

Title	Efficacy of a teat dip containing the bacteriocin lacticin 3147 to eliminate Gram-positive pathogens associated with bovine mastitis
Author(s)	Klostermann, Katja; Crispie, Fiona; Flynn, James; Meaney, William; Ross, R. Paul; Hill, Colin
Publication date	2010
Original citation	Klostermann, K, Crispie, F. Flynn, J., Meaney, W., Ross, R. Paul and Hill, C. 2010. Efficacy of a teat dip containing the bacteriocin lacticin 3147 to eliminate Gram-positive pathogens associated with bovine mastitis. <i>Journal of Dairy Research</i> , 77, pp 231-238. doi: 10.1017/S0022029909990239
Type of publication	Article (peer-reviewed)
Link to publisher's version	http://journals.cambridge.org/action/displayAbstract?fromPage=online&aid=7496876&fulltextType=RA&fileId=S0022029909990239 http://dx.doi.org/10.1017/S0022029909990239 Access to the full text of the published version may require a subscription.
Rights	© Proprietors of Journal of Dairy Research 2009
Item downloaded from	http://hdl.handle.net/10468/1100

Downloaded on 2017-02-12T13:24:00Z

Efficacy of a teat dip containing the bacteriocin lacticin 3147 to eliminate Gram-positive pathogens associated with bovine mastitis

Katja Klostermann^{1,2}, Fiona Crispie¹, Jim Flynn³, William J Meaney³, R Paul Ross^{1,2*} and Colin Hill²

¹ Moorepark Food Research Centre, Teagasc, Moorepark, Fermoy, Co. Cork, Ireland

² Department of Microbiology, University College Cork, Cork, Ireland

³ Dairy Production Research Centre, Teagasc, Moorepark, Fermoy, Co. Cork, Ireland

Received 6 March 2009; accepted for publication 29 July 2009; first published online 29 September 2009

On most dairy farms teat dips are applied to the teats of cows either before or after milking in order to prevent pathogens from gaining access to the mammary gland via the teat canal. In the present experiments, a natural teat dip was developed using a fermentate containing the live bacterium *Lactococcus lactis* DPC 3251. This bacterium produces lacticin 3147, a two-component lantibiotic which was previously shown to effectively kill Gram-positive mastitis pathogens. Lacticin 3147 activity in the fermentate was retained at 53% of its original level following storage for 3 weeks at 4 °C. In the initial experiments in vitro, 10⁵ colony-forming units/ml (cfu/ml) of either *Staphylococcus aureus*, *Streptococcus dysgalactiae* or *Streptococcus uberis* were introduced into the lacticin-containing fermentate. Neither *Staph. aureus* nor *Str. dysgalactiae* could be detected after 30 min or 15 min, respectively, while *Str. uberis* was reduced approximately 100-fold after 15 min. Following these trials, preliminary experiments were performed in vivo on teats of lactating dairy cows. In these experiments, teats were coated with each of the challenge organisms and then dipped with the lacticin-containing fermented teat dip. Following a dip contact time of 10 min, staphylococci were reduced by 80% when compared with the undipped control teat. Streptococcal challenges were reduced by 97% for *Str. dysgalactiae* and by 90% for *Str. uberis*. These trials showed that the teat dip is able to reduce mastitis pathogens on the teats of lactating cows.

Keywords: Mastitis prevention, teat dip, non-chemical, natural.

Mastitis is the most prevalent and costly disease in dairy herds, where it is associated with high somatic cell count (SCC) and decreased milk production. While many studies have tried to calculate economic losses caused by mastitis, the conclusions vary widely, as reviewed by Halasa et al. (2007). A study using national US data from 1996 found that the decrease in milk production associated with high bulk milk SCC caused a total loss of up to \$810±480 million to the US economy when consumer supply and demand were taken into account (Losinger, 2005). In the UK, the annual value of output losses or resource wastage due to mastitis was estimated to have a direct cost of £121 million (Bennett et al. 1999). The same study also estimated annual treatment costs at an average of £61.5 million.

With respect to teat disinfection, teat dipping plays a crucial role in protection against invading mastitis pathogens and consists of treating teats pre- and/or post milking. It has been estimated that the implementation of teat dipping costs £1.40 per cow per year. The same study calculated that herds using three different preventive measures for mastitis, including teat dipping, could save an average of £35 per cow per year on disease costs, which would be a yearly saving of £3500 in a cow herd averaging 100 animals (Yalcin et al. 1999). Teat dips commonly contain diluted disinfectants, teat-skin conditioners or emollients as well as surfactants (Pankey et al. 1984). The antibacterial active ingredients used in teat dips are diluted disinfectants such as iodine or chlorhexidine. Use of a post-milking teat dip containing 1% iodine led to residues of 336 µg of iodine/l of milk marking a significant rise when compared with the corresponding value of 292 µg/l resulting from using 0.1% iodine in dips (Galton

*For correspondence; e-mail: paul.ross@teagasc.ie

et al. 1986). In the 1970s concerns were raised in the USA over high iodine residues in foods, as some daily intakes exceeded the 'safe-levels' set by the National Research Council, as found in surveys by Vanderveen (1979). High iodine levels in milk and dairy products were suggested to be the major source of dietary iodine intake of the US population and the percentage of iodine provided by dairy products for adults and young children was 38–56% and 56–85%, respectively (Park et al. 1981). Use of iodine products, through either feed supplements or disinfectant usage in the dairy was subsequently monitored carefully in dairy herds and has fallen since the 1980s (Pennington et al. 1989). In recent years, further controversy over iodine residues arose when dermatologists linked the levels of iodine in dairy products to the development of teenage acne, with some clinicians advising their acne-affected patients to avoid the intake of dairy products (Arbesman, 2005; Danby, 2007). Consequently, there is a need to develop more natural antimicrobials as alternatives for incorporation into teat dips.

To date, little research has been undertaken to develop alternative formulations that would reduce the risk of disinfectant residues in milk and few attempts have been made to replace chemical teat dips with more 'natural' substances. Previously, an alternative commercial teat dip containing the bacteriocin nisin was investigated (Sears et al. 1992; Serieys & Poutrel, 1996). Additionally, experimental teat dips made from a *Lactobacillus acidophilus* preparation, from a sour milk product and a teat dip based on an Aloe Vera gel have all been examined (Oliver & Mitchell, 1985; Koskinen et al. 1996; Leon et al. 2004). The rate of new infections was insignificant compared with the diverse control groups (in most trials iodine-dipped control groups were included).

Lc. lactis DPC 3147 was originally isolated from an Irish kefir grain (Rea & Cogan, 1994) and produces the bacteriocin lacticin 3147. Bacteriocins are peptides produced by many bacteria for the purpose of destroying their competitors. Lacticin 3147 is a two-component bacteriocin classified as a lantibiotic (Class I bacteriocins) owing to its unusual lanthionine and β -methyllanthionine groups which result from post-translational modifications. These unusual groups assist the bacteriocin in forming a certain three-dimensional structure which is necessary for its binding to lipid II, a general cell wall component in Gram-positive bacteria. The binding of lacticin 3147 to lipid II leads to potassium leakage to the extracellular space, dissipating the membrane potential, and is followed by cell apoptosis (McAuliffe et al. 1998; Sit & Vederas, 2008). Lacticin 3147 is active against a wide range of Gram-positive bacteria including mastitic pathogens such as *Staph. aureus* and *Str. dysgalactiae* (Ryan et al. 1998). In previous trials, we showed that incorporation of lacticin 3147 into teat seals improved the protection of animals against both these organisms (Ryan et al. 1999; Twomey et al. 2000; Crispie et al. 2005). Subsequent trials have shown that preparations of the live producing culture

were as effective as commonly used antibiotics for the treatment of intramammary infections (Crispie et al. 2008; Klostermann et al. 2008). In the present study, we evaluated the ability of a milk-based lacticin-containing fermentate to eliminate the mastitis pathogens *Str. uberis*, *Str. dysgalactiae* and *Staph. aureus* both in vitro and in vivo on the teat surface. *Staph. aureus* and *Str. dysgalactiae* are predominant udder specific pathogens especially in Northern European countries (Barrett et al. 2005; Persson Waller et al. 2009) and the incidence rates of mastitis caused by such specific pathogens can be significantly reduced by the use of teat dipping (Whist et al. 2007). *Str. uberis* belongs to the group of environmental pathogens and is another important Gram-positive mastitis pathogen which can enter the teat canal after milking when the closure of the teat orifice is not yet established and the teat end is contaminated. The aim of the present study was to examine the bactericidal activity of a lacticin-containing fermentate against three important mastitis pathogens as a basis for the development of an effective teat dip in the future.

Materials and Methods

Bacterial strains

All bacterial strains were either selected from the culture collection at the Moorepark Food Research Centre, Teagasc, Moorepark, Fermoy, Co. Cork, Ireland or from the Bacteria Collection of the Laboratorium voor Microbiologie, Universiteit Gent, Belgium (Table 1). The lactococcal strains used in the study were *Lc. lactis* DPC 3147, the original strain found to produce the bacteriocin lacticin 3147 and *Lc. lactis* DPC 3251, another isolate which naturally overproduces the bacteriocin. *Lc. lactis* DPC 5399, a derivative of *Lc. lactis* DPC 3147 which does not contain the lacticin 3147-encoding plasmid pMRC01, was used as a lacticin negative control (Bac⁻). *Lc. lactis* subsp. *cremoris* HP was used as a lacticin 3147-sensitive indicator strain. All lactococcal strains were maintained on M17 Agar or Broth (Becton Dickinson and Company, Sparks MD, USA) supplemented with 0.5% (w/v) Lactose (LM17 Agar and LM17 Broth). The lactococcal strains were grown overnight at 30 °C.

The mastitis pathogen *Staph. aureus* DPC 5246 was isolated from a naturally occurring mastitis case and was used in previous studies as a challenge organism (Twomey et al. 2000; Crispie et al. 2005). *Str. uberis* Diernhofer 1932 and *Str. dysgalactiae* M were also isolates from mastitic milk and the latter was used as a challenge strain in experiments both in vivo and in vitro (Ryan et al. 1999). All mastitis pathogens were routinely grown in TSB (Tryptic Soy Broth, B.D. and Co., 38800 Le Pont de Claix, France) and on Blood Agar Base No. 2 (Merck, Darmstadt, Germany) supplemented with 0.1% aesculin (w/v, Sigma Chemical Company, St. Louis MO, USA) and 5–7% (v/v) whole calf blood (Aesculin Blood Agar, ABA) (IDF, 1981).

Table 1 Summary of bacterial strains

Strain	Description	Source or Reference
Lactococci		
<i>Lc. lactis</i> DPC 3251	Natural overproducer of lactacin 3147	MFRC†
<i>Lc. lactis</i> DPC 5399	Lactacin 3147 negative strain	MFRC
<i>Lc. lactis</i> subsp. <i>cremoris</i> HP	Indicator strain, sensitive to lactacin 3147	MFRC
Staphylococci		
<i>Staph. aureus</i> DPC 5246	Bovine mastitis clinical isolate	MFRC
Streptococci		
<i>Str. uberis</i> Diernhofer 1932 ^{AL}	Bovine, mastitis milk	BCCM TM /LMG‡
<i>Str. dysgalactiae</i> subsp. <i>dysgalactiae</i> M	Bovine mastitis clinical isolate	MFRC

† Moorepark Food Research Centre

‡ Bacteria Collection of the Laboratorium voor Microbiologie, Universiteit Gent, Belgium

Staphylococci were enumerated on Mannitol Salt Agar (Merck, Darmstadt, Germany) while streptococci were grown on Modified Edwards Medium (Oxoid Ltd., Hampshire, England) supplemented with 5–7% (v/v) whole calf blood (IDF, 1981).

Preparation of the teat dip

The teat dip was prepared by growing *Lc. lactis* DPC 3251 in 10% (w/v) reconstituted skim milk (10% RSM). An overnight broth culture of the above strain was used to inoculate 10% RSM and was subsequently grown for 20 h at 30 °C. The resulting fermentate was then stored at 4 °C and used as the 3251 teat dip in bactericidal assays and in experiments in vivo. For the experiments in vitro, a control fermentate produced by the bacteriocin negative strain *Lc. lactis* DPC 5399 was prepared using identical conditions to the 3251 fermentate. The fermentate of the strain *Lc. lactis* DPC 5399 (Bac⁻ fermentate) was chosen because it is a bacteriocin-negative derivative of the original lactacin producer *Lc. lactis* DPC 3147. The pH and the consistency of the 5399 fermentate were similar to fermentates produced by the lactacin-overproducing strain *Lc. lactis* DPC 3251. Both dips were always used within 24 h of production.

Lactacin production and storage stability

To assess the storage stability of the bacteriocin in the *Lc. lactis* 3251-fermentate at 4 °C, the lactacin activity of a 3251 fermentate, prepared in duplicate, was assayed over a 3-week period on days 1, 4, 7, 14 and 21. Bacteriocin activity was measured by an agar well diffusion assay, with the lactacin 3147-sensitive strain *Lc. lactis* subspecies *cremoris* HP (Table 1) as the indicator strain. Activity was measured and expressed as arbitrary units (AU/ml) as described by Ryan et al. (1996).

Preparation of challenge bacteria for bactericidal assays and teat challenges

The challenge bacteria were prepared by inoculating overnight cultures of *Staph. aureus* DPC 5246, *Str. uberis*

Diernhofer 1932 or *Str. dysgalactiae* M to 1% (v/v) in TSB and incubating at 37 °C for 16 h. Resulting cultures were then diluted to a concentration of 10⁵ cfu/ml using TSB and these dilutions were immediately used as the challenge in bactericidal assays. Initially, challenge concentrations of 10⁵ cfu/ml were also used for experiments in vivo. To ensure sufficient recoveries to allow accurate assessment of effectiveness for experiments in vivo, the concentration of challenge organisms had to be increased to >10⁷ cfu/ml.

Bactericidal assays measuring the effect of teat dips on Gram-positive mastitis pathogens

The teat dip was assessed for its ability to kill Gram-positive mastitis pathogens in vitro. The Bac⁻ fermentate served as the control dip. In each assay, 1 ml of the diluted challenge pathogen was mixed with 10 ml of the teat dip. Immediately after the addition of the challenge to the dip solution, dips were mixed thoroughly. An aliquot of the challenge strain was taken immediately (time zero, T₀) for viability counts and the mixture of teat dip and challenge pathogen was incubated at 37 °C, similarly to experiments by Koskinen et al. (1996). Ryan et al. (1998) previously showed that lactacin 3147 was generally more potent against streptococci than staphylococci and thus, as staphylococcal challenges were expected to be more resilient than streptococci, aliquots in such challenges were taken after 30, 60, 120 and 180 min (T₃₀, T₆₀, T₁₂₀ and T₁₈₀). To examine expectedly quicker changes, aliquots for streptococcal challenges were taken after 15, 30, 60 and 120 min (T₁₅, T₃₀, T₆₀ and T₁₂₀). Each aliquot was immediately diluted and 100 µl of each dilution as well as 100 µl of the original aliquot were plated in duplicate using standard microbiological procedures as described by Twomey et al. (2000). Plates were incubated overnight at 37 °C and viable challenge pathogens were counted. The detection of pathogen activity was very sensitive and pathogens ≥10 cfu/ml could be detected. If no growth was detected on any of the plates (numbers of pathogens <10 cfu/ml) then a value of zero was applied (Twomey et al. 2000), represented as 0 log₁₀ cfu/ml (Fig. 3).

Selection of animals, sampling procedures and microbiological methods for trials in vivo

For the trials in vivo, live teats rather than excised teats were used to mimic field conditions, as the Bac⁺ fermentate, and the challenge pathogens may be affected by the changing temperatures of the teat surface after milking (Isaksson & Lind, 1994) or by other conditions found on live teats, which can be overlooked using an excised teat model. Eighteen teats of eight lactating Holstein-Friesian cows were used for the teat dip trials. Of these, five teats were assigned to the trial with the staphylococcal challenge. Seven teats and a further six teats were assigned to the trials with *Str. dysgalactiae* M and *Str. uberis* Diernhofer 1932, respectively. One teat in each of the three trials served as an undipped control. To identify teats suitable for the experiments, quarter milk samples of cows were collected aseptically from all trial cows before the morning (AM) milking. For the latter sample collection, teats were cleaned by washing with water and then dried with service-paper towels. Immediately before sampling, teats were then disinfected with cotton wool swabs containing Methylated Spirits (Blackhall Pharmaceutical Distributors Ltd., Swords, Co. Dublin, Ireland). Twenty-five ml of milk were collected from each quarter. SCC of milk samples was determined using the Somacount 300[®] (Bentley Instruments Incorporated, USA). Pathogens in milk samples were detected by plating 10 µl of each sample on ABA and incubating overnight at 37 °C. As only teats of healthy quarters were used for the live challenge trials, teats were enrolled if quarter milk samples taken before the trial had a SCC of 400×10^3 cells/ml and were free of pathogens. Additionally, the selected teats were free from warts, lesions, crusts or abrasions, showed a uniform size, with smooth teat skin and no signs of inflammation (redness, tenderness or swelling).

Challenge, teat dipping procedure and recovery of challenge bacteria used in trials in vivo

Following the AM milking, the selected teats and the surrounding skin were prepared for the trial by washing with lukewarm water. Teats and udder were subsequently dried with service paper towels. Washed teats were then disinfected twice from teat base to tip with cotton wool swabs containing Methylated Spirits[®] (Blackhall Pharmaceutical Distributors Ltd). The challenge and teat dipping procedures were essentially a modified version of the procedure described by Philpot et al. 1978. The principal dipping technique is shown in Fig. 1. In brief, the washed and disinfected teats (including control teats) were dipped once in 25-ml beakers containing 12-ml volumes of the challenge culture. The teat tip had to touch the bottom of the beaker to ensure equal coating of each teat with the challenge organism. Following a 5-min drainage time, each of the teats selected for dipping with the DPC

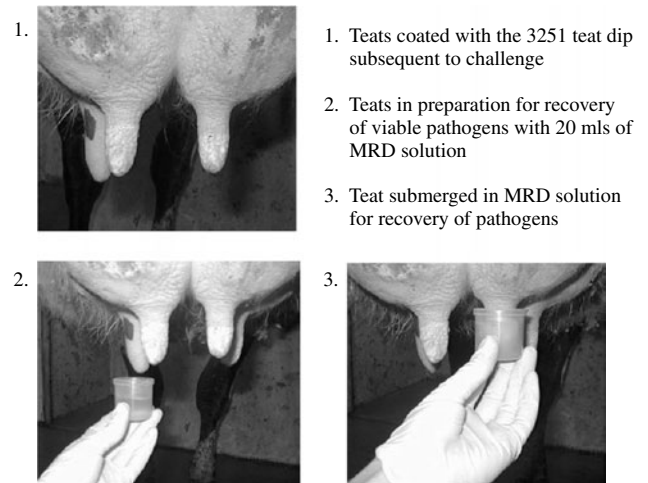


Fig. 1. Technique for challenge, teat dip, wash off and recovery on live teats

3251 teat dip was submerged into a beaker containing 20 ml of the experimental dip, while one teat per organism type remained undipped as a negative control. The contact time for the teat dip was 10 min. Following the contact of the challenge organisms with the dip, the surviving organisms were recovered by submerging the teats (control and dipped teats) into sterile beakers containing 20 ml MRD (Maximum Recovery Diluent, Oxoid Ltd., Hampshire, England). The final recovery suspension from each teat was then stored in Sterilin[®] bottles and immediately transported to the laboratory to enumerate the surviving bacterial cells. The recovery solutions were diluted and plated in duplicate within 2 h of collection. Plates were incubated overnight at 37 °C and viable challenge pathogens were counted.

Aftercare of the animals

To remove any remaining bacteria from the teat surface, all teats were disinfected with cotton wool swabs soaked in Methylated Spirits[®] after the final recoveries were taken. The teats were then washed with lukewarm water to remove any teat-dip residues. Teats were subsequently dried with service paper towels. Finally, the teats were sprayed with a commercial teat dip, Deosan Summer Teatcare Plus[®] (RTU, Diversey Lever) which was used routinely in the herd.

Results

Preparation of the fermented teat dip and lacticin content throughout storage

Teat dips used in all the experiments consisted of the described fermentate of *Lc. lactis* DPC 3251 grown in 10% RSM (Bac⁺ fermentate). Generally, teat dips produced by growing *Lc. lactis* DPC 3251 contained bacterial counts

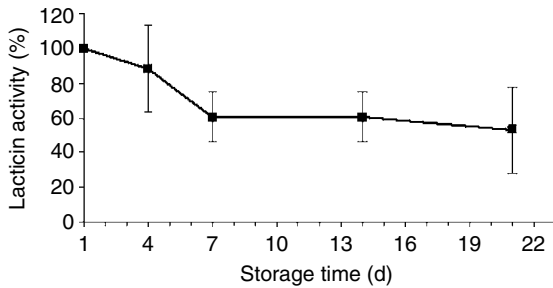


Fig. 2. Mean percentage of lacticin activities of two *Lactococcus lactis* DPC 3251 fermentates over a storage period of 3 weeks at 4 °C. Errors indicate ± 1 sd.

of 10^9 cfu/ml after 20-h incubation at 30 °C. The lacticin activity for each Bac⁺ fermentate used in bactericidal assays or live teat challenges was $\geq 10\,240$ AU/ml at a pH value of approx. 4.4. No lacticin activity was detected in Bac⁻ fermentates and the pH value of the control fermentates was always similar to that of the lacticin-containing fermentate at approx. 4.4.

The lacticin stability of two dips was assessed during storage of the dip at 4 °C for up to 21 d. The mean lacticin activity in the dips declined to 60% in the first 7 d of storage (Fig. 2) but the bacteriocin still retained 53% of its activity until the final sampling day.

Bactericidal assays against Gram-positive mastitis pathogens

The anti-bacterial efficacy of the fermented teat dip was first evaluated *ex vivo* against *Staph. aureus* DPC 5246, *Str. dysgalactiae* M and *Str. uberis* Diernhofer 1932. A control fermentate produced by the Bac⁻ strain *Lc. lactis* DPC 5399 was included in the assays. When exposed to the Bac⁺ fermentate, *Staph. aureus* DPC 5246 was rapidly killed and no live cells were recovered after 30 min of incubation (a reduction of 5.2 log₁₀ cfu/ml). In contrast, the Bac⁻ fermentate resulted in a decrease in viability of only 1.6 log₁₀ cfu/ml (Fig. 3). The Bac⁺ fermentate therefore resulted in a reduction of staphylococci of 3.6 log₁₀ cfu/ml compared with the Bac⁻ fermentate. Similarly, when *Str. dysgalactiae* M was incubated with the Bac⁺ teat dip, no viable pathogens could be detected after 15 min (a reduction of at least 5 log₁₀ cfu/ml; Fig. 3). Given that the Bac⁻ fermentate resulted in a reduction of only 0.7 log₁₀ cfu/ml we conclude that the majority of the bactericidal action of the dip is due to the lacticin content. In contrast, *Str. uberis* Diernhofer 1932 seemed to survive better in the Bac⁺ dip, where numbers were reduced by approximately 2 log₁₀ cfu/ml in the first 15 min (T_0 - T_{15}). After 120 min some live cells were still present albeit at a very low numbers (approx. 60 cfu/ml). Again, *Str. uberis* Diernhofer 1932 was only slightly reduced in the Bac⁻ control solution (Fig. 3).

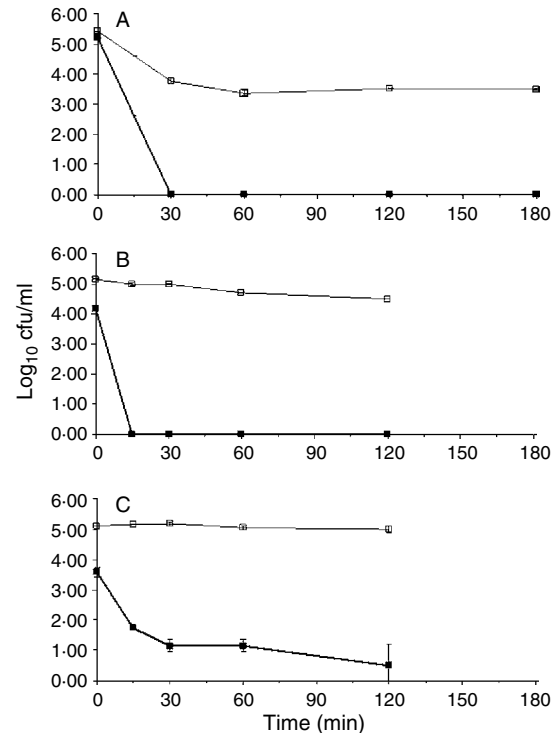


Fig. 3. Bactericidal efficacy of the fermentate of *Lactococcus lactis* DPC 3251 (black squares, ■) and a Bac⁻ fermentate as a control (white squares, □) on *Staphylococcus aureus* DPC 5246 (A), *Streptococcus dysgalactiae* M (B) and *Streptococcus uberis* Diernhofer 1932^{AL} (C). Errors indicate ± 1 sd.

Efficacy of the lacticin-based teat dip *in vivo*

To evaluate the potential value of performing large scale animal trials, we examined the short-term effect of the Bac⁺ fermentate on live teats of lactating dairy cows. For this purpose three separate, small scale teat dip trials were performed in which five teats were challenged with staphylococci, seven with *Str. dysgalactiae* and a further six with *Str. uberis*. To gain sufficient bacterial recoveries for monitoring purposes, challenge concentrations for these trials were set greater than 10^7 cfu/ml. When *Staph. aureus* DPC 5246 was used as the challenge organism, mean recoveries of viable staphylococci on the teats dipped with the DPC 3251 dip were 4.68 log₁₀ cfu/ml. Compared with the recoveries from the untreated control teat, this represented an $80 \pm 8\%$ elimination of the challenge organism on the teat surface (Fig. 4). The mean streptococcal recovery after dipping with the DPC 3251 teat dip was 2.68 log₁₀ cfu/ml for *Str. dysgalactiae* M and the mean elimination was found to be $97.5 \pm 2.5\%$ (Fig. 4). Mean recoveries from teats challenged with *Str. uberis* Diernhofer 1932 were 3.8 log₁₀ cfu/ml, representing an elimination of $90.2 \pm 5.3\%$ (Fig. 4). No animal or quarter enrolled showed any signs of infection with the pathogens used for the challenge procedure after the trial.

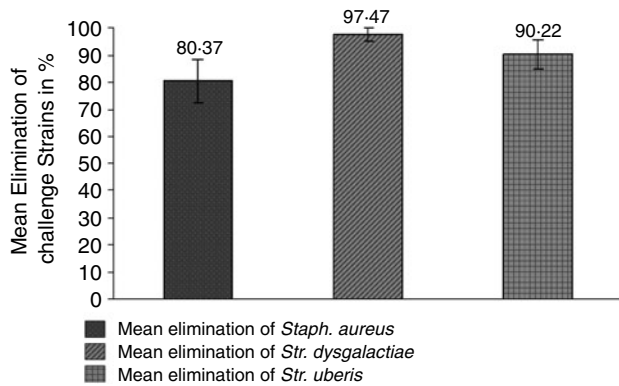


Fig. 4. Mean percent elimination of *Staphylococcus aureus* DPC 5246 (four teats), *Streptococcus dysgalactiae* M (six teats) and *Streptococcus uberis* Diernhofer 1932 (five teats) on teats of cows dipped with 3251 teat dip compared to one undipped control teat each. Errors indicate ± 1 SD

Discussion

The use of teat dips is considered to be an essential element of on-farm practice and contributes to both good milk quality and improved animal health. However, most dips are based on disinfectants of a synthetic nature, which can lead to residues in milk. Such residues may lead to quality problems in later processing such as the presence of high amounts of iodine in infant formula as discussed by Fisher (1989). Only a few studies have addressed the application of more natural products with antimicrobial properties for use as teat dips (Oliver & Mitchell, 1985; Sears et al. 1992; Koskinen et al. 1996; Serieys & Poutrel, 1996; Leon et al. 2004).

In the present study, we generated a fermented teat dip containing a bacteriocin-producing live culture and evaluated its efficacy at killing mastitic pathogens. Importantly, the resultant dip was shown to be highly effective in killing both *Staph. aureus* and *Str. dysgalactiae* as well as reducing numbers of *Str. uberis* by approx. $3 \log_{10}$ cfu/ml (Fig. 3). The effects observed in vitro were more than likely due to the bacteriocin lacticin, since initial controls using acidified RSM (data not shown) and controls using the Bac⁻ fermentate demonstrated that neither acid nor a non-producing strain could result in the reduction of $>1 \log_{10}$ cfu/ml for acidified 10% RSM (data not shown) or $>1.6 \log_{10}$ cfu/ml for the Bac⁻ fermentate in a staphylococcal challenge (Fig. 3). The incomplete elimination of *Str. uberis* by the Bac⁺ fermentate in the experiments ex vivo might have been due to its slower growth rates in milk. Indeed, we have previously shown that the lacticin peptides have a dual mechanism involving both cell wall inhibition (binding to lipid II) and pore formation, the latter of which is associated with the energy state of the cell (McAuliffe et al. 1998; Wiedemann et al. 2006). It has been shown previously that the specific udder pathogens *Staph. aureus* and *Str. dysgalactiae* are highly adapted to a milk-based environment and therefore growth rates of

these two mastitis pathogens in milk can be higher than growth rates of the environmental pathogen *Str. uberis* (Oz et al. 1986). Thus, as lacticin activity depends on the energy status of the target cell, this may explain why *Staph. aureus* and *Str. dysgalactiae* are killed more effectively than the slow-growing *Str. uberis* in the milk-based environment of the fermented dip. A longer incubation period might have resulted in a more complete elimination of *Str. uberis*. However, as *Staph. aureus* is the most frequently isolated strain in Irish dairy cows with high SCC (Barrett et al. 2005) we want to emphasize that the effects of the teat dip resulted in a complete elimination of *Staph. aureus*.

Teat dips containing nisin have been investigated previously (Sears et al. 1992; Serieys & Poutrel, 1996). Lacticin, however, has an advantage over nisin for such applications as it is more active at physiological pH. Indeed, we have previously shown that nisin was unable to inhibit the growth of *Str. dysgalactiae* at neutral pH when incorporated into a teat seal formulation, whereas lacticin was able to show a clear zone of inhibition (Ryan et al. 1998). Similarly, nisin was unable to reduce numbers of staphylococci in skim milk at neutral pH only showing best effects against *Staph. aureus* under low-pH conditions (Sobrinho-Lopez & Martin-Belloso, 2006). These results might suggest that a lacticin-based teat dip would be more effective on the teat surface than a similarly manufactured nisin-based dip, although this would need to be tested experimentally.

In a study by Oliver & Mitchell (1985), a natural preparation made from *Lactobacillus acidophilus* reduced numbers of Gram-positive mastitis pathogens on challenged teats when compared with an iodine solution. On excised teats, the *Lb. acidophilus* preparation reduced levels of *Staph. aureus* and streptococcal species by 72% and $>95\%$, respectively. These numbers are comparable to the efficacy of the 3251 fermentate in the present study, as staphylococci were reduced by approx. 80% and average streptococcal reductions were approx. 96%. Sears et al. (1992) found that purified nisin reduced *Staph. aureus* on the teat skin of live cows by $3.9 \log_{10}$ cfu/ml after an exposure of 1 min compared with a reduction of $0.7 \log_{10}$ cfu/ml for staphylococci in the present study after a contact time of 10 min. It should be emphasised, however, that a preparation based on purified or highly concentrated nisin may prove expensive for such applications. Additionally, it should be noted that as *Staph. aureus* strains can vary significantly in virulence potential (Dingwell et al. 2006) and indeed in susceptibility to lacticin 3147 itself (Ryan et al. 1998), our results cannot be compared directly with the results with nisin as different isolates of *Staph. aureus* were used.

Another factor which should be considered in the analysis of our results is the absence of any surfactants or emollients in the dips described in this study. Surfactants and emollients are used in commercial teat disinfectants in order to ensure proper coating of the teat surface and

to keep the teat skin smooth and moisturized. One such surfactant and one emollient have been integrated into the 3251 dip in other assays. Addition of the surfactant Texapon® (TEA Lauryl Sulphate, courtesy of Cross Vet, Research Laboratories, Ireland) at a concentration of 1% (v/v) or the emollient Glycerol® (Sigma Aldrich Ireland Ltd., Dublin, Ireland) at a concentration of 5% (v/v) to the lactacin teat dip did not increase the average antibacterial effectiveness in these assays when compared with results of the teat dip without additives (data not shown). However, more studies should be undertaken to determine whether other surfactants could enhance the activity of the lactacin-based dip.

It was previously found that under varying natural conditions streptococcal and staphylococcal counts on teats never exceeded approx. 3 or 4 log₁₀ cfu/ml respectively (Hogan et al. 1990). Therefore, artificial teat challenge models present a much higher bacterial count to any experimental teat dip than in conditions found under natural exposure. Therefore under natural conditions, the dip may be even more effective. Another parameter which may have influenced the results of the activity of the dips was the incubation temperature of 37 °C used when recovering pathogens. Although the resting skin surface temperature of the teat in a housed cow averages 30 °C, it has been shown that during milking, the temperature of the teat surface can be subject to change and can range from 29 °C to 38 °C (Isaksson & Lind, 1994). The surface temperature is also higher after milking when the milk flow has warmed the teat surface (Isaksson & Lind, 1994). Therefore, as teat dips are applied immediately post milking, when the milk flow has warmed the teat surface, it was decided that similarly to Koskinen et al. (1996), for the bactericidal assays in vitro, an incubation temperature of 37 °C should be used. This may also have positively selected for pathogens as this temperature is recommended by National Mastitis Council for optimal recovery of pathogens and is also substantially higher than the optimum growth temperature for *Lc. lactis* DPC 3251 of 30 °C (S Morgan, personal communication). To examine the 3251 dip on a larger scale, a natural exposure trial, also evaluating the effectiveness of the dip at varying temperatures, would therefore be recommended. In the natural exposure trial by Koskinen et al. (1996) fewer isolates of *Staph. aureus* (2.09%) or coagulase-negative staphylococci (2.52%) were found in milk from udder quarters dipped with sour milk compared with the results of undipped controls (3.09% and 4.07% of total isolates, respectively). The group dipped with an iodine-containing dip had the least number of *Staph. aureus* isolates (0.83% total isolates); however, there were no statistical significant differences between treatments. Following their natural exposure trial, Oliver & Mitchell (1985) concluded that their *Lb. acidophilus* preparation was effective in preventing intramammary infections by 57% when compared with the number of infections in the undipped control group. Additionally, no teat irritations were noticed. In

a field trial evaluation by Sériey & Poutrel (1996) a commercial dip formulation containing nisin was compared with a 0.5% iodine dip. No significant difference in new infection rates was found. Another non-chemical teat dip based on an Aloe Vera gel was also compared with an iodine dip in a field trial. Results showed that the mastitis occurrence in both groups was not significantly different (Leon et al. 2004). These results have therefore shown that to date, alternative ('non-chemical') teat dips have proven efficient in preventing mastitis.

From a food perspective, consumers are becoming more aware of the sources, contents and the production of food, which is evident from the rising demands for organic and natural dairy products (McElroy, 2008). In this respect, a natural, non-chemical teat dip containing food grade bacteria would certainly be of benefit for the dairy industry. Additionally, given that chemical teat dips can cause severe irritation of the skin (Sears et al. 1992), the search for milder agents with antimicrobial action seems to be a sensible approach. In the present study, the teat skin effect of an application of the 3251 dip was not examined, though in the long term trials by Koskinen et al. (1996) and Oliver & Mitchell (1985) using similar products, no adverse effects on the teat skin with either teat dip were mentioned.

If teat dips containing food grade bacteria were to be commercialized, problems with increases in total bacteria count (TBC) might arise as levels of, in this case, *Lc. lactis* DPC 3251 in the milk might lead to a rise in the resulting bacterial count. Dairy co-operatives, however, could use differential counting of TBC for dairy farms using such a teat dip. The effect of milk containing LAB on processing would also require investigation, though the process of pasteurization, which would eliminate any remaining lactococci, is used in most dairy processing plants and raw milks are rarely used.

In conclusion, the lactacin-containing 3251 teat dip used in this study decreased Gram-positive mastitis pathogens under laboratory conditions as well as on the teats of live cows. Further investigations in accordance with National Mastitis Council recommendations (Nickerson et al. 2004) would be required in order to produce a commercially viable product.

References

- Arbesman H 2005 Dairy and acne—the iodine connection. *Journal of the American Academy of Dermatology* **53** 1102
- Barrett DJ, Healy AM, Leonard FC & Doherty ML 2005 Prevalence of pathogens causing subclinical mastitis in 15 dairy herds in the Republic of Ireland. *Irish Veterinary Journal* **58** 333–337
- Bennett R, Christiansen K & Clifton-Hadley R 1999 Preliminary estimates of the direct costs associated with endemic diseases of livestock in Great Britain. *Preventive Veterinary Medicine* **39** 155–171
- Crispie F, Alonso-Gomez M, O'Loughlin C, Klostermann K, Flynn J, Arkins S, Meaney W, Ross RP & Hill C 2008 Intramammary infusion of a live culture for treatment of bovine mastitis: effect of live lactococci on the mammary immune response. *Journal of Dairy Research* **75** 374–384

- Crispie F, Twomey D, Flynn J, Hill C, Ross P & Meaney W 2005 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*. *Journal of Dairy Research* **72** 159–167
- Danby FW 2007 Acne and iodine: reply. *Journal of the American Academy of Dermatology* **56** 164–165
- Dingwell RT, Leslie KE, Sabour P, Lepp D & Pacan J 2006 Influence of the genotype of *Staphylococcus aureus*, determined by pulsed-field gel electrophoresis, on dry-period elimination of subclinical mastitis in Canadian dairy herds. *Canadian Journal of Veterinary Research* **70** 115–120
- Fisher DA 1989 Upper limit of iodine in infant formulas. *Journal of Nutrition* **119** 1865–1868
- Galton DM, Petersson LG & Erb HN 1986 Milk iodine residues in herds practicing iodophor premilking teat disinfection. *Journal of Dairy Science* **69** 267–271
- Halasa T, Huijps K, Østerås O & Hogeveen H 2007 Economic effects of bovine mastitis and mastitis management: a review. *Veterinary Quarterly* **29** 18–31
- Hogan JS, Smith KL, Todhunter DA & Schoenberger PS 1990 Bacterial counts associated with recycled newspaper bedding. *Journal of Dairy Science* **73** 1756–1761
- International Dairy Federation 1981 Chapter 3: Isolation and identification of mastitis bacteria. In: *Laboratory methods for use in mastitis work*, *Bulletin* **132** 19–26
- Isaksson A & Lind O 1994 Brief Communication: Milking-related changes in the surface temperature of the bovine teat skin. *Acta Veterinaria Scandinavica* **35** 435–438
- Klostermann K, Crispie F, Flynn J, Ross RP, Hill C & Meaney W 2008 Intramammary infusion of a live culture of *Lactococcus lactis* for treatment of bovine mastitis: comparison with antibiotic treatment in field trials. *Journal of Dairy Research* **75** 365–373
- Koskinen E, Rantala M & Saloniemi H 1996 The effect of sour milk as a postmilking teat dip for mastitis prevention in a dairy herd. *Acta Veterinaria Scandinavica* **37** 427–432
- Leon L, Beer C, Wacken H, Nurnberg M & Andersson R 2004 [Effect on teat skin condition and effectivity against new intramammary infections of a barrier teat dip based on aloe vera gel.] *Tierärztliche Umschau* **59** 237–244
- Losinger WC 2005 Economic impacts of reduced milk production associated with an increase in bulk-tank somatic cell count on US dairies. *Journal of the American Veterinary Medical Association* **226** 1652–1658
- McAuliffe O, Ryan MP, Ross RP, Hill C, Breeuwer P & Abee T 1998 Lacticin 3147, a broad-spectrum bacteriocin which selectively dissipates the membrane potential. *Applied and Environmental Microbiology* **64** 439–445
- McElroy B 2008 Global statistics of the organic market. Ecological Farming Conference, Pacific Grove CA, USA.
- Nickerson SC, Saxon A, Fox LK, Hemling T, Hogan JS, Morelli J, Oliver SP, Owens WE, Pawlak M & Petersson L 2004 National Mastitis Council: Recommended protocols for evaluating efficacy of postmilking teat germicides. In: *NMC Annual Meeting Proceedings* pp. 379–399
- Oliver SP & Mitchell BA 1985 Prevention of bovine mastitis by a *Lactobacillus acidophilus* preparation. *Journal of Dairy Science* **68** 271
- Oz HH, Farnsworth RJ & Cox HU 1986 Growth of Gram-positive mastogenic bacteria in normal, simulated bulk tank, and mastitic milk held at simulated fluctuating temperatures of farm bulk tank. *Journal of Dairy Science* **69** 2060–2065
- Pankey JW, Eberhart RJ, Cuming AL, Daggett RD, Farnsworth RJ & McDuff CK 1984 Uptake on postmilking teat antiseptics. *Journal of Dairy Science* **67** 1336–1353
- Park YK, Harland BF, Vanderveen JE, Shank FR & Prosky L 1981 Estimation of dietary iodine intake of Americans in recent years. *Journal of the American Dietetic Association* **79** 17–24
- Pennington JA, Young BE & Wilson DB 1989 Nutritional elements in US diets: results from the Total Diet Study, 1982–1986. *Journal of the American Dietetic Association* **89** 659–664
- Persson Waller K, Bengtsson B, Lindberg A, Nyman A & Ericsson H 2009 Incidence of mastitis and bacterial findings at clinical mastitis in Swedish primiparous cows—influence of breed and stage of lactation. *Veterinary Microbiology* **134** 89–94
- Philpot WN, Boddie RL & Pankey JW 1978 Hygiene in the prevention of udder infections. IV. Evaluation of teat dips with excised cows' teats. *Journal of Dairy Science* **61** 950–955
- Rea MC & Cogan TC 1994 Buttermilk plants: the Irish version of kefir. *Irish Scientist* **2** 7
- Ryan MP, Rea MC, Hill C & Ross RP 1996 An application in cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, lacticin 3147. *Applied and Environmental Microbiology* **62** 612–619
- Ryan MP, Meaney WJ, Ross RP & Hill C 1998 Evaluation of lacticin 3147 and a 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, reduced the incidence of mastitis after experimental challenge with *Streptococcus dysgalactiae* in nonlactating dairy cows. *Journal of Dairy Science* **82** 2625–2631
- Sears PM, Smith BS, Stewart WK, Gonzalez RN, Rubino SD, Gusik SA, Kulisek ES, Projan SJ & Blackburn P 1992 Evaluation of a nisin-based germicidal formulation on teat skin of live cows. *Journal of Dairy Science* **75** 3185–3190
- Serieys F & Poutrel B 1996 Field trial evaluation of two teat dips containing nisin or polyvinylpyrrolidone iodophor designed for use before and after milking. *Veterinary Research* **27** 295–303
- Sit CS & Vederas JC 2008 Minireview: Approaches to the discovery of new antibacterial agents based on bacteriocins. *Biochemistry and Cell Biology* **86** 116–123
- Sobrinho-Lopez A & Martin-Belloso O 2006 Enhancing inactivation of *Staphylococcus aureus* in skim milk by combining high-intensity pulsed electric fields and nisin. *Journal of Food Protection* **69** 345–353
- 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
- Vanderveen JE 1979 Estimation of total iodine consumption by Americans in recent years. Presented at: *Iodine Workshop*, American Medical Association, Scottsdale, Arizona, November 13–14
- Wiedemann I, Bottiger T, Bonelli RR, Wiese A, Hage SO, Gutschmann T, Seydel U, Deegan L, Hill C, Ross RP & Sahl HG 2006 The mode of action of the lantibiotic lacticin 3147—a complex mechanism involving specific interaction of two peptides and the cell wall precursor lipid II. *Molecular Microbiology* **61** 285–296
- Whist AC, Østerås O & Solverod L 2007 *Staphylococcus aureus* and *Streptococcus dysgalactiae* in Norwegian herds after introduction of selective dry cow therapy and teat dipping. *Journal of Dairy Research* **74** 1–8
- Yalcin C, Stott AW, Logue DN & Gunn J 1999 The economic impact of mastitis-control procedures used in Scottish dairy herds with high bulk-tank somatic-cell counts. *Preventative Veterinary Medicine* **41** 135–149