Journal of Dairy Research (2005) **72** 159–167. © *Proprietors of Journal of Dairy Research 2005*
doi:10.1017/S0022029905000816 *Printed in the United Kingdom*

The lantibiotic lacticin 3147 produced in a milk-based medium improves the efficacy of a bismuth-based teat seal in cattle deliberately infected with *Staphylococcus aureus*

Fiona Crispie[1,2], Denis Twomey[1], James Flynn[2], Colin Hill[3,4], Paul Ross[1,4] and William Meaney[2]*

[1] Teagasc, Dairy Products Research Centre, Moorepark, Fermoy, Co. Cork
[2] Teagasc, Dairy Production Research Centre, Moorepark, Fermoy, Co. Cork
[3] Department of Microbiology, University College Cork
[4] Alimentary Pharmabiotic Centre, University College Cork

Received 25 May 2004 and accepted for publication 17 August 2004

A preparation of the bacteriocin lacticin 3147 (prepared from a demineralized whey protein fermentation liquor) was combined as a powder with a bismuth-based intramammary teat seal and evaluated for its potential as an antimicrobial in non-lactating cows. The lacticin/teat seal formulation enabled significant bacteriocin release from the seal without the requirement for a surfactant. Studies *in vivo* in lactating cows demonstrated that this formulation was effective in reducing bacterial recoveries (~20-fold) from teats deliberately inoculated with *Staphylococcus aureus* after infusion. Moreover, this formulation also significantly reduced the numbers of *Staph. aureus* recovered from teats that were exposed to the challenge bacterium before the infusion of the teat seal preparation. The powdered preparation of lacticin 3147 did, however, cause some teat irritation as evidenced by associated rises in somatic cell count (SCC). However, this effect was short-lived and when the mean SCC readings pre-infusion and the final two readings post-infusion were compared, there was no significant difference in the immunological acceptance between treatments.

Keywords: Mastitis, bacteriocin, teat seal, *Staphylococcus aureus*.

It has been proposed that the widespread use of antibiotics for both human and animal applications may contribute to the spread of resistance and multi-resistance to antibiotics amongst bacterial pathogens (Chadwick & Goode, 1997). While these claims are disputed (Booth, 1997) there is, nonetheless, mounting concern worldwide regarding the use of antibiotics for prophylactic applications, particularly in veterinary medicine and as feed additives in livestock and poultry management systems (European Commission Scientific Steering Committee, 1999; National Research Council, 1999; WHO, 1994). It is envisaged that the prophylactic use of antibiotics in areas such as feed supplements and in the control of bovine mastitis will be limited in the EU in the near future and consequently alternative remedies or herd management practices will have to be adopted. A general principle underlying many of these strategies is to limit the use of antibiotics to situations where they are deemed critical, an approach which has been adopted in some of the Nordic countries where selective dry cow therapy is practised (Grave et al. 1999; Østerås et al. 1999). This has stimulated our efforts to seek an effective non-antibiotic alternative for preventing bovine mastitis during the dry period.

Sealing teats of uninfected cows at the end of lactation using an intramammary seal may provide an acceptable alternative to the widespread use of antibiotics. Teat seal (Boviseal, Bimeda, Cross VetPharm Group Ltd., Dublin, Ireland) is a non-antibiotic intramammary dry cow product that is currently sold in many countries worldwide (Orbeseal, Pfizer Animal Health). It is primarily recommended for the prevention of mastitis in low cell count cows that are considered free from infection. The formulation comprises bismuth subnitrate (65%) and liquid paraffin and is presented as a 4-g seal for intramammary application. When infused into the teat sinus and teat canal, this viscous formulation provides a physical barrier to the invasion of mastitis-causing pathogens. Infusion of teat seal without an antibiotic has been shown to be as effective as a long-acting antibiotic in preventing the

*For correspondence; e-mail: bmeaney@moorepark.teagasc.ie

Table 1. Bacterial strains used in this study

Strain	Description	Source or Reference
Lactococcus lactis		
DPC3147	Lacticin 3147 producer	DPC†
MG1363	Plasmid-free derivative of *L. lactis* subsp. *cremoris* 712	Gasson et al. 1983
DPC5011	MG1363 transconjugant containing plasmid pMRC01 which contains the lacticin 3147 production, processing and immunity genes.	DPC
HP	Cheese starter sensitive to lacticin 3147	DPC
Staphylococcus aureus		
DPC5246	Bovine mastitis clinical isolate – RAPD type 4	DPC Fitzgerald et al. 1997

† DPC; Dairy Products Research Centre culture collection at Teagasc, Moorepark, Fermoy, Co. Cork, Ireland

establishment of new intramammary infections during the dry period in cows which were infection-free and had somatic cell counts (SCC) <200 000/ml at drying off (Woolford et al. 1998; Berry & Hillerton, 2002; Huxley et al. 2002).

A potential drawback of teat seal is that it is biologically inert and does not have associated antimicrobial activity but rather relies on its physical properties to impede the entry of pathogens into the teat. Good udder hygiene is, therefore, considered critical when teat seal is infused. To help safeguard against poor udder hygiene and potentially to protect against invading pathogens, teat seal has previously been evaluated in combination with the bacteriocin, lacticin 3147 (Ryan et al. 1998, 1999; Twomey et al. 2000). This bacteriocin is composed of two component peptides which undergo a series of post-translational modifications including lanthionine bridge formation and conversion of L-serine to D-alanine (Ryan et al. 2000). Both peptides are required for optimal activity of the bacteriocin, the structure of which has recently been determined (Martin et al. 2004). Its choice as a potential agent to protect against mastitis-causing pathogens derives from a number of its properties, which include (1) it is produced by a GRAS (Generally Regarded As Safe) organism, *Lactococcus lactis*; (2) it has a broad spectrum of activity against all Gram positive bacteria including many mastitis-causing pathogens; (3) it is active at low and physiological pH; and (4) it is heat stable (Ross et al. 1999). Previous studies have shown that when lacticin 3147 was formulated with a bismuth subnitrate-based teat seal, the combination was effective in preventing new infections in dry cows after exposure to deliberate infection with *Streptococcus dysgalactiae* (Ryan et al. 1999). Furthermore, a combination of teat seal and lacticin 3147 was shown to be effective in inhibiting *Staphylococcus aureus in vitro* and also reduced the incidence of teats shedding *Staph. aureus* which had been deliberately introduced into the teat duct (Twomey et al. 2000).

The present study investigated the feasibility of producing a lacticin 3147-enriched fermentate from a milk-based (whey) medium. The lacticin-enriched powder produced was formulated with teat seal and the efficacy of this combination assessed for the prevention of mammary gland colonization with *Staph. aureus in vivo*. The study also investigated the immunological acceptance of the formulation together with a number of other lacticin 3147 formulations in lactating cows. The generation of an effective antimicrobial produced from a GRAS organism derived from a milk-based medium would provide a cost-effective method of producing large quantities of the bacteriocin while ensuring the potential problems with tissue irritancy would be minimized.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. Lactococcal strains were grown in M17 broth (Terzaghi & Sandine, 1975) (Difco, Detroit MI, USA) containing either 0·5% w/v of lactose (LM17) or glucose (GM17), and incubated overnight at 30 °C. Staphylococcal strains were grown in brain heart infusion broth (BHI, Oxoid Ltd., Basingstoke, UK) and incubated overnight at 37 °C. Aesculin blood agar (ABA) plates containing blood agar base No. 2 (Oxoid) supplemented with 7% (v/v) citrated whole calf blood and 0·1% (v/v) aesculin (Sigma, St. Louis MO, USA) were used to identify the characteristic haemolytic activity of the staphylococcal strains grown at 37 °C. Staphylococcal strains were preserved in a microbiological bead storage system (Protect; bacterial preservers, Technical Service Consultants Ltd., Lancashire, UK) and lactococcal strains were preserved in LM17 to which 40% (w/v) glycerol was added. All strains were stored at −80 °C.

Production of a lacticin 3147-enriched powder derived from demineralized whey protein

The lacticin 3147 producing strain, *Lactococcus lactis* DPC3147 was grown overnight at 30 °C in LM17. This culture (2 ml) was used to inoculate 200 ml of a 10%

https://doi.org/10.1017/S0022029905000816 Published online by Cambridge University Press

solution of demineralized whey protein (DWP; Dairygold Co-op, Michelstown, Co. Cork, Ireland) and grown overnight at 30 °C. Subsequently, 150 ml of this culture was used to seed 15 l of a 10% DWP medium which had been clarified by passing through a column containing ~1 kg of XAD16 beads (Sigma, St. Louis MO, USA). The fermentation was performed in a Biostat ED fermenter (B. Braun Biotech, Germany) at 30 °C at pH 6·5. Following fermentation, the majority of the whey proteins were precipitated by reducing the pH to 4·6 with lactic acid and heating the fermentate to 75 °C for 15 min. The precipitated whey proteins were removed by centrifugation at ~6000 ***g*** for 15 min and the resulting clarified fermentate containing lacticin 3147 was passed through columns containing XAD16 beads. Columns were washed with 40% ethanol and the bacteriocin eluted using 70% isopropanol. Isopropanol was subsequently removed using a rotary evaporator and the resulting solution was freeze dried. The freeze dried powder was milled at 18 000 rpm using a Retsch ZM100 ultracentrifugal mill (Retsch, Germany) incorporating an 80-µm ring sieve. Particle size distribution and mean particle size were determined using a Mastersizer (Malvern Instruments Ltd., Malvern, UK). The bacteriocin activity in the powder was determined by reconstituting 50 mg in 1 ml 10 mM-sodium phosphate buffer, pH 7·0 and assaying using the agar well diffusion method (Parente & Hill, 1992).

Production of a lacticin 3147-enriched powder derived from trypticase-yeast extract-glucose (TYG) medium

Lacticin 3147 was concentrated from a TYG fermentate as described by Twomey et al. (2000). The final powder obtained was either milled in a similar manner to the DWP powder (TYG powder) or solubilized in 10 mM-sodium phosphate buffer pH 7·0 plus 2% Tween 80 (TYG-L solution). Bacteriocin activity in the TYG powder was determined by reconstituting 50 mg in 1 ml 10 mM-sodium phosphate buffer at pH 7·0 and assayed using the agar well diffusion method. The solubilized preparation was assayed using the same procedure.

Formulation and assay of teat seal and lacticin 3147

Commercial intramammary teat seal, containing 65% bismuth subnitrate mixed in paraffin oil, was supplied by Cross Vetpharm Group Ltd. (Dublin, Ireland). Teat seal was blended with lacticin 3147-enriched powders (0·1 g of powder in 4 g of teat seal) derived from either DWP or TYG. Teat seal containing 1% Tween 80 was also blended with the solubilized lacticin 3147 (TYG-L) (100 µl /g of teat seal). The addition of 1% Tween 80 to the seal was previously shown to be necessary for efficient release of bacteriocin from the seal when a solubilized version of the bactericin was used (Ryan et al. 1999). The solubilized lacticin 3147 solution was filter-sterilized using 0·45-µm HVLP filters (Millipore, Cork, Ireland) prior to blending with teat seal. All the formulations were sterilized by gamma irradiation using a cobalt 60 radiation source at a dose of ~30 kGy. The antimicrobial activity of the teat seal preparations was assessed by inserting the blend into wells of an LM17 agar plate seeded with *Lc. lactis* HP, incubating overnight at 30 °C and measuring zones of inhibition using electronic calipers. Activity against mastitic staphylococcal strains was also assessed as described previously (Twomey et al. 2000). In brief, overnight cultures of *Staph. aureus* DPC5246 were subcultured using a 2% inoculum in fresh broth, and incubated with shaking at 37 °C for 4 h. Cultures were then diluted in 3 ml sterile saline to an optical density equivalent of a McFarland Standard of 0·5. The diluted bacterial suspensions were then streaked on the surface of dried Mueller-Hinton agar plates. Seal and lacticin combinations were dispensed into wells (6·4-mm diameter) on the plates. Following overnight incubation at 37 °C, zones of inhibition around the wells were measured using electronic calipers (Twomey et al. 2000).

Preparation of bacterial challenge cultures

Staph. aureus DPC5246 was used *in vivo* as the challenge organism to test the efficacy of teat seal containing the lacticin-enriched powder derived from DWP (hereafter called 'the lacticin product'). One bead of the stock culture was removed and streaked over the surface of an aesculin blood agar (ABA) plate and incubated overnight at 37 °C. Individual colonies were subcultured from the plate into 10 ml of BHI broth and incubated for 6 h at 37 °C. The number of viable cells was counted and the culture was diluted in 10% sterile antibiotic-free skim milk to the required number of cfu/ml. The diluted culture was stored at 4 °C.

Efficacy of teat seal and lacticin against artificial challenge with Staph. aureus

Before beginning the experiments *in vivo*, foremilk quarter samples were collected aseptically at three consecutive milkings from a group of cows, and these were screened for mastitis-causing pathogens by streaking 10 µl on separate quadrants on the surface of ABA plates, followed by incubation at 37 °C for 16 h. Only quarters of cows that were free of udder pathogens in all three samples and had SCC <200 000/ml were considered suitable for the experiments. Twenty-eight quarters from seven cows were thus selected, all of which met the selection criteria. Prior to the experiments, the selected cows were routinely milked daily at 7.00 and 15.00. The ability of teat seal plus lacticin DWP to inhibit *Staph. aureus* that had been deliberately introduced into teats of lactating cows was then assessed. Following morning milking, the tip of each teat was disinfected with a cotton wool swab soaked in methylated spirits. Two teats in each cow were selected at random and infused with teat seal (Control), while the two

https://doi.org/10.1017/S0022029905000816 Published online by Cambridge University Press

remaining teats were infused with teat seal containing the DWP-based lacticin-enriched powder. The seal was not manipulated in the teat after infusion to ensure that an effective plug had formed. Two hours later, all the teats were inoculated to a depth of 17 mm with a *Staph. aureus* DPC5246 challenge inoculum (1340 cfu) using a syringe with a blunted smoothed tip to prevent injury to the teat. The evening milking was omitted. Teat seals were removed 18 h later and a single foremilk sample was taken aseptically from each of the challenged quarters for microbiological analysis. Milk samples were diluted to allow accurate enumeration of bacteria and 100 µl of appropriate dilutions of each milk sample was streaked in duplicate on the surface of ABA or Baird Parker Agar plates and incubated for 24 h at 37 °C. Following incubation, *Staph. aureus* colonies were enumerated.

Ability of teat seal and lacticin to eliminate Staph. aureus *cells already present in the teat duct at the time of infusion*

A further study was performed to evaluate the effectiveness of teat seal containing the lacticin product for inhibition of *Staph. aureus* that may already be present in the teat canal at the time of infusion. Four cows were selected for this study. Using the bacteriological and SCC criteria outlined above, 16 quarters were considered suitable for this trial. Following morning milking, 100 µl of 10% skim milk containing ~740 cfu of viable *Staph. aureus* DPC5246 was inoculated into 16 teats to a depth of ~17 mm. Teat seal alone or teat seal containing lacticin product was administered to the teats immediately after inoculation. Prior to evening milking, teat seals were removed and the quarters were sampled as already described. The number of *Staph. aureus* in each sample was enumerated following growth on ABA. In both studies, log-transformed counts of surviving *Staph. aureus* were analysed by Anova using Genstat (Payne et al. 1994). Blocks were individual cows and treatments were seal plus lacticin or seal alone.

Tolerance of teat seal and lacticin 3147 in the bovine mammary gland

Three different experiments were performed to assess the tissue tolerance of the various lacticin 3147 preparations blended with teat seal. Teats of lactating cows whose foremilk was free of pathogens and had SCC <200 000 /ml over three consecutive milkings were selected for the three studies. Depending on the availability of suitable cows and products for infusion, teats were randomized and the test formulations were infused after morning milking. In the first experiment, five cows were selected and udder quarters were treated with seal only (n=5), seal plus lacticin DWP (n=5), a commercial antibiotic preparation containing sodium cloxacillin (Orbenin QR, Pfizer Animal Health, Ireland) (n=5) or left untreated (n=5). In the second experiment five cows were selected and treated with seal (n=5), seal plus lacticin DWP (n=5), seal plus lacticin TYG (n=5) or left untreated (n=5). In the third experiment, teat seal containing a solubilized preparation of lacticin 3147, similar to one used in previous studies (TYG-L), (Ryan et al. 1999; Twomey et al. 2000), was infused into teats (n=8), and compared with teats infused with seal alone (n=8) or left untreated (n=8). Approximately 8 h after treatment, seals were removed and milk samples collected. Udder quarters were sampled for the next ten consecutive milkings. Before and after infusion of the test formulation, SCC status was assessed by flow cytometry using a Somacount 300® (Bentley Instruments Inc., Chaska MN, USA) Somatic Cell Counter. To overcome the problem of seal particles interfering with the operation of the automatic somatic cell counter, the California Mastitis Test (CMT) was used as a subjective measure of SCC for the first three samplings after infusion. CMT readings were scored on a scale of 1 to 5. However, when plotting the mean CMT, the presence of clots was also taken into consideration. Milk clots were scored on a scale of 1 to 4 and this value was added to the CMT score (i.e., a sample with a CMT reading of 5 and a clot score of 2 was given a final CMT reading of 7). Using the Genstat program, the SCC data from these three experiments were analysed by Anova and CMT data were assessed using the Friedman probability test (Payne et al. 1994).

Results

Production of a lacticin 3147-enriched whey-based powder

Previously, a variety of food-based media for the production of lacticin 3147 by *Lc. lactis* DPC3147 have been used (Morgan et al. 1999). Of the culture media evaluated, a 10% reconstituted demineralized whey protein (DWP) was considered the most suitable substrate for the production of a lacticin 3147-enriched whey powder, in terms of economics and culture performance. This substrate was investigated for the production of a lacticin 3147-powder that would be suitable for blending with teat seal. To increase the specific activity of the final product, the 10% DWP medium was first clarified of the majority of hydrophobic proteins by passing through a column containing XAD16 beads. When *Lc. lactis* DPC3147 was grown in this substrate for ~24 h, the final bacteriocin concentration was ~2000 AU/ml (as compared with ~320 AU/ml generally obtained in synthetic media). The activity of lacticin 3147 in the fermentate following removal of the majority of contaminating whey proteins was ~500 AU/ml.

When lacticin 3147 was concentrated from the clarified DWP fermentate, freeze dried and milled, the mean diameter of powder particles was 26·6 µm. The maximum particle size of the resulting powder was 64 µm, with 90% of the particles less than 38 µm. When 50 mg of this powder was reconstituted in 10 mM-sodium phosphate

https://doi.org/10.1017/S0022029905000816 Published online by Cambridge University Press

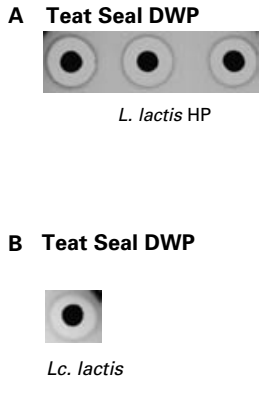

Fig. 1. (A) Assessment of the ability of teat seal containing lacticin 3147 derived from demineralized whey protein (the lacticin product) to inhibit *Lc. lactis* HP using the agar well diffusion method. (B) Demonstration of the ability of the lacticin product to inhibit *Lc. lactis* MG1363 but not *Lc. lactis* MG1363 × pMRC01 which is immune to lacticin 3147 activity using the agar well diffusion method.

buffer (pH 7·0) and assayed using the agar well diffusion method (Parente & Hill, 1992), the bacteriocin activity was 81 960 AU/ml. This indicated that activity of the powder was $\sim 1{\cdot}6 \times 10^6$ AU/g. In contrast to previous liquid preparations of lacticin 3147, which required the addition of a surfactant (Tween 80) to allow significant bacteriocin release from the teat seal, teat seal containing the DWP powder preparation displayed significant antimicrobial activity without the addition of a surfactant (Fig. 1A). The average zone diameter when assayed against *Lc. lactis* HP was 12·5 mm. The addition of 1% Tween 80 to this teat seal did not noticeably enhance bacteriocin release from the seal (results not shown). Teat seal containing the lacticin product was also assayed for activity against mastitic staphylococci and displayed significant antimicrobial activity against these pathogens (results not shown).

Confirmation that this antimicrobial activity was due to lacticin 3147 and not some other metabolite was provided by demonstrating inhibitory activity against *Lc. lactis* MG1363, whereas a transconjugant of this strain (*Lc. lactis* DPC5011, Table 1), harbouring the plasmid that encodes immunity to lacticin 3147, was not inhibited (Fig. 1B).

Effectiveness of teat seal and a milk-based lacticin 3147 powder in preventing colonization by Staph. aureus

The effectiveness of the blend of teat seal and lacticin was evaluated using deliberate bacterial challenge studies in lactating cows. Teat seal containing the lacticin product was first compared with commercial teat seal in an experiment using seven cows selected on the basis of their udder health. The number of viable *Staph. aureus* DPC5246 cells subsequently recovered from teats administered the seal containing the DWP-derived powder was significantly lower than teats administered commercial teat

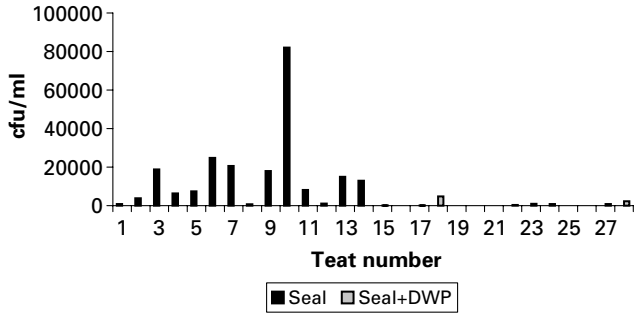

Fig. 2. Recoveries of *Staphylococcus aureus* DPC5246 18 h following the inoculation of teats with 1340 cfu after the infusion of either teat seal (black bars, Teats 1–14) or the lacticin product (grey bars, Teats 15–28).

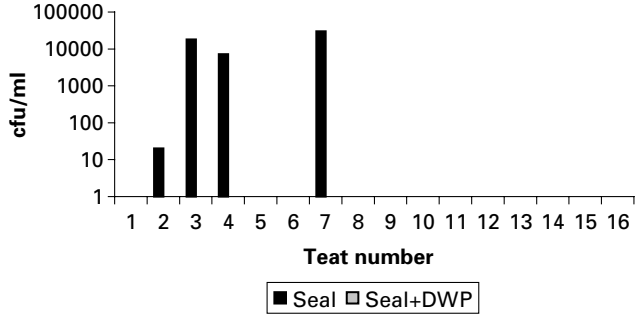

Fig. 3. Recoveries of *Staphylococcus aureus* DPC5246 from teats which were inoculated with 740 cfu prior to infusing either teat seal (black bars, Teats 1–8) or the lacticin product (grey bars, Teats 9–16). *Staph. aureus* DPC5346 were enumerated 8 h post infusion of the teat seal or lacticin product.

seal ($P<0{\cdot}001$) (Fig. 2). The average recovery of viable *Staph. aureus* from teats infused with teat seal DWP was $7{\cdot}3 \times 10^2$ cfu/ml as compared with $1{\cdot}6 \times 10^4$ cfu/ml for teat seal alone. These data indicate that the lacticin product and teat seal was capable of reducing the number of viable *Staph. aureus* cells recovered from teats pretreated with the seal plus lacticin 3147 relative to those pretreated with seal alone (Fig. 2).

Effectiveness of teat seal and a milk-based lacticin 3147 powder in eliminating Staph. aureus *cells already present in the teat duct at the time of infusion*

In a further study, the effectiveness of teat seal combined with the DWP-based lacticin–enriched powder was evaluated for its ability to reduce *Staph. aureus* levels in teats deliberately contaminated prior to infusion with the seal formulations. When inoculation of *Staph. aureus* DPC5246 was followed by the infusion of teat seal containing the DWP-based powder, viable *Staph. aureus* DPC5246 were recovered from four (50%) of the teats (Fig. 3). *Staph. aureus* was not recovered from any (0%) of the teats

https://doi.org/10.1017/S0022029905000816 Published online by Cambridge University Press

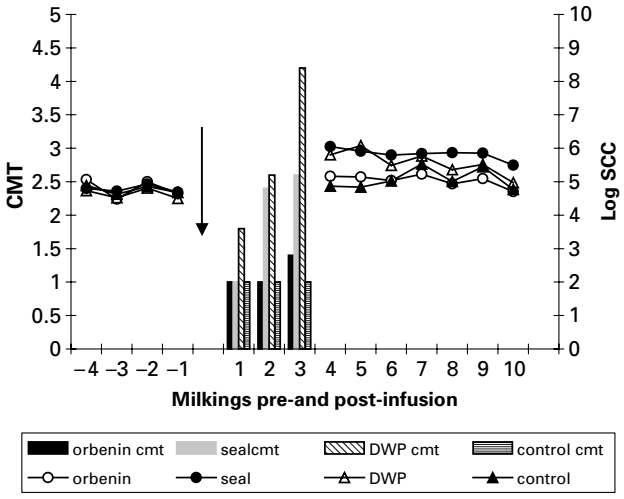

Fig. 4. Mean SCC and CMT values pre-and post-infusion from untreated teats (control), teats infused with teat seal (seal), teat seal plus the DWP-derived powder (DWP) or sodium cloxacillin (Orbenin QR). Mean CMT values are presented for the three milkings post-infusion of the sample and SCC are presented for remaining samples. The black arrow indicates the point of infusion.

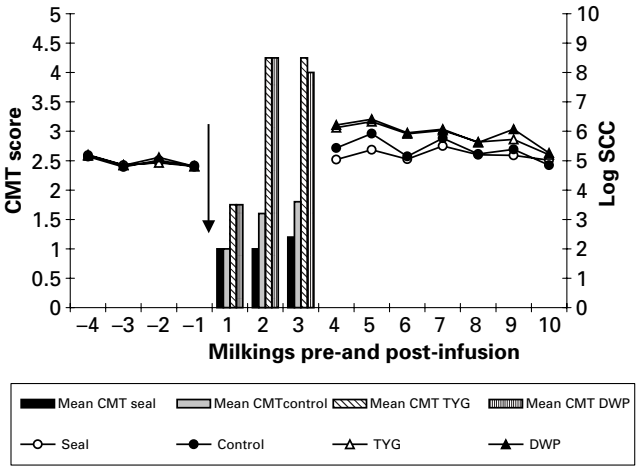

Fig. 5. Mean SCC and CMT values pre-and post-infusion from untreated teats (control), teats infused with teat seal (seal), teat seal plus the DWP-derived powder (DWP) or teat seal plus the TYG-derived powder (TYG). Mean CMT values are presented for the three milkings post-infusion of the sample and SCC are presented for remaining samples. The black arrow indicates the point of infusion.

infused with teat seal and the lacticin product (Fig. 3). The average recovery of *Staph. aureus* DPC5246 from the teats infused with teat seal was $6{\cdot}9 \times 10^3$ cfu/ml and 0 cfu/ml from the teats infused with seal plus the lacticin product. This difference was significant ($P<0{\cdot}05$). These results show that the infusion of teat seal and whey-based lacticin 3147 inhibited *Staph. aureus* pathogens *in vivo* whether they entered the teat before or after infusion of the intramammary product (Fig. 3).

Teat tissue tolerance studies

Three separate irritancy studies were performed in lactating cows to establish the effect of infusing teat seal containing the lacticin product on SCC. In the first experiment, randomized udder quarters in five cows were treated with seal only ($n=5$), seal plus lacticin DWP ($n=5$), a commercial antibiotic preparation ($n=5$) or left untreated ($n=5$). In general, control teats that were either untreated or infused with commercial teat seal, had cell counts well within accepted SCC levels (<200 000 /ml) (Fig. 4). While some of the control samples gave an occasional high cell count with no culturable pathogen present, the mean CMT of the first three milkings post-infusion was <1·5. Subsequent mean SCC values did not exceed 400 000 /ml in any of the control samples. These results indicated that teat seal was non-irritant and generally gave a similar immunological response as uninfused teats. The teats infused with commercial sodium cloxacillin ($n=5$) did not display any signs of irritation and behaved similarly to the untreated teats. Teats infused with teat seal containing the lacticin product had higher SCC than the untreated teats or teats infused with teat seal alone. CMT values for three of these samples reached the maximum value of 5 and clots were observed in some of the samples. Mean CMT values for the three samples collected immediately post-infusion peaked at 3·7 for teat seal containing the DWP-derived powder (Fig. 4). When the mean log SCC from the four samples pre-infusion and the final two samples post-infusion was compared between treatments the difference was not significant ($P=0{\cdot}352$). Additionally, when the mean log SCC post-infusion were compared, the difference between the treatments was also insignificant ($P=0{\cdot}078$) (Fig. 4).

In addition to the teat seal containing the lacticin product derived from DWP, a similar powder derived from TYG (lacticin TYG) was also blended with teat seal and assessed. In this experiment, five cows were treated with seal ($n=5$), seal plus lacticin DWP ($n=5$), seal plus lacticin TYG ($n=5$) or left untreated ($n=5$). SCC or CMT was then determined for each quarter for ten consecutive milkings. During the course of the experiment, one of the quarters treated with seal plus lacticin TYG developed mastitis as a result of a *Streptococcus dysgalactiae* infection. Additionally, one quarter treated with seal plus the lacticin product also became mastitic although no pathogen was isolated from the quarter. As a result, both of these quarters were treated with antibiotics and the data from these quarters were subsequently removed from the analysis. There was no significant difference in the mean log SCC post-infusion between treatments ($P=0{\cdot}111$). When the mean log SCC from the four samples pre-infusion and the final two samples post-infusion was compared across treatments the difference was not significant ($P=0{\cdot}331$) (Fig. 5).

https://doi.org/10.1017/S0022029905000816 Published online by Cambridge University Press

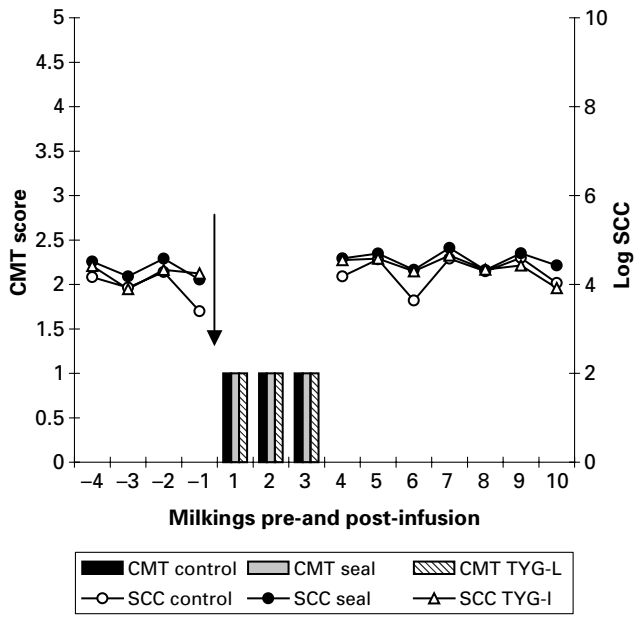

Fig. 6. Mean SCC and CMT values pre-and post-infusion from untreated teats (control), teats infused with teat seal (seal), or Teat Seal plus TYG-L (TYG-L). Mean CMT values are presented for the three milkings post-infusion of the sample and SCC are presented for remaining samples. The black arrow indicates the point of infusion.

Teat seal containing a solubilized preparation of lacticin 3147 derived from TYG (TYG-L) which was previously shown to inhibit mastitis-causing organisms *in vivo* (Ryan et al. 1999; Twomey et al. 2000) was also included in a third study to determine the immunological acceptance of the seal plus lacticin mixtures. In this experiment, teat seal plus TYG-L was infused into teats (n=8) and compared with teats infused with seal alone (n=8) or left untreated (n=8). No irritation or increase in SCC was observed in any of the quarters regardless of treatment. Mean CMT for the first three milkings post-infusion was 1·0 for all treatments and subsequent SCC readings did not reveal any sign of irritation in the teat tissue, nor was there any difference between SCC readings post-infusion for all treatments (P=0·222). There was no significant difference between treatments for the mean SCC readings of the four samples pre-infusion and the final two samples post-infusion (P=0·244) (Fig. 6).

Taken together, these results indicate that while the infusion of seal containing either the lacticin product or TYG-derived lacticin powders caused a rise in SCC, this effect was temporary with no long-term negative effects on the cows (Figs 4–6).

Discussion

The lacticin product assessed in this study offered some protection against *Staph. aureus* deliberately introduced into the teat duct following the infusion of teat seal blended with the bacteriocin. The mean number of deliberately introduced pathogens recovered was significantly reduced relative to the teats containing teat seal alone (P<0·001). As *Staph. aureus* is one of the dominant mastitis pathogens worldwide, this finding indicates that the seal and lacticin product may have potential to enhance the capacity of teat seal to prevent *Staph. aureus* mastitis. As shown in previous studies (Twomey et al. 2000), the presence of the bacteriocin was associated with a reduction in the number of pathogens recovered although in general the challenge organism was not completely eradicated from the teats. Staphylococci, including mastitis strains, are sensitive to lacticin 3147 (Ryan et al. 1998; Twomey et al. 2000). However, of the Gram positive bovine mastitis pathogens tested to date, they tend to be among the least sensitive and in general mastitic streptococci are far more susceptible to the bacteriocin. As in previous trials, the inability to eradicate the pathogen completely may be a consequence of insufficient bacteriocin present or a lack of contact between the lacticin powder and the pathogen in the animal, given that it is well known that staphylococci are capable of surviving intercellularly in macrophages. Thus the observed reduction in numbers of staphylococci recovered from the teats administered teat seal plus the lacticin product, may be due to macrophage internalization rather than true 'kill' by lacticin. If this was the case however, one would expect to see a similar decrease in staphylococci in the control group, as these would also be internalized. This did not occur, suggesting that the effect observed was due to the antimicrobial action of lacticin 3147 or due to an enhanced immune response caused by the addition of the lacticin-enriched powders.

When the challenge organism was present in the teat duct prior to infusing the teat seal containing the DWP-derived lacticin powder, the number of teats still shedding viable pathogens was reduced significantly (P<0·05) and a greater eradication of the challenge organism was observed. This *in-vivo* model was designed to facilitate greater contact between the bacteriocin formulation and *Staph. aureus* as the teat seal containing the lacticin product had to be infused through the teat duct containing the pathogen. At drying-off, teats may have a low cell count and be considered pathogen-free. However, the teat orifice may be colonized with *Staph. aureus* or another Gram positive pathogen. When the seal is infused, a pathogen may get pushed further into the teat and, since teat seal is inert, this infusion may facilitate the passage of a viable pathogen into the teat which may lead to mastitis. The ability to kill pathogens present in the teat duct, therefore, is an important consideration when a teat seal formulation is being used. These data indicate that the presence of the bacteriocin preparation in teat seal should help alleviate the risk of pathogens, particularly *Staph. aureus*, being introduced into the teat duct during seal

https://doi.org/10.1017/S0022029905000816 Published online by Cambridge University Press

infusion especially if the bacteriocin or other antimicrobial is localized within the seal.

Teat seal containing a lacticin powder derived from TYG or DWP inhibited the growth of mastitic staphylococci *in vitro*. Although the animal studies indicated that the lacticin product could offer some protection against *Staph. aureus*, the infusion of this formulation into the teats of lactating cows caused a short-lived increase in SCC. Overall, this increase was not significant. The relatively small numbers of teats assessed in this study, however, may have restricted statistical analysis of the results and larger trials in non-lactating cows would be required to substantiate the findings. Intolerance was also observed when teats of lactating cows were infused with teat seal containing a TYG-derived powder. The irritation was attributed to the presence of the powders, as teat seal alone was very well accepted and the immunological response was similar to that of untreated teats. While both of the milled bacteriocin powder preparations caused some teat irritation, a solubilized lacticin 3147 preparation derived from TYG did not cause irritation when infused. Teat seal containing the latter preparation is essentially the same as teat seal containing the powder preparation derived from TYG, except that the lacticin 3147-enriched preparation was solubilized in buffer prior to blending. This indicates that the teat irritation observed with teat seal containing lacticin powder derived from TYG or DWP was associated with the particulate nature of the powder preparations, rather than lacticin 3147 or some other substance from the processed fermentate. This finding is similar to some of the very early mastitis studies *in vivo* with the bacteriocin, nisin (Taylor et al. 1949) where relatively large particle sizes contributed to teat irritancy. Importantly, when the nisin particle size was reduced, problems with irritancy were not encountered (Taylor et al. 1949). In this respect, however, attempts to solubilize the lacticin 3147 DWP-preparation and reduce the particle size using a variety of aqueous buffers and oils were unsuccessful.

The increased SCC associated with the powder preparations may have complicated the findings from the bacterial challenge study. Although the challenge work was performed with cows that initially had low SCC prior to infusion of the test formulation, the increase in SCC may effect the survival of the challenge organism. During involution and inflammation the mammary gland is known to secrete proteins with some antimicrobial properties including a secreted lysosomal enzyme N-acetyl-β-D-glucosaminidase (NAGase). The protective role of mammary secretions is difficult to assess in these relatively short-term animal studies. As the irritation was short-lived however, it may be viewed as a positive feature of this treatment as a moderate stimulation of the immune system could also enhance the protection afforded by the lacticin product. In this respect, it is worth mentioning that it can be very difficult experimentally to assess the relative contribution of an antimicrobial versus the immune stimulatory effects of preparations that have an associated irritation. In practice, the protective effect is probably a result of both activities. It is also worth noting that lacticin 3147 only inhibits Gram positive bacteria, and thus teat seal plus lacticin should not be able to prevent Gram negative bacteria from gaining entry to the teat duct due to poor infusion technique at drying off. Gram positive bacteria, particularly *Staph. aureus*, are major problems in mastitis control, particularly in cows where teat disinfection is rarely or never used. *Staph. aureus* is also a problem in autumn-calving herds, where the cows are at pasture at drying-off. In the latter case, teats are usually relatively free of Gram negative pathogens. In spring-calving herds, however, where the cows are indoors at drying-off, the risk of infection by Gram negative bacteria is greater. The mild irritation caused by seal and lacticin however, could arguably be important in this regard. It would be interesting to determine whether the immunostimulatory effects observed could extend the protection offered by the seal and lacticin combination to Gram negative bacteria.

Veterinary products used for the treatment or prevention of disease in animals whose tissues and/or products are destined for human consumption may give rise to residues of such products or their metabolites in these food-producing animals. To ensure the safety of these pharmacologically active substances and to maintain consumer confidence, the nature and extent of these residues in foods has to be controlled. International bodies such as the European Agency for the Evaluation of Medicinal Products (EMEA), Codex Alimentarius and the Food and Drug Administration (FDA) help to evaluate risks and oversee laws relating to the levels and type of residues of veterinary products in foods. Previous reports indicate that the addition of lacticin 3147 to teat seal offers significant protection against mastitis pathogens *in vivo* (Ryan et al. 1999; Twomey et al. 2000). However, these lacticin 3147 preparations were derived from a synthetic laboratory medium and as such contain associated residues that would undoubtedly complicate their acceptance for routine use in mastitis treatment. In contrast, the lacticin product described in the present study is generated from food grade materials and would be far more cost-effective to produce. The current problems worldwide regarding antibiotic resistance and the debate regarding the prophylactic use of antibiotics in animals dictate that novel remedies and management practices are required to offset problems with bovine mastitis. It has been demonstrated in this study that protection against a deliberate challenge of *Staph. aureus* can be achieved using a combination of teat seal and lacticin. A limitation of this study is that the teat seal and lacticin combinations were only infused for a relatively short period of time ($\leqslant$18 h), and thus the results may not truly reflect what could happen in a 50–60-d dry period. Further research is therefore necessary to evaluate the efficacy of this combination under field conditions, in a large number of cows during a regular dry period. Under these conditions, the efficacy of the teat seal/lacticin

https://doi.org/10.1017/S0022029905000816 Published online by Cambridge University Press

combinations should also be compared with (a) teat seal without lacticin, (b) no treatment and (c) a long-acting antibiotic. It would also be interesting to investigate the effect on new intramammary infection by natural infection and to evaluate SCC and inflammation at and after time of calving. The results presented here indicate that using a combination of teat seal and lacticin prepared from a cheap, natural source, may provide a novel alternative to treatment with antibiotics.

The authors gratefully acknowledge the financial assistance of CrossVet Pharmaceutical, the Irish Dairy Levy Fund and the Science Foundation of Ireland.

References

Berry EA & Hillerton JE 2002 The effect of selective dry cow treatment on new intramammary infections. *Journal of Dairy Science* **85** 112–121

Booth JM 1997 [Progress in mastitis control–an evolving problem] In *Proceedings of the British Mastitis Conference* pp 3–9. Stoneleigh UK: British Mastitis Council Press

Chadwick DJ & Goode J 1997 In *Antibiotic Resistance: Origins, Evolution, Selection and Spread* New York: John Wiley & Sons

European Commision Scientific Steering Committee DG XXIV 1999 *The European Commission, Opinion of the Scientific Steering Committee on Antimicrobial Resistance* May 28th

Fitzgerald JR, Meaney WJ, Hartigan PJ, Smyth CJ & Kapur V 1997 Fine-structure molecular epidemiological analysis of *Staphylococcus aureus* recovered from cows. *Epidemiology and Infection* **119** 261–269

Gasson MJ 1983 Plasmid complementation of *Streptococcus lactis* NCDO712 and other lactic streptococci after protoplast induced curing. *Journal of Bacteriology* **154** 1–9

Grave T, Greko C, Nilsson L, Odensvik K, Mörkand T & Rönning M 1999 The usage of veterinary antibacterial drugs for mastitis in cattle in Norway and Sweden during 1990–1997. *Preventative Veterinary Medicine* **42** 45–55

Huxley JN, Green MJ, Green LE & Bradley AJ 2002 Evaluation of the efficacy of an internal teat sealer during the dry period. *Journal of Dairy Science* **85** 551–561

Martin NL, Sprules T, Carpenter MR, Cotter PD, Hill C, Ross RP & Vederas JC 2004 Structural characterisation of lacticin 3147, a two-peptide lantibiotic with synergistic activity. *Biochemistry* **43** 3049–3056

Morgan SM, Galvin M, Kelly J, Ross RP & Hill C 1999 Development of a lacticin 3147-enriched whey powder with inhibitory activity against foodborne pathogens. *Journal of Food Protection* **62** 1011–1016

National Research Council 1999 *The Use of Drugs in Food Animals: Benefits and Risks* National Research Council, Washington DC: National Academy Press

Østerås O, Edge VL & Martin SW 1999 Determinants of success or failure in the elimination of major mastitis pathogens in selective dry cow therapy. *Journal of Dairy Science* **8** 1221–1231

Parente E & Hill C 1992 Comparison of factors affecting the production of two bacteriocins from lactic acid bacteria. *Journal of Applied Bacteriology* **73** 290–298

Payne RW, Harding SA, Arnold GM, Campbell MJ, Cole TJ, Gleeson AC, Goedhart PW, MacRae KB, Morgan GW, Murray AWA, Roger JH, Thissen JTNM, Triggs CM, Trinder KI & Tunnicliffe-Wilsen G 1994 *Genstat 5 Reference Manual* Release 3, Oxford: Clarendon Press

Ross RP, Galvin M, McAuliffe O, Morgan SM, Ryan MP, Twomey DP, Meaney WJ & Hill C 1999 Developing applications for lactococcal bacteriocins. *Antonie van Leeuwenhoek* **76** 337–346

Ryan MP, Meaney WJ, Ross RP & Hill C 1998 Evaluation of lacticin 3147 and teat seal containing this bacteriocin for inhibition of mastitis pathogens. *Applied and Environmental Microbiology* **64** 2287–2290

Ryan MP, Flynn J, Hill C, Ross RP & Meaney WJ 1999 The natural food grade inhibitor, lacticin 3147 can prevent mastitis in non-lactating dairy cows. *Journal of Dairy Science* **82** 2625–2631

Ryan MP, Jack RW, Josten M, Sahl H-G, Jung G, Ross RP & Hill C 2000 Extensive post-translational modification, including serine to D-alanine conversion, in the two-component lantibiotic, lacticin 3147. *Journal of Biological Chemistry* **274** 37544–37550

Taylor JI, Hirsch A & Mattick ATR 1949 The treatment of bovine streptococcal and staphylococcal mastitis with nisin. *Veterinary Record* **61** 197–198

Terzaghi BE & Sandine WE 1975 Improved medium for lactic streptococci and their bacteriophage. *Applied Microbiology* **29** 807–813

Twomey DP, Wheelock AI, Flynn J, Meaney WJ, Hill C & Ross RP 2000 Protection against *Staphylococcus aureus* mastitis in dairy cows using a bismuth-based teat seal containing the bacteriocin, lacticin 3147. *Journal of Dairy Science* **83** 1981–1988

WHO 1994 *World Health Organization Scientific Working Group on Monitoring and Management of Bacterial Resistance to Antimicrobial Agents* Geneva, Switzerland: WHO

Woolford MW, Williamson JH, Day AM & Copeman PJA 1998 The prophylactic effect of a teat sealer on bovine mastitis during the dry period and the following lactation. *New Zealand Veterinary Journal* **46** 12–19