The Natural Food Grade Inhibitor, Lacticin 3147, Reduced the Incidence of Mastitis After Experimental Challenge with *Streptococcus dysgalactiae* in Nonlactating Dairy Cows

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ABSTRACT

Lacticin 3147 is a broad-spectrum bacteriocin produced by the food-grade organism *Lactococcus lactis*. Lacticin 3147 is active at a neutral pH and has been shown to be bactericidal to streptococci and staphylococci in vitro. The effectiveness of an intramammary teat seal formulation, and a teat seal containing lacticin 3147 was evaluated at drying off in 68 uninfected quarters of 18 cows. Following infusion of either teat seal or lacticin 3147 combined with teat seal, a deliberate infection challenge of *Streptococcus dysgalactiae* (≅1.5 × 10⁴ cfu per teat) was administered by direct inoculation into the teat sinus. During an 8-d experimental period following inoculation, 61% of control quarters and 6% of the treatment quarters either developed clinical mastitis or were shedding the challenge organism. Randomly amplified polymorphic DNA polymerase chain reaction genetic typing was used to confirm that both the new infections and the bacteria surviving in the teats at the end of the experiment were the challenge strain. The combination of teat seal and lacticin 3147 was well tolerated within the udder and elicited only a temporary increase in somatic cell count to 5.7 × 10⁵/ml (88 h after infusion) in a previously uninfected lactating udder quarter. Therefore, we concluded that this nonantibiotic approach to mastitis prevention may contribute to a reduction in the routine application of antibiotics at drying off in the future.

(Dairy cows are particularly susceptible to mastitis during the nonlactating period. For this reason, long-acting antibiotics (dry cow therapy) are routinely used for curing existing subclinical infections and for preventing new infections during the dry period (2, 7, 16, 18). Some researchers (4, 5, 15, 25) have recommended that dry cow antibiotic therapy should not be used as a routine prophylactic measure but rather restricted to treatment of infected cows. The continued use of antibiotics in the dry period for either therapeutic or prophylactic purposes has some disadvantages, including the perceived connection to the emergence of antibiotic-resistant human pathogens. Such concerns may lead to restrictions on antibiotic therapies for animal applications in the future. As a result, there is now an increased interest in alternative approaches to the prevention of intramammary infections, particularly in dry cows. Some of these alternatives include the use of internal teat sealers (12, 13, 17, 28) and bacteriocins (20, 22, 23, 24).

The bacteriocin nisin was first identified in 1928 by Rogers and Whittier (19), and early attempts to find a practical application for nisin included the treatment of bovine mastitis. In 1946, Mattick and Hirsch (10) showed that a nisin-producing strain of *Lactococcus lactis* was inhibitory to tubercle bacilli. Trials carried out by Taylor et al. (27) indicated that a single intramammary infusion of nisin was effective in treating both streptococcal and staphylococcal infections. However, infusions of the nisin preparation into the udder produced an adverse cellular response, although there was no correlation between the nisin concentration and local intolerance produced in the udder. Further experiments carried out by Bavin et al. (1) showed that nisin activity on a weight basis was less than that of penicillin against Gram-positive bacteria and less than that of streptomycin against *Mycobacterium tuberculosis*. Interest in the use of nisin as a therapeutic agent was renewed in 1989, when Broadbent et al. (3) showed that nisin was inhibitory to several Gram-positive, mastitis-causing pathogens. The practical use of nisin was inves-
Investigated by Sears et al. (23, 24) when nisin, in combination with lysostaphin, was administered by intramammary infusions. Promising results were obtained; cure rates were 66% for *Staphylococcus aureus*, 95% for *Streptococcus agalactiae*, and 100% for *Streptococcus uberis*. Nisin has since found application in two products that are currently in use for the prevention of mastitis.

Recently, it has been demonstrated that lactacin 3147, a novel bacteriocin produced by *Lactococcus lactis* ssp. *lactis* DPC3147 is also effective against a wide range of Gram-positive bacteria, including many mastitis-causing pathogens (20, 21). In contrast to nisin, however, lactacin 3147 is effective over a wide pH range, including physiological pH. The bacteriocin is composed of two subunits and is encoded on a large conjugative plasmid, pMRC01, which has recently been sequenced (6). Lactacin 3147 has a bactericidal mode of action that is elicited through the selective dissipation of the cell membrane potential of susceptible cells by the formation of K⁺-specific pores (11). In a previous study (20), a teat seal was formulated containing lactacin 3147, which exhibited very effective antimicrobial action against mastitis-causing pathogens.

The objective of the present study was to evaluate the efficacy of this teat seal containing lactacin 3147 as a means of preventing artificially induced *Streptococcus dysgalactiae* mastitis in nonlactating cows.

**MATERIALS AND METHODS**

**Teat Seal and Lacticin 3147 Formulation**

Osmonds Teat Seal 2 (Cross Vetpharm Group Ltd., Dublin, Ireland) is a commercial, oil-based intramammary formulation containing bismuth subnitrate and is used for the treatment of cows at drying off. A laboratory-scale batch of this teat seal product (Osmonds Teat Seal Non-Commercial Batch), similar to that evaluated by Woolford et al. (28) was used in this experiment. The seal was presented as a 3.5-g fill in a plastic syringe, which is similar in type to those used for standard intramammary antibiotic applications.

Lactacin 3147 was prepared as follows with a modification of the method already described by Ryan et al. (20). *Lactococcus lactis* DPC3147 was propagated in tryptone yeast extract (TY) broth [2.5 g/L of tryptone, 5 g/L of yeast extract, 10 g/L of glucose, 19 g/L of β-glycerophosphate, 0.25 g/L of MgSO₄·7H₂O, and 0.05 g/L of MnSO₄·4H₂O (pH 6.75) overnight at 30°C]. The medium had been previously cleared of contaminating proteins by passing it through 500 g of XAD-16 beads (Sigma-Aldrich Co. Ltd., Dorset, England) to which hydrophobic proteins bind. Following incubation, the culture was centrifuged at 10,000 × g to remove the bacteriocin-producing cells. The supernatant was then passed through 500 g of XAD-16 beads at a flow rate of approximately 15 ml/min, allowing the bacteriocin to bind. The column was subsequently washed with 40% ethanol, and the bacteriocin eluted with 70% propan-2-ol (10 mM acetic acid; pH 2). The propan-2-ol was removed by rotary evaporation with a Buchi apparatus (Buchi AG, Flawil, Switzerland), and the resulting bacteriocin-containing solution was dialyzed against 2 mM sodium phosphate buffer (pH 7). Following dialysis, the solution was lyophilized, and the resulting powder was resuspended in 5 ml of sterile distilled water. Tween 80 (Merck, Darmstadt, Germany) was then added to a final concentration of 2%. The activity of the solution containing lactacin 3147 was determined by using the critical dilution assay and expressed as arbitrary units (AU) per milliliter, as described by Ryan et al. (21). Subsequently, 0.25 ml of the lactacin solution (containing ~20,000 AU) was mixed with each of 3.5 g of seal to form an emulsion, which was then packed in a syringe. The Tween 80 was included to facilitate the release of the lactacin from the seal (20).

Bacteriocin activity in the teat seal was assayed by the agar well diffusion method as follows. Mueller-Hinton agar (Merck, Darmstadt, Germany) was seeded with *Strep. dysgalactiae* (laboratory reference code M), and wells (4.6 mm in diameter) were cut in the agar after it had solidified in standard 90-mm petri dishes. Aliquots (50 mg) of teat seal plus lactacin preparations were carefully dispensed into the wells so that the seals were in contact with the wall of the wells. Activity was observed as a clear zone of inhibition around the wells. In total, 35 seals containing lactacin were prepared and tested for antimicrobial activity before infusion.

**Tissue Tolerance Study**

Two lactating cows with SCC in individual quarters of ≤100,000/ml were used to assess the tolerance of the seal preparations in udder tissue. One teat of the first cow was infused with teat seal alone, and a second teat was infused with the seal plus lactacin 3147 formulation. For the second cow, one teat was infused with a commercial lactating cow antibiotic product containing 200 mg of sodium cloxacillin as a positive control, and a second teat was not infused (negative control). The antibiotic was used for comparison against the seal formulations. All infusions were administered after the evening milking. The seal preparations were removed before the next milking 16 h later. Somatic cells were counted using a Somacount 300™ (Bentley Instruments Inc., Chaska, MN) for milk samples collected from the treated and control quarters at every milking for 5 consecutive d after infusion.
Efficacy Study

Sixty-eight uninfected udder quarters were selected from 18 cows. Quarters were defined as uninfected at drying off if (1) they were free of pathogens in each of two foremilk samples collected 5 to 8 d apart and (2) no clinical mastitis was seen in either the udder or the foremilk. Cows were also selected free of teat skin lesions. After the last milking of the lactation, 33 teats were infused with seal, and 35 were infused with seal plus lactacin 3147. Four teats were not infused due to an insufficient supply of sealing material. Within-cow treatment comparisons were made using treatment pairs selected at random of either the right front and right hind or left front and left hind teats.

Challenge Culture and Inoculation Procedure

Three days after infusion, the 68 treated teats were inoculated with Strep. dysgalactiae M using approximately 1.5 × 10⁶ cfu per teat. Streptococcus dysgalactiae M was selected as the challenge organism because previous in vitro studies showed that lactacin 3147 was effective against this pathogen (14, 20). This isolate, classified as Strep. dysgalactiae ssp. dysgalactiae by SDS-PAGE total protein profiling (BCCM™ Culture Collection; Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium) was previously recovered from a case of clinical mastitis and had been preserved in a microbiological bead storage system (Protect; bacterial preservers; Technical Service Consultants Ltd., Lancashire, England) prior to the start of this study.

At the beginning of the experiment, one bead was removed from a storage vial, streaked on the surface of an aesculin blood agar plate (ABA), and incubated for 16 h at 37°C. The ABA was prepared from blood agar base #2 (Lab M, Bury, England) to which 0.1% aesculin and 7% citrated whole calf blood were added. Two hundred fifty milliliters of brain heart infusion broth (Oxoid Ltd., Hampshire, England) were inoculated with the Strep. dysgalactiae M culture and incubated at 37°C for 8 h. A total bacterial count was carried out on the 8-h stock, which was then diluted to produce a working concentration of 1.5 × 10⁶ cfu/ml in 10% sterile antibiotic-free skim milk. A 0.1-ml aliquot of skim milk containing 1.5 × 10⁴ cfu of streptococci was inoculated into treated and control udder quarters via the teat canal and deposited into the teat sinus at a depth of 17 mm from the tip of the teat. This experimental technique was used primarily to demonstrate the combined effect of the lactacin 3147 with the protection afforded by the teat seal alone.

Isolation of Pathogens from Udder Quarters

Samples of secretions (0.1 ml) collected from clinically affected udder quarters were streaked on the surface of ABA plates and incubated aerobically at 37°C for 24 h. The milk or dry period secretion samples collected from the four quarters of each cow (at the beginning and at the end of the experiment) were streaked on separate quadrants of a single ABA plate.

Differentiation of Mastitis-Causing Pathogens by Polymerase Chain Reaction

Pathogens isolated from udder quarters during the course of the experiment were typed by DNA fingerprinting using the technique outlined subsequently. Genomic DNA were isolated from the challenge strain, Strep. dysgalactiae M and from teat isolates by a modification of Hoffman and Winston as described by Gardiner et al. (8). Amplification of DNA was performed with a Perkin-Elmer DNA thermal cycler (Perkin-Elmer, Norwalk CT). Taq DNA polymerase was added during the first temperature cycle (hot start), and DNA was amplified for 35 cycles. Each cycle involved 1-min denaturation at 93°C, followed by an annealing step at 44°C for 1-min and an extension step at 72°C for 1 min. Of the final mixture, 10% was analyzed on 1.5% (wt/vol) agarose (Sigma Chemical Co. St. Louis, MO) gels with ethidium bromide staining. The arbitrary primer used had the sequence 5′ ATGTAAACGCC 3′.

Diagnosis of Clinical Mastitis

Following challenge, the cows were observed twice daily (morning and evening) for signs of clinical mastitis (swelling, hardness, heat, and pain). These observations were conducted blind; workers had no knowledge of test and control quarters. In all cases, quarters were allowed to develop definite clinical signs before a sample of secretion was taken for analysis. Inspection of the secretion from the teat to detect abnormality before collecting a sample for analysis was not an option in this study, since this would entail removal of the teat seal from the suspect quarter. The seals were removed from clinically affected quarters by hand-stripping, and samples of secretions, containing seal material were collected aseptically for microbiological analysis. Secretions containing clotted material and shedding mastitic pathogens were classified as clinical. Infected quarters were treated with intramammary antibiotics (indicated for use in lactating cows) until the clinical signs disappeared and quarters returned to normal. On the last day of the experiment, (8 d after inoculation), samples of secretion, which also contained recovered teat seals were collected both for microbiological analysis and also
to measure the persistence of the seal in the remaining uninfected quarters. All inoculated teats were infused with long-acting (dry cow) intramammary antibiotics at the end of the trial to eliminate possible residual contamination associated with the challenge strain. The presence of *Strep. dysgalactiae* in nonclinical samples of secretion collected at the end of the experiment were recorded as bacteria surviving in the teats as a result of the bacterial challenge. Representative isolates were compared with the challenge *Strep. dysgalactiae* M strain using randomly amplified polymorphic DNA polymerase chain reaction (RAPD PCR) analysis.

**Statistical Analysis**

The difference between the number of new infections between treatments was examined using the chi-square test (26). The Cochran matched-pairs test (26) was used to detect differences between treatment pairs of either the right front and left front quarters or in the right hind and left hind quarters. Time to “survival” distribution analysis was also carried out between the two treatment groups using the Cox-Mantel Test (9).

**RESULTS**

**Seal and Lacticin Formulation**

Overall, the 35 seals containing lacticin 3147 displayed effective antimicrobial action with mean diameters of the zones of inhibition of 8.3 ± 2.4 mm.

In general, the seal plus lacticin formulation was well tolerated within the udder; SCC peaked at 88 h after infusion at $7.22 \times 10^5$ and $5.71 \times 10^5$/ml for quarters treated with seal and seal plus lacticin 3147, respectively. Similar SCC trends were shown in a second cow; a maximum level of $1.008 \times 10^6$/ml was observed in the quarter infused with cloxacillin, and $6.27 \times 10^5$/ml was observed in the untreated control. The SCC data for the four quarters are presented in Figure 1. Overall, SCC trends were similar in the quarters infused with either the seal alone or with the seal plus lacticin 3147. Slightly higher SCC developed in the quarter treated with cloxacillin. Also, clinical abnormality in any of the treated quarters was not evident when observed twice each day at milking over 5 consecutive d.

**Differentiation of Mastitis-Causing Pathogens by Genetic Fingerprinting**

To aid the accurate identification of the challenge organism used in the trial, RAPD PCR fingerprints were generated with a random primer. This approach was found to be very useful for discriminating between different teat isolates, which exhibited typical morphology of *Strep. dysgalactiae* on blood agar plates, i.e., small colonies with a narrow green zone of pale discoloration. An example of this is given in Figure 2, where the genetic fingerprints of three such wild-type isolates taken from cows with mastitis (lanes 5, 6, and 7) are clearly distinct from that of the M strain (lane M). As such, this method of differentiating was chosen as a means of identifying the challenge organism throughout the course of the challenge trial.

**Trial Comparing Teat Seal Versus Teat Seal Combined with Lacticin**

In all, 16 clinical cases of mastitis developed in the sealed quarters compared with 3 in the quarters infused with lacticin 3147.
TABLE 1. Clinical mastitis and bacterial recoveries after challenge with *Streptococcus dysgalactiae* in quarters treated with either seal or seal plus lacticin 3147.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Quarters (no.)</th>
<th>Clinical infections</th>
<th><em>Strep. dysgalactiae</em> nonclinical recoveries (d-8)</th>
<th>Clinical infections and recoveries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seal</td>
<td>33</td>
<td>16 (48.5%)</td>
<td>6</td>
<td>22 (66.6%)</td>
</tr>
<tr>
<td>Seal plus lacticin</td>
<td>35</td>
<td>3 (8.6%)</td>
<td>0</td>
<td>3 (8.6%)</td>
</tr>
</tbody>
</table>

Two of the infections were caused by *Staphylococcus aureus*.

One infection was not caused by challenge with *Streptococcus dysgalactiae* M.

with seal plus lacticin 3147 (Table 1). Subsequently, *Strep. dysgalactiae* was isolated from 17 of these infected quarters (14 in sealed teats and 3 in the teats treated with seal plus lacticin 3147). *Staphylococcus aureus* was isolated from the 2 remaining infected quarters treated with the teat seal alone. When all remaining nonclinical quarters were sampled at the end of the experiment, *Strep. dysgalactiae* was recovered from 6 quarters that had been treated with seal alone but not from the quarters treated with seal and lacticin 3147 (Table 1). The daily incidence of new clinical infection caused by the challenge strain is presented for each treatment group in Figure 3. Of the 14 teats containing seal only that became infected with the challenge strain, 12 (86%) had developed clinical mastitis by d 2 following the bacterial challenge. The first clinical case of mastitis in the quarters treated with seal plus lacticin 3147, however, was detected 4 d after the introduction of the challenge.

Randomly amplified polymorphic DNA polymerase chain reaction profiles for 14 of the *Strep. dysgalactiae* isolates recovered from the quarters treated with seal and 2 of the *Strep. dysgalactiae* isolates recovered from quarters treated with seal plus lacticin 3147 confirmed that the infections were caused by the challenge strain. An example of the DNA profiles generated from these isolates is shown in Figure 2 (lanes 1, 2, 3, and 4). One of the 3 *Strep. dysgalactiae* isolates recovered from the quarters treated with seal plus lacticin 3147, however, was different from the challenge strain. Combining the information obtained from these analyses, it was shown that there were 14 clinical cases of mastitis caused by *Strep. dysgalactiae* M in sealed quarters, and only 2 cases in the quarters treated with seal containing lacticin 3147. The difference in new infection caused by the challenge strain between the treatments was significant (*P* < 0.001). When the Cochran-matched pairs test was applied to these data there was also a significant difference between treatments (*P* < 0.001). Time to survival distribution analysis between the rate of occurrence of each clinical event again showed that the treatments were different (*P* < 0.001).

The strain recovered from the 6 nonclinical sealed quarters were also confirmed to be the challenge strain. In contrast, there were no surviving bacteria at the end of the experiment in the 32 remaining uninfected quarters that had been infused with seal plus lacticin 3147. Combining these results with the clinical data, 20 sealed quarters and 2 quarters treated with seal containing lacticin 3147 were either clinical or shedding the challenge strain during the experiment (Table 1). Again, this difference was significant (*P* < 0.001). Thus, 94% of teats treated with seal plus lacticin were protected from infection with the challenge strain.

Recovery of Teat Seal

Seals were recovered from all of the 33 quarters infused with seal alone and from 33 of the 35 quarters infused with seal plus lacticin 3147. The 2 quarters that did not have seals present at the end of the experiment did not become infected and did not contain the challenge organism at the final sampling.

DISCUSSION

The generation of nonantibiotic formulations for the treatment and prevention of mastitis in cows has the potential to reduce the veterinary dependence on anti-
bacteriocins in the control of this persistent and costly disease. The trials reported in this study provide convincing evidence that the novel combination of lacticin 3147 and a bismuth-based teat seal offers very effective protection against a significant challenge with mastitic Strep. dysgalactiae. Moreover, the challenge organism could not be isolated from nonclinical cases in which the bacteriocin was present during the 8-d trial period.

The choice of the teat seal as a delivery vehicle for lacticin 3147 has a number of obvious advantages. The teat seal itself has been proven to provide an effective barrier against infection in a large-scale animal trial in New Zealand (28). That study showed that its use was as effective as a long-acting antibiotic containing 250 mg of cephalonium in preventing naturally occurring infections in 528 dairy cows that had been selected as noninfected at drying off. In the New Zealand study (28), however, the principle pathogen was Strep. uberis and the incidence of other mastitis-causing pathogens was too low to make valid comparisons. One of the main advantages in combining the seal with a broad-spectrum bacteriocin is that, in addition to the barrier protection of the seal, the seal also localizes the inhibitor in the teat sinus. This localization of bacteriocin activity should also mean that less bacteriocin will be required to prevent infection.

Although previous laboratory experiments demonstrated that lacticin 3147 (in the presence of the surfactant, 2% Tween 80) was successfully released from teat seal and could inhibit Strep. dysgalactiae seeded in an agar plate, it was necessary to establish its efficacy in vivo. Preliminary studies on the acceptance of the seal plus lacticin 3147 formulation in udder tissue of the lactating cow showed that the combination was well tolerated and also that the short-term increase in SCC was less than the SCC increase elicited after the administration of a standard commercial intramammary antibiotic formulation. These data also confirm results obtained previously when the teat seal was again found to be nonirritating (20). In addition, clinical observations in the nonlactating cows indicated that there were no adverse clinical signs in the udder quarters that remained free of clinical mastitis during the 8-d trial period or at the next calving. Hence, it can be concluded that the lacticin 3147 preparation used in this study was a nonirritant in the cows, an important finding given that some of the earlier work with nisin was abandoned for mastitis treatment because of adverse reactions in cows, which were attributed to impure preparations of the bacteriocin (27). A significant outcome of our trials is the in vivo evidence of the ability of lacticin 3147 to retain suitable activity in the teat environment during the 8-d trial period.

The efficacy of the teat seal containing lacticin 3147 could only be assessed by artificial challenges in a model that would ensure the partial failure of the teat seal alone. The impact of the challenge can be manipulated by increasing either the number of cfu in the challenge culture or by increasing the depth to which the bacteria were introduced into the teats, or by a combination of both methods. Previous experiments (W. J. Meaney et al., unpublished, 1998) had demonstrated that a Strep. dysgalactiae challenge of 1.5 × 10^4 cfu was sufficient to cause a 50% new infection rate (approximately) in teats infused with seal. In the present experiment, significant improvements in the level of protection afforded by the seal containing lacticin 3147 were observed. Only 6% of the teats that had been infused with seal and lacticin 3147 became clinically infected with the challenge organism; 48.5% of teats treated with seal only developed infection. The presence of lacticin 3147 was associated with a delayed onset of the disease in the few cases in which it occurred. For example, the first clinical case of mastitis in an udder quarter containing seal plus lacticin 3147 occurred 4 d after inoculation, at a point where 15 quarters containing teat seal alone had already become clinically infected. Possible explanations for the development of the 2 new infections in the quarters treated with the seal plus lacticin 3147 may be that the lacticin was absorbed by the tissues or that its activity was degraded during the first 4 d of the dry period and was, therefore, no longer available.

This novel approach to mastitis control harnesses an existing effective physical barrier as a method of localizing the nonantibiotic bacteriocin in the teat sinus, thereby offering further protection against new infection. In contrast, incorporation of nisin into teat seal resulted in negligible inhibition of an indicator mastitic pathogen, which was presumably due to its insolubility at a physiological pH. For this reason, lacticin 3147 was chosen as a more suitable candidate for further artificial infection studies in dairy cows. This study has shown that lacticin 3147 has potential as an effective preventative measure against the onset of mastitis during the nonlactating period. The use of lacticin 3147 has potential to limit the use of current prophylactic antibiotics treatments and should help reduce the potential health threat of emerging antibiotic-resistant organisms. Indeed, mastitis pathogens sensitive to lacticin 3147 have shown negligible resistance to this bacteriocin (20).

Treatment regimens may now be possible whereby infected cows are treated with long-acting antibiotics and uninfected cows or udder quarters are still protected from new infections with either seal or seal plus lacticin 3147 type formulations.
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