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SHORT COMMUNICATION

LysGH15 Effectively Control Murine Mastitis Caused by *Staphylococcus aureus*

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A B S T R A C T

Bovine mastitis is an inflammatory response mainly caused by *Staphylococcus aureus*. Lysin is a cell wall hydrolase encoded and synthesized by a bacteriophage, which can kill specific Gram-positive bacteria. In this study, phage lysin "LysGH15" is used to treat the mice mastitis caused by *S. aureus.* The purified lysGH15 showed strong bactericidal activity *in vitro*. When treated with 25μg/mL of the LysGH15, the bacterial counts of *S. aureus* dropped approximately 5 log units within 10 min. In the *in vivo* experiments, the administration of LysGH15 significantly ($P<0.05$) reduced the colonies of *S. aureus* and alleviated damage to the breast tissue. Also, the levels of IL-6 and TNF-α in breast tissue were significantly decreased. It indicates that the LysGH15 can effectively treat the murine mastitis caused by *S. aureus*. This study demonstrated the potential of LysGH15 as an alternative to antibiotics for treating bovine mastitis caused by *S. aureus*.

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INTRODUCTION

Bovine mastitis, an inflammatory response mainly caused by bacteria, renders huge economic losses to the world dairy industry, and brings issues like food safety for humans (Lopes *et al*., 2020). *Staphylococcus aureus* is the major etiological agent that causes bovine mastitis in the dairy industry (Sheet *et al*., 2019; Maity *et al*., 2020). Antibiotic therapy has long been the main strategy against mastitis. However, the *S. aureus* strains which show resistance to most common antibiotics, especially methicillin-resistant *S. aureus* (MRSA), have brought great challenges to the treatment of mastitis (Gomes and Henriques, 2016). Therefore, there is an urgent need for new antibacterial drugs against *S. aureus*-inducing bovine mastitis.

Lysin is a kind of cell wall hydrolase encoded and synthesized by the bacteriophage gene, that can specifically kill Gram-positive bacteria. Usually, the bactericidal activity of phage lysin is specific, and even it doesn't disturb the normal microflora. Furthermore, the lytic activity of lysin is not affected by antibiotic resistance, it has efficient bactericidal activity against multidrug-resistant bacteria (Zhang *et al*., 2018). Therefore, lysin is expected to be a new antibacterial drug for the treatment of mastitis. Our previous studies have indicated LysGH15, a lysin encoded by the phage GH15, displays efficiently lytic activity against MRSA strains and antibiotics sensitive *S. aureus* strains isolated from the clinics (Gu *et al*., 2013; Cheng *et al*., 2018). In this study, we aimed to test the activity of LysGH15 against *S. aureus* and the effect of LysGH15 on protecting the murine mastitis against *S. aureus* infection.

MATERIALS AND METHODS

Ethical statements and animals: The animal studies were approved by the Animal Welfare and Research

Ethics Committee, Jilin University. A total of 42 6~7-week-old BALB/c mice including 30 unbridled females, weighing 24-26g, and 12 mature males were procured from the Experimental Animal Center, Jilin University, Changchun, China.

Bacterial strains and LysGH15: *S. aureus* ATCC49525 were obtained from the American Type Culture Collection (ATCC), Baltimore, USA. The tryptic soy broth (TSB) was used to culture *S. aureus*. The expression, purification, and preparation of LysGH15 were according to the methods previously described (Gu *et al*., 2011).

***In vitro* bactericidal activity of LysGH15:** *S. aureus* ATCC49525 was cultured to 600 nm (OD_{600}) with an optical density of 0.8 (approximately 5×10^8 CFU/ml) and washed three times with sterile PBS. The bacterial suspension was treated with LysGH15 (25μg/mL) and incubated at 37°C for 10 min. Bacterial counts were performed every minute after incubation. An equal amount of *S. aureus* suspension was treated with PBS as a negative control.

Murine mastitis model: Murine mastitis model was constructed referring to the previous method with some modifications (Iwano *et al*., 2018). Before infecting with *S. aureus* ATCC49525, all mice were anesthetized by using a mixture of midazolam (6mg/kg), medetomidine (0.45mg/kg), and butorphanol (7.5mg/kg). The fourth pair of mammary glands (Fig. 1**)** of all mice in each group were injected with 50μL *S. aureus* ATCC49525 with 10^5 CFU/mL concentration.

Effect of LysGH15 on the mice with mastitis: Mice were randomly divided into 8 equal groups. The fourth pair of mammary glands of each mouse in each group were treated with LysGH15 10μg (group A), 30μg (group B), and 50μg (group C) at 1h post-infection or treated with LysGH15 10μg (group D), 30μg (group E) and 50μg (group F) at 8h post-infection. The negative control group treated with PBS at post-infection while the normal group was neither challenged nor lysin treated.

The bacterial loads and the levels of IL-6 and TNF-α in the mice mammary glands were detected at 48h post-infection. The L4 glands from each group were weighed and homogenized in sterile PBS. The dilution (100μL) of the homogenates per gland was used to detect the number of *S. aureus*. The remaining homogenates supernatant was used to determine the levels of inflammatory cytokines using ELISA kits (Catalogue # 431304 and 430904).

For histopathology, the R4 glands from each group were taken and immediately placed into 10% neutral buffered formaldehyde. After processing and staining with H&E, the mammary gland tissues were analyzed using a light microscope.

Statistical analysis: All the statistical analyses were conducted using SPSS version 13.0 software (SPSS, Inc., Chicago, IL, USA) and performed using Prism (GraphPad Software, La Jolla, CA, USA). One-way ANOVA (Dunnett's t-test) and Tukey's multiple comparisons test analysis were used for all experimental data analysis at significance level $P<0.05$ (*) and $P<0.01$ (**).

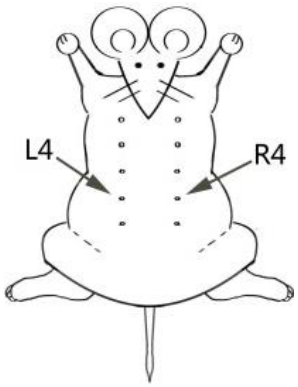

Fig. 1: The injection site for *S. aureus* inoculation. L4 and R4 indicated the left and right teats of the fourth pair of mammary glands, respectively.

RESULTS

Bactericidal activity of LysGH15: LysGH15 at the concentration of 25μg/mL showed highly efficient lytic activity on *S. aureus* ATCC49525 (Fig. 2A). The number of viable *S. aureus* in the LysGH15-treated group was reduced approximately 4-5 log units after the treatment for 10 min compared to the PBS-treated group.

Effect of LysGH15 on the mice with mastitis: The bacterial counts of *S. aureus* in the control group reached about 3×10^7 CFU/mL in the mammary gland at 48 h post-infection (Fig. 2B). The bacterial counts of *S. aureus* in the mammary glands decreased significantly in different concentrations of LysGH15 treated groups at 1h post-infection compared with that of the control group, especially in 50μg LysGH15 treatment groups reached 9.31×10^1 CFU/mL ($P<0.01$) (Fig. 2B). In addition, when the mice were treated with 30μg or 50μg LysGH15 at 8h post-infection, the number of *S. aureus* in the mammary gland was also reduced significantly ($P<0.01$), being 9.32×10^4 CFU/mL and 6.02×10^2 CFU/mL, respectively.

As shown in Fig. 3, the levels of IL-6 and TNF-α in the mammary gland tissues of the control group were significantly ($P<0.01$) increased at 48h post-infection with *S. aureus* ATCC49525 comparing with healthy mice. In contrast, the levels of IL-6 and TNF-α were significantly reduced by different concentrations of the LysGH15 at 1h post-infection and also significantly reduced by 30μg or 50μg LysGH15 treatment at 8h post-infection.

Based on the histopathological analysis, the breast acinar structure of healthy mice was intact and has no obvious pathological changes (Fig. 4A). In contrast, the mammary glands of mice suffering from mastitis caused by *S. aureus* ATCC49525 showed much inflammatory cell infiltration (Fig. 4E). Compared with the control group, the structure of acinar tissue in mammary gland tissue was gradually alleviated in different concentrations of the LysGH15 treatment group at 1h post-infection especially in 50μg LysGH15 treatment group and almost no inflammatory cell exudation could be seen (Fig. 4B, 4C and 4D). In addition, inflammation of breast tissue could not be relieved by 10μg of the LysGH15 at 8h post-infection (Fig. 4F). However, the tissue lesions were significantly alleviated by 50μg of the LysGH15, even there is still a small amount of inflammatory cell infiltration and protein exudation (Fig. 4H).

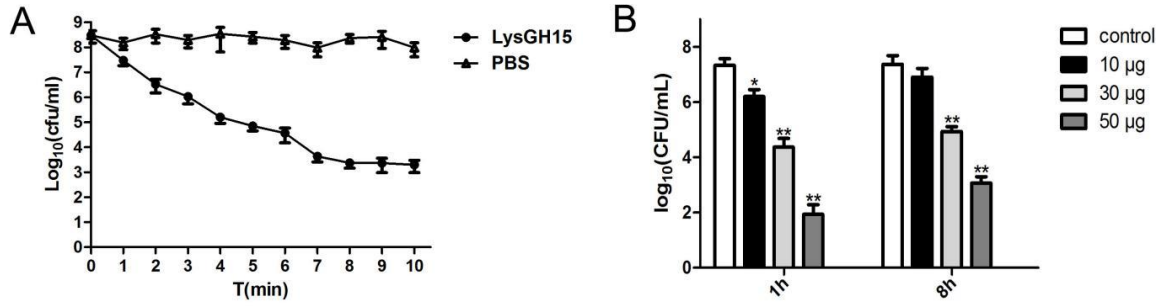

Fig. 2: Bactericidal activity of LysGH15 against *S. aureus* ATCC49525 *in vitro* and *in vivo*. A) Log (CFU/mL) decrease of the *S. aureus* ATCC49525 culture (5×10^8 CFU/mL) was used to determine the lytic activity of LysGH15 at the concentration of 25μg/mL. The control group was treated with PBS buffer. B) The mammary gland tissues were removed and homogenized at 48h after *S. aureus* strain ATCC49525 (1×10^5 CFU/mL) was injected. The values represent mean±SD (n=3). Bars bearing *(P<0.05) or **(P<0.01) differ significantly than that of control.

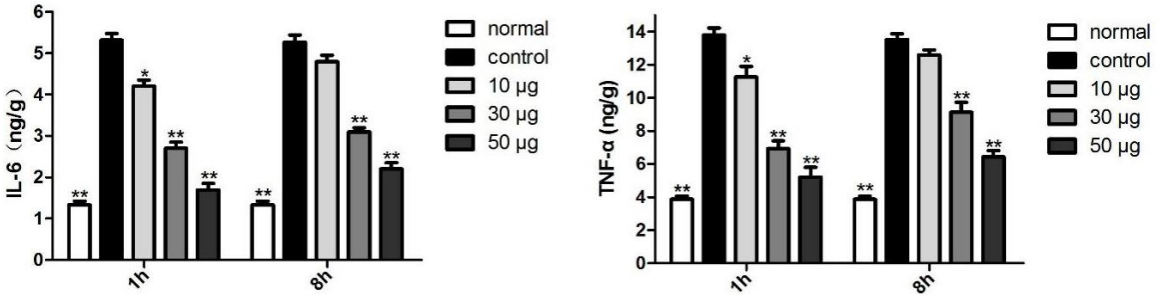

Fig. 3: The levels of IL-6 and TNF-α in mammary gland tissues. The mice infected with *S. aureus* ATCC49525 were treated with different concentrations of LysGH15 or PBS. At 48h post-infection, the levels of IL-6 and TNF-α in mammary gland tissue were detected. Bars (mean±SD) bearing *(P<0.05) or **(P<0.01) differ significantly between control and different LysGH15 doses treatment groups.

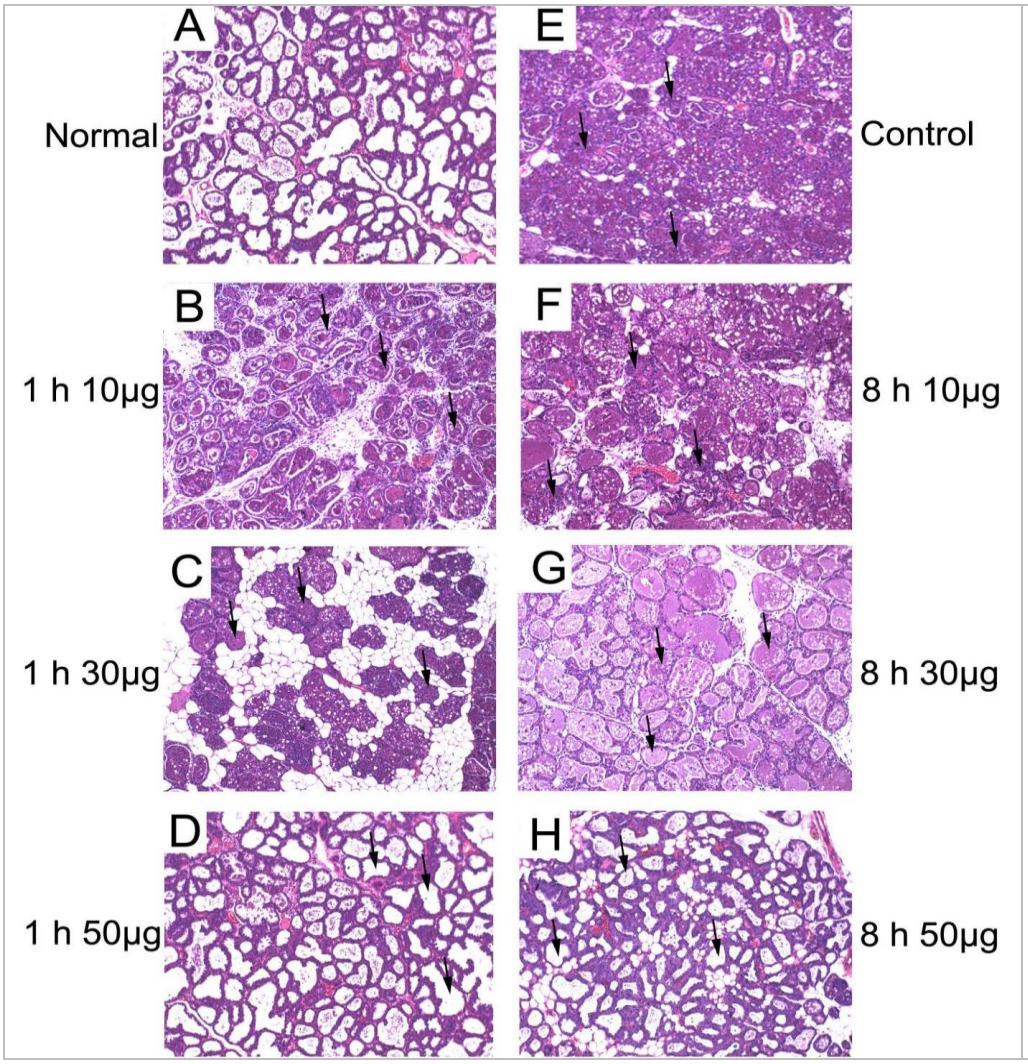

Fig. 4: Histopathology of the mammary gland tissue (100×). A) The mammary glands of the healthy mice (neither challenged nor lysin treatment). B) 10μg LysGH15 was treated at 1h post-infection. C) 30μg LysGH15 was treated at 1h post-infection. D) 50μg LysGH15 was treated at 1h post-infection. E) The mammary glands of mice suffering from mastitis (treated with PBS at post-infection). F) 10μg LysGH15 was treated at 8h post-infection. G) 30 μg LysGH15 was treated at 8h post-infection. (H) 50μg LysGH15 was treated at 8h post-infection.

DISCUSSION

In our previous study, we have found the combination of LysGH15 and apigenin (Traditional Chinese Medicine) showed a better treatment effect in a murine pneumonia model (Xia *et al.*, 2016). It is speculated that the combination of LysGH15 and apigenin will be more fruitful in the murine mastitis model (Cheng *et al*., 2018). The combination of anti-alpha-hemolysin and anti-inflammatory of apigenin and the bactericidal activity of LysGH15 could exhibit better treatment effects on the murine mastitis model than singly. On the other hand, the mastitis model established in this study is an acute infection model. The residual of *S. aureus* in feces, bedding materials, and utensils are responsible for the bovine infection (Gu *et al*., 2011). Thus, it is obligatory to carry out further studies on the mastitis prevention effect of the LysGH15. May be, LysGH15 can be used as a spray to disinfect the farm environment to reduce the bacterial load.

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Authors contribution: JG and WH conceived the idea and designed the study. YJ, FX, WZ, JD, and SL executed the experiment. XF, CS, and LL were involved in data analysis. YJ, WZ, JD, and AK wrote manuscript while critical revision and editing were carried out by JG.

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