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Research Article

Synthetic Analogs of Antimicrobial Peptides from *Limulus* Inhibit the Growth of *Listeria monocytogenes* by Increasing Cell Membrane Permeability and Suppressing Virulence Genes

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Listeria monocytogenes is a foodborne pathogen that can cause listeriosis in humans and animals. It is of significant concern to the food industry as it can grow at low temperatures. Aggravatingly, only some of the commonly used food preservatives can effectively inhibit the growth of LM. In this study, the effectiveness of synthetic analogs of antimicrobial peptides from *Limulus* in inhibiting the growth of LM was studied. As determined by the Kirby–Bauer disc diffusion method, the diameters of the inhibition zones produced by the synthetic antimicrobial peptides ranged from 8.24 mm to 8.86 mm, and the minimum inhibitory concentrations (MICs) of the peptides ranged from 40 μg/mL to 160 μg/mL. At their MICs, initially, the synthetic antimicrobial peptides exhibited bacteriostatic effects on LM. They permeabilized the cell membrane of the bacterium and suppressed virulence genes (the *inlA*, *prfA*, and *hly* genes) in the bacterium. However, the bacteriostatic effects were effective only for 1 h, after which the bacterium slowly became resistant to them. After 6 h, the bacterium resumed its growth. Although cells in treatment groups resumed their growth after 6 h, the growth of the cells was inhibited compared to the growth of cells in the control group. Further studies are necessary to reduce the resistance of LM to the antibacterial effects of the synthetic antimicrobial peptides.

1. Introduction

Listeria monocytogenes (LM) is a foodborne pathogen that can cause listeriosis in humans and animals. It is a significant concern for the food industry as it can grow at low temperatures [1]. The rod-shaped, gram-positive bacterium primarily infects people with suppressed immune function or immunodeficiency [2]. The clinical manifestations of listeriosis include vomiting, abdominal pain, meningitis, brain inflammation, sepsis, hemolytic disease of the newborn, and premature delivery [3]. Although the incidence of human listeriosis is generally low, the case fatality rate of the illness is unusually high (20–30%) [2]; thus, the contamination of food with LM is a serious concern. Unlike other mesophilic bacteria, LM can survive and reproduce in a 4°C environment; thus, ordinary refrigerator temperatures facilitate rather than inhibit the growth of LM [4]. In view of the ability of LM to multiply under various conditions and environments and to contaminate a variety of foods, the food industry views the pathogen as a constant concern [5]. Despite the availability of various antibacterial agents, due to safety reasons, only some of them have been approved as food preservatives. Among the approved food preservatives, only some of them are effective at inhibiting the growth of LM. Therefore, finding a safe food preservative that is effective against LM is the focus of the current research.

The Chinese horseshoe crab (*Tachypleus tridentatus*), which is distributed throughout Malaysia, is a species of the

genus *Limulus*. It is a natural source of antimicrobial peptides known as *Limulus* antimicrobial peptides (LAMPs). The first LAMP, Tachyplesin I, was isolated by Nakamura et al. [6] from the hemocytes of *Tachypleus tridentatus*. The antimicrobial peptide contains 17 amino acids and two disulfide linkages, and its secondary structure is the antiparallel β-sheet. LAMPs inhibit the growth and reproduction of bacteria, invasion of viruses, reproduction of fungi, and apoptosis of tumor cells [7–10]. Several studies have shown that LAMPs inhibit the growth of a variety of pathogenic bacteria. Hong et al. [11] found that Tachyplesin I causes the death of *Escherichia coli* (*E. coli*) cells by destroying the integrity of the cell membranes of the cells and inactivating intracellular esterases. Qi et al. [12] revealed that Tachyplesin III inhibits coinfection by *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. However, it is not yet clear whether LAMPs are bacteriostatic or bactericidal antibacterial agents.

This research studies the effectiveness of synthetic analogs of LAMPs (sLAMPs; five sLAMPs in total) to inhibit the growth of LM and determines the minimum inhibitory concentrations (MICs) of the sLAMPs. Moreover, the modes of action of the sLAMPs are determined. This research provides a theoretical basis for the antibacterial applications of sLAMPs.

2. Materials and Methods

2.1. Determination of MICs of Five sLAMPs. Five sLAMPs, including the synthetic analog of Tachyplesin I (sTac-I), the synthetic analog of Tachyplesin II (sTac-II), the synthetic analog of Tachyplesin III (sTac-III), cysteine-deleted Tachyplesin (CDT), and the synthetic analog of Polyphemusin I (sPol-I), were synthesized by Shanghai Jixi Biotechnology Co., Ltd. (Shanghai, China). The amino acid sequences of the sLAMPs are shown in Table 1. A standard strain of LM (ATCC 19115) was purchased from the Guangdong Institute of Microbiology. For the determination of the MICs of the sLAMPs by the Kirby–Bauer disc diffusion method [13], different concentrations (640, 320, 160, 80, 40, 20, 10, and 5 μg/mL) of each of the five sLAMPs were prepared. Phosphate-buffered saline (PBS; pH 7.4) was used as a negative control.

2.2. Time-Kill Kinetics of the Five sLAMPs. At its MIC, an sLAMP (sTac-I, sTac-II, sTac-III, CDT, or sPo-I) was added to tryptone soy broth containing 1×10^5 colony forming units (CFU)/mL of LM, and the LM bacterial suspension was incubated in a 37°C incubator. At time intervals of 0, 0.5, 1, 2, 3, and 6 h, aliquots were taken from the bacterial suspension and inoculated onto tryptone soy agar. The LM cultures were incubated at 37°C for 24 h. Time-kill curves were obtained by measuring the CFU/mL of LM at the above time intervals.

2.3. Effects of the Five sLAMPs on the Morphology of LM. At its MIC, a sLAMP (sTac-I, sTac-II, sTac-III, CDT, or sPo-I) was added to a 1×10^5 CFU/mL LM bacterial suspension, and the bacterial suspension was incubated at 37°C for 6 h. The suspension was centrifuged 3 times, each at $200 \times g$ for 10 min, to obtain a bacterial pellet. The pellet was washed 3 times with PBS (pH 7.4) and fixed with glutaraldehyde. Then, the pellet was washed with PBS (pH 7.4) and kept at −20°C for 4 h. Finally, the pellet was freeze-dried for 4 h and observed with a scanning electron microscope.

2.4. Effects of the Five sLAMPs on the Permeability of the Cell Membrane of LM. An alkaline phosphatase assay kit (Nanjing Jianchen, China) was used to detect the activity of alkaline phosphatase (AKP) in an LM bacterial suspension. The number of viable cells in the bacterial suspension was adjusted to 1×10^5 CFU/mL using physiological saline. At its MIC, an sLAMP (sTac-I, sTac-II, sTac-III, CDT, or sPo-I) was added to the bacterial suspension, and the suspension was incubated for 6 h. Then, LM cells were collected by centrifuging the suspension at $200 \times g$ for 10 min. Then, the alkaline phosphatase assay was performed according to the manufacturer's instructions. The 1×10^5 CFU/mL LM bacterial suspension was used as a negative control.

2.5. Inhibitory Effects of the Five sLAMPs on the Expression of Virulence Genes in LM. The number of viable cells in an LM bacterial suspension was adjusted to 1×10^5 CFU/mL using physiological saline. At its MIC, an sLAMP (sTac-I, sTac-II, sTac-III, CDT, or sPo-I) was added to the bacterial suspension, and the suspension was incubated for 6 h. Then, LM cells were collected by centrifuging the suspension at $200 \times g$ for 10 min. According to the manufacturer's instructions, TRIzol reagent (Invitrogen, USA) was used to isolate total RNA from the LM cells. A microvolume spectrophotometer (Analytik Jena, Germany) was used to detect the concentration of the total RNA. After reaching an A_{260}/A_{280} ratio (the ratio of the absorbance at 260 nm to the absorbance at 280 nm) of 1.8–2.0, the RNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent kit (Takara Bio, China).

An Applied Biosystems (ABI) 7500 real-time PCR system (Thermo Fisher Scientific, USA) and SYBR® Premix Ex Taq™ II (Takara Bio, China) were used for real-time quantitative reverse transcription (QRT-PCR) experiments. The thermocycling conditions for the QRT-PCR experiments were as follows: an initial denaturation step at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, 60°C for 34 s, 60°C for 60 s, and 95°C for 15 s. Three replicates of each cDNA sample were prepared. The changes in the relative expression of three virulence genes (the *inlA*, *prfA*, and *hly* genes) in LM were calculated using the $2^{-\Delta\Delta Ct}$ method. The *16S rRNA* gene was used as an internal reference gene. Table 2 shows the primers used to detect the three virulence genes. The primers were synthesized by Sangon Biotech Co., Ltd. (China).

2.6. Statistical Analyses. GraphPad Prism 5 was used for data analysis. Results were expressed as means ± standard errors ($X \pm$ SE) from three replicates. A two-tailed Student's t-test was used to evaluate significant differences in results (CFU/

Table 1: The amino acid sequences of the sLAMPs used in this study.

Antimicrobial peptides	Purity (%)	Amino acid sequences
sTac-I	95	NH_2-K-W-C-F-R-V-C-Y-R-G-I-C-Y-R-R-C-R-$CONH_2$
sTac-II	95	NH_2-R-W-C-F-R-V-C-Y-R-G-I-C-Y-R-K-C-R-$CONH_2$
sTac-III	95	NH_2-K-W-C-F-R-V-C-Y-R-G-I-C-Y-R-K-C-R-$CONH_2$
CDT	95	NH_2-K-W-F-R-V-Y-R-G-I-Y-R-K-R-$CONH_2$
sPol-I	95	NH_2-R-R-W-C-F-R-V-C-Y-R-G-F-C-Y-R-K-C-R-$CONH_2$

Table 2: The sequences of the primers used to detect three virulence genes in LM.

Gene names	Primer sequences (5′-3′)
16S rRNA	F 5′-CTGCTTGTCCCTTGACGGT-3′ R 5′-CTTCGCCACTGGTGTTCCT-3′
inlA	F 5′-ACCGCACTCACTAACTT-3′ R 5′-TAGCCAATGGTGTAAGAT-3′
prfA	F 5′-CGGGAAGCTTGGCTCTATTTG-3′ R 5′-GCTAACAGCTGAGCTATGTGC-3′
hly	F 5′-CGGGAAGCTTGGCTCTATTTG-3′ R 5′-GCTAACAGCTGAGCTATGTGC-3′

mL, the activity of AKP, and the relative expression of virulence genes in LM) between two groups, and one-way analysis of variance (ANOVA) or the least significant difference (LSD) method was used to analyze significant differences in results among groups. A p value less than 0.05 was considered statistically significant.

3. Results

3.1. MICs of the Five sLAMPs. As depicted in Table 3, the diameters of the inhibition zones produced by sTac-I, sTac-II, sTac-III, CDT, and sPo-I are 8.86 mm, 8.51 mm, 8.24 mm, 8.31 mm, and 8.37 mm, respectively. On the other hand, the MICs of sTac-I, sTac-II, sTac-III, CDT, and sPo-I are 40 μg/mL, 80 μg/mL, 40 μg/mL, 160 μg/mL, and 40 μg/mL, respectively.

3.2. Analysis of Time-Kill Curves. As shown in Figure 1, all of the five sLAMPs exhibit antibacterial effects on LM: the addition of the sLAMPs to LM bacterial suspensions significantly reduces the CFU/mL of LM, especially within 0.5 h of the time-kill assay. Interestingly, after 1 h, LM begins to adapt and slowly resumes its growth. After 2-3 h, LM enters a logarithmic growth phase. The number of viable LM cells stabilizes after 6 h. Therefore, the five sLAMPs initially produce a favorable bacteriostatic effect on LM. However, the bacterium slowly develops resistance that allows it to resume its growth after 6 h. However, after 6 h, the number of viable LM cells in the treatment groups (the sTac-I group, sTac-II group, sTac-III group, CDT group, and sPo-I group) is lower than that of viable LM cells in the control group. Therefore, for subsequent experiments, at their MICs, the five sLAMPs were added to LM bacterial suspensions, and the suspensions were incubated for 6 h.

3.3. Effects of the Five sLAMPs on the Morphology of LM Cells. The effects of the five sLAMPs on the morphology of LM cells were studied by scanning electron microscopy (SEM).

Table 3: The diameters of the inhibition zones produced by the five sLAMPs and the MICs of the synthetic antimicrobial peptides.

Antimicrobial peptides	Diameters of inhibition zones (mm)	MICs (μg/mL)
sTac-I	8.86	40
sTac-II	8.51	80
sTac-III	8.24	40
CDT	8.31	160
sPo-I	8.37	40

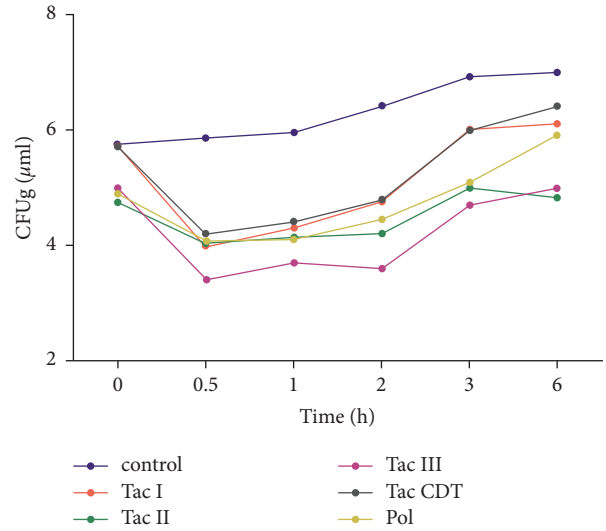

Figure 1: Time-kill curves depicting the antibacterial activity of the five sLAMPs against LM.

The cells in the control group were intact, short, and rod-shaped (Figure 2(a)). The surface of the cells was smooth and flat, and the distribution of intracellular substances was favorable. On the other hand, the degrees of damage to LM cells treated with the sLAMPs varied according to the

sLAMPs. For example, treating LM cells with sTac-I (Figure 2(b)) or sTac-III (Figure 2(d)) resulted in the rupture of the cell membranes of the LM cells and the leakage of cellular contents from the cells. Moreover, following treatment with sTac-II (Figure 2(c)), CDT (Figure 2(e)), or sPol-I (Figure 2(f)), LM cells were damaged, and most of the cells shrank. These results indicate that the five sLAMPs produce different degrees of damage to the cell membrane of LM.

3.4. Effects of the Five sLAMPs on the Activity of AKP in LM Bacterial Suspensions. As depicted in Figure 3, the activity of AKP in LM bacterial suspensions containing the five sLAMPs is significantly higher than that of AKP in control LM bacterial suspensions, which do not contain any of the sLAMPs. AKP is an intracellular enzyme [14]. Therefore, the increases in the activity of AKP in LM bacterial suspensions containing the sLAMPs indicate that the sLAMPs damage the cell membrane of LM and cause the leakage of AKP from LM cells.

3.5. Effects of the Five sLAMPs on the Expression of Virulence Genes in LM. The effects of the five sLAMPs on the expression of three virulence genes in LM (the *inlA*, *prfA*, and *hly* genes) were verified by QRT-PCR. The results of QRT-PCR experiments showed that the degrees of the inhibitory effects of the five sLAMPs on the virulence genes were different (Figure 4). The expression of the *inlA* gene was significantly inhibited by sTac-I and sPol-I, and the expression of the *prfA* gene was significantly inhibited by sTac-I, sTac-III, and CDT. On the other hand, the expression of the *hly* gene was significantly inhibited by sTac-I, sTac-II, and CDT.

4. Discussion

This study determines the MICs of the five sLAMPs against LM, the time-kill kinetics of the sLAMPs, and the antibacterial mechanisms of the antimicrobial peptides. The structures of the sLAMPs were linear, lacking disulfide bonds, and β-sheets. Shi et al. [15] reported that disulfide connectivity significantly affects the secondary structure, antibacterial activity, and hemolytic activity of the isomers of Tachyplesin I. Rao [16] suggested that a balance between hydrophobic and hydrophilic groups, rather than the presence of disulfide bonds, determines the antibacterial activity of Tachyplesin. Hirakura et al. [17] reported that the affinity of a cyclic β-sheet antimicrobial peptide (Tachyplesin I) with lipopolysaccharide is 280 times greater than the affinity of the peptide with an acidic phospholipid. On the other hand, the affinity of a linear α-helical peptide with lipopolysaccharide is similar to the affinity of the peptide with an acidic phospholipid. Hence, disulfide bonds play an important role in maintaining the stability of the secondary structures of LAMPs (Tachyplesins and Polyphemusins). The amphiphilic secondary structures of LAMPs are generally associated with the antibacterial activity of the antimicrobial peptides. However, the current study found that the linear structures of the five sLAMPs exhibited antibacterial activity against LM.

The antibacterial activity of LAMPs is generally associated with the permeabilization of the bacterial cell membrane [18]. LAMPs interact with bacterial cell membranes via ionic and amphiphilic interactions and alter the permeability or rupture the cell membranes [19, 20]. Interactions of LAMPs with bacterial cell membranes are the main mechanisms for the antibacterial activity of the antimicrobial peptides. After positively charged LAMPs interact with bacterial cell membranes via electrostatic interactions, they form pores on the cell membranes and cause the leakage of intracellular materials, ultimately leading to bacterial cell death [21]. Via SEM, the current study examined the effects of the five sLAMPs on the cell membrane of LM. The sLAMPs caused varying degrees of damage to the cell membrane of LM (Figure 2). However, the mechanisms by which the sLAMPs damaged the cell membrane of LM appeared to differ significantly. Thus, the specific mechanism by which each of the five sLAMPs damages the cell membrane of LM should be studied in follow-up experiments. Furthermore, the current study tested the activity of AKP in LM bacterial suspensions containing the sLAMPs to verify the effects of the sLAMPs on the permeability of the cell membrane of LM. As an intracellular enzyme, AKP only leaks from bacterial cells when the cell walls and cell membranes of the cells are compromised [14]. The addition of the five sLAMPs to LM bacterial suspensions significantly increased the activity of AKP in the bacterial suspensions (Figure 3), indicating that the sLAMPs permeabilize the cell membrane of LM and cause the leakage of AKP through the cell membrane.

In addition to interactions between LAMPs and cell membranes, interactions between LAMPs and intracellular DNA are important to the antibacterial activity of the LAMPs [22]. Powers et al. [23] labeled Polyphemusin I with biotin and observed the biotin-labeled Polyphemusin I in *E. coli* cells via a confocal fluorescence microscope. Although the cell membranes of the *E. coli* cells are intact, a large amount of Polyphemusin I accumulates in the cytoplasm and aggregated genomic DNA of the cells. Using DAPI staining, confocal fluorescence microscopy, and DNA footprint analysis, Yonezawa et al. [22] found that the bacteriostatic effect of Tachyplesin I is resulted from the binding of the antimicrobial peptide to the minor groove of the DNA double helix, which prevents the replication or transcription of the DNA. The results reported by Powers et al. [23] and Yonezawa et al. [22] indicate that the antibacterial effects of LAMPs are facilitated by interactions between the antimicrobial peptides and bacterial DNA. In this study, the effects of the five sLAMPs on the expression of three virulence genes in LM (the *inlA*, *prfA*, and *hly* genes) were verified by QRT-PCR experiments to discover the mechanism for the antibacterial activity of the sLAMPs against LM. As a transcription factor, Prfa, which belongs to the Crp/Fnr family, can regulate the expression of the other virulence genes in LM [24]. The *inlA* gene plays a key role in the adhesion and invasion of LM [25]. The *hly* gene is involved in the growth and spread of LM [26]. The *hly* gene

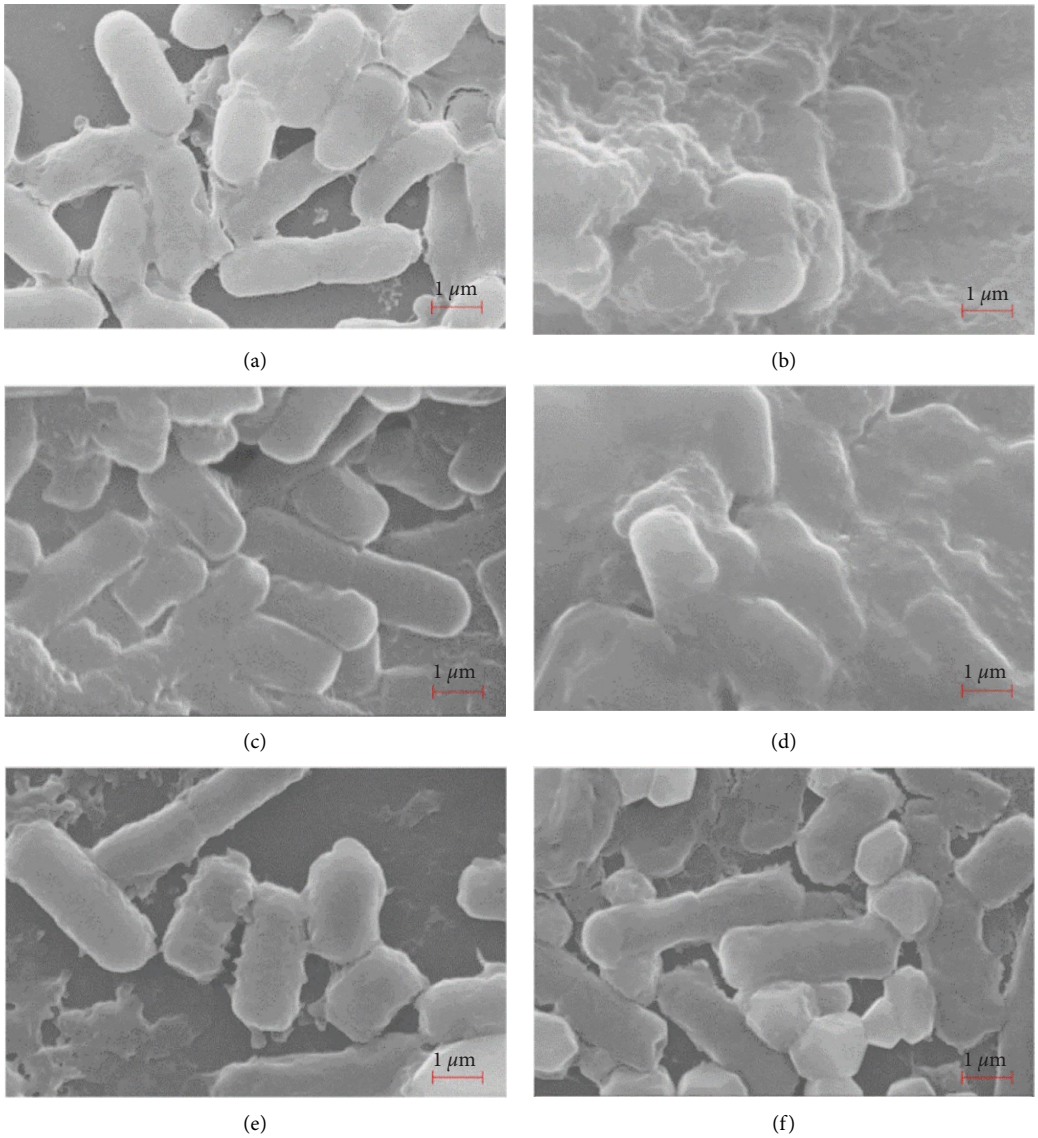

Figure 2: The scanning electron micrographs of (a) control (untreated) LM cells, (b) LM cells treated with sTac-I, (c) LM cells treated with sTac-II, (d) LM cells treated with sTac-III, (e) LM cells treated with CDT, and (f) LM cells treated with sPo-I.

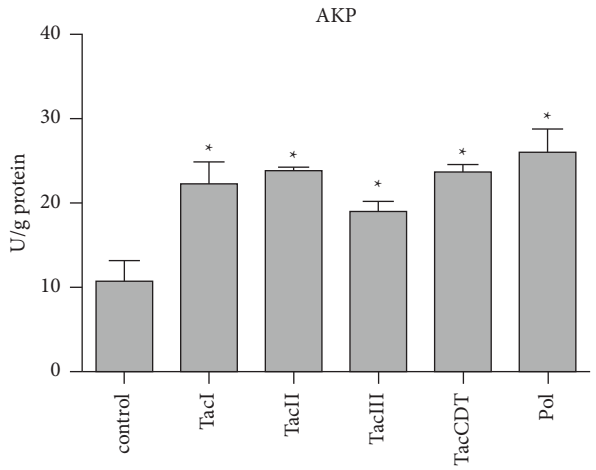

Figure 3: The activity of AKP in control LM bacterial suspensions and LM bacterial suspensions containing the five sLAMPs. $^{*}p < 0.05$.

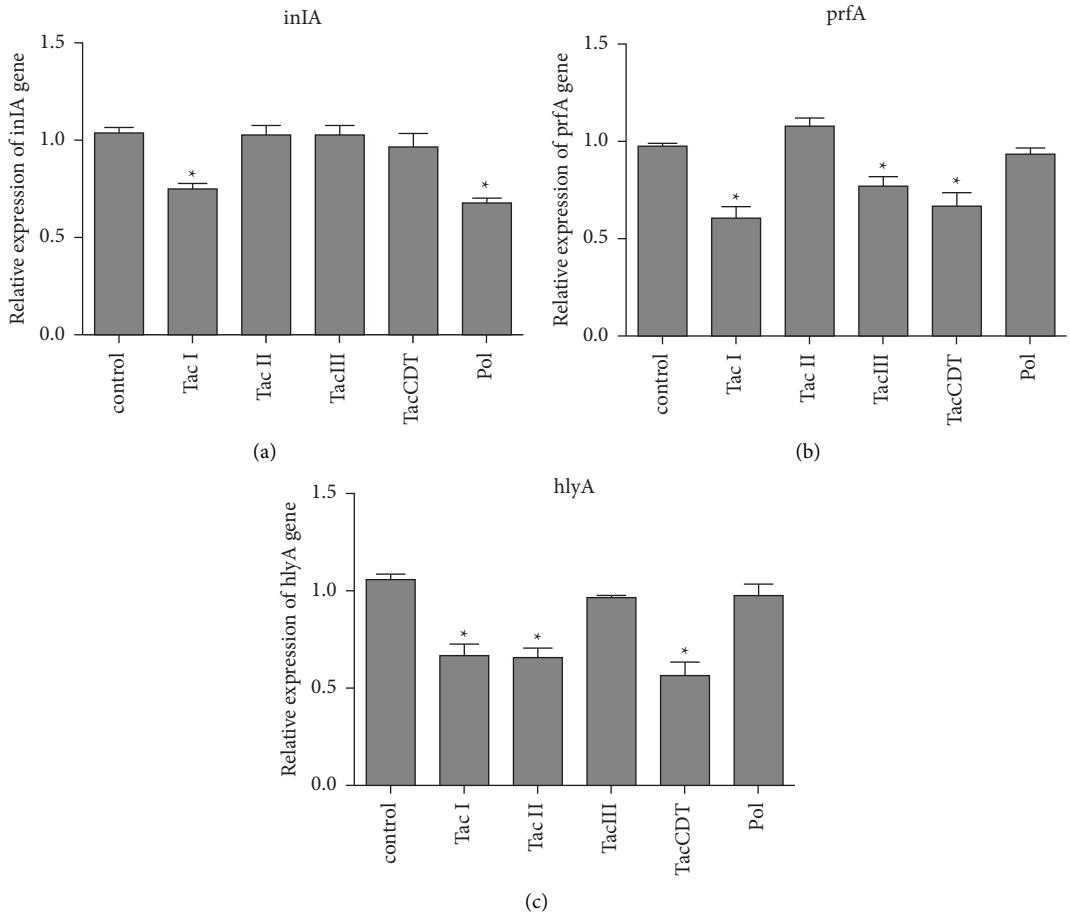

Figure 4: The effects of the five sLAMPs on (a) the relative expression of the *inlA* gene, (b) relative expression of the *prfA* gene, and (c) relative expression of the *hly* gene. The genes are virulence genes in LM. $^{*}p < 0.05$, $^{**}p < 0.01$.

encodes listeriolysin O, a protein that mediates the lysis of the membranes of phagocytic vacuoles and the release of LM cells [15]. Although all of the five sLAMPs exhibited antibacterial effects on LM, the effects were short-lived as LM developed resistance in a few hours and resumed its growth after 6 h. Thus, further investigation is required to reduce the resistance of LM to the antibacterial effects of the sLAMPs.

5. Conclusions

This study demonstrated the antibacterial effects of the five sLAMPs on LM. Initially, the sLAMPs effectively inhibited the growth of LM by permeabilizing the cell membrane of the bacterium and suppressing virulence genes in the bacterium. However, the inhibitory effects were effective only for 1 h, after which the bacterium slowly became resistant to them. After 6 h, the bacterium continued its growth. Interestingly, although cells in treatment groups continued their growth after 6 h, the growth of the cells was limited compared to the growth of cells in the control group. Thus, further research is required to overcome the resistance of LM to the antimicrobial effects of the sLAMPs.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Haiwei Xie performed supervision, project administration, funding acquisition, and wrote the original draft. Mingyang Wang performed visualization, developed the methodology, performed data curation, software, formal analysis. Zhongling Wu performed investigation, developed the

methodology. Zhantu Lai performed investigation, formal analysis. Liping Luo, Fengcai Lin, and Jia Lei performed investigations. Haiwei Xie and Mingyang Wang contributed equally to this work.

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