

RESEARCH ARTICLE

Antibacterial Effect of CpAtt Against *Streptococcus agalactiae* by Increasing the Cell Membrane Permeability

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Abstract

This study aimed to explore the in vitro antibacterial effect of CpAtt, a novel attacin from *Conogethes punctiferalis*, against *Streptococcus agalactiae*. The minimum inhibitory concentration (MIC) was measured via broth microdilution method. Scanning electron microscopy (SEM) was applied to assess the alterations in the overall cell envelope of *S. agalactiae* upon CpAtt treatment. Changes in membrane permeability were characterized by detecting the leakage of intracellular nucleic acids, proteins, alkaline phosphatase (ALP), and β -galactosidase, combined with propidium iodide (PI) staining. Additionally, intracellular reactive oxygen species (ROS) levels were quantified in CpAtt-treated *S. agalactiae*. Additionally, intracellular reactive oxygen species (ROS) levels were detected in the CpAtt-treated *S. agalactiae*. The results showed that the MIC of CpAtt against *S. agalactiae* was 18.8 μ g/mL. After 1 h of treatment with CpAtt at the concentration of 2 MIC, SEM revealed bacterial aggregation, ruptured and collapsed cell walls. CpAtt induced the massive leakage of intracellular nucleic acids and proteins in *S. agalactiae*. Even at low concentrations, CpAtt significantly increased extracellular activities of ALP and β -galactosidase activities, alongside elevated intracellular PI fluorescence intensity, which indicates enhanced membrane permeability of *S. agalactiae*. Furthermore, CpAtt significantly downregulated intracellular ROS levels. Therefore, CpAtt exerts antibacterial activity against *S. agalactiae* by increasing bacterial membrane permeability. These findings suggest that CpAtt is a promising candidate for developing novel antibacterial agents targeting *S. agalactiae*.

Keywords: *Streptococcus agalactiae*, Attacin, Antibacterial activity, Minimum inhibitory concentration, Cell membrane permeability

INTRODUCTION

Streptococcus agalactiae, also known as Group B *Streptococcus* (GBS), a Gram-positive facultative anaerobic zoonotic coccus, was first identified as the causative agent of bovine agalactia mastitis. It remains an important pathogen associated with human perinatal disorders, bovine mastitis and aquatic streptococcosis [1,2]. In fish, *S. agalactiae* induces septicemia and meningoencephalitis, posing a severe threat to tilapia aquaculture [3]. It impairs milk production and milk quality in dairy cows, increasing farming costs and restricting dairy development [4]. Furthermore, *S. agalactiae* infection in young and pregnant livestock may lead to septicemia, polyarthritis, and endometritis, resulting in growth retardation, high culling rates, abortion and stillbirth, thereby imposing substantial economic losses on animal husbandry [5].

Currently, β -lactams are the first choice for treating *S. agalactiae* infections, while macrolides are used for allergic individuals [6]. Long-term and extensive application of penicillins and quinolones in veterinary and aquaculture practices has caused a statistically notable rise in *S. agalactiae* isolation and elevated rates of penicillin non-susceptibility [7]. Zhao et al. [8] analyzed 497 mastitis milk samples collected in China and reported that streptococcal isolates exhibited high levels of antimicrobial resistance and harbored multiple virulence genes of concern. The growing prevalence of multidrug-resistant *S. agalactiae*, together with high resistance to macrolides and tetracyclines, narrows therapeutic choices for human perinatal infections, increases maternal and infant health risks, and compromises the efficacy of conventional antibiotics for bovine mastitis and aquatic streptococcosis. Drug-resistant strains from animals can



also spread to other hosts and threaten public health [9-12]. To address this challenge, alongside standardized antibiotic stewardship, priorities include enhanced resistance surveillance, commercial vaccine development, improvement of rearing environments, and discovery of novel natural antibacterial agents with a low propensity to drive bacterial resistance.

CpAtt, a glycine-rich antimicrobial peptide, was identified from *Conogethes punctiferalis* in our laboratory [13]. Insect antimicrobial peptides are central effector molecules of insect humoral immunity [14]. Most of them are small cationic amphipathic polypeptides, featuring prominent advantages with excellent thermal stability, broad antibacterial spectrum, and low tendency to induce bacterial resistance [15,16]. Therefore, insect antimicrobial peptides are well known as ideal substitutes for antibiotics [17]. Our preliminary assays confirmed that CpAtt exerted remarkable antibacterial activity against Gram-positive bacteria [13]. Building on these findings, the present study further characterizes the antibacterial mode of action of CpAtt against *S. agalactiae*, aiming to provide experimental evidence for the future development of antibacterial agents targeting this pathogen.

MATERIAL AND METHODS

Ethical Statement

This study did not require ethical approval.

Test Strains

S. agalactiae ATCC 13813 were preserved in the Henan Joint International Research Laboratory of Veterinary Biologics Research and Application.

Main Reagents

O-Nitrophenyl β -D-galactopyranoside (ONPG) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. Alkaline phosphatase (ALP) assay kit and enhanced ATP assay kit were obtained from Beyotime Biotechnology Co., Ltd. Triton X-100 was supplied by Thermo Fisher Scientific (China) Co., Ltd. Propidium iodide (PI) was purchased from Beijing Dingguo Biotechnology Co., Ltd. The antimicrobial peptide CpAtt was previously prepared and stored in our laboratory [13]. Briefly, CpAtt was expressed in an *E. coli* expression system and purified by affinity chromatography. The purity of CpAtt was confirmed by SDS-PAGE, and the purity was >95%. The CpAtt stock solution was prepared in sterile PBS, with a stock concentration of 150 μ g/mL. The protein solution was aliquoted and stored at -80°C to avoid repeated freezethaw cycles before use.

MIC Determination of CpAtt against *S. agalactiae*

The MIC of CpAtt against *S. agalactiae* was determined

by the broth microdilution method according to the CLSI M07 standard. An overnight culture of *S. agalactiae* was diluted with sterile THB broth to a final concentration of 2×10^5 CFU/mL and used as the inoculum. Aliquots of 100 μ L of the bacterial suspension and 100 μ L of the two-fold serially diluted attacin solution were added to each well of a 96-well microtiter plate, resulting in a total volume of 200 μ L per well and an inoculum of approximately 2×10^4 CFU/mL. CpAtt was prepared in sterile PBS at 150 μ g/mL and then serially two-fold diluted in the wells to final concentrations of 75, 37.5, 18.75, 9.37, 4.68, 2.34, and 1.17 μ g/mL, with three replicates per concentration. The MIC value of Kanamycin sulfate against *S. agalactiae* was 125 μ g/mL, so Kanamycin sulfate (500 μ g/mL) and PBS served as the positive and negative controls, respectively. After static incubation at 37°C for 16 h, the optical density at 600 nm (OD_{600}) of each well was measured using a microplate reader. The MIC was defined as the lowest attacin concentration at which no significant increase in OD_{600} was observed, i.e., consistent with that of the negative control. Kanamycin sulfate (final concentration 500 μ g/mL) served as the assay validity positive control. OD_{600} measurements from the microplate confirmed complete growth inhibition of *S. agalactiae* under these experimental conditions, verifying the reliability of the MIC system. This concentration of kanamycin has been widely used as a positive inhibition control in related published work [13]. Independent kanamycin susceptibility testing for this strain was not performed in the current study.

SEM Observation

Bacterial cells in the logarithmic growth phase were collected by centrifugation, washed and resuspended with PBS. A total of 600 μ L bacterial suspension was mixed with an equal volume of CpAtt solution to reach a final concentration of 2 MIC. Sterile PBS was used as the negative control. The mixture was incubated in a water bath at 37°C for 1 h. After PBS washing, the bacterial samples were fixed with 2.5% (v/v) glutaraldehyde solution and transported under low temperature to Wuhan Servicebio Technology Co., Ltd. for sample preparation and observation via SEM.

Detection of Nucleic Acid and Protein Leakage

Bacterial cells in the logarithmic growth phase were collected by centrifugation, and washed twice with PBS. The supernatant was discarded, and the pellet was resuspended in sterile PBS to adjust the bacterial concentration to 2×10^8 CFU/mL. Four groups were treated with CpAtt at the concentrations of 0 MIC, 0.5 MIC, 1 MIC, and 2 MIC, respectively. After incubation in a 37°C water bath for 1 h, samples were centrifuged at 12,000 rpm for 1 min. The absorbance values of the supernatant

at 260 nm (A_{260}) and 280 nm (A_{280}) were determined using an ultra-micro spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, USA)

Determination of ALP Activity

Bacterial suspensions were treated with 0 MIC, 0.5 MIC, 1 MIC, and 2 MIC CpAtt, respectively, while 0.3% (v/v) Triton X-100 was used as the positive control. All mixtures were incubated in a 37°C water bath for 1 h, followed by centrifugation at 12,000 rpm for 1 min. The supernatant was collected and assayed in accordance with the instructions of the ALP assay kit. The OD value of each well was detected at 405 nm using a microplate reader, and the ALP activity of samples were calculated based on the standard curve.

Determination of β -Galactosidase Activity

S. agalactiae was cultured overnight, harvested by centrifugation, washed, and resuspended in sterile PBS buffer. The bacterial suspension of *S. agalactiae* was treated with CpAtt at final concentrations of 0 MIC, 0.5 MIC, 1 MIC, and 2 MIC, respectively. Under dark conditions, 100 μ L supernatant was added to each well of a 96-well plate, followed by 4.5 μ L ONPG solution with a final concentration of 3 mmol/L. The mixture was gently pipetted and blended, then incubated at 37°C for 30 min. The optical density at 420 nm was measured using a microplate reader. All measured values were normalized to the corresponding bacterial density at OD₆₀₀, and each assay was performed in triplicate with independent biological replicates.

PI Staining Assay

Bacterial suspensions were treated with CpAtt at final concentrations of 0 MIC, 0.5 MIC, 1 MIC, and 2 MIC, respectively. Meanwhile, 0.3% (v/v) Triton X-100 was used as the positive control. All mixtures were incubated in a 37°C water bath for 1 h. After incubation, the bacterial cells were centrifuged, washed and resuspended with sterile PBS to a fixed final volume. Subsequently, 4 μ L of PI stock solution (1 mg/mL) was added to each bacterial suspension in the dark. After thorough mixing, 100 μ L sample was transferred to 96-well plate and incubated at 37°C for 30 min. The fluorescence intensity was measured with a microplate reader at an excitation wavelength of 536 nm and an emission wavelength of 617 nm, respectively. All fluorescence values were normalized to the OD₆₀₀ of the corresponding bacterial suspension to eliminate the interference of bacterial density differences among groups.

Detection of Intracellular ROS Level

Bacterial suspensions were prepared according to Section of “Detection of Nucleic Acid and Protein Leakage”. DCFH-DA probe was added to the samples to a final

concentration of 10 μ M. After incubation at 37°C for 20 min, the bacteria were washed three times with PBS and resuspended. The bacteria were then treated with CpAtt at the concentrations of 0 MIC, 0.5 MIC, 1 MIC and 2 MIC. All samples were incubated at 37°C for 30 min. The fluorescence intensity was detected by a microplate reader at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Statistical Analysis

All data were analyzed and plotted using GraphPad Prism 9.3. Each experiment was performed in triplicate with technical replicates of the same biological sample, and results were presented as mean $\pm$ standard deviation (SD). For experiments with a single independent variable (e.g., concentration gradient only), inter-group comparisons were performed using one-way analysis of variance (one-way ANOVA). $P < 0.05$ indicated a significant difference; $P < 0.01$ indicated a statistically significant difference. For experiments containing two independent variables (concentration and incubation time), statistical analysis was conducted using two-way ANOVA followed by Tukey's post-hoc multiple comparison test; $P < 0.05$ compared with the 0MIC group at the same incubation time; ns indicates no significant difference between 30min and 60min groups under the same concentration ($P > 0.05$).

30 min and 60

RESULTS

MIC of CpAtt Against *S. agalactiae*

As shown in Fig. 1, robust growth of *S. agalactiae* was observed in the PBS-treated growth control group,

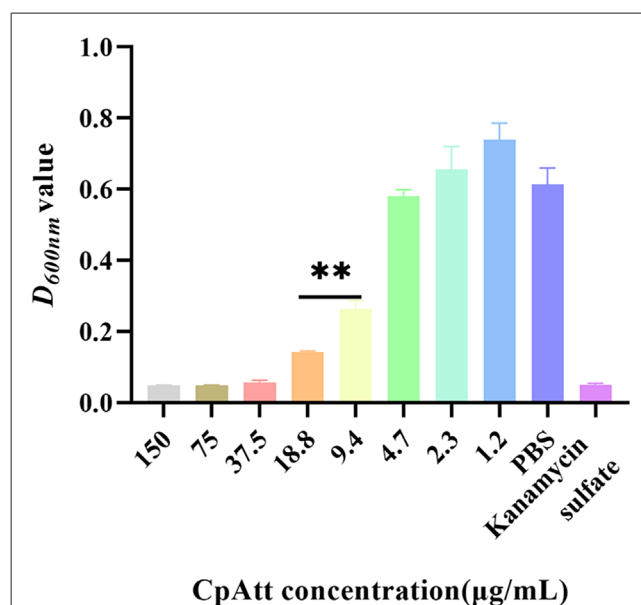


Fig 1. MIC of CpAtt against *S. agalactiae*. PBS served as the sterile negative control, and kanamycin sulfate (500 μ g/mL) was used as the positive control. ** $P < 0.01$ indicated a statistically significant difference

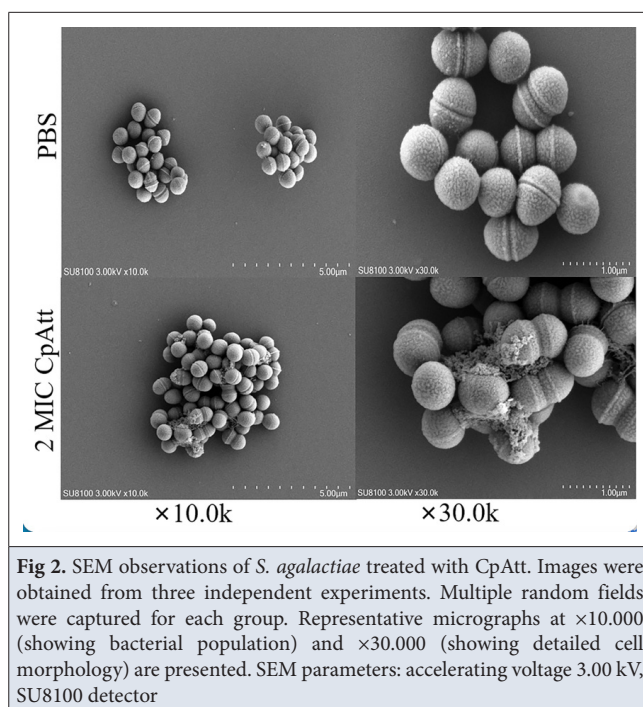


Fig 2. SEM observations of *S. agalactiae* treated with CpAtt. Images were obtained from three independent experiments. Multiple random fields were captured for each group. Representative micrographs at $\times 10,000$ (showing bacterial population) and $\times 30,000$ (showing detailed cell morphology) are presented. SEM parameters: accelerating voltage 3.00 kV, SU8100 detector

whereas bacterial growth was fully suppressed in the kanamycin-treated positive control group. Weak bacterial growth was detected at a CpAtt concentration of 9.4 $\mu\text{g/mL}$. Accordingly, the MIC of CpAtt against *S. agalactiae* was determined to be 18.8 $\mu\text{g/mL}$.

SEM Observation

After treatment with CpAtt at a concentration of 2 MIC, a large number of *S. agalactiae* cells aggregated and adhered to form dense bacterial clumps under low magnification. The typical short-chain arrangement of normal bacteria disappeared, and severe adhesion among bacterial cells was observed. Under high magnification, the bacterial surface appeared wrinkled and uneven. Cell-envelope damaged and collapsed with ruptured cell margins, and

the fragmented debris from lysed bacterial cells could be seen nearby (Fig. 2).

Detection of Nucleic Acid and Protein Leakage

After 1 h of treatment with CpAtt, compared with the 0 MIC group, the 2 MIC group showed statistically significant increase ($P < 0.01$) of nucleic acid leakage in *S. agalactiae* (Fig. 3-A). Similarly, the protein leakage increased markedly ($P < 0.05$) in the 2 MIC group (Fig. 3-B), suggesting that CpAtt could increase the cell membrane permeability of *S. agalactiae*, leading to the leakage of intracellular contents.

Determination of ALP Activity

As shown in Fig. 4, compared with the 0 MIC group, the ALP level gradually increased with the CpAtt concentration increasing in the culture supernatant of CpAtt-treated *S. agalactiae*, indicating that CpAtt destroyed the cell wall of *S. agalactiae* to cause the ALP leakage.

Determination of β -galactosidase Activity

As shown in Fig. 5, the β -galactosidase leakage gradually increased with the rise of CpAtt concentration in all CpAtt-treated groups. The OD_{420} value of the 2 MIC CpAtt-treated group was roughly equivalent to that of the positive control group treated with 0.3% Triton X-100, indicating severe permeabilization of the bacterial membrane.

Results of PI Staining Assay

Compared with the 0 MIC group, the 2 MIC CpAtt treatment significantly increased the PI fluorescence intensity of *S. agalactiae* (Fig. 6), indicating that CpAtt elevates the cell envelope permeability of *S. agalactiae*.

Effects of CpAtt on Intracellular ROS Levels in *S. agalactiae*

As shown in Fig. 7, compared with the 0 MIC group, the fluorescence intensity of all CpAtt-treated groups

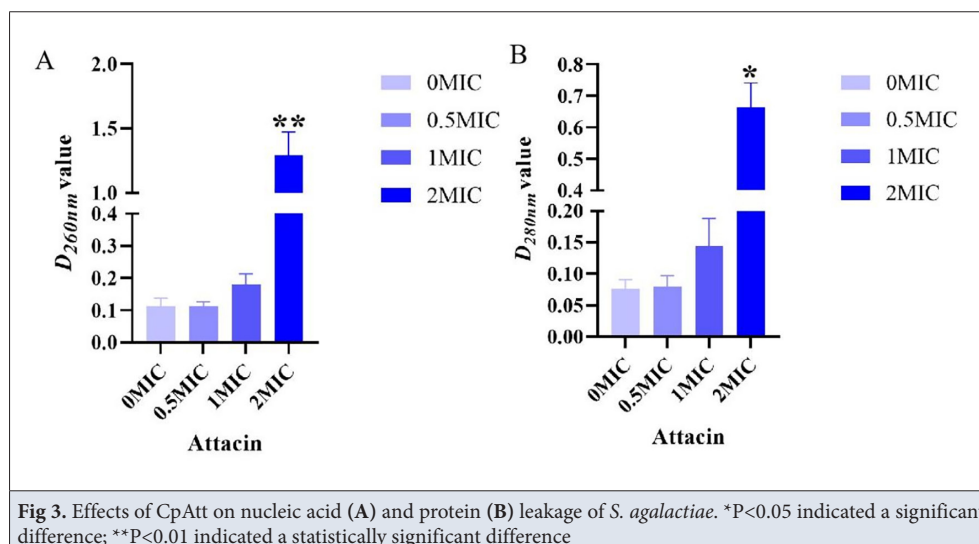


Fig 3. Effects of CpAtt on nucleic acid (A) and protein (B) leakage of *S. agalactiae*. * $P < 0.05$ indicated a significant difference; ** $P < 0.01$ indicated a statistically significant difference

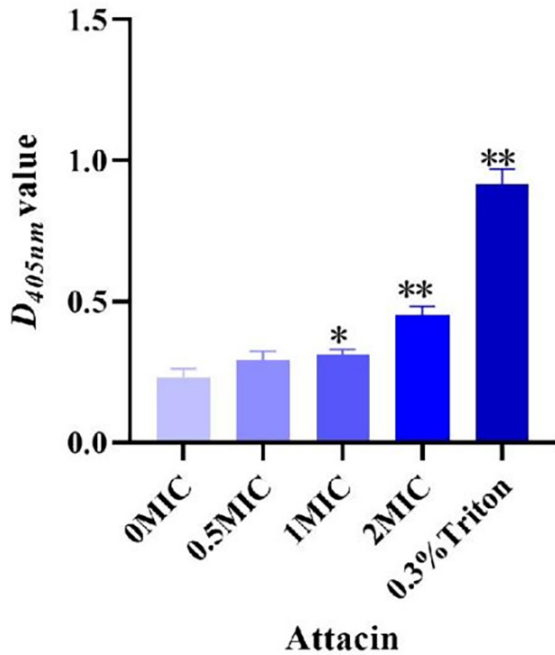


Fig 4. Effects of CpAtt on the ALP leakage in *S. agalactiae*. * $P < 0.05$ indicated a significant difference; ** $P < 0.01$ indicated a statistically significant difference

decreased significantly. Moreover, for all CpAtt-treated groups, the fluorescence intensity after treatment for 60 min did not significantly increase than the one of after

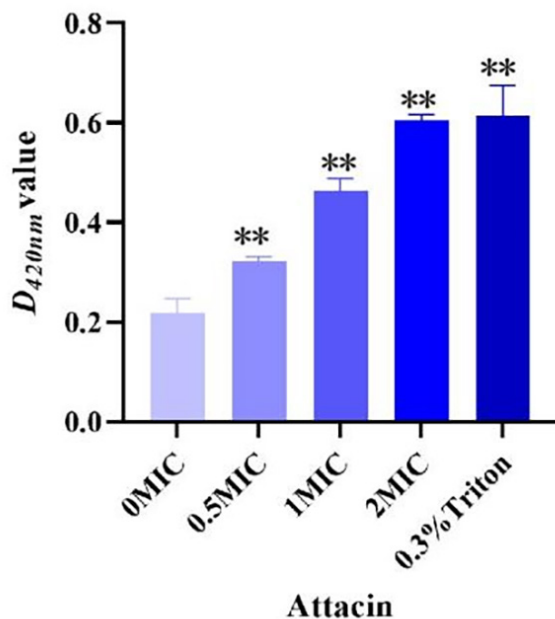


Fig 5. Effects of CpAtt on the β -galactosidase leakage in *S. agalactiae*. ** $P < 0.01$ indicated a statistically significant difference

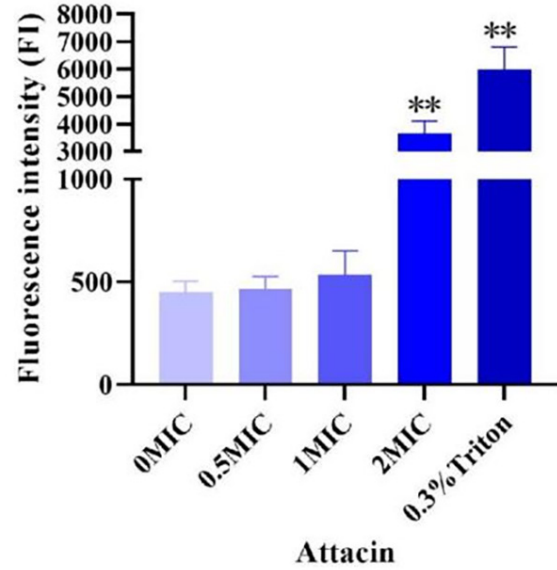


Fig 6. PI fluorescence intensity of *S. agalactiae* after CpAtt treatment. *S. agalactiae* was incubated with CpAtt at 0, 0.5, 1, and 2 MIC. PI fluorescence intensity was determined to evaluate bacterial cell envelope permeability. A broken y-axis was used to clearly visualize subtle variations among low-concentration groups without compressing the signal of the 2 MIC group. Data are presented as mean $\pm$ SD from three independent biological replicates. Significant difference was observed only at 2 MIC compared with the 0 MIC group. ** $P < 0.05$

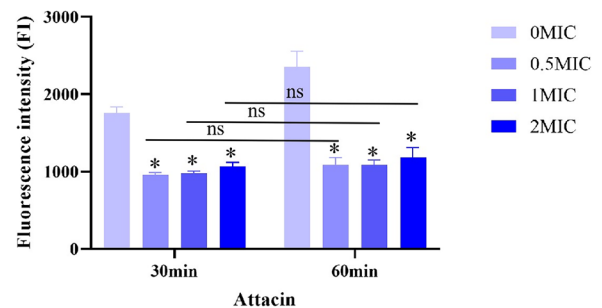


Fig 7. Effects of CpAtt on intracellular ROS Levels in *S. agalactiae*. Intracellular ROS was detected using DCFHDA staining. Fluorescence intensity was normalized to OD_{600} to correct for differences in bacterial density. *S. agalactiae* was treated with CpAtt at 0, 0.5, 1, and 2 MIC for 30 min and 60 min, respectively. Data are presented as mean $\pm$ SD from three independent biological replicates. * $P < 0.05$ compared with the 0 MIC group at the same incubation time by two-way ANOVA followed by Tukey's posthoc test; ns indicates no significant difference between 30min and 60min groups under the same CpAtt concentration ($P > 0.05$)

treatment for 30 min, suggesting that CpAtt did not induce the ROS accumulation.

DISCUSSION

S. agalactiae represents a serious hazard to the health of aquaculture species and livestock, with a continuous increase in antimicrobial resistance. This pathogen

has become an urgent challenge for public health and aquaculture sectors [18]. Therefore, discovering natural antibacterial agents with novel scaffolds, high potency, low toxicity and low risk of resistance induction, together with systematic characterization of their antibacterial mode of action, is a key research priority for the green prevention and control of pathogenic bacteria. Attacins constitute a unique family of insect-derived antibacterial proteins, with favorable attributes including broad-spectrum activity, high biosafety and low potential to select for bacterial resistance. Accordingly, attacins hold great promise for developing novel antibacterial biopreparations as alternatives to conventional antibiotics [19]. Attacins originating from different insect species display substantial differences in antibacterial activity and molecular targets [14,15,20]. Attacin from *Hermetia illucens* specifically binds to LPS, disrupts the outer membrane and forms membrane pores, thereby exerting dual antibacterial effects against methicillin-resistant *Staphylococcus aureus* (MRSA) and Gram-negative bacteria [21]. Recombinant attacin A from *Drosophila melanogaster*, expressed in *E. coli*, targets bacterial cell membranes and inhibits the growth of the Gram-negative strain *E. coli* DH5 α [22]. Given these interspecies differences, it is essential to characterize the antibacterial activity and associated mode of action of CpAtt, an attacin derived from *Conogethes punctiferalis*, against *S. agalactiae*.

The bacterial cell membrane acts as an essential physiological barrier that maintains morphological integrity, osmotic homeostasis, and stable substance metabolism in bacteria [23,24]. Structural destruction and permeability dysfunction of the cell envelope represent the primary and canonical mode of action by which natural antimicrobial peptides and proteins exert antibacterial activity. In this study, multiple detection approaches, including morphological observation, fluorescent staining, intracellular macromolecule leakage analysis, and characteristic enzyme activity determination, were integrated to systematically demonstrate that CpAtt effectively impairs cell envelope integrity and elevates the membrane permeability of *S. agalactiae*. SEM provides intuitive visual evidence for evaluating bacterial cell envelope damage. Microscopic results revealed that treatment with 2 MIC CpAtt induced obvious bacterial agglutination in *S. agalactiae*, accompanied by shrunken and irregular cell surfaces, collapsed and damaged cell envelopes, blurred cell margins, and even complete bacterial lysis. These morphological alterations confirm that CpAtt induces irreversible structural damage to the *S. agalactiae* cell envelope.

Nucleic acids and proteins are indispensable intracellular macromolecules that cannot freely diffuse out of bacterial cells under normal physiological conditions with intact

membrane barriers. In the present study, treatment with 2 MIC CpAtt significantly increased extracellular nucleic acid and protein contents in the bacterial supernatant, indicating that CpAtt disrupts bacterial membrane integrity and triggers the release of intracellular components, thereby interfering with bacterial metabolism and proliferation. ALP is a typical marker enzyme, while β -galactosidase is a cytoplasmic enzyme; both are rarely released extracellularly when the bacterial cell envelope remains intact. Our results showed that the extracellular ALP level was significantly elevated after treatment with 1 MIC CpAtt, and β -galactosidase activity increased markedly even under 0.5 MIC CpAtt intervention. These biochemical results further verify that CpAtt alters cell envelope permeability and impairs membrane barrier function in *S. agalactiae*. PI is a nucleic acid-specific fluorescent dye that cannot penetrate intact membranes of viable bacteria. Therefore, increased PI fluorescence exclusively indicates membrane damage and enhanced membrane permeability. In this study, the PI fluorescence intensity of *S. agalactiae* was significantly enhanced after 2 MIC CpAtt treatment, further confirming the loss of membrane barrier properties. Collectively, consistent evidence obtained from morphological observation, fluorescent staining, and quantitative biochemical assays demonstrates that CpAtt inhibits *S. agalactiae* growth primarily by disrupting bacterial cell envelope integrity and increasing membrane permeability, which represents the core antibacterial mode of action of CpAtt against *S. agalactiae*.

Furthermore, intracellular ROS detection further elucidated the unique antibacterial characteristics of CpAtt, which differs from the action pattern of conventional antibacterial agents. Most antibacterial substances exert bactericidal activity by inducing excessive intracellular ROS accumulation, triggering oxidative stress damage and degrading intracellular biomacromolecules [25]. In contrast, the present study showed that intracellular ROS levels in *S. agalactiae* were markedly decreased after 30 min of 1 MIC CpAtt treatment, and this regulatory effect was not obviously dependent on treatment duration. Notably, the reduction in fluorescent intensity cannot be exclusively attributed to decreased ROS levels, as it may also be affected by changes in bacterial viability and metabolic activity. Taken together, these results suggest that the antibacterial activity of CpAtt against *S. agalactiae* is not mediated by ROS accumulation and oxidative stress activation.

In summary, this study suggested that CpAtt exerted the antibacterial effects on *S. agalactiae* by increasing the cell membrane permeability to induce in the leakage of intracellular substances. These results offer a preliminary mechanistic basis, although further studies with additional clinical isolates, toxicity testing and in vivo experiments are needed to explore its potential antibacterial application.

DECLARATIONS

Availability of Data and Materials: Data and materials in this research are available upon request.

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Competing Interests: The authors declare no competing interests.

Declaration of Generative Artificial Intelligence (AI): The authors confirm that no generative AI created the manuscript text, tables or figures.

Ethical Compliance Declaration: The study was conducted in accordance with all applicable animal welfare and research ethics principles. The authors accept full responsibility for the ethical integrity of the study.

Author Contributions: KQ L, and RH P conceptualized and designed the study. LL Z, KQ L, YH W, SM M, and YK L performed experiments and analyses. LL Z, and KQ L wrote the manuscript. RH P edited the manuscript. All authors approved the manuscript.

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