Antibacterial activity and action target of phenyllactic acid against Staphylococcus aureus and its application in skim milk and cheese

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ABSTRACT

Phenyllactic acid (PLA) has been demonstrated to possess antibacterial activity and capacity to prolong food shelf life. However, studies on the performance of PLA in inhibiting Staphylococcus aureus and its effectiveness when applied to dairy products are largely lacking. Here, antibacterial activity (planktonic and biofilm states) of PLA against S. aureus CICC101145 (S. aureus_45) were investigated. The results showed that PLA inhibited growth of S. aureus_45 and formation of S. aureus_45 biofilm. Next, the antibacterial action target of PLA was uncovered from both physiological and phenotypic perspectives. The results showed that PLA decreased cell metabolic activity and cell viability, damaged cell membrane integrity, triggered leakage of intracellular contents (DNA, proteins, and ATP), and caused oxidative stress damage and morphological deformation of S. aureus_45. In practical application, the antibacterial activity of PLA against S. aureus_45 cells was further confirmed in skim milk and cheese as dairy food models, and the antibacterial effects can be adequately maintained during storage for 21 d, at least at 4°C. These findings suggested that PLA could be a potential candidate for controlling S. aureus outgrowth in dairy foods.

Key words: phenyllactic acid, Staphylococcus aureus, antibacterial activity, action target, dairy foods

INTRODUCTION

Staphylococcus aureus is a foodborne pathogen that primarily causes staphylococcal foodborne disease and food poisoning, and is of great concern to the dairy industry (Kadariya et al., 2014; Xie et al., 2020; Jiang et al., 2022a). Staphylococcus aureus is considered a highly important pathogen in dairy products, such as ice cream, pasteurized milk, and raw milk (Jørgensen et al., 2005; Xie et al., 2020; Rainard et al., 2021). For instance, a staphylococcal food poisoning outbreak occurred among school children in the United States due to the consumption of staphylococcal enterotoxin-contaminated chocolate milk (Wu and Su, 2014), indicating that S. aureus could contaminate dairy products and affect human health. In addition, when the mammary glands of cows are infected with S. aureus, it damages the milk-secreting tissues and eventually leads to reduced milk production, altered milk composition, increased connective tissue, and high production of white blood cells (Ruegg, 2017; Rainard et al., 2021; Phiri et al., 2022). Thus, the negative effect of S. aureus on dairy production and public health has been receiving increasing attention worldwide.

Milk and milk derivatives are a key component in human diets (Diana et al., 2014; Verruck et al., 2019). However, as a high-protein food, milk and milk derivatives can be easily contaminated with S. aureus, which can threaten consumers’ health via consumption of dairy products and contact with dairy processing equipment (Krishnamoorthy et al., 2021). Staphylococcus aureus has the ability to grow and produce heat-stable toxins in dairy products that, if consumed, can cause staphylococcal food poisoning, a common and widespread foodborne disease, as previous surveys have shown that staphylococcal food poisoning events often occur in countries around the world (Argudín et al., 2010; Kadariya et al., 2014; Ma et al., 2016). For examples, S. aureus caused an estimated 241,000 illnesses per year in the United States (Kadariya et al., 2014). Food poisoning caused by S. aureus also accounted for 20 to 25% of bacterial food poisoning incidents in China (Ma et al., 2016). Additionally, S. aureus has the ability to attach, multiply, and form biofilms on the surface of milk products and milk processing equipment, leading to persistent contamination (Jørgensen et al., 2005; Rall et al., 2008; Pacha et al., 2021). Hence, the development of efficient antimicrobial agents that could inhibit or eliminate S. aureus in dairy products and dairy processing equipment is paramount to mini-
mize contamination. Several technologies have been proposed and preliminarily applied to reduce or prevent contamination of *S. aureus* in dairy products, which included pasteurization, high hydrostatic pressure, modified atmosphere packing, and antibiotics-based strategies (Syed et al., 2014; Necidova et al., 2016; Ahmed et al., 2020). However, the application in dairy foods of natural agents with antimicrobial activity against *S. aureus* in planktonic or biofilm states has not been fully explored.

Phenyllactic acid (PLA), a type of natural organic acid and direct precursor from phenylpyruvic acid, can be produced by certain lactic acid bacteria strains via lactate dehydrogenase (Li et al., 2015; Debonne et al., 2020; Rajanikar et al., 2021). Phenyllactic acid showed a broad-spectrum antimicrobial activity against both gram-positive and gram-negative bacteria, as well as against fungi (Ning et al., 2021b; Kleinvächter et al., 2021; Jiang et al., 2022b). Phenyllactic acid possesses good hydrophilicity, and thus is able to easily diffuse into the food matrix (Ning et al., 2017, 2021b). In addition, PLA has strong antibacterial activity and a sour smell that is less perceptible compared with that of other organic acids, such as acetic acid and lactic acid (Ning et al., 2017, 2021b). Furthermore, PLA can be produced efficiently and at a low cost by modulating fermentation using lactic acid bacteria strains (Mu et al., 2009; Huang et al., 2021). Therefore, PLA can be considered as a promising natural antimicrobial agent to be used in the food industry.

Although the antibacterial activity of PLA against several foodborne pathogenic bacteria, such as Escherichia coli, Salmonella enterica, Shigella flexneri, and Listeria monocytogenes, has been well documented (Dienlevex et al., 1998; Ning et al., 2017; Jiang et al., 2022b), to the best of our knowledge, the antibacterial activity of PLA against *S. aureus* has not yet been fully explored. Furthermore, no reports are available concerning the application of PLA for the control of *S. aureus* contamination in dairy products. Therefore, the aims of this study were (1) to evaluate the antibacterial activity of PLA against *S. aureus* in both planktonic and biofilm states, (2) to explore the potential action target underlying the antibacterial activity of PLA against *S. aureus* cells, and (3) to assess the antibacterial performance of PLA against *S. aureus* in skim milk and cheese.

**MATERIALS AND METHODS**

**Ethical Statement**

No human or animal subjects were used, so this analysis did not require approval by an Institutional Animal Care and Use Committee or Institutional Review Board.

**Bacterial Strains and Culture Conditions**

*S. aureus* strain CICC10145 (*S. aureus* 45) isolated from flavored yogurt was purchased from the China Center of Industrial Culture Collection. The strain was cultured at 37°C for 24 h in Luria-Bertani (LB) broth (pH 6.5; Solarbio), until the logarithmic phase was reached, approximately 10⁷ cfu/mL, and was used in subsequent experiments. Determination of bacterial concentration in the downstream experiments was performed by the gradient dilution coating method and the MacFarland scale (Zapata and Ramirez-Arcos, 2015; Chen et al., 2019).

**Determination of Antibacterial Activity of PLA**

**Determination of Minimum Inhibitory Concentration.** Minimum inhibitory concentration values of PLA against *S. aureus* 45 planktonic cells were determined using the broth microdilution assay, as previously described (Li et al., 2021; Jiang et al., 2022b). Briefly, *S. aureus* 45 planktonic cells were precultured in LB broth at 37°C for 24 h, and 2-fold serial dilutions of PLA were prepared in sterile LB broth. Subsequently, 20 μL of diluted PLA was mixed with 180 μL of LB broth containing *S. aureus* 45 planktonic cells (approximately 10⁷ cfu/mL), and incubated at 37°C for 24 h. Growth of *S. aureus* 45 planktonic cells were assessed by measuring absorbance at 595 nm in triplicate in a microplate reader (MR-96A, Mindray). Minimum inhibitory concentration value was defined as the lowest concentration of PLA that resulted in no visible bacterial growth.

**Growth Curve and Time-Kill Kinetics Assays.** The growth curve assay was conducted to determine the inhibitory effects of PLA on *S. aureus* 45 planktonic cells (approximately 10⁷ cfu/mL) over time (i.e., 0–16 h), as previously described (Yi et al., 2020; Luo et al., 2021). Briefly, *S. aureus* 45 planktonic cells were grown at 37°C to an approximate optical density at 600 nm (OD600) of 0.5 to 0.7. Then, PLA was added to the cultures to achieve different final concentrations (1 × MIC and 2 × MIC). After mixture, bacterial suspensions were incubated at 37°C, and aliquots were taken every 2 h, until 16 h of treatment with PLA. The OD600 values of *S. aureus* 45 suspensions were measured in a microplate reader. Phenyllactic acid-free cultures were used as a control. The experiment was independently repeated 3 times, and each sample was evaluated in triplicate.
Time-kill kinetics assay was conducted to determine the killing effects of PLA on *S. aureus* planktonic cells over a 2 h period, as previously described (Qiao et al., 2020; Luo et al., 2021). Briefly, PLA was mixed with *S. aureus* planktonic cells (approximately 10^7 cfu/mL) to achieve different final concentrations (1 × MIC and 2 × MIC), incubated at 37°C for 0, 0.5, 1, 1.5, and 2 h. Subsequently, 100 μL of PLA-treated cultures at each sampling point was obtained and spread onto LB solid medium after serial dilution. After incubation at 37°C for 24 h, colonies of *S. aureus* formed on plates were enumerated. Phenyllactic acid-free cultures were used as a control. The experiment was independently repeated 3 times, and each sample was evaluated in triplicate.

**Determination of Inhibition of Biofilm Formation.** The effect of PLA on *S. aureus* biofilm formation was assessed as previously described (Li et al., 2021; Luo et al., 2021). Briefly, 100 μL of cell suspension of *S. aureus* planktonic cells were transferred to a 96-well plate, and an equal volume of PLA dilutions were added to wells to achieve different final concentrations of PLA (1 × MIC and 2 × MIC), incubated at 37°C for 24 h. Subsequently, non-adhered cells were discarded, and plates were gently washed 3 times with PBS buffer, thereby exposing formed *S. aureus* biofilms, which were stained using a MycoLight Live Bacteria Fluorescence Imaging Kit (AAT Bioquest) according to the manufacturer’s instructions. Excess dye was removed by washing with PBS buffer. Biofilms were visualized under a fluorescence microscope (Boschida, Boschida; excitation/emission: 488/530) at 100× magnification. Subsequently, 200 μL of PBS buffer was added to each well, the contents were thoroughly mixed, and absorbance (OD_{595}) of *S. aureus* biofilms were determined using a microplate reader. Phenyllactic acid-free cultures were used as a control. The experiment was independently repeated 3 times, and each sample was evaluated in triplicate.

**Cell Proliferation Assay.** Metabolic activity of PLA-treated *S. aureus* planktonic cells were determined using the XTT assay kit (Abcam), as previously described (Li et al., 2021; Fang et al., 2022). Briefly, *S. aureus* planktonic cells (approximately 10^5 cfu/mL) and 20 μL of PLA (at final concentration of 1 × MIC and 2 × MIC) were transferred to a 96-well plate containing 160 μL of LB broth, followed by mixing and incubation at 37°C for 1 h. Subsequently, 20 μL of the prepared XTT mixture was added to each well, followed by incubation at 37°C for 30 min. Absorbance of all samples was measured at 450 nm in a microplate reader. Phenyllactic acid-free samples were used as a control. The experiment was independently repeated 3 times, and each sample was evaluated in triplicate.

**Viability of *S. aureus* planktonic cells treated with PLA was assessed using the Cell-Check Viability/Cytotoxicity Kit for Bacteria Cells (ABP Biosciences Inc.), as previously described (Rahman et al., 2020; Fang et al., 2022). Briefly, *S. aureus* planktonic cells (approximately 10^7 cfu/mL) were treated with PLA (at final concentration of 1 × MIC and 2 × MIC) at 37°C for 30 min, followed by centrifugation at 7,104 × g for 2 min at 4°C and resuspension in 0.85% NaCl solution. Subsequently, 3.0 μL of the mixture composed of fluorescent dyes NucView Green and propidium iodide (PI) was added to bacterial suspensions according to the manufacturer’s instructions, and reacted in the dark for 30 min. All samples were evaluated on slides in a fluorescence microscopy (excitation/emission: NucView Green: 500/530; PI: 535/617 nm, 200× magnification). Phenyllactic acid-free samples were used as a control. The experiment was independently repeated 3 times, and each sample was evaluated in triplicate.

**Flow Cytometric Analysis.** To further investigate the effects of PLA on cell viability of *S. aureus* planktonic cells, a PI uptake assay was performed in a flow cytometer, as previously described (Ning et al., 2017; Yi et al., 2020). Briefly, *S. aureus* planktonic cells were cultured to exponential phase, collected by centrifugation at 7,104 × g for 5 min at 4°C, and then resuspended in 0.85% NaCl solution to obtain a concentration of approximately 10^6 cfu/mL. Phenyllactic acid was added to bacterial suspensions to achieve final concentration of 1 × MIC and 2 × MIC, incubated at 37°C for 30 min. Subsequently, PI solution (10 μg/mL) was added to bacterial suspensions, and reacted for 30 min in the dark. Propidium iodide uptake was determined using a FACScan Flow Cytometer (Becton Dickinson). Data were analyzed using the WinMDI software (version 2.9; Scripps Research Institute). Phenyllactic acid-free samples were used as a control. The experiment was independently repeated 3 times, and each sample was evaluated in triplicate.

**Determination of the Content of Extracellular DNA and Protein.** Leakage of intracellular DNA, ATP, and proteins can be used as a proxy to determine the degree of integrity of cell membrane and, consequently, the degree of cell damage (Kang et al., 2019; Ning et al., 2020). To elucidate the potential of PLA in determining leakage of DNA and protein in *S. aureus* planktonic cells, the contents of extracellular DNA and protein were determined, as previously described (Kang et al., 2019; Luo et al., 2021). Briefly,
S. aureus_45 planktonic cells were cultured to exponential phase, collected by centrifugation at 7,104 × g for 2 min at 4°C, and resuspended in 0.85% NaCl solution to a concentration of approximately 10^7 cfu/mL. Phenyllactic acid was added to bacterial suspensions to result in final concentration of 1 × MIC and 2 × MIC, followed by incubation at 37°C for 12 h. Subsequently, all samples were centrifuged at 7,104 × g for 5 min at 4°C, and the supernatant was collected. Absorbance of extracellular DNA in samples was measured at 260 nm using an UV spectrophotometer (Unico Instruments Co. Ltd.). The content of extracellular protein in PLA-treated S. aureus_45 cells was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Phenyllactic acid-free samples were used as a control. The experiment was independently repeated 3 times, and each sample was evaluated in triplicate.

**Determination of Extracellular ATP Content.** The extracellular-ATP content in S. aureus_45 planktonic cells treated with PLA was determined using an ATP Assay Kit (Grace Biotechnology Co. Ltd.), as previously described (Kang et al., 2019; Luo et al., 2021). Briefly, S. aureus_45 planktonic cells were cultured to exponential phase, collected by centrifugation at 7,104 × g for 2 min at 4°C, and resuspended in 0.85% NaCl solution to a concentration of approximately 10^7 cfu/mL. Phenyllactic acid was added to the bacterial suspensions to a final concentration of 1 × MIC and 2 × MIC, followed by incubation at 37°C for 30 min and centrifugation at 7,104 × g for 5 min at 4°C. The supernatant was then collected and kept on ice until measurements were conducted. The content of extracellular ATP in all samples was determined by measuring absorbance of samples at 700 nm using a microplate reader and according to the manufacturer’s instructions. Phenyllactic acid-free samples were used as a control. The experiment was independently repeated 3 times, and each sample was evaluated in triplicate.

**Assays of Cell Oxidative Stress.** To investigate the effects of PLA on oxidative stress of S. aureus_45 planktonic cells, the activity of superoxide dismutase (SOD) and lactate dehydrogenase (LDH) were assessed using the SOD and LDH Assay Kit (Jiancheng Biotechnology Institute), as previously described (Wu et al., 2020; Yan et al., 2021). Briefly, S. aureus_45 planktonic cells were cultured to exponential phase, collected by centrifugation at 7,104 × g for 2 min at 4°C, and resuspended in 0.85% NaCl solution to a concentration of approximately 10^7 cfu/mL. Phenyllactic acid was added to the bacterial suspensions to a final concentration of 1 × MIC and 2 × MIC, followed by incubation at 37°C for 6 h and centrifugation at 7,104 × g for 5 min at 4°C. The supernatant was then collected and kept on ice until measurements were conducted. Afterward, levels of SOD and LDH release in samples were quantified using a microplate reader with specific absorbances at 550 nm and 490 nm, respectively. Phenyllactic acid-free samples were used as controls. The experiment was independently repeated 3 times, and each sample was evaluated in triplicate.

**Scanning Electron Microscopy Analysis.** Morphological changes in S. aureus_45 induced by exposure to PLA were determined by scanning electron microscopy, as previously described (Peng et al., 2021; Jiang et al., 2022a). Briefly, 1 mL of S. aureus_45 planktonic cells culture (approximately 10^7 cfu/mL) was centrifuged at 7,104 × g for 5 min at 4°C. Bacterial cells were collected and washed thrice using PBS buffer. Subsequently, cells were treated with PLA at concentrations of 0 × MIC (control), 1 × MIC, and 2 × MIC, and incubated at 37°C for 1 h. Phenyllactic acid-treated bacterial cells were then collected by centrifugation at 7,104 × g for 5 min at 4°C, washed 3 times with PBS buffer, and fixed in 2.5% glutaraldehyde at 4°C for 8 h. All samples were dehydrated in a concentration gradient of ethanol solutions (30, 50, 70, 80, and 95%) for 30 min each. Subsequently, all samples were transferred to polished silicon wafers (10 × 10 mm), dried at room temperature, and covered by gold powder to be imaged in a Regulus 8220 SEM (Hitachi). The experiment was independently repeated 3 times, and each sample was evaluated in triplicate.

**Antibacterial Activity of PLA Against S. aureus_45 in Dairy Food Models**

**Inhibitory Effect of PLA in Skim Milk.** The antibacterial activity of PLA on S. aureus_45 planktonic cells in skim milk was assessed as previously described (Chen et al., 2019; Kang et al., 2020). Briefly, 1 mL of S. aureus_45 planktonic cell culture (approximately 10^7 cfu/mL) was centrifuged at 7,104 × g for 5 min at 4°C. Subsequently, the collected bacterial cells were washed 3 times with PBS buffer, and 100 mL of commercially available UHT skim milk was incorporated to the cells, reaching a final concentration of approximately 10^5 cfu/mL of bacterial cells. Six 5-mL sets of samples were prepared and treated with different concentrations of PLA as follows: 0 × MIC (control), 1/8 × MIC, 1/4 × MIC, 1/2 × MIC, 1 × MIC, and 2 × MIC, followed by incubation at 4°C for 21 d. Aliquots were taken every 3 d until 21 d of treatment, and enumeration of viable cells of S. aureus_45 in skim milk was determined by LB solid plate counting. The experiment was independently repeated 3 times, and each sample was evaluated in triplicate.

**Inhibitory Effect of PLA in Cheese.** The antibacterial activity of PLA on S. aureus_45 planktonic
cells in cheese was assessed as previously described (Lin et al., 2018; Meng et al., 2021). Briefly, S. aureus_45 planktonic cells (approximately 10^6 cfu/mL) was diluted with PBS buffer to a final concentration of approximately 10^4 cfu/mL. Subsequently, fresh cheese samples (25 × 25 × 15 mm) were inoculated with S. aureus_45 suspension cells to obtain an initial concentration of approximately 10^4 cfu/mL. Then, the samples were kept for 10 min under sterile conditions at room temperature. The inoculated samples were then sprayed with PLA solutions (0 × MIC (control), 1/8 × MIC, 1/4 × MIC, 1/2 × MIC, 1 × MIC, and 2 × MIC) at a dosage of 1 mL/100 g. All samples were packaged in sterile bags and stored at 4°C for 21 d. Aliquots were taken every 3 d until 21 d of treatment, and enumeration of viable cells of S. aureus_45 in cheese was determined by LB solid plate counting. The experiment was independently repeated 3 times, and each sample was evaluated in triplicate.

**Statistical Analysis**

All experiments were conducted in triplicate, and results are presented as mean ± standard deviation of 3 replicates. Statistical significance was determined by one-way ANOVA and least significant difference test in SPSS 22.0 statistical software (IBM Software Inc.). P-values <0.05 were considered to indicate a significant difference.

**RESULTS**

**Antibacterial Activity of PLA Against S. aureus**

**Antibacterial Activity of PLA Against S. aureus Cells.** Minimum inhibitory concentration value of PLA against S. aureus_45 cells was 3.5 mg/mL. Growth of S. aureus_45 cells decreased continuously when treated with PLA at 1 × MIC and 2 × MIC from 6 to 16 h posttreatment (hpt; Figure 1A). Compared with the control samples (OD_600 = 1.69 ± 0.09 at 16 hpt), OD_600 value of S. aureus_45 cells treated with PLA at 1 × MIC and 2 × MIC at 16 hpt significantly (P < 0.05) decreased to 1.14 ± 0.05 and 0.62 ± 0.05, respectively. Furthermore, the number of viable cells of S. aureus_45 gradually decreased when treated with PLA at 1 × MIC and 2 × MIC from 0 to 2 hpt (Figure 1B). Compared with the control samples (8.17 ± 0.18 log_{10} cfu/mL at 2 hpt), the number of viable cells of S. aureus_45 treated with PLA at 1 × MIC and 2 × MIC significantly (P < 0.05) decreased to 6.54 ± 0.19 log_{10} cfu/mL and 4.52 ± 0.15 log_{10} cfu/mL at 2 hpt, respectively.

**Inhibitory Activity of PLA on Biofilm Formation.** Antibiofilm activity of PLA against S. aureus_45 is shown in Figure 2. Compared with the control samples (Figure 2A), density of S. aureus_45 biofilm was significantly reduced after treatment with PLA at 1 × MIC (Figure 2B) and 2 × MIC (Figure 2C). Quan-

![Figure 1](https://example.com/figure1.png)

*Figure 1. Changes in (A) growth pattern and (B) time-kill kinetics of Staphylococcus aureus_45 exposed to phenyllactic acid (PLA) at different concentrations [0× MIC (control), 1× MIC, and 2× MIC]. Differences between the experimental group and the control were compared by one-way ANOVA coupled to least significant difference test. OD = optical density. Error bars represent the SD values of 3 independent experiments.*
Quantitative analysis determined that biofilm density after exposure to 1 × MIC and 2 × MIC PLA significantly (P < 0.01) reduced to 62.89 and 51.10% of control samples, respectively (Figure 2D).

**Determination of Antibacterial Mechanism of PLA Against S. aureus_45 Cells**

**Effects of PLA on Cell Metabolic Activity and Viability.** The effects of PLA on metabolic activity and viability of S. aureus_45 cells are shown in Figure 3. The XTT assays revealed that absorbance of S. aureus_45 cell cultures treated with PLA at 1 × MIC and 2 × MIC significantly (P < 0.01) decreased to 61.83 and 47.19% of control samples, respectively (Figure 3A). Compared with control samples (Figure 3B), the cell viability of S. aureus_45 cells treated with PLA at 1 × MIC (Figure 3C) and 2 × MIC (Figure 3D) was remarkably reduced, as demonstrated by the predominance of red-stained cells, which are indicative of loss of cell viability. Moreover, quantitative analysis by flow cytometry (Figure 4) showed that, compared with in control samples (1.02%), the percentage of red-stained S. aureus_45 cells in samples treated with 1 × MIC and 2 × MIC PLA was significantly (P < 0.05) increased (by 24.60 and 58.60%, respectively).
Effects of PLA on the Leakage of Cell Constituents. The effects of PLA on determining leakage of *S. aureus* 45 cell constituents are shown in Figure 5. The content of extracellular DNA in *S. aureus* 45 cells treated with PLA at 1 × MIC and 2 × MIC for 12 h significantly (*P* < 0.01) increased by approximately 2.2-fold (OD$_{260}$ = 0.42 ± 0.02) and 4.0-fold (OD$_{260}$ = 0.76 ± 0.05), respectively, compared with control samples (OD$_{260}$ = 0.19 ± 0.03) (Figure 5A). The content of extracellular protein in *S. aureus* 45 cells treated with PLA at 1 × MIC and 2 × MIC for 12 h significantly (*P* < 0.01) increased by approximately 2.0-fold (60.61 ± 3.44 μg/mL) and 2.7-fold (84.72 ± 5.86 μg/mL), respectively, compared with control samples (30.93 ± 3.02 μg/mL) (Figure 5B). Furthermore, the content of extracellular ATP in *S. aureus* 45 cells treated with PLA at 1 × MIC and 2 × MIC for 30 min (*P* < 0.01) significantly increased by approximately 4.2-fold (0.46 ± 0.04 μmol/L) and 10.5-fold (1.16 ± 0.01 μmol/L), respectively, compared with control samples (0.11 ± 0.03 μmol/L) (Figure 5C).

Effects of PLA on Cell Oxidative Stress. The effects of PLA on the activity of oxidative stress enzymes of *S. aureus* 45 cells are shown in Figure 6. The activity of SOD in *S. aureus* 45 cells treated with PLA at 1 × MIC and 2 × MIC for 6 h significantly (*P* < 0.01) increased by approximately 1.7-fold (2.44 ± 0.06 μmol/L) and 3.1-fold (4.67 ± 0.06 μmol/L), respectively, compared with control samples (1.41 ± 0.05 μmol/L) (Figure 6B). Furthermore, the activity of GSH-Px in *S. aureus* 45 cells treated with PLA at 1 × MIC and 2 × MIC for 6 h significantly (*P* < 0.01) increased by approximately 1.9-fold (1.23 ± 0.04 μmol/L) and 2.5-fold (2.04 ± 0.05 μmol/L), respectively, compared with control samples (0.64 ± 0.03 μmol/L) (Figure 6C).
The activity of LDH at 1 × MIC and 2 × MIC PLA for 6 h significantly \((P < 0.01)\) increased by approximately 12.3-fold (0.14 ± 0.01 mM) and 17.0-fold (0.19 ± 0.02 mM), respectively, compared with controls (0.01 ± 0.01 mM) (Figure 6B).

**Effects of PLA on Cell Morphology.** The effects of PLA on cell morphology of *S. aureus* _45_ cells are shown in Figure 7. Scanning electron microscopy observations revealed that untreated *S. aureus* _45_ cells were evenly spherical, had smooth surface, neat edges, clear outline, and intact structure (Figure 7A). In contrast, after treatment with 1 × MIC PLA, *S. aureus* _45_ cell surface was rough, appeared shriveled, and had shrunk (Figure 7B). Furthermore, treatment with 2 × MIC PLA exacerbated alterations onto *S. aureus* _45_ cells, leading to cell disruption, leakage of cell content, and cell disintegration (Figure 7C).

**Application of PLA in Dairy Food Models**

**Antibacterial Activity of PLA in Skim Milk.** The antibacterial effects of PLA for *S. aureus* _45_ cells in skim milk are shown in Figure 8A. *Staphylococcus aureus* _45_ cell count in the control samples increased from 5.03 ± 0.05 log10 cfu/mL on d 0 to 5.87 ± 0.07 log10 cfu/mL on d 21; *S. aureus* _45_ cell count in skim milk treated with 1/8 × MIC PLA increased slightly from 4.99 ± 0.07 log10 cfu/mL on d 0 to 5.81 ± 0.06 log10 cfu/mL on d 21; and *S. aureus* _45_ cell count in skim milk treated with 1/4 × MIC PLA increased from 4.97 ± 0.06 log10 cfu/mL on d 0 to 5.67 ± 0.07 log10 cfu/mL on d 21. Notably, compared with the counts of *S. aureus* _45_ cells in control samples on d 21 (5.87 ± 0.07 log10 cfu/mL), the number of viable *S. aureus* _45_ cells in skim milk treated with 1/2 × MIC PLA, 1 × MIC and 2 × MIC PLA significantly \((P < 0.05)\) decreased, reaching 4.73 ± 0.04 log10 cfu/mL, 4.25 ± 0.06 log10 cfu/mL, and 3.05 ± 0.03 log10 cfu/mL, respectively, on d 21.

**Antibacterial Activity of PLA in Cheese.** The antibacterial effects of PLA for *S. aureus* _45_ cells in cheese are shown in Figure 8B. *Staphylococcus aureus* _45_ cell count in the control samples in cheese increased from 4.04 ± 0.05 log10 cfu/mL on d 0 to 4.82 ± 0.06 log10 cfu/mL on d 21; *S. aureus* _45_ cell count in cheese treated with 1/8 × MIC PLA increased slightly from 3.99 ± 0.07 log10 cfu/mL on d 0 to 4.80 ± 0.04 log10 cfu/mL on d 21; *S. aureus* _45_ cell count in cheese treated with 1/4 × MIC PLA increased from 3.95 ± 0.05 log10 cfu/mL on d 0 to 4.69 ± 0.05 log10 cfu/mL on d 21; and *S. aureus* _45_ cell count in cheese treated with 1/2 × MIC PLA increased from 3.94 ± 0.05 log10 cfu/mL on d 0 to 4.03 ± 0.04 log10 cfu/mL on d 21. Interestingly, compared with the counts of *S. aureus* _45_ cells in control samples in cheese on d 21 (4.82 ± 0.06 log10 cfu/mL), the number of viable *S. aureus* _45_ cells in cheese treated with 1 × MIC and 2 × MIC PLA significantly \((P < 0.05)\) decreased, reaching 3.61 ± 0.04 log10 cfu/mL and 3.30 ± 0.03 log10 cfu/mL, respectively, on d 21.

**DISCUSSION**

In the present study, the MIC value for PLA against *S. aureus* _45_ cells was 3.5 mg/mL, and this value was similar that against other foodborne pathogens in previous studies (Ning et al., 2021c; Zhang et al., 2021). For instance, the MIC of PLA was 2.25 mg/mL against *L. monocytogenes*, 3.2 mg/mL against *Vibrio parahaemolyticus*, and 2.5 mg/mL against *E. coli* (Ning et al., 2017; Fang et al., 2022). Moreover, growth of *S. aureus* _45_ cells was significantly inhibited when exposed to PLA in a time-dependent and dose-dependent manner, thus indicating its effective inhibitory activity against *S. aureus* cells. Additionally, PLA had inhibitory effect on *S. aureus* _45_ biofilm formation; in particular, the inhibitory rate of 2 × MIC PLA was 48.90%. This find-
ing indicates the excellent activity of PLA against S. aureus biofilm. Collectively, these findings suggest the potential of PLA as an antibacterial agent to combat the growth and biofilm formation of S. aureus. To date, understanding of the mechanisms underlying the antibacterial activity of PLA for S. aureus remains largely scant. In the present study, a significant decrease in the metabolic activity of S. aureus_45 cells was detected in the XTT assays when exposed to PLA. Although studies on the metabolic activity of PLA against foodborne pathogens are lacking, the effect of other antimicrobial agents on the metabolic activity of foodborne pathogens has been previously investigated. For instance, treatment with bacteriocin XJS01 for 2 h led to a decrease in the metabolic activity of S. aureus and S. flexneri by 47 and 42.98%, respectively (Li et al., 2021; Jiang et al., 2022b); additionally, treatment with bacteriocin DF01 for 2 h reduced metabolic activ-

**Figure 5.** Content of extravasated (A) DNA, (B) proteins, and (C) ATP of Staphylococcus aureus_45 cells treated with phenyllactic acid (PLA) at control, at 1× MIC, and at 2× MIC. Differences between the experimental group and the control were compared by one-way ANOVA coupled to least significant difference test. OD = optical density; **P < 0.01. Error bars represent the SD values of 3 independent experiments.

**Figure 6.** Changes in the activity of (A) superoxide dismutase (SOD) and (B) lactate dehydrogenase proliferation of Staphylococcus aureus_45 cells treated with phenyllactic acid (PLA) at control, at 1× MIC, and at 2× MIC. Differences between the experimental group and the control were compared by one-way ANOVA coupled to least significant difference test. **P < 0.01. Error bars represent the SD values of 3 independent experiments.

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ity of *E. coli* and *Salmonella* Typhimurium by 30 and 31%, respectively (Kim et al., 2019). These evidences indicated that antibacterial activity of PLA, similar to other antimicrobial agents, can partly be linked to reduced metabolic activity of *S. aureus* cells. Furthermore, after treatment with PLA, decreased cell viability of *S. aureus* was observed. *Staphylococcus aureus* cells exposed to 1 × MIC and 2 × MIC PLA were largely inactivated, more than 24 and 58% dead cells, respectively. These findings indicate that the antibacterial activity of PLA against *S. aureus* cells can be partially attributed to decreased cell metabolic activity and cell viability.

In the present study, leakage of the intracellular content (including DNA, ATP, and proteins) and changes in the activity of SOD and LDH were remarkable in PLA-treated groups compared with untreated control groups, indicating lost membrane integrity and oxidative stress damage of *S. aureus* cells after PLA treatment. Furthermore, scanning electron microscopy observations revealed that *S. aureus* cells exposed to different concentrations of PLA had morphological and structural alterations, such as wrinkling, rupture, and perforated cell membrane. Previous studies suggested that antimicrobial agents induce DNA damage and metabolic disturbances of key kinases, which could lead to deformed cell phenotype, loss of cell membrane integrity, and leakage of cytoplasm content in foodborne pathogen cells (Wu et al., 2017; Yi et al., 2018; Kang et al., 2019; Zhu et al., 2021). Therefore, the proposed antibacterial mode of PLA against *S. aureus* cells can be considered as follows: PLA initially destroys membrane integrity of *S. aureus* cells, resulting in cell deformation and oxidative stress damage; simultaneously, PLA also targets cytoplasmic proteins, energy metabolites (e.g., ATP), DNA in *S. aureus* cells, which disrupts normal physiological and biochemical activities in the cell. Hence, the phenotypical and physiologi-

![Figure 7](image_url)

**Figure 7.** Scanning electron micrographs of *Staphylococcus aureus* cells treated with phenyllactic acid (PLA) at different concentrations. (A) untreated cells; (B) cells treated with PLA at 1× MIC; (C) cells treated with PLA at 2× MIC.

![Figure 8](image_url)

**Figure 8.** The antibacterial activity of phenyllactic acid (PLA) at different concentrations against *Staphylococcus aureus* cells during growth in (A) skim milk and (B) cheese. Differences between the experimental group and the control were compared by one-way ANOVA coupled to least significant difference test. Error bars represent the SD values of 3 independent experiments.
cal mechanisms underlying the antibacterial activity of PLA against *S. aureus* were partially elucidated for the first time in this study.

Antimicrobial agents with good inhibitory and killing effects on foodborne pathogens found in milk and milk derivatives are important for the development of dairy industries (Kim et al., 2008; Chen et al., 2019). Therefore, over recent years, several antimicrobial agents against foodborne pathogens in dairy foods has been reported, such as myristic acid that inhibits staphylococci in milk for 10 d at 4°C and 10°C (Chen et al., 2019), actobionic acid that inhibits *Pseudomonas fluorescens* in milk for 12 d at 4°C (Kang et al., 2020), bacteriocin *NX371* that inhibits *Salmonella Enteritidis*, *L. monocytogenes*, *E. coli*, and *S. aureus* in artificially contaminated milk and mozzarella cheese for 7 d at 25°C and 35°C (Meng et al., 2021), and ε-Poly-lysine that inhibits *L. monocytogenes* in cheese for 15 d and *S. aureus* in pasteurized milk for 5 d at 4°C (Lin et al., 2018; Ning et al., 2021a). In the current study, PLA at different concentrations (particularly higher than 1/2 MIC) significantly inhibited number of viable cells of *S. aureus* in skim milk and cheese. Notably, PLA showed inhibitory effects against *S. aureus* cells in these 2 dairy food models during storage for 21 d at 4°C (this is the temperature adopted in most residential refrigerators), which was longer than that found for most antimicrobial agents against other foodborne pathogens in milk and milk derivatives. These results demonstrated that PLA retained its antibacterial activity against *S. aureus* when applied as an antimicrobial agent, and the advantage of applying PLA to control *S. aureus* contamination in dairy foods.

**CONCLUSIONS**

The present study demonstrated the antibacterial activities of PLA against *S. aureus* in planktonic and biofilm states. The underlying inhibitory activity of PLA against *S. aureus* cells can be attributed to decreased cell metabolic activity, reduced cell viability, as well as induced leakage of DNA, proteins, ATP, oxidative stress damage, and cell deformation. In addition, PLA inhibits the growth of *S. aureus* in dairy food models under common home storage conditions. To the best of our knowledge, this study is the first comprehensive exploration of antibacterial roles of PLA against *S. aureus* in dairy foods. However, further studies using molecular docking and omics are required to elucidate the antibacterial molecular mechanisms of PLA. Meanwhile, the combined use of PLA and other antibacterial agents (such as antibacterial peptides, other organic acids, and lyases) in a synergetic manner is important to achieve a better antibacterial effect in the future.

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