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Litopeidin$_{28-51}$, a novel antimicrobial peptide from *Litopenaeus vannamei*, combats white spot syndrome virus infection through direct virus lysis and immunomodulatory effects

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ABSTRACT

White spot syndrome virus (WSSV) poses a critical threat to crustacean aquaculture, particularly shrimp, causing widespread pandemics. In crustaceans, hemocytes function as a key component of the innate immune system and play a pivotal role in both cellular and humoral immune responses by producing various immune factors, such as antimicrobial peptides (AMPs), to defend against pathogenic microorganisms. In this study, an uncharacterized functional gene named *Litopeidin* was identified in Pacific white shrimp (*Litopenaeus vannamei*). It exhibited heightened expression in hemocytes and demonstrated a significant response to WSSV infection. Further, a truncated peptide, Litopeidin$_{28-51}$, derived from this gene, was characterized and identified as a novel AMP with robust antibacterial and antifungal properties, especially against common aquatic pathogens, including Vibrio spp. Moreover, Litopeidin$_{28-51}$ significantly suppressed the expression of viral genes (*IE1* and *VP28*, WSSV replication-related genes) and the VP28 protein, as well as reduced viral copy numbers in hematopoietic tissue (Hpt) cells following WSSV infection. Mechanistic studies revealed that Litopeidin$_{28-51}$ exhibited a direct virucidal effect on WSSV and significantly upregulated immune-related gene expression (including *Relish, ALF, Crustin,* and *LYZ1*) in Hpt cells. Notably, in *Cherax quadricarinatus* and *L. vannamei*, either co- or pre-treatment with Litopeidin$_{28-51}$ markedly reduced animal mortality and viral replication in tissues. Collectively, the findings suggest that Litopeidin$_{28-51}$, a newly identified AMP with potent antibacterial activity, effectively inhibits WSSV replication by disrupting the viral envelope and regulating the cellular antiviral responses, making it a promising candidate for developing anti-infective agents or immunostimulants in aquaculture.

1. Introduction

Shrimp, recognized for their high nutritional value, are abundant in protein and fatty acids, and they hold considerable economic importance in the aquaculture sector [1]. Nevertheless, the rapid growth of the shrimp farming sector has encountered challenges from various bacteria, fungi, and viruses. Among these, white spot syndrome virus (WSSV), responsible for shrimp white spot disease (WSD), poses a particularly severe threat [2,3]. WSSV is a DNA baculovirus with an envelope capable of both vertical and horizontal transmission [3–5]. Classified as a notifiable disease by the World Animal Health Organization (OIE) due to its highly contagious nature, WSD significantly impacts the industry [6]. To mitigate the impact of animal viral diseases, several strategies have been employed, including chemotherapeutics, vaccinations, and immunostimulants [7]. Although some chemotherapeutics, such as chloroquine, ribavirin, and lopinavir/ritonavir are effective in inhibiting the proliferation of mammalian viruses, their actual efficacy and safety remain unclear [8,9]. Additionally, the unique living environment of aquatic animals poses challenges to the application of chemotherapeutics, which may threaten non-target species, disrupt microbial

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Table 1
Primer sequences were used in this study.

Primer	Accession no.	**Sequence (5′-3′)**	Application
Litopeidin-F	OR161970	ATGAATCGTAAGAATGTAGAACGTGATAG	PCR
Litopeidin-R		TCAACGGAACACATGAATCACTTTAATG	PCR
5′-Litopeidin-R1		CAAGCCAAGCAGGCACGC	PCR
5′-Litopeidin-R2		GCGACGGTATTGAATGGCACA	PCR
3′-Litopeidin-F1		CGCTCTATGACGAGAACCTTCACCTG	PCR
3′-Litopeidin-F2		CACTGGGCTGGCTTTGCCTTTG	PCR
Litopeidin-qF		GTGCCATTCAATACCGTCGC	qPCR
Litopeidin-qR		GGCTCGTGATGTTGTAGTCGC	qPCR
β-actin-qF	AF300705.2	CACCGCCGAGCGAGAAAT	qPCR
β-actin-qR		AGCGAGGAGGAGGAAGCA	qPCR
GAPDH-qF	KM538172.1	TCAATGAGATGAAACCAGAAAAC	qPCR
GAPDH-qR		CAGAGGGGGCAGAGATAACTAC	qPCR
IE1-qF	AF332093.2	GGGAGGTATTGGAGGAGTT	qPCR
IE1-qR		CCGTCCTTCCGCTTCGTG	qPCR
VP28-qF	AF332093.2	AAGTGACTGTGGGGCAGAATC	qPCR
VP28-qR		CATCCTCATCAATAGAGACGGG	qPCR
Relish-qF	ASM3850222v1	CCCTATCGCCCACATCCG	qPCR
Relish-qR		CCGAGGTTGCTGAAGACGC	qPCR
ALF-qF	KX083340.1	TCGTCTGTGTTAGTGAGCG	qPCR
ALF-qR		GAAACTTCTTGATGGTAGG	qPCR
Crustin-qF	KF773763.1	ATGGTAGTGGTGGTTGTGG	qPCR
Crustin -qR		AGGGTGTTTCTGTTCTGGA	qPCR
LYZ1-qF	XM_070097654.1	GGAGAAACCTTTGCGGGATA	qPCR
LYZ1-qR		CTTCAGGGCACAGGAGACG	qPCR

communities, and lead to harmful residue accumulation [10,11]. Unlike mammals and other vertebrates, crustaceans lack acquired immunity and therefore poorly protected by vaccinations [12]. Consequently, immunostimulants - including nutritional factors, cytokines, and antimicrobial peptides (AMPs) - have emerged as promising strategies to enhance resistance to infections in shrimp. AMPs, in particular, play a vital role in the health management of these organisms, contributing significantly to disease control [10,13,14].

AMPs have garnered significant attention due to their crucial role in eliminating pathogenic microorganisms and modulating host immune responses [15–18]. As a fundamental component of innate immunity, they serve as the first line of defense against microbial infections [19,20]. In crustaceans, hemocytes, as important immune cells, produce several types of AMPs, such as crustin, anti-lipopolysaccharide factors (ALFs), Penaeidins, and hemocyanin, which make important contributions to crustacean innate immunity. These AMPs exhibit various modes of action and broad-spectrum activity against pathogens. For example, ALFs have been identified in numerous crustaceans; some exhibit not only potent antibacterial properties but also significantly inhibit the multiplication of WSSV [21–25]. Penaeidin5 from *Fenneropenaeus chinensis* has antimicrobial activities [26]. Inhibition of Penaeidin5 expression in *Penaeus monodon* increased the susceptibility of fish to WSSV infection [27]. Additionally, hemocyanin, the main protein component of hemolymph, protects against bacterial and fungal invasion and can inhibit WSSV proliferation in shrimp [28]. However, studies on AMPs specifically targeting WSSV remain limited, highlighting the need to continue exploring novel AMPs to meet the growing demand for antiviral drug development.

In this study, we identified a previously uncharacterized functional gene, *Litopeidin*, from *Litopenaeus vannamei* and examined its tissue distribution and expression pattern in response to WSSV infection. Through physicochemical analysis and AMP database prediction, we screened and chemically synthesized the truncated peptide Litopeidin$_{28\text{-}51}$ and evaluated its antibacterial and antifungal activities to explore its potential as an AMP. To investigate its antiviral properties, we conducted assays using a hematopoietic tissue (Hpt) cell infection model to assess the impact of Litopeidin$_{28\text{-}51}$ on WSSV infection and cellular immune responses. WSSV destruction was further examined via transmission electron microscopy and micrococcal nuclease digestion assays. Additionally, the antiviral efficacy of Litopeidin$_{28\text{-}51}$ was evaluated *in vivo* using WSSV infection models of *C. quadricarinatus* and *L. vannamei*. Our study aimed to elucidate the physicochemical properties and antimicrobial activities of the novel AMP Litopeidin$_{28\text{-}51}$, highlight its antiviral effect against WSSV, and uncover the underlying mechanisms, with the goal of developing a potentially effective antiviral agent for future aquaculture applications.

2. Methods and materials

2.1. Experimental animals and Hpt cells

Red claw crayfish (*C. quadricarinatus*) and Pacific white shrimp (*L. vannamei*) were obtained from aquaculture companies in Fuzhou, Fujian Province, China. Both species were acclimated in freshwater and artificial seawater, respectively, and fed twice daily at 5 % of their total biomass. Random PCR tests confirmed that these animals were free of WSSV.

Hpt cells, which serve as a good model for the study of WSSV infection, were isolated from were isolated from adult *C. quadricarinatus* and subsequently cultured, as previously described [29,30].

2.2. Pathogenic microorganisms

WSSV was generously supplied by Prof. Hai-Peng Liu and quantified using absolute quantitative real-time PCR (qPCR), as previously described [3,31].

Gram-negative bacteria (*Acinetobacter baumannii* CGMCC 1.6769, *Escherichia coli* CGMCC 1.2389, *Vibrio alginolyticus* CGMCC 1.1833, *Vibrio parahaemolyticus* CGMCC 1.1615, *Vibrio fluvialis* CGMCC 1.1609 and *Vibrio harveyi* CGMCC 1.1593), gram-positive bacteria (*Staphylococcus aureus* CGMCC 1.2465), and fungi (*Cryptococcus neoformans* CGMCC 2.1563, *Aspergillus niger* CGMCC 3.316, *Fusarium solani* CGMCC 3.584, *Fusarium graminearum* CGMCC 3.4521 and *Fusarium oxysporum* CGMCC 3.6785) were purchased from the China General Microbiological Culture Collection Center (CGMCC).

2.3. Cloning of full-length cDNA of Litopeidin

To amplify the full-length cDNA sequence of *Litopeidin*, cDNA was extracted from shrimp hemocytes. 5′/3′- RACE PCR-template was prepared using a SMARTer RACE 5′/3′ Kit (Takara, Japan). The PCR products of the amplified *Litopeidin* fragments were sequenced by

Sangon Biotech Co., Ltd (Shanghai, China). The primers used are listed in Table 1.

2.4. Sequence analysis and peptide synthesis

Similarity and homology analysis of the *Litopeidin* sequence was verified by the BLAST website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Structural domain analysis of the peptide was performed with the SMART tool (https://smart.embl.de/). Multiple sequence alignment was carried out using the DNAMAN software. I-TASSER (https://zhanggroup.org/I-TASSER/) predicted the three-dimensional (3D) structure of peptides, which can be viewed in PyMOL. ExPASy website (https://web.expasy.org/protparam/) was used to predict the peptide physicochemical properties, including net positive charge, hydrophobicity, theoretical isoelectric point, and molecular weight. The amino acid sequences were analyzed through the Collection of Anti-Microbial Peptides ($CAMP_{R3}$; http://www.camp3.bicnirrh.res.in) to predict antimicrobial regions within Litopeidin. The truncated peptide $Litopeidin_{28\text{-}51}$ (RRHRLIIRRRMRNSFWSSRACLAW) was synthesized by Genscript (Nanjing, China), purified through RP-HPLC (>95 %), and characterized using MALDI-TOF MS. The powdered peptide was remained at −80 °C, while the stock peptide solution was maintained at −20 °C.

2.5. Tissue distribution and expression profiles of Litopeidin

The expression profiles of *Litopeidin* were determined by qPCR in different tissues of *L. vannamei* and at different stages of WSSV infection. Tissues (hemocytes, eyestalks, heart, nerves, gills, swimming legs, muscles, hepatopancreas, stomach, and intestine) from healthy shrimps (*L. vannamei*) were collected. A total of 200 shrimp were randomly divided into two groups. The control group was injected with 100 μL of normal saline per shrimp, and the WSSV group was injected intrathoracically with WSSV at a dose of 1×10^3 copies per shrimp. Hemocytes of the shrimps were collected at 3, 6, 12, 24, 48, and 72 h post-infection (pi) to analyze the expression profile of *Litopeidin*.

TB Green Premix Ex *Taq*II (TaKaRa) was used for qPCR, with cycling conditions set to 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 34 s. The distribution of *Litopeidin* in different tissues of *L. vannamei* was analyzed by absolute qPCR and displayed as copies/μL. Relative expression levels of *Litopeidin* were calculated using the $2^{-\Delta\Delta CT}$ method [32] after normalization to *β-actin* (AF300705.2) [33]. Used primers are listed in Table 1.

2.6. Antimicrobial activity assay

The antimicrobial activity of $Litopeidin_{28\text{-}51}$ was assessed using the broth microdilution method [34]. Logarithmic-growth phase bacteria were collected and diluted in Muller-Hinton broth (HKM, China) to ~10^6 colony-forming units (CFU)/mL. Different concentrations of $Litopeidin_{28\text{-}51}$ (3–48 μM) were added to the bacteria in 96-well polystyrene flat-bottom plates (NEST, China), with MilliQ water serving as the control. The microplates were incubated statically at 37 °C, and the minimum inhibitory concentration (MIC) was determined. The minimum bactericidal concentration (MBC) was detected through colony counting at 24 h.

2.7. Cell viability assay

Hpt cells were seeded in 96-well plates and incubated at 27 °C overnight. The experimental groups were treated with different concentrations of $Litopeidin_{28\text{-}51}$ (1, 5, 10, 15, 25, and 50 μM), while the control group was treated with crayfish phosphate-buffered saline (CPBS). Cytotoxicity of $Litopeidin_{28\text{-}51}$ was determined after 24 h of treatment using the CellTiter 96®Aqueous Non-Radioactive Cell Proliferation Assay (Promega, USA), following the manufacturer's instructions.

2.8. QPCR analysis of viral genes

Based on previous experiments [3], WSSV (5×10^6 copies/mL) and 10 μM $Litopeidin_{28\text{-}51}$ were added simultaneously to Hpt cells (24-well plates) at 27 °C. After treatment, the fresh culture medium was added to the cells. Hpt cells were collected for total RNA at 6 h pi and for genomic DNA at 6, 12, and 24 h pi. RNA and DNA were extracted using TRIzol reagent (Invitrogen, USA) and the TIANamp Genomic DNA Kit (TIANGEN, China), respectively. QPCR was carried out to analyze the expression of the glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*; KM538172.1; an internal reference gene), the immediate early (*IE1*), and the envelope protein 28 (*VP28*). The viral load was determined using the VP28 recombinant plasmid to establish a standard curve for measuring WSSV copy numbers [35].

2.9. Western blot analysis

Following previous methods [3], WSSV (5×10^6 copies/mL) and $Litopeidin_{28\text{-}51}$ (2.5 and 10 μM) were added simultaneously to Hpt cells (24-well plates) at 27 °C and the cells were cultured for 24 h. The proteins of samples from different treatments were extracted and then analyzed by Western blot. The primary antibodies of VP28 (1:5000) and β-actin (1:5000; Proteintech Group, USA) were used [36]. IRDye®800CW Goat anti-Mouse IgG (1:5000; Li-Cor, USA) was employed as the secondary antibody. The result was analyzed by the Odyssey Infrared Imaging System (Li-Cor).

2.10. Time-of-addition assay

$Litopeidin_{28\text{-}51}$ (10 μM) was applied in three experimental setups based on different stages of viral infection: (1) Pretreat virus, (2) Pretreat cells, and (3) Post-treatment. Refer to the figure legends for a detailed description of each experimental setup. According to previous reports, the *IE1* gene was highly expressed 5 h post-WSSV infection, while the *VP28* gene peaked at 6 h [37]. WSSV genome replication began at 12 h pi and rapidly replicated within 24–48 h pi [38]. Consequently, cells were collected at 6 h pi to measure the expression of *IE1* and *VP28* using relative qPCR, and at 24 h pi to assess viral DNA accumulation through absolute qPCR.

2.11. Inactivation of cell-free virions

$Litopeidin_{28\text{-}51}$ (5 and 15 μM) were incubated with WSSV (5×10^6 copies/mL) at 4 °C for 1 h, and then the procedures were performed according to the previous report [3]. PEG-6000 (Sigma-Aldrich, USA) was added to the mixture and incubated at 4 °C for an additional 1 h. After centrifugation, the supernatant was discarded, and the pellet was washed twice with 3 % PEG-6000 containing 10 mg/mL BSA. Finally, the pellet was resuspended in the medium and added to Hpt cells. Following 24 h of culture at 27 °C, the viral loads were measured by absolute qPCR.

2.12. Transmission electron microscopy (TEM) analysis

Following the protocol described by Lin et al. [39], sample preparation for electron microscopy was performed. WSSV (10^7 copies) was incubated with $Litopeidin_{28\text{-}51}$ at concentrations of 5 and 15 μM for 1 h at room temperature (RT). Samples were further fixed in paraformaldehyde (1 %) for 20 min and placed onto carbon-coated copper grids. They were subsequently stained with sodium phosphotungstate (2 %) for 5 min and allowed to dry at RT for at least 3 h before undergoing TEM analysis using a Tecnai G2 Spirit BioTwin (FEI, USA).

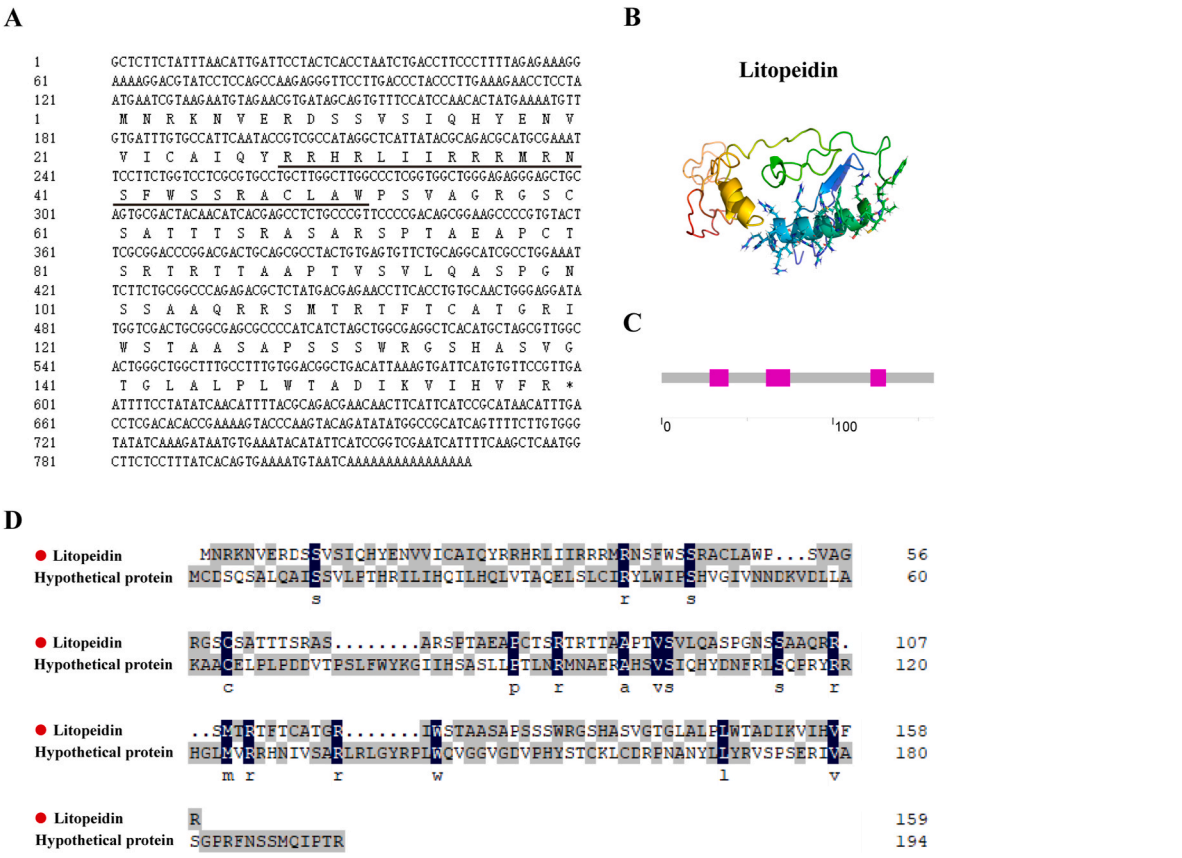


Fig. 1. Bioinformatic analysis of Litopeidin. (A) Full-length cDNA and deduced amino acid sequence of Litopeidin. The underline indicates the sequence of the synthetic Litopeidin$_{28\text{-}51}$. (B) The protein structure of the Litopeidin was predicted using the I-TASSER server. (C) Structural domain analysis of Litopeidin. (D) Sequence alignment analysis of Litopeidin with the homologous protein. The accession numbers in GenBank are as follows: *Litopenaeus vannamei* OR161970 (Litopeidin); *Petrolisthes manimaculis* KAK4303894.1 (Hypothetical protein Pmani_024144).

2.13. Micrococcal nuclease digestion

Concerning the report of Lok et al. [40], digestion experiments were conducted to evaluate the damage of Litopeidin$_{28\text{-}51}$ to virions. Purified WSSV (10^8 copies) was incubated with Litopeidin$_{28\text{-}51}$ (at a series of multiple dilution concentrations) for 1 h at 27 °C. This was followed by digestion of the mixtures with micrococcal nuclease (New England BioLabs, USA) for an additional hour at 37 °C. After digestion, WSSV genomic DNA was extracted and subsequently quantified to determine copy numbers using absolute qPCR.

2.14. The expression analysis of immune-related genes

Hpt cells were incubated with 10 μM Litopeidin$_{28\text{-}51}$ or CPBS at 27 °C for 1 h and then infected with WSSV (5×10^6 copies/mL). The cells were collected at 3 h, 6 h, 12 h, and 24 h pi, and qPCR was performed for the determination of gene expression (*GAPDH*, *Relish*, *ALF*, *Crustin*, and *LYZ1*). The dissociation curves for each gene were plotted to confirm the specificity of the PCR product. The cycling conditions and calculation methods were consistent with those described above. The specific primers of genes were shown in Table 1.

2.15. Antiviral assay in vivo

Co-stimulation and protection experiments were designed to evaluate the antiviral effect of Litopeidin$_{28\text{-}51}$ on *C. quadricarinatus* and *L. vannamei*. In the co-stimulation experiment, animals were randomly divided into two groups. (1) Control group (Mock): 50 μL of normal saline was injected into animals using a syringe with a 29-gauge needle. (2) Experimental group: 0, 0.5, and 2 μg/g Litopeidin$_{28\text{-}51}$ and WSSV inoculum (10^4 copies/crayfish or 10^3 copies/shrimp) were simultaneously injected into animals.

In the protection experiment, *C. quadricarinatus* and *L. vannamei* were similarly randomized into two groups, respectively. (1) Control group (Mock): 50 μL of normal saline was injected into animals, and the same dose of normal saline (without WSSV) was injected again 1 h later. (2) Experimental group: animals were injected with 0, 0.5, and 2 μg/g Litopeidin$_{28\text{-}51}$, and 1 h later, they were infected with prepared WSSV inoculum (10^4 copies/crayfish or 10^3 copies/shrimp).

As previously reported, the gill tissue is the main site susceptible to WSSV infection [41]. At 48 h pi, the gill tissues of animals were collected for DNA extraction, and then the viral genomic DNA was quantified by absolute qPCR. Meanwhile, the number of surviving animals was recorded every 12 h, and the survival curve was plotted.

2.16. Data analysis

Each independent assay was performed in triplicate, and data were displayed as the mean ± standard deviation (SD). The statistical significance was analyzed using Prism version 8 software (GraphPad, USA).

3. Results

3.1. Sequence analysis of Litopeidin

The full-length cDNA sequence of *Litopeidin* was obtained, measuring 826 bp in total, and has been assigned the GenBank accession number OR161970. It consisted of a 120 bp 5′-untranslated region (UTR), a 226

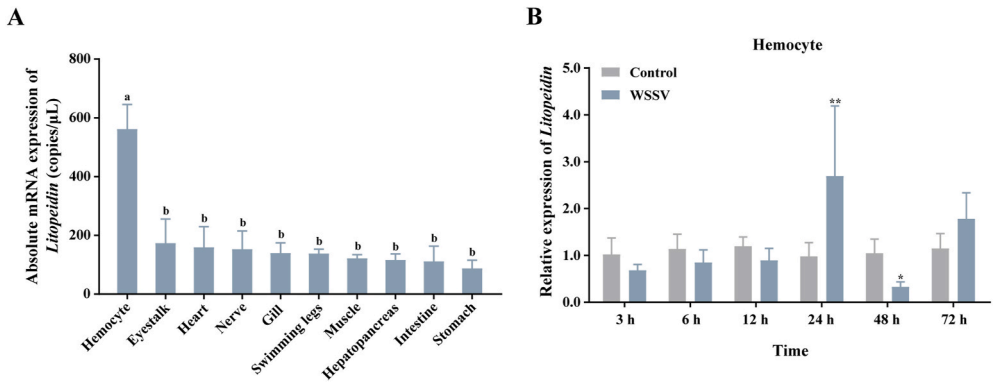


Fig. 2. Tissue distribution and expression profiles of *Litopeidin* (A) The distribution of *Litopeidin* in different tissues *of L. vannamei* was analyzed by absolute qPCR. Differences in different tissues were indicated with the letter "a", "b" or "c". (B) The expression pattern of *Litopeidin* in hemocytes after WSSV infection. The expression levels of *Litopeidin* were normalized using the *β-actin* expression. $*p < 0.05$ and $**p < 0.01$. An unpaired two-tailed Student's t-test was used for comparisons between two groups, while a two-way analysis of variance (ANOVA) was employed for multiple groups. Data was expressed as the mean ± SD (n = 3).

Table 2
Sequence prediction information.

Name	SVM Result	RF Result	ANN Result
Litopeidin	1 (AMP)	0.826 (AMP)	AMP
Litopeidin$_{28\text{-}51}$	0.837 (AMP)	0.6915 (AMP)	AMP

SVM Result: Results with Support Vector Machine (SVM) classifier; RF Result: Results with Random Forest Classifier; ANN Result: Results with Artificial Neural Network (ANN) classifier.

bp 3′-UTR, and a 480 bp ORF encoding a 159-amino acid peptide (Fig. 1A). The predicted structure of Litopeidin showed that the two typical α-helical structures of this peptide were located at Arg31-Ala47 and Ser102-Met109, respectively (Fig. 1B). Structural domain and sequence alignment analyses indicated that Litopeidin lacked conserved domains and showed 66 % sequence similarity and 31.48 % homology to the hypothetical protein Pmani_024144 (KAK4303894.1) from *Petrolisthes manimaculis* (Fig. 1C and D). This suggested that it may represent an uncharacterized functional peptide.

3.2. Tissue-specific expression and expression profile after viral infection

The tissue distribution of *Litopeidin* was analyzed using absolute qPCR, with results expressed as copies/μL. The analysis revealed that *Litopeidin* was ubiquitously expressed across all collected tissues, with the highest expression found in the hemocytes (Fig. 2A). Additionally, the expression of *Litopeidin* was markedly upregulated at 24 h pi (Fig. 2B), suggesting that it may be an immune-related gene in response to viral infection. Using the Collection of Anti-Microbial Peptides (CAMP$_{R3}$; http://www.camp3.bicnirrh.res.in) for prediction, Litopeidin and Litopeidin$_{28\text{-}51}$ were found to be potential AMPs (Table 2).

3.3. Structure prediction and physicochemical properties analysis of Litopeidin$_{28\text{-}51}$

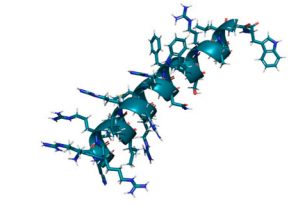
The predicted 3D structure of Litopeidin$_{28\text{-}51}$ was shown in Fig. 3A. Litopeidin$_{28\text{-}51}$ was derived from Litopeidin and contained a predominant α-helix structure. The key physicochemical parameters of Litopeidin$_{28\text{-}51}$ were shown in Fig. 3B, with a hydrophobicity of 33.4 % and a total net charge of +8.

3.4. Litopeidin$_{28\text{-}51}$ exhibited broad-spectrum antimicrobial activity

The antimicrobial activity of Litopeidin$_{28\text{-}51}$ was examined. The results showed that Litopeidin$_{28\text{-}51}$ inhibited the growth of Gram-positive

Table 3
Antimicrobial activities of synthetic Litopeidin$_{28\text{-}51}$.

Microorganisms	MIC (μM)	MBC (μM)
Gram-negative bacteria		
Acinetobacter baumannii	6–12	12–24
Escherichia coli	3–6	6–12
Vibrio alginolyticus	6–12	12–24
Vibrio harveyi	6–12	12–24
Vibrio fluvialis	6–12	6–12
Vibrio parahaemolyticus	6–12	12–24
Gram-positive bacteria		
Staphylococcus aureus	6–12	12–24
Fungi		
Cryptococcus neoformans	6–12	12–24
Fusarium oxysporum	6–12	12–24
Fusarium solani	6–12	12–24
Fusarium graminearum	12–24	24–48
Aspergillus niger	12–24	24–48



B

Physicochemical Parameters	Litopeidin$_{28\text{-}51}$
Number of amino acids	24aa
Molecular weight	3128.72 Da
Isoelectric point	12.48
Total net charge	+8
Hydrophobicity	33.4%

Fig. 3. Analysis of structure and physicochemical properties of peptides. (A) The 3D structure of the Litopeidin$_{28\text{-}51}$ was predicted using the AlphaFold II. (B) The key physicochemical parameters of Litopeidin$_{28\text{-}51}$.

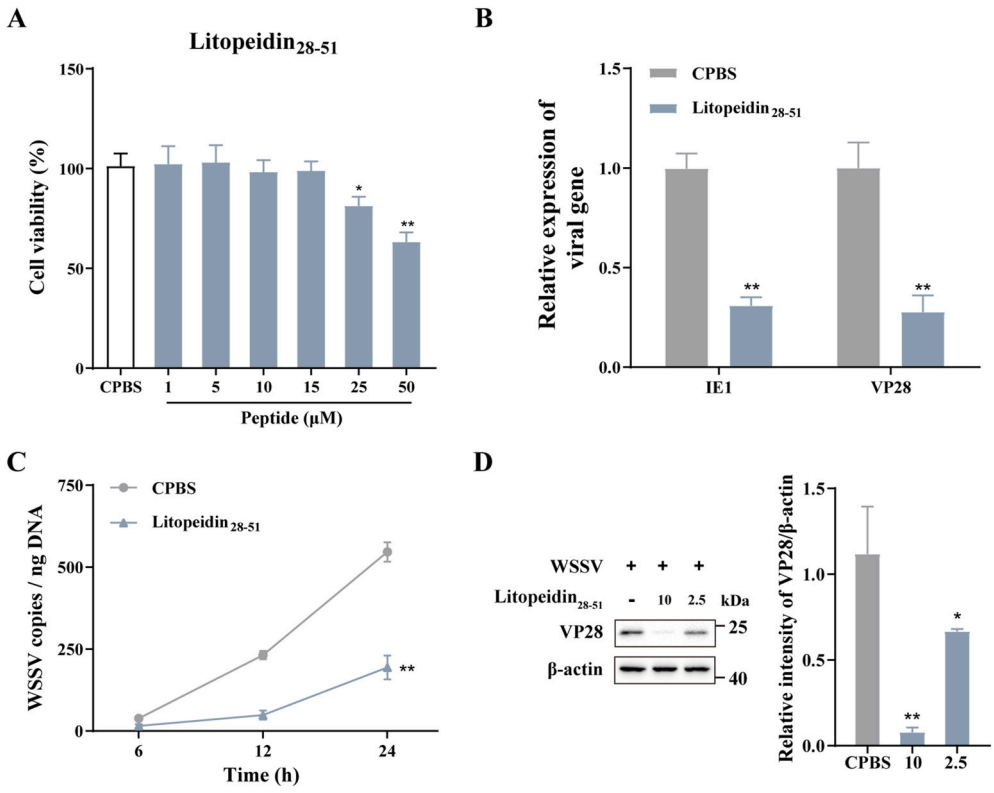


Fig. 4. Litopeidin$_{28\text{-}51}$ efficiently inhibited WSSV replication in Hpt cells. (A) The toxicity test of Litopeidin$_{28\text{-}51}$ in Hpt cells. (B) Total RNA was extracted 6 h after WSSV infection, and qPCR was used to detect the levels of viral gene transcription after Litopeidin$_{28\text{-}51}$ treatment. *IE1* and *VP28* gene expressions were normalized to *GAPDH*. (C) Genomic DNA was extracted at 6 h, 12 h, and 24 h post-WSSV infection to assess the effect of Litopeidin$_{28\text{-}51}$ treatment on WSSV replication, with absolute qPCR used to measure the viral load at each time point. (D) Viral protein expression at 24 h pi after WSSV treated with Litopeidin$_{28\text{-}51}$ at different concentrations was detected by Western blot. An unpaired two-tailed Student's t-test was used for comparisons between two groups, while a two-way analysis of variance (ANOVA) was employed for multiple groups. Results were expressed as the mean ± SD (n = 3). *$p < 0.05$, **$p < 0.01$.

and Gram-negative bacteria (*S. aureus*, *A. baumannii*, *E. coli*, *V. alginolyticus*, *V. harveyi*, *V. fluvialis*, *V. parahaemolyticus*), whose MIC values ranged from 3 to 12 μM and MBC values were below 24 μM. In addition, Litopeidin$_{28\text{-}51}$ also exhibited potent antifungal activity against *C. neoformans*, *F. oxysporum*, *F. solani*, *F. graminearum*, and *A. niger* with MIC values of 6–24 μM (Table 3).

3.5. Litopeidin$_{28\text{-}51}$ reduced viral replication in Hpt cells

Before the antiviral assay, it is essential to evaluate the safety of Litopeidin$_{28\text{-}51}$. To this end, we evaluated the cytotoxicity of Litopeidin$_{28\text{-}51}$ on the Hpt cells. As shown in Fig. 4A, Litopeidin$_{28\text{-}51}$ at concentrations below 15 μM was non-toxic to cells cultured for 24 h according to the MTS test. The viability of cells did not exhibit significant differences compared to the untreated control. Further, we evaluated the inhibitory effects of Litopeidin$_{28\text{-}51}$ on intracellular WSSV transcription levels. In the co-stimulation group, 10 μM Litopeidin$_{28\text{-}51}$ significantly reduced the transcription levels of IE1 and VP28, which were reduced by approximately 69.0 % and 72.3 %, respectively, compared with the control group (Fig. 4B). Meanwhile, the copies of WSSV were decreased by 78.9 % at 12 h and remained decreased by 64.5 % at 24 h (Fig. 4C). In addition, 10 μM of Litopeidin$_{28\text{-}51}$ significantly down-regulated the protein level of VP28 compared with the control group (Fig. 4D).

3.6. Litopeidin$_{28\text{-}51}$ exerted antiviral action during the WSSV infection process

We performed a time-of-addition assay to investigate the mechanism of action of Litopeidin$_{28\text{-}51}$, with the schematic diagram outlining each treatment presented in Fig. 5A. The results showed that Litopeidin$_{28\text{-}51}$ significantly down-regulated the transcription levels of *IE1* and *VP28* genes, as well as the viral DNA accumulation in both the pretreated virus group and the pretreated cell group, compared to the CPBS group, with a stronger effect on the pretreated virus group (Fig. 5B–D). For the accumulation of viral DNA, the viral inhibition rate of Litopeidin$_{28\text{-}51}$ in the pretreated cell group was above 50 % (Fig. 5D). These results suggested that Litopeidin$_{28\text{-}51}$ may inhibit viral infection by interacting with virions and play a critical role in the immunomodulation of cells.

3.7. Litopeidin$_{28\text{-}51}$ directly destroyed WSSV particles

Antiviral cationic peptides have been reported to show antiviral activity by directly inhibiting viral replication through interaction with virions [42]. To determine whether Litopeidin$_{28\text{-}51}$ exhibited direct antiviral activity against WSSV, we conducted a cell-free virion inactivation assay, with the schematic diagram illustrated in Fig. 6A. It was found that cell-free virions pretreated with Litopeidin$_{28\text{-}51}$ lost their infectivity toward Hpt cells, suggesting that the peptide may directly act on the virions (Fig. 6B). WSSV virions were co-incubated with CPBS or Litopeidin$_{28\text{-}51}$ (5 and 15 μM) for 1 h, then the morphological changes of WSSV were observed by TEM after negative staining with paraformaldehyde. Unenveloped WSSV virions are thinner and longer than intact virions, are less infectious, and have a segmented appearance [43, 44]. Similarly, as shown in Fig. 6C, the envelope of virions was destroyed by Litopeidin$_{28\text{-}51}$ compared to the control WSSV virions. Furthermore, the intact WSSV particles exhibited resistance to micrococcal nucleases, whereas Litopeidin$_{28\text{-}51}$ destroyed virions in a dose-dependent manner, allowing the viral genomes to be digested by nucleases (Fig. 6D). These results implied that Litopeidin$_{28\text{-}51}$ destroys

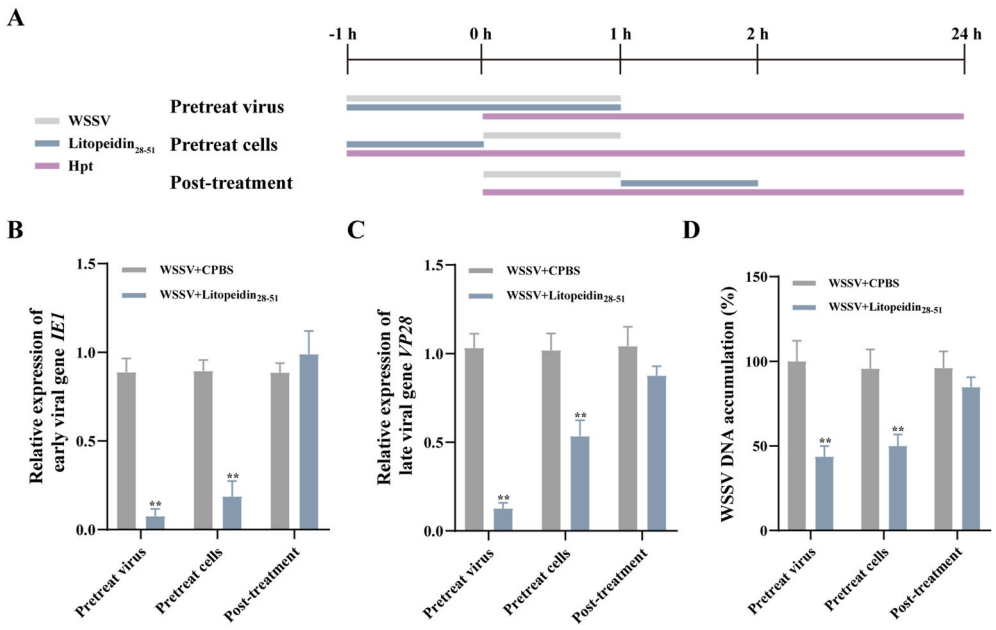


Fig. 5. The role of Litopeidin$_{28\text{-}51}$ in different stages of viral infection. (A) Schematic of different treatment assay workflow: (1) Pretreat virus: WSSV (5×10^6 copies/mL) was incubated with Litopeidin$_{28\text{-}51}$ (10 μM)/CPBS at 27 °C for 1 h. This mixture was then applied to cells for another hour, after which the cells were washed thrice with CPBS and supplied with fresh culture medium; (2) Pretreat cells: Cells were treated with Litopeidin$_{28\text{-}51}$ (10 μM)/CPBS at 27 °C for 1 h. Following this, they were washed three times with CPBS before being infected with WSSV (5×10^6 copies/mL) for 1 h. The cells were then washed again and fresh medium was added; (3) Post-treatment: Cells were first infected with WSSV (5×10^6 copies/mL) for 1 h at 27 °C, followed by three washes with CPBS. They were subsequently treated with Litopeidin$_{28\text{-}51}$ (10 μM) for 1 h, washed with CPBS, and then provided with fresh culture medium. (B and C) Total RNA was extracted 6 h after WSSV infection, and the effects of Litopeidin$_{28\text{-}51}$ on gene transcription levels at different stages of virus infection were detected by qPCR. *IE1* and *VP28* gene expressions were normalized to *GAPDH*. A two-way analysis of variance (ANOVA) for multiple groups. (D) Genomic DNA was extracted at 24 h after WSSV infection, and the effects of Litopeidin$_{28\text{-}51}$ on viral load at different stages of virus infection were detected by absolute qPCR. A two-way analysis of variance (ANOVA) was used for multiple groups. Data was expressed as the mean ± SD (n = 3). $^{**}p < 0.01$.

the structure of WSSV and produced a direct antiviral effect.

3.8. Effect of Litopeidin$_{28\text{-}51}$ on immune-related genes

By pretreating the cells, Litopeidin$_{28\text{-}51}$ was also able to reduce viral load. Therefore, we speculated that Litopeidin$_{28\text{-}51}$ might regulate the host immune response to resist viral infection. Further experiments for verification were designed, as shown in Fig. 7A. We analyzed the transcriptional expression of *Relish*, *ALF*, *Crustin*, and *lysozyme 1* (*LYZ1*) after WSSV infection in the presence of Litopeidin$_{28\text{-}51}$. Expression profiles of the immune-related genes were determined in the Hpt cells, and the results showed different changes occurred between the three groups after infection (Fig. 7B–E). At 3 h pi, the transcriptional level of *Relish* in the peptide pretreat cell group was significantly higher than that in the WSSV group. Additionally, *ALF*, *Crustin*, and *LYZ1* genes were significantly upregulated at 12 h pi compared to the WSSV group. These results indicated that Litopeidin$_{28\text{-}51}$ better activated the expression of immune-related genes, suggesting that it has indirect antiviral capabilities through immunomodulatory effects.

3.9. The efficient anti-infection of Litopeidin$_{28\text{-}51}$ in vivo

To evaluate the *in vivo* antiviral effects of Litopeidin$_{28\text{-}51}$, we assessed the survival rates and WSSV copy numbers in *C. quadricarinatus* and *L. vannamei* during the WSSV challenge. Schematic diagrams illustrating the treatment of both species with WSSV and Litopeidin$_{28\text{-}51}$ were shown in Figs. 8A and 9A. In the co-treatment mode, the viral copy numbers in the gill tissues of *C. quadricarinatus* and *L. vannamei* treated with 2 μg/g Litopeidin$_{28\text{-}51}$ were significantly reduced compared with the control group (Fig. 8B and C), and survival rates increased to 70.0 % (0 μg/g, 36.7 %) and 46.7 % (0 μg/g, 16.7 %) at 5 d pi, respectively (Fig. 8D and E). In the pretreatment mode, where animals were treated with 2 μg/g Litopeidin$_{28\text{-}51}$ before WSSV infection, the viral replication in the gill tissues was significantly lower than the control group (Fig. 9B and C), and survival rates increased to 63.3 % (0 μg/g, 33.3 %) and 30.0 % (0 μg/g, 13.3 %) at 5 d pi, respectively (Fig. 9D and E).

4. Discussion

Antimicrobial peptides (AMPs) are one of the essential components in the innate immune system, protecting against bacteria, fungi, viruses, and parasites [45]. While much of the past research has concentrated on their antimicrobial activity [46], recent investigations have revealed that AMPs also exhibit notable antiviral activities [17,47–49].

In crustaceans, major immune responses occur in hemolymph, and hemocytes play key roles in both cellular and humoral immunity, serving as a crucial component of innate immune defenses [50,51]. *Litopeidin* is highly expressed in hemocytes and exhibits a significant response to WSSV infection, suggesting it may function as an immune-related gene. During WSSV infection, the viral genome replicates rapidly from 24 to 48 h [38]. The expression of *Litopeidin* notably increased at 24 h pi, indicating its involvement in responding to WSSV infection and combating the virus [27]. The significant decrease in the expression of this gene at 48 h pi may be attributed to the gradual clearance of the virus by the immune system, which maintains homeostasis and prevents excessive inflammatory responses by down-regulating the expression of some genes [52]. These findings highlight *Litopeidin*'s role as an effector in the immune system. The previously discovered histones are important immune-related proteins produced by hemocytes, and a truncated peptide derived from histone H2A is considered an AMP with significant antimicrobial activity [53,54]. Our study also obtained a truncated peptide consisting of 24 amino acid residues (Litopeidin$_{28\text{-}51}$) through physicochemical properties and protein structure analysis. It is characterized as a positively charged,

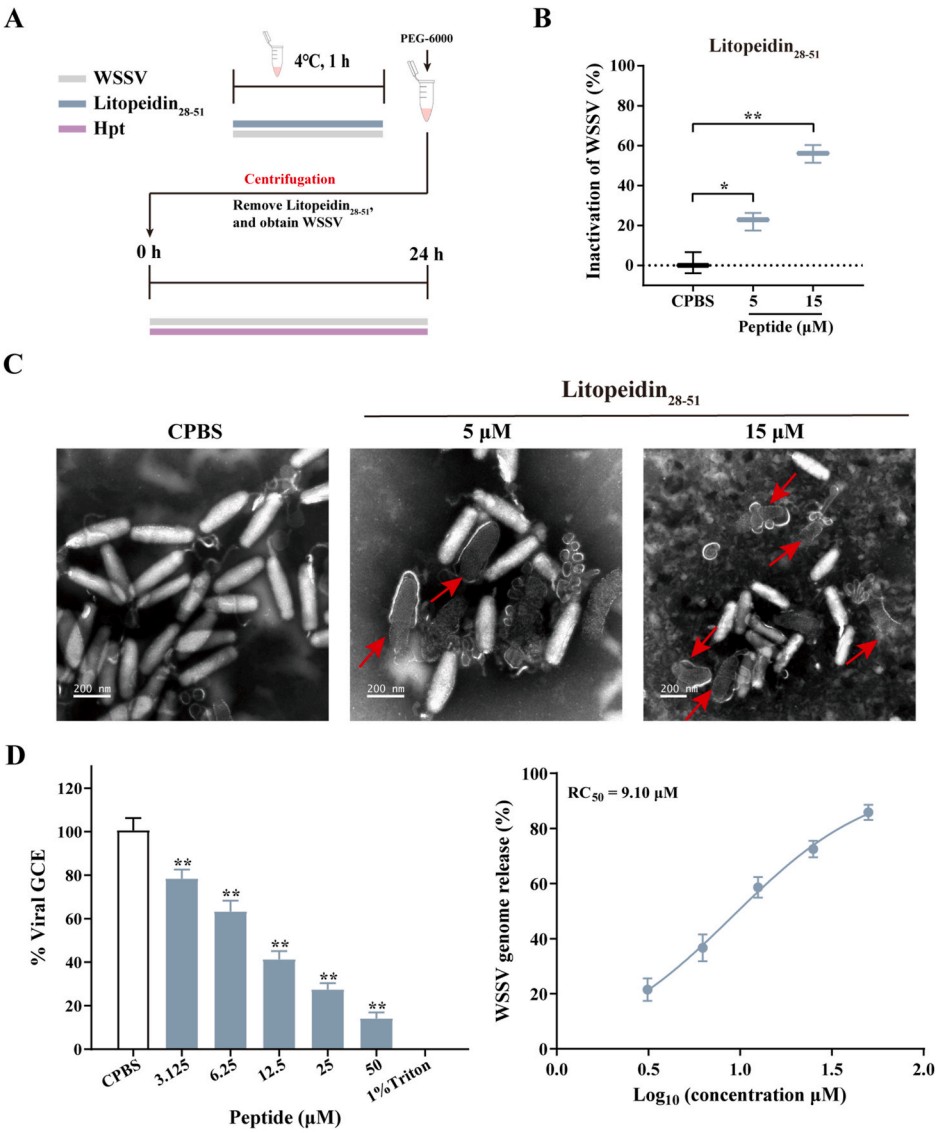


Fig. 6. Litopeidin$_{28\text{-}51}$ disrupted the structure of the WSSV virion. (A) Schematic workflow for the inactivation of cell-free virions. (B) Litopeidin$_{28\text{-}51}$ inactivated the cell-free WSSV. (C) TEM analysis of WSSV exposed to Litopeidin$_{28\text{-}51}$ (5 μM and 15 μM, scale bar = 200 nm). The red arrows indicate ruptured virions. (D) MNase digestion experiment was used to detect the genomic DNA unreleased by WSSV treated with Litopeidin$_{28\text{-}51}$. A two-way analysis of variance (ANOVA) was used for multiple groups. The data were expressed as the mean ± SD (n = 3). $^{**}p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

hydrophobic α-helical peptide, with these properties contributing to its biological activities [55,56]. As an AMP, possessing antimicrobial activity is essential; thus, this study first explored the antibacterial and antifungal effects of Litopeidin$_{28\text{-}51}$ *in vitro*. We demonstrated a bacteriostatic and/or bactericidal action of Litopeidin$_{28\text{-}51}$ against various Gram-positive, Gram-negative bacteria, and fungi, indicating that Litopeidin$_{28\text{-}51}$ is a novel AMP.

Recent reports have indicated that there are no great parametric differences between AMPs and antiviral peptides (AVPs) and that AMPs with antiviral activity are an important source of AVPs [57,58]. Given *Litopeidin*'s significant response to WSSV infection, we have investigated the potential antiviral activity of Litopeidin$_{28\text{-}51}$. *In vitro* anti-WSSV assay showed that Litopeidin$_{28\text{-}51}$ significantly inhibited WSSV replication (including intracellular viral RNA and DNA levels) in Hpt cells, demonstrating its strong antiviral activity against WSSV infection. Similarly, a defensin found in *L. vannamei* (named LvDBD) has been shown to disrupt bacterial membranes, and interact with viral envelope proteins, thereby inhibiting WSSV proliferation [59]. To further explore the antiviral mechanism of Litopeidin$_{28\text{-}51}$, we have observed the structural changes of WSSV virions after co-incubation with Litopeidin$_{28\text{-}51}$. Most WSSV virions pre-incubated with Litopeidin$_{28\text{-}51}$ have been found in the non-intact state, lacking the viral envelope compared to control group. Previous studies have shown that many antiviral peptides interact with viral particles through direct contact, resulting in their inactivation [39,60,61], which is a key antiviral mechanism. For example, ALFPm3 in *P. monodon* [62] and FcALF5 in *F. chinensis* inhibited the WSSV replication by interacting with its coat proteins [25]. Therefore, it is reasonable to infer that Litopeidin$_{28\text{-}51}$ directly binds to WSSV virions and disrupts the envelope integrity to reduce viral infectivity.

On the other hand, our data showed that the viral load was significantly lower in the Litopeidin$_{28\text{-}51}$ pretreated group compared to the control group. Therefore, we have speculated that Litopeidin$_{28\text{-}51}$ may exert not only a direct antiviral effect but also an indirect effect by affecting the expression of certain immune effectors. As we all know, invertebrates lack adaptive immunity and that these immune effectors are essential for antiviral immunity in invertebrates [63–65]. Some AMPs (such as ALF, crustin, and LYZ) have been identified as key

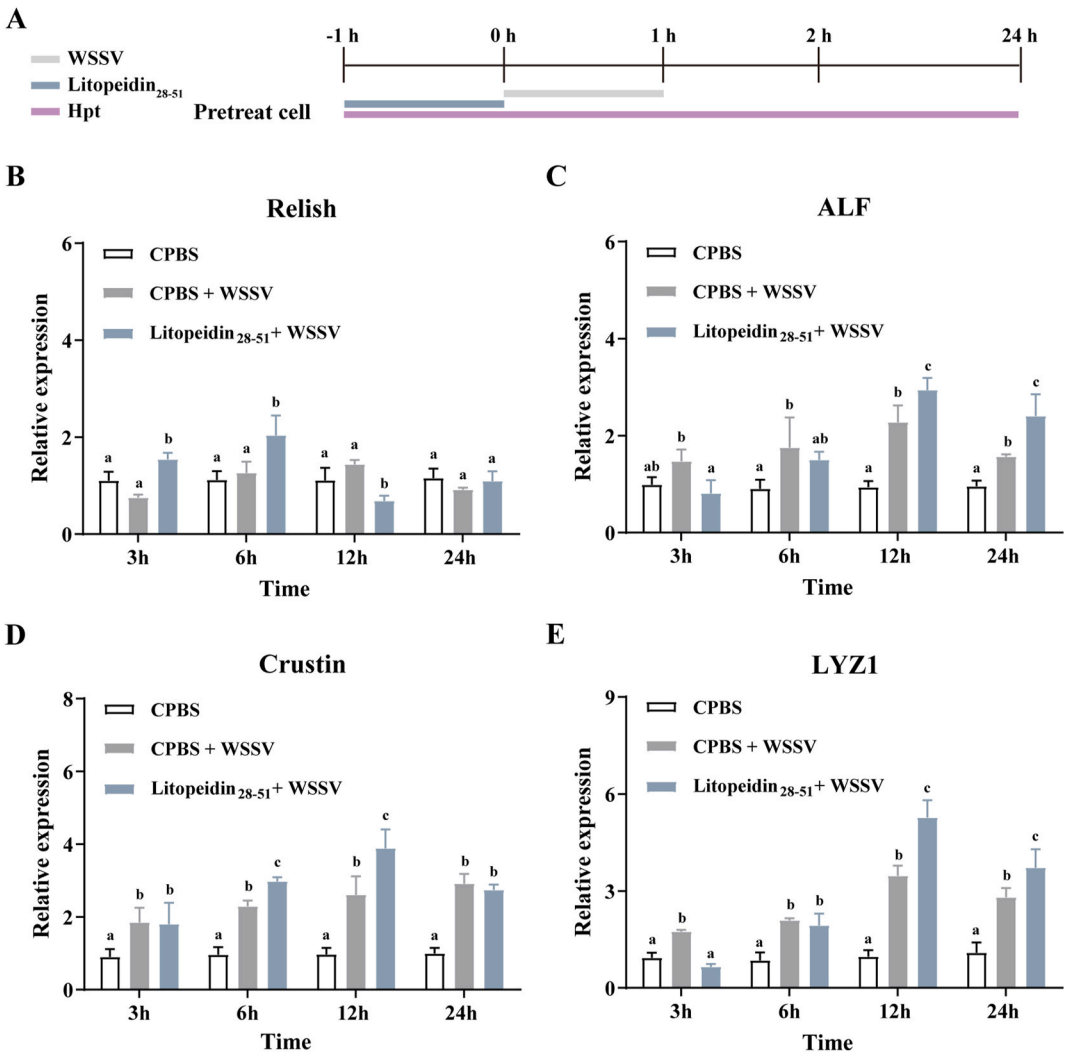


Fig. 7. Effect of Litopeidin$_{28\text{-}51}$ on immune-related genes. (A) Schematic of pretreat cell way workflow. (B–E) Relative expression levels of *Relish* (B), *ALF* (C), *Crustin* (D), and *LYZ1* (E) in Hpt cells. The immune-related gene expressions were normalized using the *GAPDH* expression. A two-way analysis of variance (ANOVA) was used for multiple groups. The data were expressed as the mean ± SD (n = 3). Differences in different times were indicated with the letter "a", "b" or "c".

immune effectors with antiviral properties against WSSV [66–68]. By qPCR experiments, we found that, the expression levels of these effectors were significantly increased in the Litopeidin$_{28\text{-}51}$ pretreated group compared with the WSSV infection group. It has been established that nuclear factor-κB (NF-κB) could regulate the expression of these effectors [69–71]. Our findings showed that *Relish*, a member of the NF-κB family [72], was differentially expressed at different time points in the Litopeidin$_{28\text{-}51}$ pretreated group, suggesting that Litopeidin$_{28\text{-}51}$ may modulate the NF-κB pathway. This regulation may enhance antiviral activity by influencing the expression of AMPs [41,73,74], aligning with previous studies such as the peptide MjRPS27, which was found to activate the NF-κB signaling pathway and regulate AMPs expression in shrimp post-infection, inhibiting WSSV replication [75]. Therefore, our findings, together with other studies, support the notion that Litopeidin$_{28\text{-}51}$, as an AMP, may enhance the host immune response and warrant further *in vivo* studies to explore its practical application value.

Survival is one of the most important indicators of commercial production of aquaculture [76]. By the simultaneous treatment assay, we have found that Litopeidin$_{28\text{-}51}$ increased the survival rate of infected crayfish and shrimps by 33.3 % and 30 %, respectively, which opens up the possibility for preventive applications. Currently, there are limitations to the use of agents as prophylactic treatment in aquaculture [77]. Encouragingly, pretreatment with Litopeidin$_{28\text{-}51}$ also increased the survival rate of crayfish and shrimps against WSSV by 30 % and 16.7 %, respectively, and compared with the control group, the copy numbers of WSSV were significantly lower, indicating its potential for preventing WSSV infection. It is worth noting that during actual aquaculture, microorganisms such as viruses, bacteria, and fungi co-exist in the water environment, and their co-infection will complicate the treatment and prognosis of diseased shrimp. Future studies should consider selecting various pathogenic microorganisms for infection to further evaluate the antimicrobial activity of Litopeidin$_{28\text{-}51}$ under combined infection conditions. We believe that further studies will provide new insights into infection control in aquaculture, and make Litopeidin$_{28\text{-}51}$ a potential new antimicrobial agent.

5. Conclusion

In summary, we identified a novel antimicrobial peptide, Litopeidin$_{28\text{-}51}$, from *L. vanamei,* which was found to have potent antibacterial and antiviral activity. We found that Litopeidin$_{28\text{-}51}$ played an active and important role in crayfish resistance to WSSV. Furthermore, our study revealed that Litopeidin$_{28\text{-}51}$ not only directly inactivated the WSSV virions but also effectively regulated the cellular immune response, thereby improving the survival rate of WSSV-infected crayfish and shrimp. Our study highlighted the potential application of a novel AMP

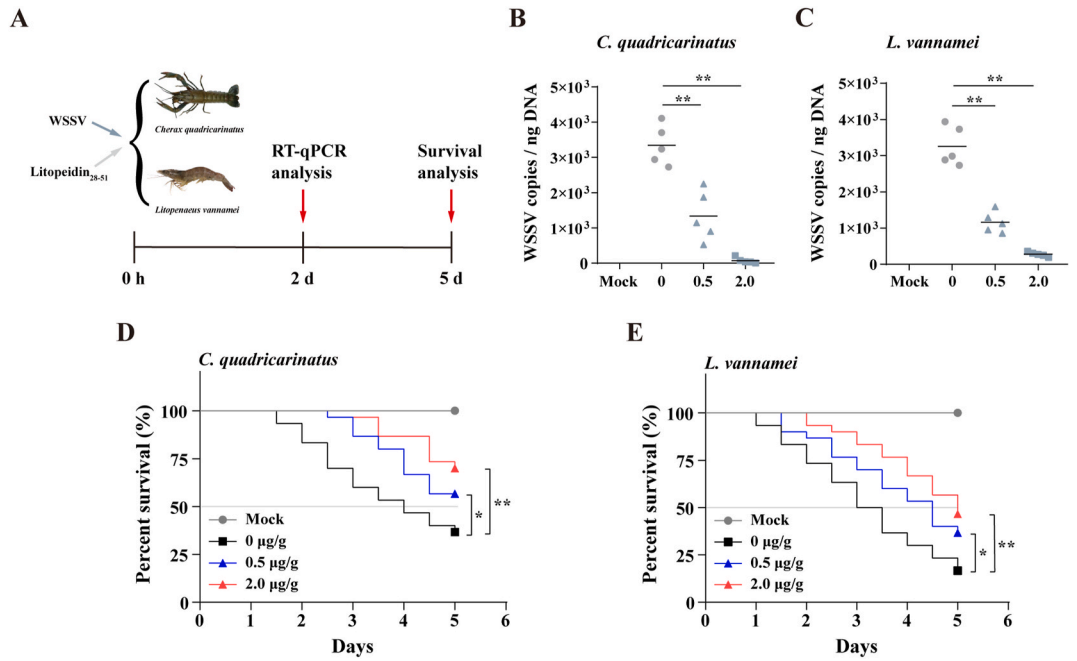


Fig. 8. Litopeidin$_{28\text{-}51}$ exhibited antiviral infection *in vivo*. (A) Schematic illustration of the experimental procedures of *C. quadricarinatus* and *L. vannamei* in a co-stimulation way. (B and C) After 2 d pi, the WSSV DNA copy numbers in the gill tissues of animals (n = 5) were quantified by absolute qPCR. A two-way analysis of variance (ANOVA) was used for comparing multiple groups. (D and E) Survivorship curves of animals injected with WSSV and Litopeidin$_{28\text{-}51}$ (n = 30). The Log-rank (Mantel-Cox) test was specifically employed for analyzing survival data. $^{*}p < 0.05$ and $^{**}p < 0.01$.

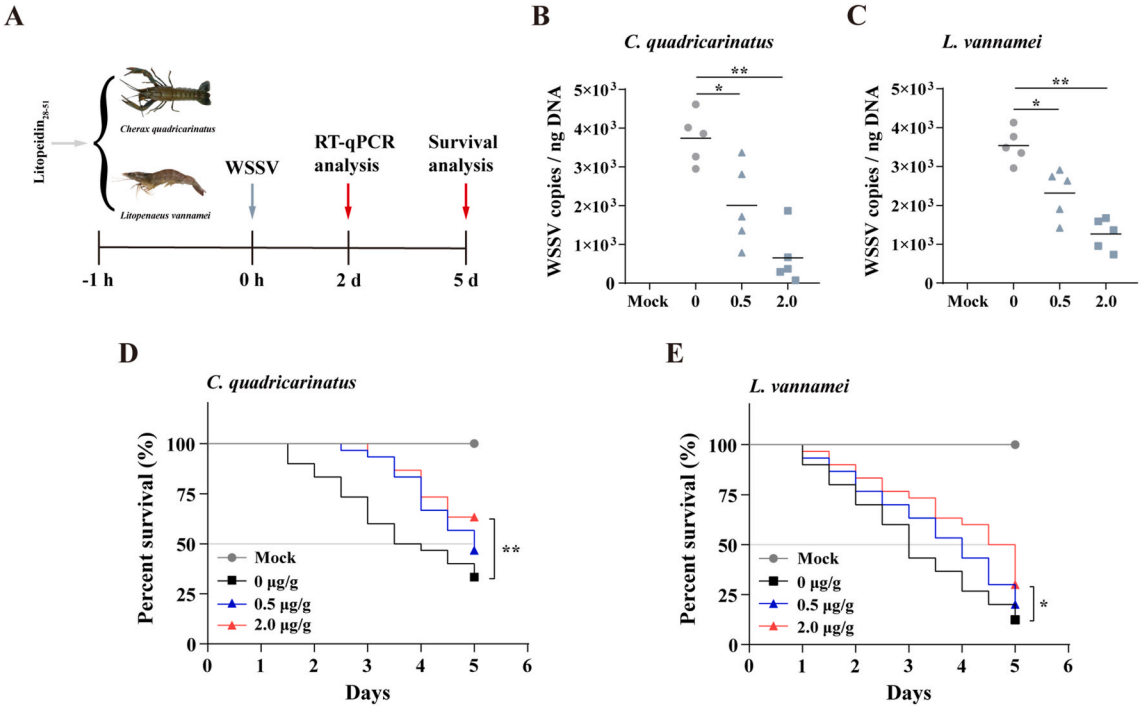


Fig. 9. Litopeidin$_{28\text{-}51}$ protected the host from WSSV infection *in vivo*. (A) Schematic illustration of the experimental procedures of *C. quadricarinatus* and *L. vannamei* in a protection way. (B and C) After 2 d pi, the WSSV DNA copy numbers in the gill tissues of animals (n = 5) were quantified by absolute qPCR. A two-way analysis of variance (ANOVA) was used for comparing multiple groups. (D and E) Survivorship curves of animals injected with WSSV and Litopeidin$_{28\text{-}51}$ (n = 30). The Log-rank (Mantel-Cox) test was specifically employed for analyzing survival data. $^{*}p < 0.05$ and $^{**}p < 0.01$.

Litopeidin$_{28\text{-}51}$ against WSSV infection in aquaculture.

CRediT authorship contribution statement

Xin-Zhan Meng: Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **Yingyi Duan:** Writing – review & editing, Validation, Software, Methodology, Formal analysis, Data curation. **Yuqi Bai:** Methodology. **Weibin Zhang:** Methodology. **Chang Zhang:** Methodology. **Ke-Jian Wang:** Writing – review & editing, Supervision, Resources, Project administration,

Funding acquisition, Conceptualization. **Fangyi Chen:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization, All authors have read and approved the final work.

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Data availability

Data will be made available on request.

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