Efficacy of lacticin NK34 against bovine intramammary infections caused by *Staphylococcus aureus* and *Streptococcus agalactiae*

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**Abstract:** Lacticin NK34 is a small nisin-like bacteriocin present in the supernatants of an isolate of *Lactococcus lactis* from *jeotgal*, a salted and fermented Korean food made with seafood such as shrimp, oysters, and fish. Recently, we demonstrated that a partially purified NK34 is highly effective against various *Staphylococcus* species in a murine infection model. In this study, the two major bacterial pathogens associated with bovine mastitis, *Streptococcus aureus* and *S. agalactiae*, were evaluated for their susceptibility to NK34 in vitro using a standard teat-dip assay as well as in vivo using mastitic cows infected with one of these pathogenic strains. The experimental analyses showed a significant decrease (up to 98 times) in the bacterial numbers between the NK34-treated and -untreated teats. Moreover, a dramatic reduction in somatic cell counts was observed at 3 days post-treatment with 10 ml of NK34 administered directly into the mastitic cows. Neither *S. aureus* nor *S. agalactiae* were recovered. Taken together, these results imply that lacticin NK34 is an alternative antimicrobial substitute for the treatment of bovine mastitis, caused especially by either *S. aureus* or *S. agalactiae*.

**Key words:** *Lactococcus lactis*, lacticin NK34, bacteriocin, antimicrobial substitute, bovine mastitis

INTRODUCTION

Bovine mastitis is one of the most common and devastating diseases in many countries [14]. Technical and scientific developments have not been able to eradicate the disease from the world. One third of all cows are believed to have mastitis in at least one of the breasts; however, most farmers are unaware of the prevalence of this disease [12, 25]. The economic loss due to bovine mastitis has been estimated to be about $1.8 billion per year in the United States [26] due to reduced milk production, degraded milk quality, increased replacement costs, discarded milk, drug costs, veterinary fees, and labor costs for the farmers [6, 10].

Various management programs for bovine mastitis have contributed to a reduction in the incidences of intramammary infections in cows in recent years [29]. However, the modern methods for the control, therapy, and prophylaxis of bovine mastitis are still focused on the usage of antimicrobial agents. Unfortunately, this method is sometimes ineffective in the case of chronic infections [28], and can promote bacterial resistance to antimicrobials [17, 19, 20]. In addition, the amount of antimicrobials needed for bovine mastitis can inhibit the growth of lactic streptococci, which is essential in cheese- or yogurt-making processes. Thus, it is highly possible that the antimicrobials may remain in the food chain, which is a threat to consumers [4, 9]. Therefore, alternative antibiotics based on naturally occurring substances with antimicrobial activities such as bacteriocin, bacteriophages, probiotics, and antimicrobial peptides have been considered as antimicrobial substitutes [7, 27]. The advantages of these substitutes include their high or equivalent antimicrobial activities as well as non-toxicity to humans. For example, nisin has been approved as a natural food preservative in many countries around the world [1].

Lacticin NK34, a nisin-like bacteriocin previously known as a product of *Lactococcus lactis* isolated from *jeotgal* (a salted and fermented Korean food made with seafood), has been characterized and has demonstrated antimicrobial
activity against various bacterial spp., yeasts, and molds in our previous study [16]. In addition, NK34 has been applied in vivo to a murine infection model with various strains of Staphylococcus spp. [12]. Although it is well known that over 150 different contagious or environmental microorganisms are associated with bovine mastitis [15], we aimed to evaluate the antimicrobial effect of NK34 against the two major bacteria species responsible for contagious bovine mastitis: S. aureus and S. agalactiae [3, 8] both in vitro and in vivo bovine model systems.

**MATERIALS AND METHODS**

Preparation of lacticin NK34

The preparation of the Lactococcus lactis-produced bacteriocin NK34 was carried out according to the method of Kim et al. [12].

Preparation of the indicator strains

Milk samples were collected by herd owners or personnel from several dairy farms in Korea. Samples with somatic cell counts (SCCs) greater than 5 × 10⁵ cells/mL (determined using a Bentley Soma count 150 [Bentley Instrument; Chaska, MN]) were suspected to be from cows with mastitis.

Bacterial examination of milk samples was conducted with standard laboratory techniques [21]. All the selected samples were streaked onto 5% blood agar plates (Komed; Sungnam, Korea) and incubated at 37°C for 18–24 hrs. Examination of colony morphology and Gram staining were performed for the primary identification of Staphylococcus spp. or Streptococcus spp. and further confirmed by the Vitek II System (BioMerieux; Durham, NC) [19].

**Determination of the antimicrobial effect of NK34 against bovine mastitis in vitro using a teat dip assay**

For the teat dip experiment in vitro, 12 teats were collected from slaughtered cows. They were immediately transported in a frozen state to the laboratory. Preparation consisted of thawing in water, removal of excess skin and tissue, washing in mild detergent solution, rinsing in water, drying, dipping in 70% ethanol, and singeing [22].

For the experiment, the teats were divided into two groups of 6 each. They were dipped once into a depth of 15 mm of trypticase soy broth (Becton Dickinson and Co; Sparks, MD) containing S. aureus or S. agalactiae in a concentration of 1 × 10⁶ CFU/ml for 12 min and then dried for 5 min. Next, only 5 teats from each group were dipped into a depth of 20 mm of lactic in NK34 solution (6,150 AU/mL) for 10 min. The remaining one teat from each group was used as a positive-control. Following the contact of the challenge organisms, the teats were dipped into a depth of 20 mm of aquncher solution (Maximum Recovery Diluent, MRD) for 10 min [14] (Fig. 1). 200 μL of diluted MRD was plated on 3 M petrifilm plates (3 M; St. Paul, MN) and incubated for 24 hat 37°C. Since the positive control plates resulted in too many colonies to count, the half-logarithmic dilution was performed once with the recovery solution. In addition, the MDR was streaked on 5% blood agar plates (Komed; Sungnam, Korea) to check for contamination.

**Determination of the antimicrobial effect of NK34 in the mastitic cows**

To evaluate the in vivo effectiveness of NK34 in the mastitic cows, bovine raw milk samples were collected by herd owners or personnel from several dairy farms in Gyeonggi province, South Korea, from cows with SCCs higher than 3.5 × 10⁵ cells/mL, an indicator of subclinical and clinical mastitis. All these samples were then examined for the gram-positive S. aureus or S. agalactiae by culturing on 5% blood agar plates at 37°C for 24 hrs. The suspected colonies were further confirmed by the Vitek II bacterial identification System (Bio Merieux; Durham, NC). According to the previously established antimicrobial treatment scheme [29], 3, 5 and 10 ml of lacticin NK34 (6,150 AU/mL) were injected once a day for 3 days using a syringe after milking during the evening when the papillary pores were opened. The raw milk samples were then analyzed for SCCs and the

![Fig. 1. Schematic presentation of the in vitro bovine teat dip assay with purified lacticin NK34.](image-url)
bacterial pathogens examined during the following week (Fig. 2).

RESULTS

In vitro antimicrobial effect of NK34 determined by teat dip assay

The comparative experimental results between the NK34-treated and the un-treated groups showed that the NK34 treatment in the groups with *S. aureus* or *S. agalactiae* reduced the numbers of the challenged bacteria by 16 to 98 times and 17 to 36 times, respectively, compared to those of the un-treated groups (Fig 3). There was no visible contamination by other bacteria species observed in this study (data not shown).

*In vivo* antimicrobial effect of NK34 against the mastitic cows with *S. aureus* or *S. agalactiae*

Our SCC analyses of all the raw milk samples from the teats of the subclinical (having SCCs of $3.5 \times 10^5$ cells/mL) or clinical (having SCCs of $5 \times 10^5$ cells/mL) mastitic cows with *S. aureus* or *S. agalactiae* demonstrated an increase in SCCs until the 3rd (in the groups treated with 5 or 10 mL of NK34) or 4th (in the groups treated with 3 mL of NK34) day post-treatment with NK34 (Fig. 4). However, after reaching a peak, the SCCs were reduced in a dose-dependent manner after lacticin NK34 was injected (Fig. 4). Indeed, about 39.5% of the SCCs were decreased in the groups treated with 10 mL of NK34 on the 7th day post-treatment, compared to the 3rd day post-treatment (Fig. 4). Similar results were observed in the other groups (Fig. 4).

Antimicrobial activity of NK34 on other groups and negative bacterial pathogens

The standard spot-on-lawn assay was performed to determine the antimicrobial activity of lacticin NK34 against various gram-positive bacteria including: *S. epidermidis* (n=20), *S. pseudintermedius* (n=48), *Enterococcus faecium* (n=20), *E. faecalis* (n=18), *Listeria monocytogenes* (n=33), *Streptococcus* spp. (excluding *S. agalactiae* n=34), *Clostridium perfringens* (n=10), and *Bacillus cereus* (n=20). The assay was also performed on Gram-negative bacteria including: *Enterobacter sakazakii* (n=4), *Escherichia coli* (n=4), *Salmonella* spp. (n=4), *Shigella* spp. (n=4), *Vibrio cholera* (n=4), *Pseudomonas* spp. (n=4), *Aeromonas* spp. (n=4), *Klebsiella* spp. (n=4), and *Actinobacillus* spp. (n=4). The bacteria were collected from raw milk samples, small animals, chickens and racing horses [12]. Our results demonstrated that lacticin NK34 was significantly effective against most gram-positive bacteria tested (Table 1) but not against all the gram-negative bacteria evaluated in this study (data not shown).

DISCUSSION

Bovine mastitis is a serious cow disease that can cause considerable economic damage to dairy farms [5]. The
Table 1. Antibacterial activity of NK34 to various Gram-positive bacterial pathogens

<table>
<thead>
<tr>
<th>Gram positive strain</th>
<th>Remarks</th>
<th>Number of sensitive* and tested strains (percent, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Bacillus cereus</td>
<td>Strains isolated from bovine mastitis milk and racing horses</td>
<td>20/20 (100)</td>
</tr>
<tr>
<td>2 Clostridium perfringens</td>
<td>Strains isolated from pigs</td>
<td>10/10 (100)</td>
</tr>
<tr>
<td>3 Enterococcus faecalis</td>
<td>Strains isolated from chickens</td>
<td>18/18 (100)</td>
</tr>
<tr>
<td>4 Enterococcus faecium</td>
<td>Strains isolated from chickens (VRE included)</td>
<td>20/20 (100)</td>
</tr>
<tr>
<td>5 Listeria monocytogenes</td>
<td>Strains isolated from chickens, reference strains</td>
<td>29/33 (87.9)</td>
</tr>
<tr>
<td>6 Staphylococcus epidermidis</td>
<td>Strains isolated from small animals and bovine mastitis milk</td>
<td>16/20 (80)</td>
</tr>
<tr>
<td>7 Staphylococcus pseudintermedius</td>
<td>Strains isolated from small animals (MRSI included)</td>
<td>47/48 (97.9)</td>
</tr>
<tr>
<td>8 Streptococcus spp. (except S. agalactiae)</td>
<td>Strains isolated from bovine mastitis milk</td>
<td>22/34 (64.7)</td>
</tr>
</tbody>
</table>

* Sensitive strains were determined as 3,200 AU/mL or less.

damages caused by the disease have been estimated to be twice as much as the damages due to infertility or genital diseases [24]. The economic loss caused by bovine mastitis can be attributed to treatment costs, medicine costs, the disposal of infected cows, and discarded milk from cows on medication [25]. However, many farmers are not fully aware of the prevalence of the disease [18], especially in the case of subclinical mastitis, because there are no visible clinical signs, just an elevated SCC in the milk. Therefore, most dairy farmers are not even aware of the pervasiveness of subclinical mastitis among their own livestock [13].

*S. aureus* is one of the major contagious pathogens responsible for clinical and subclinical infections in lactating cows [11]. The microorganism usually spreads through infected milk, bacterial colonization in a teat canal, or wounds in teats [2]. This forms micro-abscesses in breasts which are protected by fiber tissues, making them difficult to treat with antibiotics [23]. *S. agalactiae* is known to be another bacterial agent mainly involved in the disease [3].

The in vivo teat dip technique with NK34 revealed its effectiveness against two major contagious bacterial pathogens significantly involved with bovine mastitis: *S. aureus* and *S. agalactiae*. Compared to the positive control, the number of *S. aureus* and *S. agalactiae* colonies recovered from the petrifilm plates was reduced up to 98 and 36 times, respectively. Using these findings, the in vivo effectiveness was determined with mastitic cows carrying either *S. aureus* or *S. agalactiae* with SCCs higher than $3.5 \times 10^{5}$ cells/mL (see Materials and Methods). The results clearly demonstrated that the amount of NK34 injected plays an important role in the duration of pathogen recovery. This demonstrates the potential use of lacticin NK34 on the dairy farm as an alternative natural substitute for antimicrobials commonly used. The use of teat dips in NK34 is considered to be an essential element for on-farm practice and contributes to both good milk quality and improved animal health [14]. The safety of lacticin NK34 has already been proven in our previous study [12].

It is known that bacteriocins display different antimicrobial activities as well as various durations of protection depending on the type of microorganism [14]. Our screening revealed that lacticin NK34 displays antibacterial activity against most gram-positive bacterial pathogens but not the Gram-negative strains tested in this study. This finding suggests that NK34 may be effective on meticillin-resistant *S. intermedius* and vancomycin-resistant enterococci.

In conclusion, lacticin NK34, a small nisin-like bacteriocin present in the supernatant of an isolate of *Lactococcus lactis* from *jeotgal*, a salted and fermented Korean food made with seafood, has a strong antimicrobial activity against gram-positive bacteria. Thus, NK34 might be used not only for treatment of gram-positive bacterial infections such as bovine mastitis but also to prevent the propagation of those bacterial pathogens on farms.

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