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Intramammary infusion of a live culture of *Lactococcus lactis* for treatment of bovine mastitis: comparison with antibiotic treatment in field trials

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A treatment containing a live food-grade organism, *Lactococcus lactis* DPC3147, was compared with conventional antibiotic therapy for its potential to treat bovine chronic subclinical or clinical mastitis in two separate field trials. Effects on disease symptoms and bacteriology were monitored in response to infusion with the culture in each trial. In the first trial, the live culture treatment was compared with an intramammary antibiotic (n=11 quarters for each treatment). Results from this small trial demonstrated that the live culture had potential to be as effective at eliminating chronic subclinical infections as an antibiotic treatment. By day 12, 7 of the 11 quarters treated with the live culture were pathogen-free compared with 5 of the 11 antibiotic-treated infected quarters. Somatic cell counts (SCC) remained relatively unchanged regardless of treatment: average log SCC pre- and post-treatment in the lactococci-treated group were 6·33±0·41 (day 0) and 6·27±0·43 cells/ml (day 12) and average log SCC pre- and post-treatment in the antibiotic-treated group were 6·34±0·37 and 6·22±0·46 cells/ml on day 0 and on day 12, respectively. In the second trial, the live culture was compared with an intramammary antibiotic for the treatment of naturally occurring clinical mastitis cases (n=25 quarters for each treatment). Following a 14-d experimental period, similar bacteriological responses were observed in 7 out of 25 live culture treated quarters and 9 out of 25 antibiotic-treated quarters. Additionally, 15 of 25 cases treated with the culture and 18 of 25 cases treated with the antibiotic did not exhibit clinical signs of the disease following treatment. The results of these trials suggest that live culture treatment with *Lc. lactis* DPC3147 may be as efficacious as common antibiotic treatments in some instances.

Keywords: Live, *Lactococcus*, treatment, mastitis.

Mastitis is considered the most persistent disease in dairy cows and is of great economic importance as it is associated with decreased milk production, expensive treatment costs, extra labour and an increased rate of culling (Klaas et al. 2004). Treatment of the disease accounts for the most common cause of antibiotic use in dairy cows, where it can lead to antibiotic residues in milk with concomitant economic losses due to discarding milk during and post treatment (Kaneene & Miller, 1992; Costello, 2004). In addition, some studies suggest that antibiotics may be relatively ineffective for the treatment of pathogens like *Staphylococcus aureus*, which can internalize in eukaryotic host cells, evading the immune system (Barkema et al. 2006). An effective, non-antibiotic mastitis treatment could reduce costs associated with antibiotic therapy and would also relieve some of the pressures facing the agricultural and veterinary sectors to limit the use of antibiotics. Several alternative approaches have therefore been used in the treatment of mastitis, including milking the infected quarter several times a day (Roberson et al. 2004), hydrotherapy, intramammary infusions of glucose solutions (Reinhold et al. 1986) and the use of lysostaphin (Oldham & Daley, 1991) and nisin (Cao et al. 2007; Wu et al. 2007). Other remedies have included the use of casein hydrolysates (Silanikove et al. 2005), hot and cold packing, ultrasonic therapy, topically applied liniments (Knight et al. 2000), drugs such as the milk

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ejection hormone oxytocin (Guterbock et al. 1993), antihistamines, steroids, non-steroidal anti-inflammatories and homoeopathic preparations (Varshney & Naresh, 2004). Furthermore, the intramammary treatment of subclinical mastitis with *Lactobacillus* species through competitive elimination has been studied by Greene et al. (1991).

In the animal trials reported in this paper, a novel non-antibiotic treatment, consisting of a live culture suspension of *Lactococcus lactis* DPC3147, was examined for its ability to treat naturally occurring mastitis. *Lc. lactis* DPC3147 belongs to a family of bacteria known collectively as the lactic acid bacteria. These organisms are widely used in the dairy industry as starters for fermentative processes and, as such, are food grade. *Lc. lactis* DPC3147 produces the bacteriocin lacticin 3147, a two-component lantibiotic (Ryan et al. 1999b), which inhibits a broad spectrum of Gram-positive bacteria including mastitis-causing bacteria such as streptococci and staphylococci (Ryan et al. 1998). Indeed, it was previously shown that the bacteriocin itself can reduce the incidence of mastitis after experimental challenge in non-lactating dairy cows when incorporated in a Teat Seal preparation (Ryan et al. 1999a). More recently, a whey containing lacticin 3147 formulation in a bismuth-based teat seal was effective in reducing bacterial recoveries from teats deliberately infected with *Staphylococcus aureus* in lactating dairy cows (Crispie et al. 2005).

In the present study, a live culture of *Lc. lactis* was assessed for efficacy in the treatment of bovine mastitis. Naturally infected animals were used in two trials, limiting case numbers and study size to small, preliminary trials of a size comparable to that reported previously (Greene et al. 1991). In Trial 1, the live culture treatment was used in subclinical cases caused mostly by *Staph. aureus*, whereas in Trial 2, the efficacy of infusing a live culture of *Lc. lactis* DPC3147 for the treatment of acute clinical mastitis in lactating dairy cows was assessed.

Materials and Methods

Selection of animals and clinical assessment

Cows for both trials were selected from four adjacent herds mainly consisting of Holstein-Friesian cows as well as small numbers of New Zealand Friesians, Norwegian Reds, Normandes and Montbelliards. Following selection of suitable quarters, Trial 1 was undertaken over a 2-week period in July 2003. Detection of naturally occurring clinical cases of mastitis and enrolment of quarters in Trial 2 was undertaken over a period of 8 months beginning in January 2005. All cows enrolled in the study were routinely milked twice a day where pre-milking udder preparation consisted of washing with water, forestripping and drying teats with service paper towels. Following milking, teats were sprayed with Deosan Summer Teatcare Plus® (RTU, Diversey Lever) a ready-to-use teat skin disinfectant containing chlorhexidine. At the end of their lactation period, the teats of all cows were infused with long-acting antibiotics (dry cow treatment).

In Trial 1, chronic subclinical mastitis cases were used to evaluate the treatment. Selection of animals was made using a combination of SCC and bacteriological data from quarter milk samples. Composite milk of individual cows was also sampled at monthly intervals throughout the lactation. SCC in the milk samples was determined using a Somacount 300® (Bentley Instruments Incorporated, USA). Similarly to previous studies (Berry & Meaney, 2006; Kivaria et al. 2007; Sandgren et al. 2008) cases were defined as 'chronic subclinical' when the SCC was elevated ($>3\times10^5$ cells/ml) in at least two samples and a pathogen was present or when the SCC was elevated ($>3\times10^5$ cells/ml) in at least three samples and no pathogen was present. There was no swelling of the udder or visible abnormalities in the milk of chronic subclinically infected quarters. Twenty-two chronic subclinical infections were selected in 12 cows in Trial 1. Eleven cases were infused with culture and 11 cases were infused with an intramammary antibiotic.

In Trial 2, newly acquired clinical cases of mastitis were detected by trained dairy personnel during the process of forestripping. Quarters were classified as having clinical mastitis when an udder quarter was inflamed and visible abnormalities such as clots were present in the milk. Clinical cases were subsequently assessed by a member of the study team as to the severity of the individual case and classified as either mild or severe based on milk and udder appearance and rectal temperature. If the clots in the foremilk were small to medium in size and the milk and udder showed slight abnormalities, such as 'watery' consistency and slight swelling and tenderness of the udder quarter, the case was enrolled as mild. Mild cases had rectal temperatures ranging from 38·5 °C to 39·5 °C. In contrast, if clots were large and the foremilk appeared abnormal in character, the disease was classified as severe. In severe cases the udder quarter was swollen, hot and painful to the touch and rectal body temperatures were 39·5 °C or higher. Overall in Trial 2, 50 cases of clinical mastitis were identified in 48 cows and randomly assigned at the time of detection to either culture or antibiotic treatment respectively. Two cows showing clinical mastitis in two different quarters at different time points were assigned once to culture treatment and once to antibiotic treatment. Udder condition, rectal temperatures and SCC were assessed throughout the trial by trained farm staff and veterinary personnel.

Preparation of live culture treatments

The live culture was prepared as follows: *Lc. lactis* DPC3147 (1%, v/v) was grown at 30 °C for 16 h in M17 broth (DIFCO, Becton, Dickinson and Company, Maryland, USA), containing 0·5% lactose (w/v) (LM17). In Trial 1, 2 ml of this overnight culture was mixed with 3 ml of sterile Water for Injection B.P.® (Antigen

Pharmaceuticals Ltd., Roscrea, Ireland) and the 5 ml diluted culture/water mix was used as the treatment.

In Trial 2, in an effort to develop a commercially viable live culture product, a washed cell suspension was infused. Treatments were prepared in this trial by incubating *Lc. lactis* DPC3147 for 16 h at 30 °C in LM17. The cells were subsequently harvested by centrifugation at 3500 ***g*** for 15 min and the supernatant discarded. The cell pellet was then washed with sterile water, centrifuged again and resuspended in sterile water. This suspension was divided into 5-ml volumes and frozen at −80 °C. The frozen culture was then freeze dried in a Modulyo 4K Freeze drier (Edwards, Sussex, UK). Before treatment, the freeze-dried cell pellet was resuspended in 5 ml of sterile Water for Injection B.P.® (Antigen Pharmaceuticals Ltd., Roscrea, Ireland). The resuspended live culture was then used as a single treatment. This suspension contained approximately $9{\cdot}1 \pm 0{\cdot}5$ $\log_{10}$ cfu/ml (colony forming units, average of 12 different counts) of live *Lc. lactis* DPC3147.

Treatment protocol

To compare the efficacy of treatment with lactococci *v.* antibiotics, two trials were conducted, involving cows with chronic subclinical or newly acquired clinical mastitis. Infected quarters in each trial were treated with live culture or with antibiotics. As it is unethical to withhold treatment from animals that are known to be infected, untreated controls could not be used in the experiments.

In both trials, treatments were infused in an aseptic manner after milking. For the infusion of the cultures, a syringe with a blunted smoothed tip was used to prevent injury to the teat. In Trial 1, the culture and water mixture was infused after the morning milking on day 1 and day 2 of the trial, with a 24-h interval between treatments. The antibiotic control treatment was administered at 12-h intervals as per the manufacturer's instructions, with administration following the morning and evening milkings on day 1 and following the morning milking on day 2. As *Staph. aureus* is one of the major causes of subclinical mastitis in Ireland and >80% of *Staph. aureus* isolates from Irish dairy herds exhibit a significant level of resistance towards penicillin G (Barrett, 2004), the control treatment was a commercial intramammary antibiotic formulation containing amoxycillin (200 mg), clavulanic acid (50 mg) and prednisolone (10 mg) (Synulox®, Pfizer Animal Health, New York, USA). Milk for human consumption was withheld for five milkings after the last treatment as instructed by the manufacturer.

In Trial 2, the resuspended culture was infused on day 1, day 2 and day 3 with 24-h intervals between treatments. To standardize treatment intervals for the two treatments and to provide a broad-spectrum treatment for naturally occurring clinical cases, the antibiotic treatment in this trial was a commercial formulation containing 150 mg penethamate hydriodide, 150 mg dihydrostreptomycin (as sulphate), 50 mg framycetin sulphate and 5 mg prednisolone (Leo Yellow®, Leo Laboratories Limited, Ireland). This product was infused at 24-h intervals on day 1, day 2 and day 3 as recommended by the manufacturer. Milk from cows treated with the antibiotic was withheld from the bulk supply for seven milkings after the last treatment.

Milk sampling protocol

Milk samples in both trials were taken prior to treatment and on day 12 and day 14 after treatment in Trial 1 and Trial 2, respectively, to monitor the progress of the infused quarter. Before sample collection, teats were cleaned by washing with water and then dried with service-paper towels. Immediately before sampling, teats were disinfected with cotton wool swabs containing methylated spirits (Blackhall Pharmaceutical Distributors Ltd., Swords, Co. Dublin, Ireland). Samples (20–25 ml) were collected in single-use sterile plastic containers. In Trial 1, as the treated quarters had chronic subclinical infections, SCC was measured and used as an indication of disease. In Trial 2, the California Mastitis Test (CMT) and the presence of clots were used as subjective measures of SCC as described previously (Crispie et al. 2005). A clinical response in Trial 2 was defined as a day 14 post-treatment sample that showed no visible clots or flakes, whose udder condition appeared normal and that had SCC $<9 \times 10^6$ cells/ml. The presence or absence of pathogens was not taken into account when classifying clinical response.

Microbiological assessment and bacterial enumeration

Milks obtained from animals in the studies were analysed microbiologically following recommendations of the International Dairy Federation (1981). In brief, 10 µl of each milk sample was surface plated on aesculin blood agar (ABA). ABA was prepared from Blood Agar Base No. 2 (Merck®, Darmstadt, Germany), containing 0·1% aesculin (w/v) (Sigma-Aldrich® Ireland Ltd., Dublin, Ireland) and 5–8% (v/v) whole calf-blood. Plates were incubated overnight at 37 °C and bacteria were identified on colony appearance, growth characteristics, morphology and on type of haemolysis. For analysis of the bacterial counts in Trial 1, arbitrary values were assigned to staphylococcal counts in a manner similar to that outlined by Zadoks et al. 2003.

Bacteriological eliminations in Trials 1 and 2 were defined as the absence of the infecting bacteria in the last sample post-infusion (day 12 and day 14 respectively). In Trial 2, a bacteriological response was defined as a day 14 post-treatment sample with SCC $<1 \times 10^6$ cells/ml and a pathogen count <500 cfu/ml. These values were in a similar range to those used by Corlett et al. (1984) as a threshold to define infected from non-infected quarters in a study examining the effect of an intramammary device in the control of mastitis.

In cases where the same bacterial species isolated on day 1 persisted throughout the experimental period in Trial 2, bacteria were purified after isolation and cultures were stored in 80% glycerol at −20 °C. These isolates were later subjected to 16s rRNA sequencing which was carried out essentially as described by Simpson et al. (2002).

Statistical analysis and data processing

The overall number of cases showing a clinical or a bacteriological response in Trial 2 was analysed using Chi-Square analysis, allowing a comparison of cases treated with the culture with cases in the control group (intramammary antibiotic treatment). Fisher's Exact Probability Test was used for a comparison of clinical responses and bacteriological responses in the subgroups.

SCC was available in precise counts up to $9{\cdot}99 \times 10^6$ cells/ml milk. Beyond this value, the somatic cells in a sample were estimated using CMT score and clinical appearance.

Results

Given that we had previously demonstrated that the bacteriocin lacticin 3147 was effective in preventing both staphylococcal and streptococcal mastitis (Ryan et al. 1998, 1999a; Twomey et al. 2000; Crispie et al. 2005) we decided to test the efficacy of the producing culture for treatment of the disease. It is important to emphasize that in both these trials high numbers of lactococci could be found in most quarters at the first milking post infusion but the culture did not survive for longer than 14 d in any of the treated udder quarters (data not shown).

Trial 1

In Trial 1, 22 chronic subclinical infections in 12 cows were treated with live culture or an intramammary antibiotic and the effects on pathogen survival and on SCC were observed. Most cows had two infected quarters and in these animals, one infected quarter was treated with *Lc. lactis* and the other with antibiotic. All cows were in late lactation, 6 cows were in 1st or 2nd lactation and 6 were in greater than 3rd lactation. Overall, pathogens were eliminated from four quarters treated with *Lc. lactis* and from three *Staph. aureus* infected quarters treated with amoxycillin/clavulanic acid (Table 1). SCC levels remained relatively unchanged in both groups.

Bacteriology

Of the 22 chronic subclinical infections, most were caused by *Staph. aureus*. The level of pathogen found and the SCC of milk samples on days 0 and 12 are shown (Table 1). Overall, on day 12, 7 of 11 quarters treated with *Lc. lactis* and 5 of 11 treated with antibiotics were pathogen-free. On day 12, 3 of the 6 quarters treated with *Lc. lactis* DPC3147 that had originally been infected with *Staph. aureus* were no longer shedding staphylococci or any other pathogen. One quarter in the culture treated group that had been chronically infected with *Streptococcus uberis* also showed no bacterial growth on day 12. In the group treated with antibiotics, 3 of the 8 quarters originally infected with *Staph. aureus* were pathogen-free on day 12. In each treatment group, culture and amoxycillin/clavulanic acid, one case that had no pathogen isolation on day 0 showed staphylococcal growth at the last sampling (Table 1).

Table 1. Bacteriological and SCC response to intramammary infusion of *Lc. lactis* DPC3147 or an intramammary antibiotic containing amoxycillin/clavulanic acid in chronic subclinical infections in Trial 1

	Bacteriology‡		(SCC × 10^{-3}) cells/ml	
Case No.†	Day 0	Day 12	Day 0	Day 12
1	0	0	4759	6138
2	+	0	5227	2399
3	++§	0	592	1358
4	0	0	2388	2697
5	++	++	3690	3939
6	++	0	3638	138
7	+	0	5601	2604
8	0	++	390	1124
9	++	+	2892	2998
10	+++	+++	2057	2388
11	0	0	661	1568
12	+	+	7659	5318
13	++	0	761	406
14	++	0	1735	1141
15	+++	++	2160	2862
16	+	+	2999	1539
17	0	0	428	854
18	0	0	1602	206
19	+	0	3371	6052
20	0	+	3030	2730
21	+++	++	1653	1885
22	+	++	6684	4281

† Cases 1–11 were treated with *Lc. lactis* and cases 12–22 were treated with the antibiotic control treatment

‡ Bacteria were enumerated and scored according to the following: 0 = Absence of pathogens + = < 40 cfu/10 µl, ++ = 40–400 cfu/µl and +++ = > 400 cfu/µl

§ *Str. uberis* infection

SCC response

Overall, in Trial 1, SCC remained relatively unchanged regardless of treatment. On the final day of sampling (day 12), one quarter in each treatment group had an SCC $< 3 \times 10^5$ cells/ml (Table 1). Average log SCC pre- and post-treatment in the lactococci-treated group were $6{\cdot}33 \pm 0{\cdot}41$ (day 0) and $6{\cdot}27 \pm 0{\cdot}43$ cells/ml (day 12) and average log SCC pre- and post-treatment in the antibiotic

treated group were 6·34±0·37 (day 0) and 6·22±0·46 cells/ml (day 12).

Trial 2

Fifty cases of clinical mastitis in 48 cows were selected for Trial 2, with two cows presenting clinical mastitis in two different quarters at different time points. The parity, lactation status and previous mastitis history of the cows enrolled in Trial 2 are shown in Table 2. To mimic normal farming conditions, where farmers treat clinical cases 'blindly' as they appear throughout the lactation period, cases were enrolled and randomized at the time of detection, immediately prior to treatment. Using this technique, the treatment groups could not be stratified either on parity, lactation status or mastitis history. The effect of the treatments on bacteriology and disease symptoms was assessed in the 50 cases as described in Materials and Methods.

Bacteriology

Table 3 shows the pathogen types recovered from milk samples taken on day 0 and day 14 for both culture and antibiotic treated groups, respectively. Pathogens were present in 35 of the day 0 samples, culture (n=17) and antibiotic (n=18). Overall bacteriological elimination rates were 61% (11/18) and 47% (8/17) for antibiotic and live culture treatments respectively (Table 3).

Eighteen of the 35 pathogen positive cases on day 0 still showed growth of pathogens in the live culture (n=11) and antibiotic treated groups (n=7) on day 14. Based on colony morphology and haemolysis as well as 16s rRNA sequencing (data not shown), it was concluded that in all but two cases, the pathogen isolated from the final sample was the same as the pathogen isolated on day 0, indicating a persisting infection or re-infection with the same pathogen. The two cases where a new infection occurred were both in quarters treated with the culture. In one of these cases, *Streptococcus dysgalactiae* was isolated on day 0 and a non-haemolytic staphylococcus was isolated on day 14. In the second case, a mixture of *Streptococcus uberis* and yeasts was isolated on day 0 and *Staph. aureus* was isolated on day 14. These two cases were therefore classified as showing bacteriological elimination, as the original infecting organism was no longer present (Table 3).

The treatment groups were also divided on the basis of severity of mastitis in addition to showing pathogen growth on day 0, i.e. culture mild (n=7), culture severe (n=10), antibiotic mild (n=8) and antibiotic severe (n=10) subgroups. In the group treated with the culture, two of the mild (2/7) and four of the severe cases (4/10) had no detectable pathogen on day 14. In the antibiotic-treated group, 4 of the 8 mild pathogen-positive cases and 7 of the 10 severe cases were negative on day 14.

Overall, 16 of the 50 treated quarters (32%) showed a bacteriological response, i.e. had a day 14 post-treatment sample with an SCC $<1\times10^6$ cells/ml and a pathogen count of <500 cfu/ml. In the group treated with *Lc. lactis*, this was achieved in 7 of 25 quarters (28%), whereas 9 cases responded to the antibiotic treatment (38%) (Table 3). The difference in overall bacteriological response rates between the *Lc. lactis* treated group and the antibiotic treated group was not statistically significant (P=0·5443). Treatment groups were subdivided according to the severity of mastitis on day 0 (mild and severe). In the mild clinical group, 4 of 13 cases treated with *Lc. lactis* and 4 of 12 cases treated with the antibiotic showed a bacteriological response. In the severe cases, 3 of 12 quarters treated with the culture and 5 of 13 quarters treated with the antibiotic showed a bacteriological response (Table 3).

In 15 of the day 0 samples, no pathogen growth was observed following overnight incubation at 37 °C. Of these, 10 remained negative on day 14: 5 had been treated with antibiotic (3 in the mild group, 2 in the severe group) and a further 5 had been treated with *Lc. lactis* (4 in the mild group and 1 in the severe group). Interestingly, 5 of the 15 day 0 pathogen negative samples showed bacterial growth on day 14. *Str. uberis* (n=2) and *Str. dysgalactiae* (n=1) were isolated from three of these cases treated with the culture. Both *Str. uberis* infected quarters were associated with mild clinical symptoms, whereas the *Str. dysgalactiae* infection was classified as severe. *Staph. aureus* was isolated from one mild and one severe mastitis case in the antibiotic treated group on day 14.

Table 2. Cow parity, days in milk (DIM) and previous history of mastitis at the time of enrolment in Trial 2

	Number of cases treated with live culture	Number of cases treated with antibiotic control treatment
Lactation No. at enrolment		
Lactation1	6	11
Lactation2	3	3
Lactation3	4	2
Lactation>3	12	9
Total	25	25
DIM at enrolment		
Early lactation (⩽90 d)	16	18
Mid lactation(90–180 d)	8	5
Late lactation (⩾180 d)	1	2
Total	25	25
Previous mastitis history of enrolled quarters		
Total	6	3

Clinical response

Fifty quarters were diagnosed with mastitis and selected for the trial. There were 13 and 12 out of the 25 mild cases

Table 3. Bacteriological elimination and bacteriological responses in clinical quarters treated with live culture (Top) or antibiotics (Bottom) in Trial 2

Pathogen type	Day 0 (no. of cases)	Day 14 (no. of cases showing bacterial elimination)	Day 14 (no. of cases showing bacteriological responses)
No pathogen isolated†	8	5	4
Staph. aureus (haemolytic)	7	3	2
Staph. aureus (non-haemolytic)	1‡	1	0
Str. dysgalactiae	5	2§	1
Str. uberis	1	0	0
Other streptococci	1	1	0
Escherichia coli (non-haemolytic)	1	0	0
Others (Yeasts, *Arcanobacterium pyogenes*)	1¶	1§	0
Total no. of cases	25	13	7
No pathogen isolated ††	7	5	5
Staph. aureus (haemolytic)	8	4	1
Str. dysgalactiae	5	2	1
Str. uberis	3	3	1
Escherichia coli (non-haemolytic)	1‡	1	0
Others (Yeasts, *Arcanobacterium pyogenes*)	1	1	1
Total no. of cases	25	16	9

† Cases treated with live culture
‡ quarter dried off before day 14, secretions plated
§ one quarter showed elimination of the pathogen isolated on day 0 and showed a different pathogen on day 14, indicating a new infection with a different organism had occurred
¶ mixed infection with *Str. uberis* and yeasts
††Cases treated with antibiotic

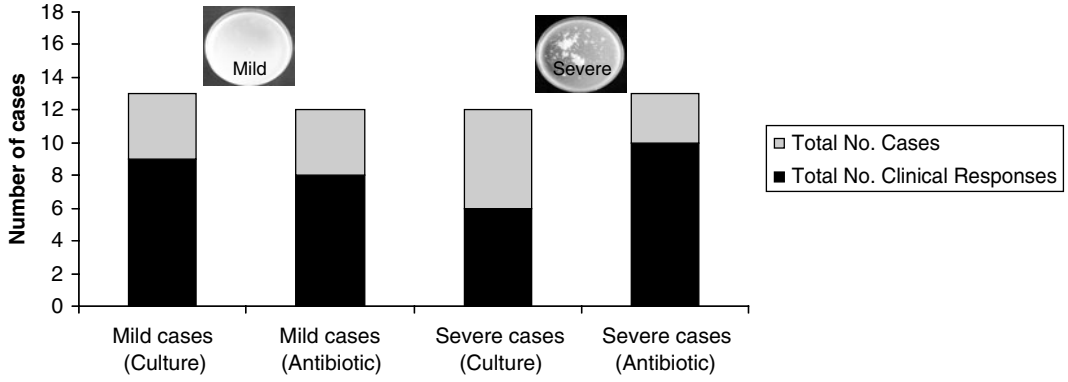

Fig. 1. Trial 2: Clinical responses on day 14 in culture and antibiotic treated quarters with photographs of a milk sample on day 0 from one mild clinical and one severe clinical case.

in culture or antibiotic treated groups, respectively. Furthermore, 12 cases treated with the culture and 13 cases treated with the antibiotic were diagnosed as severe on day 0. An illustration of udder secretions from mild and severe cases on day 0 is shown in Fig. 1. Overall, 15 of the 25 cases treated with the culture and 18 of the 25 cases treated with the antibiotic showed a clinical response at day 14. This difference was not significant (P=0·5443). Nine of the 13 mild clinical quarters treated with *Lc. lactis* and 8 of 12 mild cases treated with the antibiotic showed a clinical response at the final sampling. In the severe cases, 6 of 12 cases treated with the culture responded clinically on day 14, compared with 10 of 13 cases treated with the antibiotic (Fig. 1).

Discussion

The aim of the two studies reported here was to evaluate the effectiveness and safety of a live culture of *Lc. lactis*

DPC3147 for the treatment of clinical and chronic subclinical cases of naturally occurring mastitis.

In Trial 1, the efficiacy of the live culture treatment was examined in cows with chronic subclinical *Staph. aureus* mastitis. Although some studies consider treating chronic subclinical cases during lactation to be beneficial (Swinkels et al. 2005) it is generally regarded as controversial and most farmers do not attempt it. Usually, best treatment results for chronic subclinical mastitis are achieved when affected quarters are treated during the dry period. If dry cow therapy fails, it has been suggested that the best option is to cull the animals (Sandgren et al. 2008). The mode of action of the culture treatment is believed to be very different from conventional antibiotic treatments (Crispie et al. 2008), however, and for this reason, it was decided initially to examine the effect of the treatment on subclinical mastitis cases in lactating cows from both safety and efficacy perspectives. Of the cases initially shedding *Staph. aureus*, no staphylococci could be isolated at the last sampling in 4 of 7 infected quarters (57%) treated with the culture and in 3 of 8 infected quarters (38%) treated with antibiotics. Previously, bacteriological cure rates for *Staph. aureus*-induced subclinical mastitis was found to be 43% for amoxycillin and cephapirin (Wilson et al. 1999) and thus the bacteriological elimination rate appeared to be similar to cure rates of some antibiotics, in this small preliminary trial. Cured quarters, however, are usually defined as not only being pathogen free, but also having an SCC $<2 \times 10^5$ cfu/ml. As SCC was not considered in our definition of bacterial elimination, a direct comparison cannot be made, but our results imply similar trends for both treatments. If the similar results found for live culture and antibiotic treatment were confirmed in a larger study, the treatment of subclinical mastitis with the culture during lactation could be considered beneficial, as no cost through milk withdrawal would arise. In cases of treatment success, the major financial loss due to culling would also be prevented.

The results of Trial 2 showed that the difference between the overall bacteriological response rate and the clinical response rate for the culture treatment *v*. the antibiotic treatment was not statistically significant ($P=0{\cdot}5443$ and $P=0{\cdot}5443$ for bacteriological responses and clinical responses, respectively). In terms of bacterial elimination, 11 of 18 (61%) and 6 of 17 (35%) cases treated with antibiotic and culture treatment, respectively, were pathogen free at the end of Trial 2. At first glance, this would suggest that the antibiotic treatment seemed to compare favourably with the culture treatment. However, a further two cases treated with the live culture, although not pathogen free on day 14 had in fact eliminated the original infecting organism, bringing the elimination rate for this treatment to 47% (8/17). Although the numbers are relatively small and again SCC was not taken into consideration, indications are that the live culture may therefore be as effective as the antibiotic treatment.

Bacteriological elimination of *Staph. aureus* infections in Trial 2 was achieved for 3 of 7 and 4 of 8 cases in the culture and antibiotic group, respectively. Owens et al. (1997) previously found that using a combination of penicillin and novobiocin, bacteriological cure rates for clinical mastitis caused by *Staph. aureus* could be as high as 70% if treatment was initiated at the early stage of infection (<28 d) and could be as low as 35% for animals with chronic infections. These figures compare favourably with the bacteriological elimination rates achieved with *Lc. lactis* and the antibiotic control used in Trial 2, especially if we consider that some of the *Staph. aureus* treated cases may have been chronic. It is well known that *Staph. aureus* causes microabscesses inside the mammary gland and therefore the pathogens may be protected from antibiotic access and shed in a cyclical manner. Consecutive sampling is sometimes recommended for a more accurate diagnosis of the mastitis event (Sears et al. 1990). Since this level of sampling was not carried out in Trial 2, it is possible that some of the 15 day-0 pathogen-negative samples may well have been associated with chronic *Staph. aureus* infections and the intermittent shedding pattern of these pathogens.

Other pathogens isolated in the trials were *Str. uberis* (1 and 4 cases in Trials 1 and 2, respectively) and *Str. dysgalactiae* (10 in Trial 2). Bacteriological cure rates for both streptococcal strains when treated with penicillin alone were reported to be 60–90% (Sandholm et al. 1995) and when treated with a combination of penicillin and novobiocin were 90% and 91%, respectively (Owens et al. 1997). Three out of five *Str. dysgalactiae* related cases persisted in each treatment group in Trial 2, therefore elimination rates for both treatments were lower than the published cure rates. The three cases of *Str. uberis* mastitis treated with the antibiotic in Trial 2 showed no pathogen growth on day 14. The elimination rate for this pathogen by antibiotics in Trial 2 is therefore similar to the published cure rates. In contrast, the only *Str. uberis* case in Trial 2 treated with the culture persisted on day 14. *Str. uberis* is a mastitis pathogen that can internalize into epithelial cells for up to 120 h without affecting the viability of the host cell (Almeida et al. 2005). Trials by Crispie et al. (accompanying paper) have shown that *Lc. lactis* DPC3147 causes an immediate, short-lived effect on the immune system and therefore a longer interval between doses might be more effective in treating slow progressing *Str. uberis* type infections.

As it is unethical to withhold treatment from animals that are known to be infected, untreated controls could not be used in the experiments and thus the results of the experiments must be considered in conjunction with spontaneous recovery. Most spontaneous recoveries occur in quarters with mild or recent infections and only rarely in the case of well-established or chronic infections such as those encountered in Trial 1. However, some cows recover from clinical mastitis spontaneously: the rate of spontaneous recovery of untreated mastitis caused by

Staph. aureus is approximately 15–20% and in untreated cases of streptococcal mastitis is approximately 20–25% (Sandholm et al. 1995; Nickerson et al. 1999). The small numbers of cases in these studies make comparison difficult but it appears that the rate of elimination of pathogens observed is higher than that expected from spontaneous recovery. Moreover, the rates were comparable across treatment, i.e the live culture appeared to be as effective as the antibiotic treatments. Factors such as age, and previous history of mastitis can also effect recoveries as it has been shown that mastitis in older cows, or in cows with a previous history of mastitis, is more difficult to treat than in younger animals (Sandgren et al. 2008). In Trial 2, cases of clinical mastitis were randomly assigned to culture or antibiotic treatment as they arose, without knowledge of factors such as parity, days in milk at the time of enrolment and previous mastitis history. When these data were examined retrospectively, it was observed that overall, cows in the culture-treated group were older than in the antibiotic group. Additionally, twice as many cases in the culture group had a previous history of mastitis or high SCC, compared with the antibiotic group. In a larger trial involving more cases, the culture treatment might therefore show better results than in the present study.

Like many other intramammary antibiotics, the formulations used in this study contained a mixture of antibiotics and the anti-inflammatory component prednisolone. Prednisolone belongs to a group of substances called steroidal anti-inflammatories and mainly aids the reduction of swelling and related pain in intramammary treatments (Lees, 1991). Indeed, the better accessibility of the antibiotics to the pathogens due to lower inflammation possibly led to a quicker recovery. This may be an explanation for the numerical differences encountered in response rates of the severe cases for both treatments in Trial 2. In contrast, treatment with *Lc. lactis* DPC3147 seems to be associated with transient elevated SCC. This effect on the immune system might aid in the recovery of an inflamed quarter, especially as the mammary gland immune response in *Staph. aureus* related mastitis is evidently suppressed and down-regulated (Alluwaimi, 2004). In support of this, recent results from our laboratory have confirmed that the infusion of live cultures into quarters of low-cell-count animals is associated with a rapid influx of neutrophils in the first 2 d post infusion (Crispie et al. 2008). It is believed that this influx of PMN may enhance the intramammary immune response. The influx of PMN was also associated with a short-lived rise in SCC; however SCC rapidly decreased to pre-treatment levels, probably reflecting the inability of *Lc. lactis* to establish in the treated quarters, and no lasting adverse effects from the treatment were observed in any of the animals in any of the trials, demonstrating the safety of the treatment.

Alternative treatments such as the live culture treatment may have an application in the growing organic sector where the use of antibiotic treatments is limited or even prohibited. The live culture treatment also has potential to reduce the costs associated with the disease, as is estimated that approximately 5·7% of the cost of mastitis is due to discarding of milk containing antibiotic residues (Costello, 2004). *Lc. lactis* DPC3147 is used in food production (Ryan et al. 1998) and it is likely that as a result, milk from quarters treated with this organism would only have to be withheld until normal milk character is restored. Additionally, the ongoing discovery of more multiple resistant strains of pathogens, which is well documented through the risk posed by methicillin and vancomycin resistant *Staph. aureus* (MRSA and VRSA), will require a reduction in the use of antibiotics in every sector in the future. It would now be interesting to compare antibiotic therapies with this more natural approach in the larger field trials that would be required for regulatory approval.

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