

In Vitro Antimicrobial, Antibiofilm and Hemolytic Activity of Nisin Against *Staphylococcus aureus* Isolates from Bovine Mastitis: a Brief Report

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Short Report

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Abstract

Bovine mastitis is a major disease affecting dairy cattle, with *Staphylococcus aureus* being an important etiological agent due to antimicrobial resistance and biofilm formation. This study evaluated the antimicrobial and antibiofilm activities of nisin against 25 *S. aureus* isolates from bovine mastitis. Antimicrobial susceptibility was assessed by disk diffusion, while nisin activity was evaluated by minimum inhibitory and bactericidal concentrations, biofilm assays, and erythrocyte hemolysis. The isolates showed high resistance to β -lactam antimicrobials. Nisin exhibited *in vitro* antimicrobial activity, with variable inhibitory and bactericidal concentrations among isolates. Nisin inhibited biofilm formation and slightly reduced preformed biofilms. Hemolysis was concentration-dependent, but negligible at antibacterial concentrations. These findings indicate that nisin has promising *in vitro* antimicrobial and antibiofilm activities against *S. aureus* associated with bovine mastitis, with low hemolytic activity at antibacterial concentrations, supporting its potential as an alternative approach for controlling bovine mastitis and contributing to future effective mastitis control and prevention.

Introduction

Bovine mastitis stands out as the most frequent and economically relevant disease in dairy farming due to its direct negative impact on milk yield, composition, and quality. *S. aureus* plays a central role in bovine mastitis due to its high capacity to acquire antimicrobial resistance and to form biofilms, characteristics that favor intramammary persistence, recurrence and progression to chronic conditions (Suthovski et al. 2023).

Nisin is a lantibiotic bacteriocin that has emerged as a promising candidate for mastitis control due to its antimicrobial activity against Gram-positive pathogens, as well as its low toxicity and potential efficacy against biofilm (Bennett et al. 2021; Małaczewska and Kaczorek-Łukowska 2021; Kaczorek-Łukowska et al. 2025). However, variations in bacterial susceptibility and biofilm-forming capacity highlight the need for further investigation of nisin. Therefore, this study aimed to evaluate the toxic, antimicrobial, and antibiofilm activities of nisin against *S. aureus* isolates from bovine mastitis, contributing to the development of strategies for disease control.

Methods

2.1 Isolates:

A total of 25 isolates compatible with *S. aureus* obtained from milk samples with suspected bovine mastitis were included. The isolates were registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN), under registration number AF10531.

2.2 Preparation of the nisin stock solution:

The nisin stock solution was prepared according to the manufacturer's instructions, achieving initial concentrations of 10.000 IU/mL, 4.000 IU/mL and 1.000 IU/mL.

2.3 Disk diffusion:

Antimicrobial susceptibility testing was performed using the disk diffusion technique, according to Clinical and Laboratory Standards Institute (CLSI, 2015). The antimicrobials selected included oxacillin, penicillin, ampicillin, tetracycline, enrofloxacin, gentamicin, ciprofloxacin, Sulfamethoxazole Trimethoprim and Erythromycin. Breakpoints were interpreted according to CLSI (2023), and when mastitis-specific breakpoints for *S. aureus* were unavailable, general CLSI interpretive criteria for *S. aureus* were adopted (CLSI, 2023).

2.4 Minimal Inhibitory Concentration (MIC):

Nisin antimicrobial activity was evaluated by broth microdilution, using concentrations ranging from 5000 to 78.12 IU/mL and 2000 to 62.5 IU/mL, respectively. MHB-only and inoculated wells were used as negative and positive controls. After incubation, 15 μ L of 0.01% resazurin (R7017, Sigma-Aldrich®, Germany) was added, followed by incubation for 1 h at $35 \pm 1^\circ\text{C}$. MIC was defined as the lowest concentration remaining blue (Kumar et al. 2018). MIC₅₀ and MIC₉₀ were defined as the lowest concentrations inhibiting 50% and 90% of isolates, respectively (CLSI, 2015).

2.5 Minimum bactericidal concentration (MBC):

MBC was determined by subculturing samples from the MIC and adjacent concentrations onto agar plates. MBC was defined as the lowest concentration without visible bacterial growth. MBC₉₀ and MBC₅₀ values were defined as the lowest antimicrobial concentrations capable of killing 90% and 50% of the isolates, respectively (CLSI, 1999). An MBC/MIC ratio ≤ 4 was considered bactericidal (Pankey and Sabath 2004).

2.6 Biofilm formation, inhibition (MBIC), and eradication (MBEC):

Biofilm formation was assessed using the microplate crystal violet method. Isolates were classified as strong, moderate, weak or non-biofilm producers. For biofilm inhibition, nisin was added at the MIC to strong biofilm producer. To evaluate eradication of preformed biofilm, nisin was added after 24 h of initial incubation (Stepanovic et al. 2000).

2.7 *In vitro* toxicity test:

Nisin-induced hemolysis was evaluated following the method described by Da Paz et al. (2022), with modifications. Briefly, human erythrocytes were exposed to different nisin concentrations, ranging from 900 to 56.25 IU/mL and hemolysis was quantified by spectrophotometric measurement of the supernatant at 540 nm. The hemolysis rate was calculated according to Da Paz et al. (2022).

2.8 Statistical analysis

Antimicrobial susceptibility, MIC, MBC, and biofilm formation data were analyzed descriptively. Biofilm inhibition and eradication were compared between control and nisin-treated groups using the paired Wilcoxon test. Statistical significance was set at $p < 0.05$, and effect size was estimated using the r coefficient.

Results

High resistance to β -lactams was observed, with no isolates susceptible to oxacillin (0%; 0/6), penicillin (0%; 0/18), or ampicillin (0%; 0/17). High susceptibility was observed to enrofloxacin (91.3%; 21/23), ciprofloxacin (95.5%; 21/22), and gentamicin (100%; 18/18). Tetracycline showed 78.3% susceptibility (18/23). Sulfamethoxazole–trimethoprim showed 52.4% (11/21). Erythromycin exhibited low susceptibility (18.2%; 4/22).

Nisin inhibited all evaluated isolates. MIC values ranged from 156.25–250 IU/mL (median: 250 IU/mL; average: 238.74 ± 31.11 IU/mL), while MBC values ranged from 250–625 IU/mL (median: 500 IU/mL; average: 460.94 ± 110.29 IU/mL). MIC₅₀ and MIC₉₀ were both 250 IU/mL, whereas MBC₅₀ and MBC₉₀ were both 500 IU/mL. Most isolates (92%) had a MIC of 250 IU/mL and 68% had an MBC of 500 IU/mL. All isolates showed an MBC/MIC ratio ≤ 4 (average: 1.94), indicating bactericidal activity.

Of the 25 isolates evaluated, 80% ($n = 20$) were biofilm producers, including 7 strong, 4 moderate, and 9 weak producers. Nisin markedly reduced biofilm production in all strong biofilm-producers, ($p < 0.0001$, $r = 0.63$), indicating an inhibitory effect. In the eradication assay, nisin partially reduced preformed biofilm after 24 h in 3 isolates ($p = 0.0395$; $r = 0.1$).

Finally, nisin induced concentration-dependent hemolysis. At 900 IU/mL, hemolysis reached approximately 100%, whereas 500 IU/mL resulted in 27.1% hemolysis. Concentrations of 250 IU/mL or lower showed negligible hemolytic activity. The estimated Hemolytic Concentration 50% (HC₅₀) was 625.5 IU/mL.

Discussion

The resistance profile observed is consistent with previous reports of *S. aureus* from bovine mastitis, particularly the high resistance to β -lactams. Global analyses identify penicillin as the antimicrobial with the highest resistance prevalence in intramammary infections (Molineri et al. 2021).

Previous studies have reported variable nisin activity against pathogens associated with bovine mastitis. For *S. aureus*, MIC values for wild-type nisin A were approximately 1–2 $\mu\text{g/mL}$, while the nisin A M17Q variant showed up to fourfold greater potency (Field et al. 2021). Commercial nisin showed MICs of 1–100 $\mu\text{g/mL}$ against *S. aureus*, with MBCs consistently 1–4 times higher than MICs, indicating bactericidal activity (Bennett et al. 2021). Although our values were slightly higher, this may reflect previous antimicrobial exposure.

Studies evaluating nisin against *S. aureus* biofilms indicate that its efficacy depends on concentration, exposure time, and cell viability. In bovine mastitis isolates, nisin showed limited ability to eradicate preformed biofilms (Ceotto-Vigoder et al. 2016). Under continuous-flow conditions, nisin rapidly penetrated the biofilm matrix and damaged bacterial membranes (Godoy-Santos et al. 2019). The limited activity against mature biofilms may also result from the EPS architecture, which restricts antimicrobial diffusion.

Finally, previous studies have demonstrated a concentration-dependent hemolytic effect of nisin, with absent or low hemolysis below 50 µg/mL and nearly complete hemolysis at 400 µg/mL (Soltani et al. 2022). Similarly, nisin showed low hemolytic activity (6.6%) at 40 µM (~134 µg/mL) (Paiva et al. 2012). In the present study, hemolysis was also concentration-dependent, with negligible activity at antibacterial concentrations and an estimated HC₅₀ of 625.5 IU/mL, suggesting a preliminary therapeutic window.

Conclusion

Nisin showed *in vitro* bactericidal activity against *S. aureus* from bovine mastitis and inhibited biofilm formation, with negligible hemolytic activity at antibacterial concentrations. These findings support its potential as an alternative or adjunctive strategy for controlling *S. aureus*-associated bovine mastitis, particularly in early infection.

Declarations

Author Contribution

Bruna Alves Ottobeli: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Visualization, Writing – original draft, Writing – review & editing. Naiara Vitoria Ferreira Cortes Koprovski: Investigation, Methodology, Writing – review & editing. Denis Aurelio Lopes de Oliveira: Investigation, Methodology, Writing – review & editing. Juliana Rozendo Barbosa: Investigation, Methodology, Writing – review & editing. André Marcos Dezan Bieniek: Investigation, Methodology, Writing – review & editing. Vitória Karolini Betim Fieldkircher Caus: Investigation, Methodology, Writing – review & editing. Edineia Paula Sartori Schmitz: Methodology, Writing – review & editing. Maiara Garcia Blagitz: Resources, Writing – review & editing. Karina Ramirez Starikoff: Supervision, Writing – review & editing. Adalgiza Pinto Neto: Resources, Writing – review & editing. Letícia Trevisan Gressler: Supervision, Writing – review & editing. Dalila Moter Benvegnú: Conceptualization, Formal analysis, Visualization, Supervision, Project administration, Funding acquisition, Writing – review & editing.

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