

Functional Analysis of Plantaricin E and Its Mutant by Heterologous Expression in *Escherichia coli*

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Abstract Plantaricins are a group of ribosomally synthesized antimicrobial peptides in *Lactobacillus plantarum* that exert antimicrobial activities against some foodborne pathogens. In this study, we observed that plantaricin E in *L. plantarum* 163 was missing 19 amino acids (plnE mutant amino acid sequence: FNRGGYNFGKSVRH, plnE amino acid sequence: FNRGGYNFGKSVRHVVDAIGSVAGIRGILKSIR). In order to study the effects of mutant *plnE*, *plnE* mutant genes with and without the signal peptide were cloned from the *L. plantarum* 163 genome, linked to the pET32a vector, and expressed via a fusion protein (thioredoxin) in *Escherichia coli* BL21 (DE3). All target proteins were purified using Ni-NTA, SP FF columns, and RP-HPLC. The purified proteins were stable in an acidic environment and at temperatures below 80 °C, but they were easily degraded under alkaline conditions and by protease treatment. They showed antimicrobial activity against gram-positive bacteria such as *Micrococcus luteus*, *Staphylococcus epidermidis*, *Lactococcus lactis*, *Lactobacillus paracasei*, and *Listeria innocua*. In addition, SP-plnE and PlnE exerted stronger activity than nisin. The signal peptide had a positive effect on the activities of PlnE and PlnEm. Thus, these purified proteins may have potential applications in the food industry to control foodborne pathogens.

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Keywords Plantaricin · Antimicrobial peptides · *plnE* mutant · Mechanism model

Abbreviations

| | |
|-----------------|---|
| <i>plnE</i> | synthesized plantaricin E gene |
| <i>plnEm</i> | mutant of <i>plnE</i> in <i>Lactobacillus plantarum</i> 163 |
| <i>sp-plnE</i> | <i>plnE</i> gene with the signal peptide |
| <i>sp-plnEm</i> | <i>plnEm</i> gene with the signal peptide |

Introduction

Antibiotic resistance is becoming a major public health concern worldwide. “Superbugs” may cause many serious infections that currently available antibiotics cannot treat [3, 17, 35]. Plantaricins, which are considered nontoxic and are relatively safe for use as food preservatives and drugs, are novel antimicrobial peptides with the potential to control “superbugs” [23, 29]. Based on the statistics presented in the antimicrobial peptide database (<http://bactibase.pfba-lab-tun.org/main.php>), approximately 40% of known bacteriocins are produced by *Lactobacillus*.

In addition, as many of the species of the genus *Lactobacillus* have a “generally regarded as safe” status, including *Lactobacillus plantarum*, they may have applications as probiotics [23]. Many plantaricins are produced by various strains of *L. plantarum*. For example, plantaricin SIK83 [2] was purified and identified from *L. plantarum* SIK-83; this peptide inhibits some lactic acid bacteria belonging to *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*. Additionally, plantaricins A, C, S, E, F, J, K, SA6, and UG1 [7, 9, 11, 16, 24, 26, 27, 31] have been purified and detected from the culture supernatants of *L. plantarum*. To date, more than 36 plantaricins [4, 12, 13, 18, 19, 28, 34, 36, 39, 45] have been identified in *L. plantarum*, and 16 of these plantaricins are included in the bacteriocin database (<http://aps.unmc.edu/AP/>).

Plantaricin E is a two-peptide bacteriocin that depends on the complementarity of two different antimicrobial peptides (plantaricin E and plantaricin F) to function [21]. The *PlnE* gene was cloned from *L. plantarum* WCFS1, C11, and J51 and was found in the soil metagenome [1, 22, 24, 33]. This gene was then heterologously expressed in *Escherichia coli* to determine its antimicrobial activity. The results indicated that this molecule had activity against *Listeria innocua*, *Micrococcus luteus*, and some lactic acid bacteria [24]. The yield of plantaricin E can be as high as 1.2–1.5%, which is much higher than that in the native strain (0.3–0.7%) [25]. The three-dimensional structures of plantaricin E have been analyzed by nuclear magnetic resonance spectroscopy (NMR) in the presence of dodecylphosphocholine (DPC) micelles. Plantaricin E has an α -helix in the N-terminal (residues 10–21) and an α -helix-like structure (residues 25–30) and a small random coil (residues 31–33) in the C-terminal [10]. The mechanism of plantaricin E has been extensively studied, and many hypotheses have been proposed. These studies have indicated that plantaricins E and F have complementary effects and form pores in the cytoplasmic membranes of target cells. Plantaricins E and F dissipate the pH gradient (ΔpH) and electrical potential ($\Delta\psi$) [21]. Recent research results have shown that plantaricins E and F interact in an antiparallel manner and that the C-terminal of plantaricin E and N-terminal of plantaricin F are localized to the outer part of the target cell membrane, whereas the N-terminal of plantaricin E and C-terminal of plantaricin F are localized to the inner part of the membrane [8].

In the present work, we isolated an *L. plantarum* strain having antimicrobial activity against some gram-positive bacteria [14]. A genome framework map was analyzed to identify the bacteria-associated gene. A plantaricin-associated locus was found; this locus contained five operons: pln123, plnRL, plnEFI, plnABD, and plnGHSTUVW. The function of pln123 was tested and verified in our previous work [20]. In this study, we found that the *plnE* expressed in *L. plantarum* 163 lost five bases, resulting in a stop codon (TAG) and shortening of PlnE to 14 amino acids, compared with the 33-amino acid sequence of PlnE in *L. plantarum* WCSF1 [33] (gene ID of *plnE* 1064171). To evaluate the influence of the mutant PlnE, the protein was expressed as a fusion protein in *E. coli*. In addition, the effects of the signal peptide on plantaricin activity were also studied.

Material and Methods

Microbial Strains and Culture Medium

The indicator strains were *Lactococcus lactis*, *L. innocua* CICC 10417, *Lactobacillus paracasei* CICC 20241, methicillin-resistant *S. aureus* (MRSA), methicillin-resistant *Staphylococcus epidermidis* (MRSE), *M. luteus* CMCC63202, and *S. epidermidis*. *E. coli* DH5 α and *E. coli* BL21 (DE3) strains used for cloning and protein expression were grown in LB medium at 37 °C. Lactic acid bacteria were grown in MRS medium at 30 °C, *Listeria* were grown in brain–heart infusion medium at 30 °C, and all other indicator strains were grown in LB medium at 37 °C.

The protein marker, recombinase exnase II, high-fidelity enzyme DNA polymerase, and *E. coli* strains DH5 α and BL21 (DE3) were purchased from Vazyme Biotech (Nanjing, China). A low-molecular-weight protein ladder and restriction endonuclease were purchased from Thermo Scientific (MA, USA). Cation exchanger (HiTrap SP FF) columns and nickel-nitrilotriacetic acid (Ni-NTA) were purchased from GE Healthcare (Pittsburgh, PA, USA). Dialysis bags were purchased from Viskase (IL, USA). Primers were designed as indicated in Table 1.

Table 1 Primers used in this study

| Primer name | Sequence |
|----------------|---|
| Enterokinase-F | GGACAGCCCAGATCTGGGTACCGACGACGACGACAAGG |
| plnE-F | cgacgacgacgacaaggCAACCGTGGTGGTTACAAC |
| plnE-R | tcgacggagctcgaattcACGGATAGATTTTCAGGATA |
| plnEm-F | cgacgacgacgacaaggtTAATCGGGGCGGTTATAAC |
| plnEm-R | tcgacggagctcgaattcTCAATGTCGA ACACTTTTA |
| sp-plnE-F | cgacgacgacgacaaggATGCTACAGTTTGAGAAG |
| sp-plnE-R | tcgacggagctcgaattcTTAACGAATACTTTTCAAAAT |
| sp-plnEm-F | cgacgacgacgacaaggATGCTACAGTTTGAGAAGTT |
| sp-plnEm-R | tcgacggagctcgaattcTCAATGTCGAACACTTTTAC |

Lowercase letters indicate the recombinant sequence within the pET32a vector. The pET32a vector was digested with *KpnI* and *BamHI*, using PCR (enterokinase-F) to rebuild the sequence from the *KpnI* site to the lysine codon of the enterokinase site. This ensured that the plantaricins had the native N-terminal

Construction of Recombinant Plasmids

The genomes of *L. plantarum* 163 and STIII were extracted as previously described [30]. *PlnE* and *sp-plnE* genes were cloned from *L. plantarum* STIII, and *PlnEm* and *sp-plnEm* were cloned from *L. plantarum* 163 by high-fidelity enzyme DNA polymerase. After a hotstart at 95 °C for 3 min, 30 cycles of the following program were run: denaturation at 95 °C for 20 s, primer annealing at 45 °C for 20 s, and elongation at 72 °C for 30 s, with a final elongation at 72 °C for 10 min. The sequence of the enterokinase site was removed with *KpnI* and *BamHI* and then reconstructed by PCR (using the primer enterokinase-F with the reaction conditions described above). Four genes were linked to linearized pET32a (digested with *KpnI* and *BamHI*) directly using the infusion method (recombinase exnase II) and transformed into *E. coli* DH5 α as described previously [30]. The plasmid of the positive clone was extracted and transformed into *E. coli* BL21 (DE3) for protein expression after induction with 0.5 mM IPTG.

Expression and Purification of Recombinant Proteins

One liter of culture was induced with 0.5 mM IPTG (OD₆₀₀ of 0.6), and the cells were cultured for an additional 8 h at 25 °C. After washing with phosphate-buffered saline (PBS, pH 7.4), cells were suspended in binding buffer (20 mM Na₃PO₄, 8 M urea, pH 7.8) and then crushed by sonication for 15 min. The supernatant was loaded onto the Ni-NTA resin and eluted with binding buffer (containing 200 mM imidazole). The imidazole, phosphate, and urea were removed by dialysis in enterokinase buffer (10 mM Tris-HCl, pH 8.0, with 10 mM CaCl₂) at 4 °C overnight. The fusion protein was removed by enterokinase at 37 °C overnight and analyzed by Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein was then purified using a Ni-NTA column again to remove the fusion protein and histidine tag. The flow-through, which contained recombinant plantaricins, was purified using a cation exchanger (SP FF) column in an AKTA purifier UPC10 (GE Healthcare). Proteins were collected, and the antimicrobial activity was tested after desalting by dialysis in citrate buffer (0.1 M, pH 5.0). The concentrations of target proteins were determined using Bradford assays. For further purification, active samples were applied to an AICROMBond C18 (250 × 4.6 mm) column using an Ultimate 2000 HPLC system (Dionex, CA, USA).

Inhibitory Activities of Purified Proteins

Target protein activities were quantified with a 96-well microtiter plate assay. The density of the indicator bacteria was adjusted to 2×10^6 CFU/mL. Fifty microliters each of the indicator bacteria and gradient dilutions (1, 10, 20, 30, 40, and 50 μ M) of target proteins was added to the 96-well plates. Alternatively, 50 μ L nisin (0.1, 0.5, 1, 5, 10, or 15 μ M) was added as a positive control, and 50 μ L citrate buffer (0.1 M, pH 5.0) was added as a negative control. More accurate results of minimum inhibitory concentrations (MICs) were tested by further dilutions by using more refined concentrations. Three replicates were carried out for each experiment. The MICs were defined as the minimum concentrations for which the OD₆₀₀ did not change significantly ($P < 0.05$) compared with the initial OD₆₀₀ value.

Protein Stability in the Presence of Proteases and Variations in pH and Temperature

All target proteins were adjusted to a concentration of 10 μM (pH 5.0). The indicator bacterium was *L. innocua*. Proteins were then treated with proteinase E, proteinase k, pepsin, or papain in appropriate buffers and temperatures for 1 h. The relative activities, which depended on the diameter of the inhibition zone, were then tested and calculated after the pH was adjusted to 5.0. Additionally, the buffer for the protein was changed to acidic (pH 1, 2, 3, or 4) or alkaline (pH 11, 12, 13, or 14) for 2 h, followed by adjustment to pH 5.0 to calculate the relative stability of the protein under different pH conditions as described above. Moreover, plantaricins were treated by heating (60, 80, 100, or 120 $^{\circ}\text{C}$) for 30 min, and relative activities of the proteins were calculated as described above. All analyses were carried out in triplicate, and results are the averages of three reactions. The standard deviations were calculated using the “Descriptive Statistics Std. deviation” function in IBM SPSS Statistic 19. A fitting equation between zone diameter and antimicrobial activity was established using Origin 9.0. The relative activity was defined as the ratio of post-treatment activity to untreated activity. The activities of all positive controls were set as 100%.

Time-Killing Kinetics Assay, Growth Inhibition, and Cellular Content Leakage

The time-killing kinetics assays of recombinant plantaricins were carried out using *M. luteus* as described previously [15, 41]. The initial concentration of the indicator bacteria was 1×10^6 CFU/mL. One MIC of plantaricins was added after culturing at 37 $^{\circ}\text{C}$ for 2 h, and cells were then cultured for an additional 6 h. The OD₆₀₀ was detected every hour using a microplate reader. The viable cells of all samples were counted by dilution plate counting.

To study the effects of recombinant plantaricins on the cell membrane, the leakage of intercellular contents (DNA and protein) was examined. Overnight cultures were washed twice in PBS (pH 7.4). One MIC of recombinant plantaricins was added, and cells were then cultured at 37 $^{\circ}\text{C}$ for an additional 1 h. Citrate buffer (0.1 M, pH 5.0) was added as the negative control. The values of OD₂₆₀ and OD₂₈₀ were detected to measure leakage of protein and DNA, respectively, from the indicator bacteria.

Electron Microscopy Study

To investigate whether recombinant plantaricins affected the overall cell morphology and to visualize the bacterial membrane, we examined cell morphology after treatment with one MIC of plantaricins by using scanning electron microscopy (SEM). The indicator bacterium (*M. luteus*) was grown at 37 $^{\circ}\text{C}$ for 8 h. Cultures were then washed twice in PBS (pH 7.4) and adjusted to 1×10^6 CFU/mL using fresh LB medium. One MIC of plantaricins was added, and cells were then cultured for an additional 2 h. Next, cells were harvested and washed twice in PBS (pH 7.4). Finally, samples were treated for SEM as previously described [37].

Results and Discussion

Analysis of plnE and Its Mutant

The synthetic gene of plantaricin E was mutated in *L. plantarum* 163 compared with that in *L. plantarum* WCFS1, C11, and NC8 [6, 19, 33] (gene ID of *plnE* 1064171). There were five

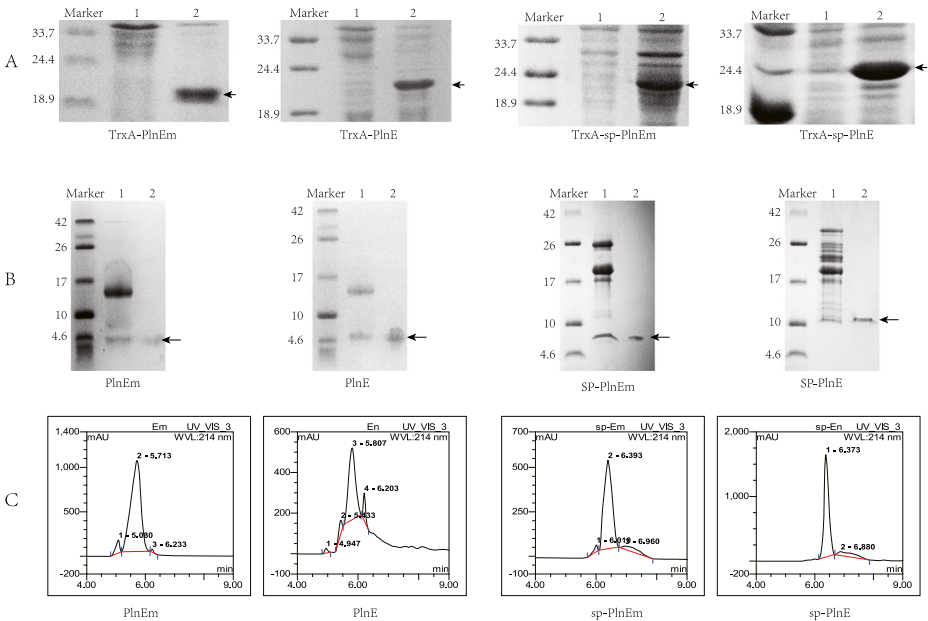


Fig. 2 Expression and purification of target genes. **a** Protein expression results for plnEm, plnE, sp-plnEm, and sp-plnE. Lane 1 indicates supernatants from ultrasonic disruption of positive clones before induction by IPTG and lane 2 indicates supernatants from ultrasonic disruption of positive clones after induced by IPTG. **b** Cleavage and purification results of PlnEm, PlnE, SP-PlnEm, and SP-PlnE, respectively, in Tricine-SDS-PAGE. Lane 1 indicates recombinant proteins that were digested by enterokinase and lane 2 indicates recombinant plantaricins that were purified by cation exchange column (Hitrap SPFF). **c** Results of PlnEm, PlnE, SP-PlnEm, and SP-PlnE analyses by RP-HPLC. Analysis of the peak areas showed that more than 92.19, 75.17, 88.78, and 85.74% of the proteins were objective proteins, respectively

For further purification, the supernatants were subjected to Ni-NTA affinity chromatography. The bound proteins were eluted in 200 mM imidazole. The proteins were detected by Tricine-SDS-PAGE after enterokinase cleavage (Fig. 2b). The cleavage mixture was then subjected to Ni-NTA affinity chromatography again to remove fusion protein and further purified using a cation exchange column (SP FF column). The proteins were analyzed by Tricine-SDS-PAGE after desalting by dialysis in citrate buffer (0.1 M, pH 5.0).

For further purification, target proteins were purified by RP-HPLC; the target proteins were eluted at about 6 min. The percentages of target protein yields were analyzed by peak calculus and found to be 92.19, 75.17, 88.78, and 85.74% for PlnEm, PlnE, SP-PlnEm, and SP-PlnE, respectively (Fig. 2c).

Antimicrobial Activities of Plantaricin E and Its Mutant

The results of our study showed that PlnEm, PlnE, SP-PlnEm, and SP-PlnE had different antimicrobial activities against gram-positive bacteria (Table 2). All target proteins had activity against *M. luteus* CMCC 63202, *S. epidermidis*, *L. lactis* NZ3900, *L. paracasei* CICC 20241, and *L. innocua* CICC 10417 at different concentrations. PlnE and SP-PlnE had activity against *S. aureus* ATCC29213 and MRSA, whereas PlnEm and SP-PlnEm did not. Additionally, PlnEm also showed no activity against MRSE.

Table 2 Antimicrobial spectrum and MICs of objective proteins and nisin

| Indicator bacteria | Source | PlnEm (μM) | PlnE (μM) | SP-PlnEm (μM) | SP-PlnE (μM) | Nisin (μM) |
|-----------------------|--------------|-------------------------|------------------------|----------------------------|---------------------------|-------------------------|
| <i>M. luteus</i> | CMCC 63202 | 30 \pm 2.5a | 3.1 \pm 0.34e | 7 \pm 2b | 2.3 \pm 0.2d | 5.9 \pm 0.8c |
| <i>S. aureus</i> | ATCC 29213 | N | 11.3 \pm 1.6b | N | 8.2 \pm 1.1a | 14.2 \pm 1a |
| <i>S. epidermidis</i> | JSPH | 18.3 \pm 1.5a | 2 \pm 0.06e | 5.4 \pm 0.5b | 1.8 \pm 0.09d | 5.4 \pm 0.44c |
| MRSE | JSPH | N | 4.2 \pm 0.42d | 9.8 \pm 0.88a | 3.7 \pm 0.23c | 9.4 \pm 0.51b |
| <i>L. lactis</i> | Lab isolated | 12.2 \pm 0.92a | 1.4 \pm 0.05d | 2.8 \pm 0.1b | 1.1 \pm 0.01c | 0.2 \pm 0.005e |
| <i>L. paracasei</i> | CICC 20241 | 18.3 \pm 1.3a | 3.7 \pm 0.5d | 5.6 \pm 0.25b | 2.4 \pm 0.2c | 0.45 \pm 0.02e |
| <i>L. innocua</i> | CICC 10417 | 7 \pm 0.25a | 1 \pm 0.01d | 1.6 \pm 0.01b | 0.8 \pm 0.02b | 0.42 \pm 0.02e |
| MRSA | JSPH | N | 24 \pm 1.5b | N | 19.4 \pm 1.4a | N |

Values in the same line with different letters indicate significant differences (overall $P < 0.05$, Duncan correction) in antimicrobial activity

N no inhibition, ATCC American Type Culture Collection, CMCC China Center of Medicine Culture Collection, AS China General Microbiological Culture Collection Center, JSPH Jiangsu Province Hospital, MRSE methicillin-resistant *S. epidermidis*, MRSA methicillin-resistant *S. aureus*

Overall, PlnE had better activity than PlnEm, indicating that the mutant had a significant influence on the activity of PlnE, which may be explained by the length of the alpha helix and the positive charge. Compared with the positive control (nisin), PlnEm and SP-PlnEm had reduced activity for all indicator bacteria. However, PlnE and SP-PlnE had better activities against *M. luteus*, *S. aureus*, *S. epidermidis*, MRSE, and MRSA than nisin. This difference may be associated with the distinct structure and positive net charge of the mutants. For example, plnE had a greater positive net charge than plnEm, which may affect the interaction between peptides and the bacterial phospholipid bilayer during the initial stage. Moreover, plnE had a more hydrophobic region than plnEm, altering hydrophobic interactions and binding strength. Therefore, the missing amino acids of plnE decreased the efficiency and strength of the interaction between plantaricins and the cell membrane.

In previous studies [24, 42], almost all of the heterologous expressed bacteriocins were analyzed without consideration of the influence of the signal peptide. It is commonly thought that the signal peptide has no effect or a negative effect on bacteriocin activity. However, the signal peptide of plnE has a positive effect on the activity of plnEm. We found that SP-plnEm had higher activity than plnEm. The role of the signal peptide may be attributed to its large hydrophobic domain, which could bind to the lipid bilayer, and the +4 positive charge, which could play a role in initial membrane binding to enhance the binding stability of PlnEm [5].

Stability of Plantaricin E and Its Mutant

The antimicrobial activities of PlnEm, PlnE, SP-PlnEm, and SP-PlnE were reduced when exposed to acidic/alkaline conditions or heat. Additionally, stability was enhanced as the peptide length increased. SP-PlnE showed the best stability, whereas PlnEm showed the worst stability. All target proteins had more than 87% relative activity when treated in an acidic environment (pH 1.0), demonstrating good stability under these conditions. However, relative activity was reduced when exposed to alkaline conditions; specifically, the activity of PlnEm was only around 78% when

the protein was exposed to an alkaline environment at pH 14.0 for 2 h. The activities of PlnEm, PlnE, SP-PlnEm, and SP-PlnE were rapidly reduced after heat treatment at temperatures above 100 °C but were stable at below 80 °C. Indeed, PlnEm had only 68% of the original activity after processing at 120 °C for 1 h (Fig. 3). Additionally, the proteins did not have activity after treatment with proteolytic enzymes, such as proteinase E, proteinase K, pepsin, or papain.

Time-Killing Kinetics Assays and SEM Results

Some models for antimicrobial peptide membrane lysis have been proposed [40, 43]. Based on the variability of the microbial membrane ultrastructure, a specific peptide may have different mechanisms under different membrane environments. Many models have shown that antimicrobial peptides result in the formation of membrane pores, leakage of ions and intercellular substances, changes in metabolism, and cell death [40].

In order to study the activities of plantaricins against indicator bacteria, time-killing kinetics assays and growth inhibition assays were conducted. The results showed that four plantaricins could inhibit the growth of the indicator bacteria when the plantaricin was added (Fig. 4b). The time-killing kinetics assays showed that the number of viable cells of indicator bacteria did not decrease when treated with plantaricins (Fig. 4a). These results indicated that the four recombinant plantaricins exerted their inhibitory effects by inhibiting bacterial growth.

The leakage of intercellular contents and SEM were conducted to evaluate the destruction of the cell membrane. The OD₂₆₀ and OD₂₈₀ values were significantly different between treated samples and the negative control (Fig. 4c). These data indicated that proteins and DNA leaked from the cell; thus, the cell membrane was damaged. However, SEM images showed that the cell morphology was not substantially altered (Fig. 4d). We could not find “holes” or specific occurrences of leakage.

Cell growth was inhibited when cells were treated with the four plantaricins. The number of dividing cells (dumbbell-shaped cells) was reduced significantly, as shown in our SEM images. Notably, the better the bacteriocin, the greater the inhibitory effect on cell division (Fig. 4d). The cells barely divided when treated with SP-PlnE.

Overall, the bacteriostasis mechanisms of these four plantaricins caused irreversible breakage of the cell membranes. Peptides may create membrane pores, leading to ion and metabolite

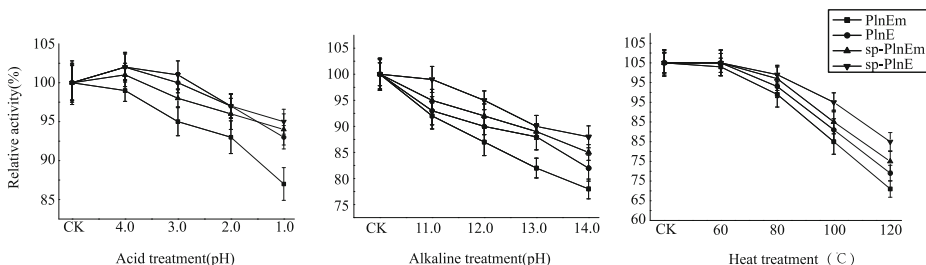


Fig. 3 Protein stability at varying pH values and temperatures. All assays were carried out against *Micrococcus luteus* CMCC 63202. The relationship between zone diameter and antimicrobial activity was analyzed to establish a fitting equation using Origin 9.0. The relative activity was defined as the ratio of post-treatment activity to untreated activity. $Y = 0.395X + 1.881$ and $r^2 = 0.996$; Y is $\log C$, C is the concentration of target protein ($\mu\text{M}/\text{mL}$), and X is the ring diameter of the inhibition zone (mm). The activity of all positive controls was set to 100%

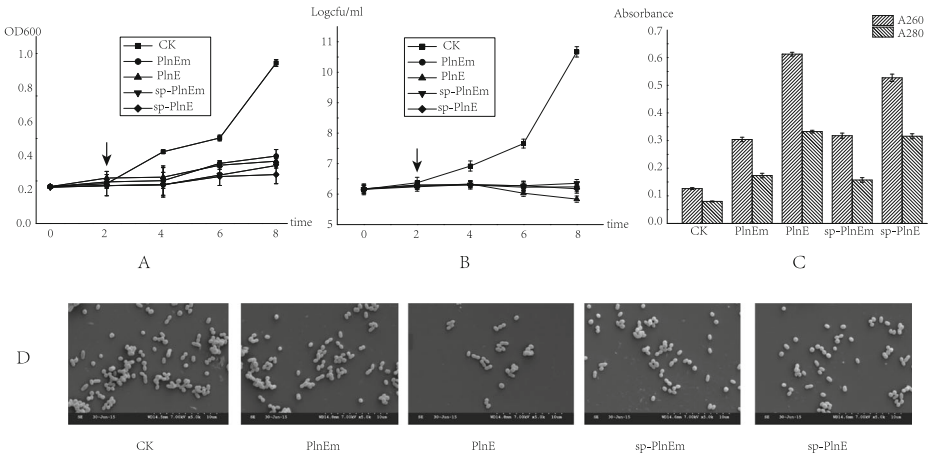


Fig. 4 Time-killing kinetics assays and SEM results. **a** Growth inhibition of recombinant plantaricins to *Micrococcus luteus* CMCC 63202. The plantaricins were added after 2 h of culture. The OD₆₀₀ was detected every 2 h using a microplate reader. **b** The CFU were counted by diluted coating in plates at the same time. **c** Results of leakage of intercellular contents following treatment with plantaricins. A260 and A280 represent the amounts of protein and nucleic acids, respectively, that leaked from the indicator bacteria. **d** SEM images after plantaricin treatment. The acceleration voltage was 7.0 kV, and the magnification was $\times 5000$

leakage, membrane depolarization, pH gradient (ΔpH) and electrical potential ($\Delta\psi$) dissipation, and ultimately cell death [21, 43]. Recent studies have shown that plantaricins E and F have complementary effects; plantaricins E and F interact in an antiparallel manner and the C-terminal of plantaricin E and N-terminal of plantaricin F are localized to the outer part of the target cell membrane, whereas the N-terminal of plantaricin E and C-terminal of plantaricin F are localized to the inner part of the membrane. The preference for an aromatic residue at position 6 (tyrosine) in plantaricin E suggests positioning of this residue in or near the membrane interface inside the cells [8].

PlnEm had the main $G_5 \times \times \times G_9$ motif, which is critical for the antimicrobial activity of the protein, but showed loss of the $G_{20} \times \times \times G_{24}$ motif, which has a secondary role in antimicrobial activity. Therefore, although PlnEm had antimicrobial activity, the activity was poorer than that of PlnE. When the indicator bacteria were treated with PlnE or PlnEm alone (without PlnF), the ability to create pores was weakened; many small pores may have been created, allowing leakage of ions and metabolites. However, these changes did not cause cell death, and the cell morphology was not changed significantly. Additionally, these plantaricins may influence cell metabolism, thereby affecting the growth of the indicator bacteria.

Conclusion

Currently, staphylococcal infections and other resistant bacteria account for a significant proportion of hospital-acquired infections, with *S. epidermidis* infections being the most common cause of bacteremia in hospitals [32, 44]. Many antimicrobial peptides had activities against resistant bacteria, providing new approaches for the treatment of resistant bacterial infections. Moreover, the potential use of purified plantaricins as food biopreservatives may result in the replacement of synthetic

chemical preservatives in the future. Currently, nisin is the only approved bacteriocin that can be used as a food additive [38]. In this study, PlnE and SP-PlnE had better activities against *M. luteus*, *S. aureus*, *S. epidermidis*, MRSE, and MRSA than nisin. Thus, these antimicrobial peptides may have applications as new food additives and may provide new approaches to overcoming antibiotic resistance in food production.

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