (Lv et al., 2018) were mainly

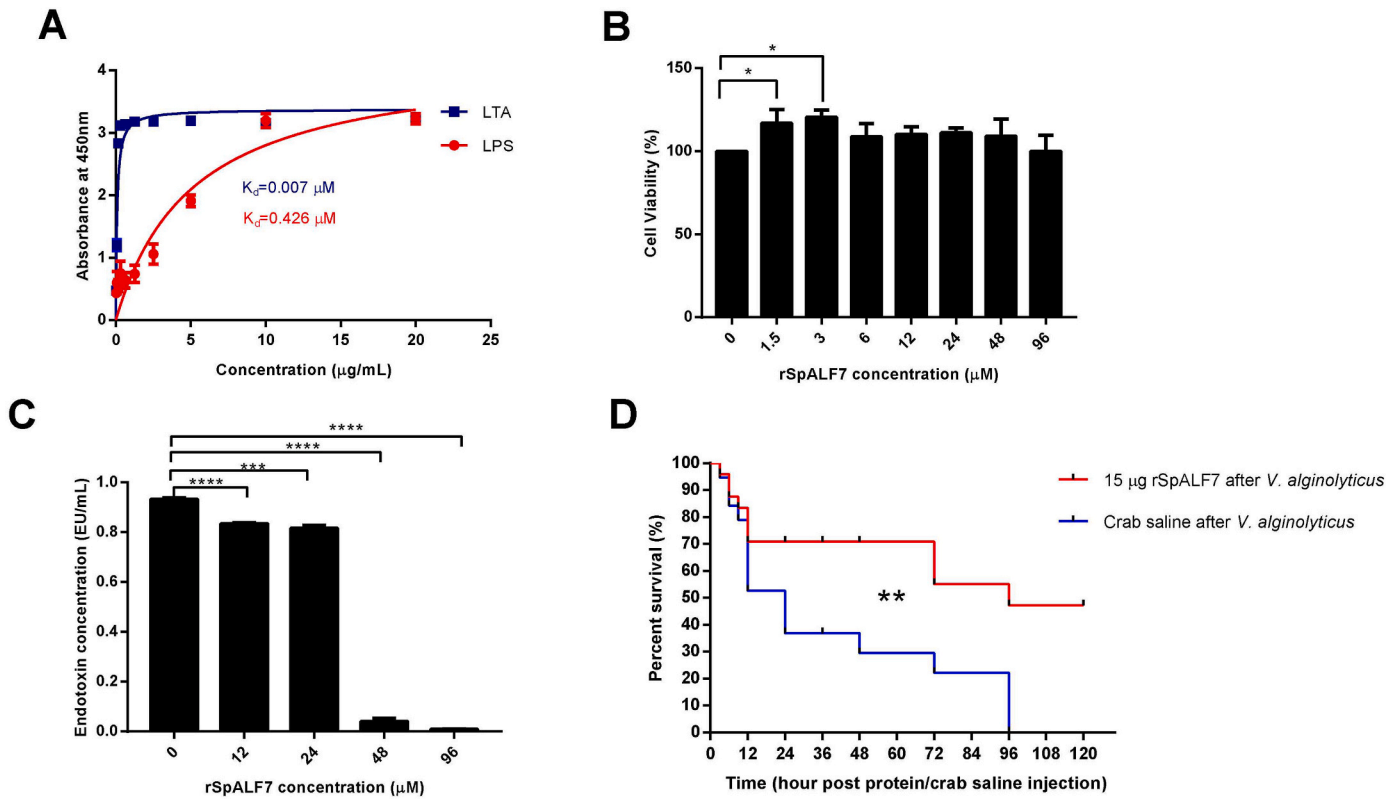

Fig. 6. Evaluation of *in vitro* cytotoxicity and *V. alginolyticus* endotoxin after rSpALF7 treatment and the *in vivo* protective effect of rSpALF7 on *S. paramamosain* infected by *V. alginolyticus*. (A) The binding affinity of rSpALF7 to LPS and LTA. (B) Cytotoxicity effect of rSpALF7 on hemocytes isolated from male crabs. They were incubated with culture medium supplemented with various concentrations of rSpALF7, and the cell viability was determined by MTS method. Data represent the means ± SD from three independent experiments. (C) Endotoxin production of *V. alginolyticus* treated with different concentrations of rSpALF7. (D) *In vivo* protective effect of rSpALF7. Crabs were challenged with *V. alginolyticus* at 3×10^6 CFU mL^{-1}, and injected with rSpALF7 at 15 μg at 1 h post-bacterial challenge ($n = 15$ for each group). The survival curve of each group was analyzed using the Kaplan–Meier log-rank test.

found in hemocytes. Interestingly, our study showed that SpALF7 was most highly expressed in the seminal vesicles of male mud crabs, which was different from other mud crab ALFs and other crustacean ALFs. In our previous reasearch work, it was reported that AMPs scygondin (Peng et al., 2012; Peng et al., 2010; Wang et al., 2007; Xu et al., 2011), SCY2 (Qiao et al., 2016) and scyreprocin(Yang et al., 2020) were highly expressed in the ejaculatory ducts and testis of male mud crabs. Scygondin exhibited potent activities against Gram-negative, Gram-positive bacteria and even WSSV. SCY2 also showed activity against several Gram-negative bacteria. The recombinant scyreprocin exerted potent, broad-spectrum antifungal, antibacterial and anti-biofilm activity. In addition, they were further confirmed to play vital roles in the reproductive immunity of *S. paramamosain*. Similar phenomenon has also been found in other species, such as, the epididymis-specific AMP Bin1b, which is involved in sperm maturation and could prevent microorganisms from entering the adjacent testis where sperm are produced (Li et al., 2001). Andropin, as a cecropin with antibacterial properties, has a protective effect on male reproductive tract and semen, which was strongly induced during the mating process and was strictly limited to the ejaculatory duct of adult male *Drosophila melanogaster* (Samakoviis et al., 1991). Spag11e/Bin1b, which belongs to β-defensins family is specifically expressed in rat epididymis, and can not only kill bacteria, but also trigger sperm movement by activating Ca^{2+} channels (Zhou et al., 2004). The antimicrobial peptide β-defensin, which is specifically expressed in the pituitary gland and testis of the brown spotted grouper (*Epinephelus coioides*), has been shown to be effective in killing Gram-negative bacteria, and even the *Rana grylio* virus (Jin et al., 2010). All these indicated that the AMPs highly expressed in the gonads might have important physiological functions in addition to their antimicrobial activity. Since SpALF7 was also an AMP and had a high transcription level in male gonad, whether it is also involved in the immune defense process during reproduction is worthy of further investigations.

The up-regulation of ALFs expression after various immune challenges appeares to be a common defense strategy for crustaceans to rapidly eliminate invading pathogens (details shown in Table S1). In *M. nipponense*, when challenged with *Aeromonas hydrophila*, the expression of all five MnALFs were significantly increased in gills, intestine, hepatopancreas and hemocytes. (Wang et al., 2015). MjALF-E1 and MjALF-E2 were upregulated in the hemocytes of *M. japonicus* with injection of Gram-positive (*S. aureus*) and Gram-negative (*V. anguillarum*) bacteria (Jiang et al., 2015). In addition, the expression of ALF*Pm*3 (*P. monodon*) was upregulated upon injection of *V. harveyi* (Tharntada et al., 2008). After *P. monodon* was challenged with WSSV and *V. harveyi*, the ALF*Pm*6 transcripts increased significantly at 6 hpi (Ponprateep et al., 2012). LPS, PGN and Poly I:C greatlyup-regulated the expression of PcALF in the hepatopancreas of *P. clarkii* (Zhu et al., 2019). At24 h post-injection with *V. harveyi* and WSSV, the SpALF4 transcripts in the hemocytes of *S. paramamosain* increased remarkably (Zhu et al., 2014). Moreover, after *V. parahemolyticus*, WSSV or PolyI:C challenges, the SpALF5 mRNA levels in the hemocytes of mud crab were significantly upregulated at 6 hpi or 12 hpi (Sun et al., 2015). In the hemocytes, the temporal expression of SpALF6 reached the highest level at 2 h after *S. aureus* challenge and were doubled by *V. parahemolyticus* challenge at 12 hpi (Hou et al., 2017). In this study, the immune challenge assay also showed that SpALF7 could be significantly upregulated at 6 hpi and 12 hpi when injected with LPS and *V. alginolyticus*, which indicated that SpALF7 might involved in the immune response of *S. paramamosain*.

Interestingly, our results showed that the antibacterial spectrum of synthetic peptide sSpALF7 was broader than that of rSpALF7. And for

S. freundii and *P. schneiderii*, the activity of rSpALF7 (MIC 3–6 μM; 1.5–3 μM) was stronger than that of sSpALF7 (MIC 24–48 μM; 12–24 μM). As we all know, recombinant protein expressed by the eukaryotic expression system can form a certain spatial structure and undergo post-translational modifications, which would better mimic the existing form of the recombinant protein *in vivo* and the structure and modification play important roles in protein function. The amino acid sequences of rSpALF7 and sSpALF7 both have two cysteines.The predicted tertiary structure of SpALF7 is composed of three α-helices and four β-sheets. In this structure, the two cysteines might form a single disulfide bond similar to other ALFs (Schmitt et al., 2016b). And the synthesized peptide might have its distinct structure. Therefore, the structure of the two may be one of the reasons for the difference in their antibacterial activity. In addition, the net positive charge and hydrophobicity of AMPs have been proved to be important factors affecting their antibacterial activity. Such as, ALF*Pm*8 (one net positive charge) has limited antibacterial activity, compared to ALF*Pm*3 (six net positive charges) (Somboonwiwat et al., 2005; Zhou et al., 2019a). MjALF-E1 and E2 (three net positive charges) can only inhibit the growth of Gram-negative bacteria (Jiang et al., 2015), while, the LBDs of SpALF-1 and SpALF-2 (seven net positive charges) have a broader antimicrobial spectrum and stronger antibacterial activity (Liu et al., 2012a). Similarly, SpALF5 (six net positive charges) exhibit potent activity against several Gram-negative bacteria, Gram-positive bacteria and yeast *S. cerevisiae* (Sun et al., 2015), while SpALF6 (two net positive charges) was not active against the microorganisms tested in the study (Hou et al., 2017). Moreover, the hydrophobicity of the non-polar face of the peptides was shown to correlate with peptide helicity. The peptideP analogues with greater hydrophobicity (separated by reverse phase-HPLC, the retention time t_R ranging from 32.59 to 47.25 min) showed stronger anticancer activity (Huang et al., 2011). Based on HPRP-A1, ten peptides with different hydrophobicity (t_R ranging from 30.2 to 40.6 min) were designed. The increase in hydrophobicity made the peptides have better antibacterial activity against *Candida albicans* and *Candida krusei* (Zhao et al., 2013). Six synthetic peptide analogues with varying hydrophobicity (t_R =15.68–24.4 min) were composed of the backbone sequence $(LLKK)_2$. As the hydrophobicity of the peptide increased, it caused greater perturbation of the bacterial membrane, and ultimately killed the susceptible and drug-resistant *Mycobacterium tuberculosis* (Khara et al., 2015). In our study, sSpALF has higher net positive charges than rSpALF (+6 for sSpALF, +2 for rSpALF), and the hydrophobicity of sSpALF7 (51.4%) was greater than that of rSpALF7 (41.5%), which may also be one of the reasons why sSpALF7 had a broader antibacterial spectrum.

AMPs usually interact with bacterial membranes and kill bacteria in different modes (Malmsten, 2014). Such as, some AMPs directly disrupt the membrane through barrel stave/toroidal pore formation or carpet-like mechanism, causing cytoplasmic leakage (Vineeth Kumar and Sanil, 2017). Some AMPs can penetrate the cell membrane and bind to intracellular targets, thereby inhibiting the biological activity of microorganisms (Vineeth Kumar and Sanil, 2017). The addition of 100–200 μg/mL rSsALF could cause a large amount of fluorescencently labeled liposomes to leak, which was time-dependent (Yedery and Reddy, 2009). rSp-ALF1 or rSp-ALF2 treatment resulted in the membrane disruption of the tested *E. coli* cells (MC1061), causing the bacteria to release luciferase and react with the substrate in the medium to emit light, thereby a strong signal peak was detected (Liu et al., 2012a). The membrane permeabilization activity of ALF*Pm*3 was analyzed, and a significant increase in A260 was observed within 5 min, which implied that ALF*Pm*3 might enhance the permeability of the bacterial membrane to achieve the bactericidal or bacteriostatic effects (Jaree et al., 2012). The bactericidal mechanism of ALF*Pm*11 was through destroying the bacterial membrane, and flowing out the cell contents, resulting in the death of the bacteria (Zhou et al., 2019b). Similarly in this study, combined with the results of bactericidal kinetics analysis, SEM observation and SYTO9 and PI staining assay, it was also found that rSpALF7 disrupted the bacterial cell membrane by changing the morphology of the bacterial surface, and the cytoplasmic contents leaked, which eventually led to the death of the bacteria.

The main chemical component of bacterial endotoxins are lipopolysaccharide (LPS), a kind of pyrogen. LPS exists in Gram-negative bacteria and are released when cells die or during cell division and cell growth (Buttenschoen et al., 2010; Dullah and Ongkudon, 2017). As reported, various types of biomolecules including lipopolysac charide binding protein (LBP), bactericidal/permea bility-increasing protein (BPI), amyloid P component, cationic proteins, enzymes utilized in the biological endotoxin assay (anti-LPS), lysozyme and lactoferrin, can bind to and interact with endotoxins (LPS), causing a variety of effects, especially activating cell-specific responses and masking biomolecules, thus changing their overall physicochemical properties (Ongkudon et al., 2012). Under aquaculture condition, mud crabs are frequently infected by *V. alginolyticus*, which is the main cause of disease outbreaks, leading to huge economic losses in mud crab production in China (Zhang et al., 2019), which maybe closely realted to the production of bacterial endotoxin. Some ALFs have been reported to exhibit antibacterial activity to *V. alginolyticus*, such as FcALF2 (Li et al., 2015), FcALF5 (Yang et al., 2015), EcALF1 (Lv et al., 2017), EcALF4–5 (Lv et al., 2018), PtALF5–7 (Liu et al., 2013a; Liu et al., 2012b; Liu et al., 2013b), Sp-ALF1and Sp-ALF2 (Liu et al., 2012a), SpALF4 (Zhu et al., 2014), SpALF5 (Sun et al., 2015), SpALF6 (Hou et al., 2017). However, in this study, we found that rSpALF7 and its LBD were not resistant to *V. alginolyticus*. We used a LAL-based endotoxin assay to detect whether rSpALF7 would affect the endotoxin expression level of *V. alginolyticus*. The results showed that the endotoxin level of the experimental group was significantly lower than that of the control group. Similarly, thymoquinone, trans-cinnamaldehy de(TC) and citral inhibited the production of endotoxin from *Cronobacter sakazakii* (Amalaradjou et al., 2014; Shi et al., 2017a; Shi et al., 2017b). Thanatin exhibited concentration-dependent pattern on the inhibition rate of endotoxin isolated from *E. coli* (Shi et al., 2017b). It is preliminary proved that although rSpALF7 was not resistant to *V. alginolyticus*, it could reduce the bacterial endotoxin level, which provided theoretical data support for the subsequent *in vivo* immune protection experiment of rSpALF7.

The immunoprotective effect of AMPs has been confirmed in several studies, including ALFs. Such as, when rMnALF4 was injected into prawns (*M. nipponense*) before *E. coli* challenge, the protein could protect the prawns from infection (Tang et al., 2020). After incubating with rMjALF-E2 and *V. anguillarum* or *S. aureus* for a certain period of time, they were co-injected into the shrimps (*M. japonicus*), and the number of bacteria in the hemolymph of shrimp was detected. The results showed that the number of bacteria in the MjALF-E2-*V. anguillarum* group was obviously less than the control group (Jiang et al., 2015). Similarly, the injection of PcALF1 into crayfish, *P. clarkii*, could enhance the elimination of bacteria *in vivo* (Zhu et al., 2019). The WSSV replication in *E. carinicauda* after infection with pre-incubated WSSV in LBD1, LBD2, LBD5, LBD7, modified peptides sLBD2, LBD2-K and LBD4+K isolated from seven ALF isoforms from the Chinese shrimp *F. chinensis* were obviously inhibited compared with that in control group (Li et al., 2015). Similarly, mFcALF2 from *F. chinensis* could reduce the WSSV infection *in vivo* by pre-incubating with the virus (Yang et al., 2016). Incubation of WSSV with synthetic LBD peptides of EcALF3, EcALF4, and EcALF5 could reduce the *in vivo* viral copy number in WSSV-infected *E. carinicauda* (Lv et al., 2018). Our previous research work in several AMPs (SCY2, SpHyastatin and scyreprocin) also showed similar immunoprotective effect in *S. paramamosain*. For example, *S. paramamosain* pre-treatment with SpHyastatin at 1 h prior to *V. parahaemolyticus* infection exhibited a higher survival rate than those of crabs pre-treated with PBS (Shan et al., 2016). When a male-specific AMP rSCY2 was incubated with *V. parahaemolyticus* at room temperature for 1 h, and then co-injected into crabs, the results showed that rSCY2 could significantly increase the survival rate of *S. paramamosain* (Xu et al., 2011). Another male-specific AMP, rScyreprocin from *S. paramamosain*,

significantly improved the survival of *V. harveyi*-infected marine medaka, and injection of rScyreprocin combined with rSCY2 could increase the survival rate of fish with a synergistic protective effect *in vivo* (Yang et al., 2020). Our studies also revealed that rSpALF7 significantly improved the survival rate of *S. paramamosain* after *V. alginolyticus* challenge. As for the immunoprotective effect mechanism of rSpALF7, since rSpALF7 has no anti-*V.alginolyticus* activity and *V. alginolyticus*-agglutinating effect (data not shown), however, it exhibited strong binding activity to LPS, and significantly reduced the endotoxin level of *V. alginolyticus*. Therefore, we speculated that this might be one of the reasons why rSpALF7 exert an immunoprotective effect on *S. paramamosain* and its underlying *in vivo* regulation mechanism needs more in-depth study.

5. Conclusion

In the present study, a new homologous ALF gene named SpALF7 was identified from the mud crab *S. paramamosain*, and the full leghth cDNA and genomic DNA of SpALF7 were obtained. The expression pattern analysis showed that SpALF7 was widely distributed throughout the entire embryonic and larval developmental stages and various tissues of adult *S. paramamosain*, with the highest mRNA transcription level in the seminal vesicles of male crabs. In addition, injection of LPS or *V. alginolyticus* could significantly induce the expression of SpALF7 gene in hepatopancreas. Both the recombinant protein rSpALF7 which was successfully obtained by eukaryotic expression in the yeast *P. pastoris* and the synthetic peptide (sSpALF7) with the putative LPS binding domain had an potent activity against the Gram-positive bacteria and Gram-negative bacteria tested to varying degrees. The bactericidal kinetic curve unveiled that rSpALF7 could effectively kill *S. flexneri* and *P. stutzeri* in a short time. After treatment with rSpALF7, the cell lysis of *S. flexneri* and *P. stutzeri* could be observed by SEM observation. The SYTO9 and PI stainting assay showed that rSpALF7 could destroy cell membrane of the bacteria, allowing PI dye to enter the cells analyzed by flow cytometry assay. Additionally, the endotoxin level of *V. alginolyticus* decreased remarkably after treatment with rSpALF7 *in vitro*, which might be one of the reasons why rSpALF7 could significantly improve the survival rate of *S. paramamosain in vivo*. Take together, our study indicated that SpALF7 could be used as an immunoprotective agent in mud crab aquaculture, and it is worthy of further investigation as a potential antibacterial agent.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

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