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Intramammary infusion of a live culture for treatment of bovine mastitis: effect of live lactococci on the mammary immune response

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In the accompanying article, we demonstrated that a live culture of *Lactococcus lactis* compares favourably with antibiotics for treatment of bovine mastitis in two initial field trials. In an effort to explain the mechanism involved, this study investigated the effect of culture administration on the local immune response. In this respect we initially observed that infusion of the live culture *Lactococcus lactis* stimulated substantial recruitment of polymorphonucleocytes (PMN) and lymphocytes to the udder. For instance, in one assay, quarters infused with the probiotic experienced a dramatic increase (~20 000-fold) in neutrophils over the first 48-h period from an average value of 83·6 cells/ml pre-treatment to $1{\cdot}78 \times 10^6$ cells/ml 48 h post-infusion. Levels of the acute phase proteins haptaglobin and milk amyloid A were also elevated significantly in comparison with controls following infusion of the culture. The results of flow cytometric assays also demonstrated that while infusion of a live lactococcal culture led to an enhanced recruitment of PMN to the udder (from $1{\cdot}85 \times 10^4$ cells/ml pre-infusion to $1{\cdot}45 \times 10^6$ cells/ml 24 h post-infusion) cell-free supernatant from the same culture was not able to do so, indicating that live *Lc. lactis* can specifically trigger the mammary immune response to elicit PMN accumulation. These results suggest that the mechanism responsible for this probiotic treatment of mastitis is associated with stimulation of the host intramammary immune system.

Keywords: *Lactococcus*, mastitis, treatment, immune response.

Mastitis, defined as 'inflammation of the udder', is the most prevalent disease of dairy cows. In addition to causing considerable distress to the animal, mastitis is the cause of major economic losses on dairy farms worldwide. Mastitis usually arises as a result of a bacterial infection. In Irish herds the main causative agents of mastitis include *Staphylococcus aureus, Streptococcus uberis* and *Str. dysgalactiae*. Current treatments for bovine mastitis rely heavily on the use of antibiotics, both for prophylaxis and therapy. This strategy is not only very costly, it is also frequently ineffective and *Staph. aureus*, in particular, has proved very difficult to control with antibiotics (Barkema et al. 2006; reviewed in Melchior et al. 2006). This can lead to a persistent bacterial reservoir within a herd, causing chronic, recurrent infections. Additionally, there are significant concerns regarding the over-use of antibiotics in veterinary medicine. To ensure the absence of antibiotic residues in dairy products, milk has to be withheld from the dairy for a period after therapy, further increasing the cost to the dairy sector. Consumer demands for more 'organic' products, produced by healthy animals with minimal use of antibiotics and drugs, have increased dramatically in recent years. It is therefore timely to investigate new, innovative methods for treatment and prevention of mastitis.

One of the mechanisms underlying the concept of probiotic bacteria is the use of bacterial antagonism and immunomodulation to control several infectious, inflammatory and immunologic conditions. It has been proposed

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that production of suppressive factors like bacteriocins, and toxicity of end metabolic products may contribute to the antibacterial effect of probiotics (Alvarez-Olmos & Oberhelman, 2001; Cross, 2002). Previously, we demonstrated that lacticin 3147, a bacteriocin produced by *Lactococcus lactis* DPC3147, could inhibit several mastitis-causing pathogens in vitro (Ryan et al. 1998). Furthermore, when lacticin 3147 was combined with a bismuth-based teat seal, the combination prevented infection with *Str. dysgalactiae* in dry cows (Ryan et al. 1999) and *Staph. aureus* in lactating cows (Twomey et al. 2000; Crispie et al. 2005). In the accompanying study, a probiotic therapy involving infusion of a resuspended freeze-dried preparation of *Lc. lactis* DPC3147 was shown to be as effective as an antibiotic in curing clinical mastitis cases (Klostermann et al. 2008). It appeared that infusion of the culture led to an immunomodulatory effect, given that it was associated with a transient increase in SCC levels. In the present study, we demonstrate that administration of the live lactococcal culture to uninfected animals is associated with recruitment of PMN and lymphocytes to the infused quarters.

Materials and Methods

Selection of cows for treatment

Holstein-Friesian cows were selected from four adjacent herds attached to the Moorepark Research Centre, Fermoy, Co. Cork, Ireland. The animals were grazed on pasture for approximately 8 months of the year. Cows 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.

Before in-vivo experiments commenced, foremilk quarter samples were collected in an aseptic manner from all prospective cows and these were screened for mastitis-causing pathogens as described previously (Crispie et al. 2005). Somatic cell counts (SCC) were determined as described previously (Crispie et al. 2005) for each quarter before treatment. Milk samples were taken immediately before milking. Samples were collected the day before infusion (day −1), immediately prior to infusion (day 0) and post-infusion as outlined below. Uninfected cows with low SCC were selected for all the experiments so that clear comparisons between values in control and infused quarters could be made. Three cows were used in all experiments to allow for variation between different cows in immunological response. SCC and bacteriology were also monitored at every sampling as described previously (Crispie et al. 2005). Infusions and milk sampling were performed under licence from the Irish Department of Agriculture and Food, and cows' health was subsequently monitored by trained farm staff and veterinary personnel.

Infusion techniques

All treatments were infused directly into the teat sinus via the streak canal. The treatments were inoculated to a depth of 17 mm using a syringe with a blunted smoothed tip to prevent injury to the teat. Infusions were performed after the morning milking.

Analysis of leucocytes present in milk

All milk samples for leucocyte analysis were stored at room temperature or on ice following milking and were analysed within 3 h of collection. A protocol was developed (Alonso-Gomez et al. 2005) whereby milk cells were directly labelled in small volumes of whole milk instead of isolating purified cells. The samples were labelled with antibodies according to the manufacturer's (VMRD, USA) instructions. In brief, 5 μg of the primary antibodies CD45 (CACTB51A, VMRD, USA) and CD3 (MM1A, VMRD, USA) were mixed with 1 ml of whole milk, to label PMN and lymphocytes, respectively. Following incubation at 4 °C for 1 h, cells were centrifuged at 250 ***g*** for 10 min and washed twice with cold phosphate-buffered saline (PBS). One-hundred microlitres of a 1 : 100 dilution in PBS of rabbit $F(ab')_2$ antimouse IgG conjugated with flourescein isothiocyanate (FITC) secondary antibody (STAR9B, Serotec, UK) was added to the pellet, mixed and incubated at 4 °C for 30 min in the dark. Cells were then washed with 1 ml of cold PBS and centrifuged at 250 ***g*** for 10 min. The pellets were then resuspended in 200 μl of cold FACSFlow Sheath Fluid (BD-BioSciences, UK) and mixed with 2 μl propidium iodide (Sigma-Aldrich, Ireland; 1 mg/ml). Negative controls in each experiment included unlabelled samples and samples labelled with secondary antibody alone. Samples were analysed using a BD™ LSR flow cytometer (BD-BioSciences, UK) acquiring 10 000 events per sample. The percentage of FITC-positive events was determined using the acquisition and analysis program, Cell Quest (BD-Biosciences).

Analysis of acute phase protein levels in milk

Levels of the acute phase proteins (APP), milk amyloid A (MAA) and haptoglobin (Hp) were also analysed in response to various treatments. For the APP analysis, milk samples were taken on day 0 (immediately before infusion) and on days 1 and 2 post-infusion. All samples for APP analysis were frozen immediately at −20 °C until analysis. Concentrations of the proteins were determined using Enzyme Linked Immuno Sorbent Assays (ELISA) (Phase, TriDelta Development Limited, Co.Wicklow, Ireland). Absorbance was measured using an automatic

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plate reader (ELx808iu, Biotek Inc., UK) at 450 nm with a reference at 630 nm. The colour intensity observed was proportional to the concentration of the different APP present in the milk samples. The limit of detection according to the manufacturer was 0·05 mg/ml for Hp and 0·10 mg/ml for MAA.

Immunological response to infusion with Lc. lactis *or an antibiotic*

Lc. lactis DPC3147 was routinely propagated at 30 °C in M17 broth (Difco Laboratories, Detroit, USA) supplemented with 0·5% lactose (LM17). To investigate the effect of *Lc. lactis* DPC3147 on the mammary immune responses, the lactococcal culture (2 ml, $\sim 10^9$ cfu/ml) was mixed with 3 ml sterile, pyrogen-free water for injection (Antigen Pharmaceuticals Ltd, Roscrea, Ireland) and one quarter in each cow was inoculated with this preparation. A second quarter was infused with the contents of one intramammary antibiotic syringe containing 250 mg of neomycin sulphate, 100 mg of procaine penicillin, 10 mg of oxytetracycline hydrochloride and 10 mg of prednisolone (Multimast L.C., Bimeda Ltd., Dublin). One quarter was left untreated as a control. Milk samples were taken 24 h prior to infusion (day −1), immediately prior to intramammary injections on day 0, and on days 1, 2, 5 and 7 post-infusion.

Immunological response to infusion with heat-killed cells or cell-free supernatant

The effect of infusing dead *Lc. lactis* DPC3147 cells on the intramammary immune response of cows was also investigated. The teats were infused with live or heat-killed *Lc. lactis* DPC3147, culture supernatant or were left as untreated controls. The live lactococci infusion mixtures were prepared as described above. Heat-killed cells were prepared by growing *Lc. lactis* DPC3147 as described above, followed by boiling at 100 °C for 10 min. The heat-killed culture was mixed with sterile, pyrogen-free water for injection in a ratio of 2 : 3, and this mixture (5 ml) was used for infusion as described above. In each cow, a quarter was also infused with cell-free supernatant from an overnight culture of *Lc. lactis* DPC3147. To prepare the supernatant, 1·5 ml of the overnight culture was centrifuged at 10 000 ***g*** and the supernatant removed by pipetting. The supernatant was then filtered through a 0·22-µm syringe filter and diluted with sterile water for injection in a ratio of 2 : 3, and this mixture (5 ml) was used for infusions. Flow cytometric analysis using Live/Dead® *Bac*Light™ bacterial viability kit (PI/Syto9) (Molecular Probes, Leiden, The Netherlands) was performed to ensure the heat-treated cells were all killed and to ensure the supernatant was cell-free. A fourth quarter was left untreated in each cow as a negative control. Milk samples were taken on days −1, 0, 1, 2, 3, and 7. All samples were then analysed for leucocyte phenotype as described above. Milk samples were taken on days 0, 1 and 2 and used to determine the levels of APP present as described above.

Immunological response to infusion with freeze-dried Lc. lactis *DPC 3147*

LM17 medium (500 ml) was inoculated with 1% (v/v) *Lc. lactis* DPC3147 and incubated overnight at 30 °C. The cells were then harvested by centrifugation at 4000 ***g*** at 4 °C in a Sorvall RC 3Cplus centrifuge, washed in sterile distilled water and finally resuspended in ∼50 ml sterile distilled water. The resuspended cells were then freeze-dried overnight using a Modulyo freeze dryer (Edwards Modulyo, Crawley, Sussex, England). A sample of the resulting powder was then resuspended in sterile distilled water as a 10% (w/v) solution and bacteria were enumerated by plating dilutions on LM17 agar and incubating overnight at 30 °C. Appropriate amounts of powder were then added to 5 ml of sterile water for injection such that the final concentration was equivalent to 10^9 cfu/ml approximately. Powder resuspended in this way was then used as an infusion mixture.

A 16-h broth culture was prepared as described above. Both cultures used for infusion were enumerated by plating 100 µl in duplicate on LM17 agar overnight. Three cows were used and milk samples were taken pre- and post-infusion on days −1, 0, 1, 2, 3 and 6. Milk cells from these samples were labelled directly in whole milk as described above. Milk samples were taken on days 0, 1 and 2 and used to determine the levels of APP present as described above.

Immunological response to high and low levels of lactococci

A freeze-dried powder preparation of *Lc. lactis* DPC3147 was prepared as described above and subsequently resuspended in sterile water at levels appropriate to yield concentrations of $\sim 10^3$ cfu/ml and $\sim 10^8$ cfu/ml. Selected quarters in three cows were infused with either a high level (10^8 cfu/ml) or a low level (10^3 cfu/ml) of lactococci, sterile water or left untreated. Milk samples were taken pre- and post-infusion from the three cows on days −1, 0, 1, 2, 3 and 7. Milk cells from these samples were labelled directly in whole milk as described above. Milk samples were taken on days 0, 1 and 2 and used to determine the levels of APP present as already described.

Statistical analysis

Differences amongst treatments were determined by analysis of variance, in which three factors (cow, treatment and day of infusion) were assessed. Means were compared by the Duncan method after a logarithmic transformation

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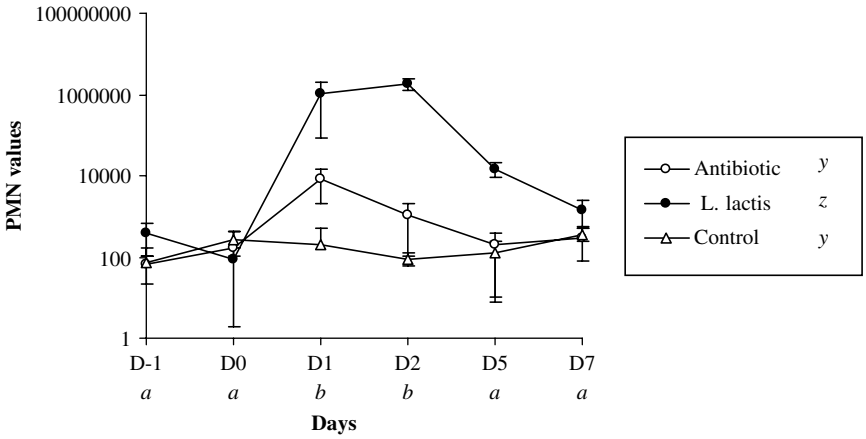

Fig. 1. Mean PMN values recruited in cows before and after treatment with *Lc. lactis* DPC3147, intra-mammary antibiotic, or untreated control. Groups with different letters differ significantly from each other ($P<0{\cdot}05$).

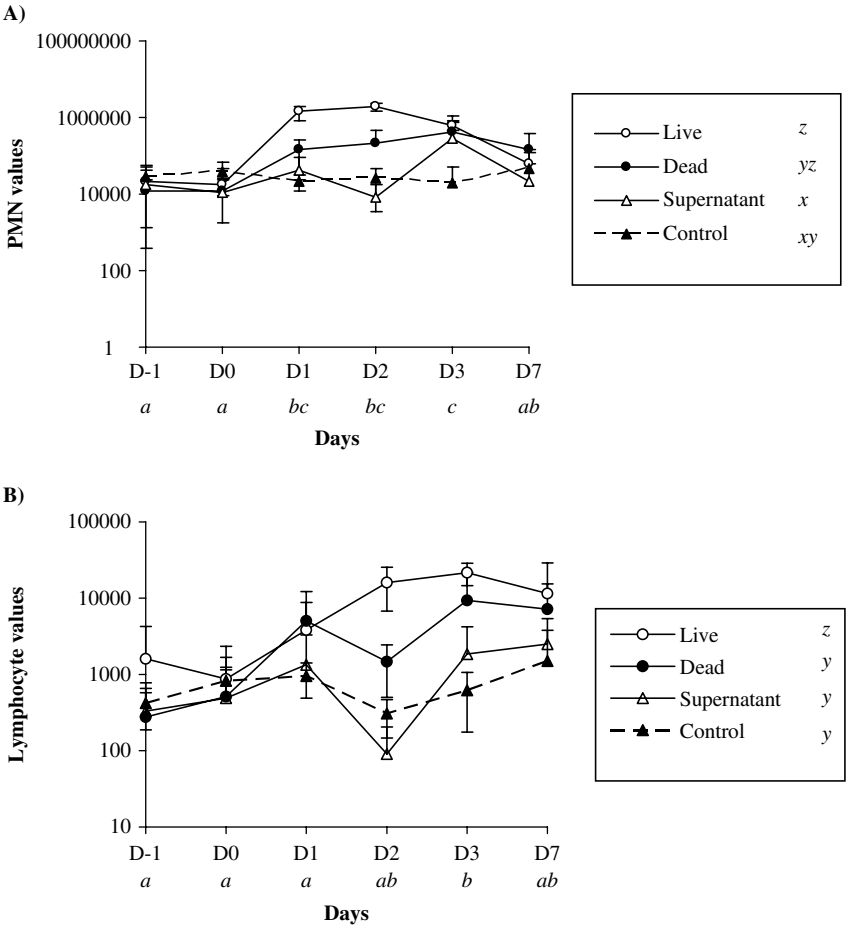

Fig. 2. Mean (A) PMN and (B) lymphocyte values recruited in cows before and after treatment with live *Lc. lactis* DPC3147 (Live), heat-killed *Lc. lactis* DPC3147 (Dead), or supernatant (Supernatant). One quarter in each cow was left untreated (control). Groups with different letters differ significantly from each other ($P<0{\cdot}05$).

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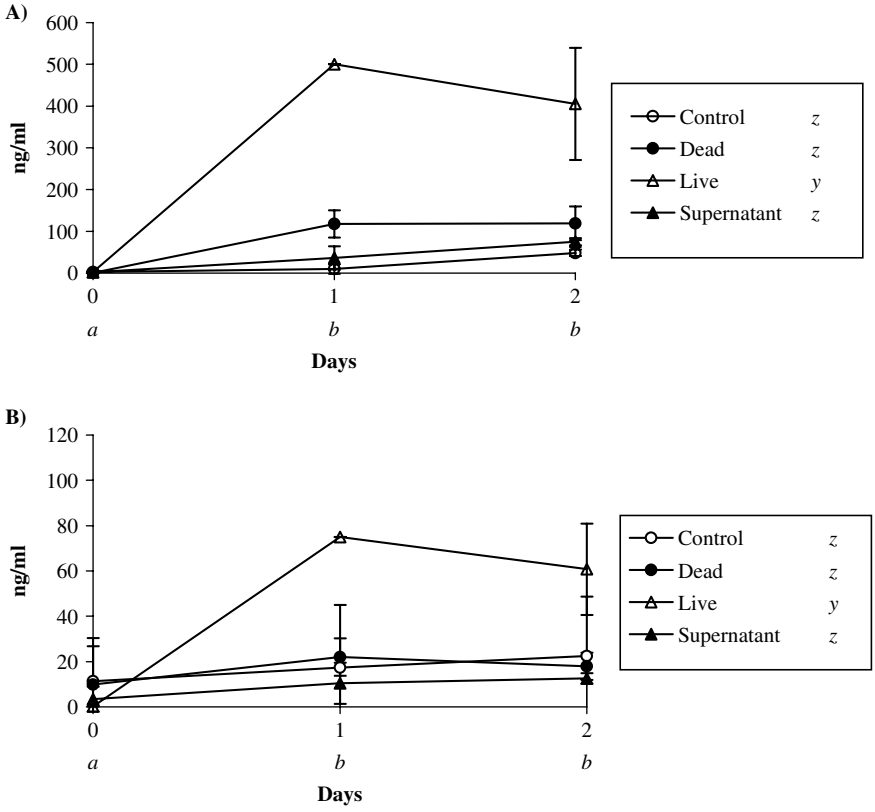

Fig. 3. (A) Mean haptoglobin and (B) mean milk amyloid A concentrations in response to treatment with live *Lc. lactis* DPC3147 (Live), Heat-killed *Lc. lactis* DPC3147 (Dead), or cell-free supernatant from the live culture (Supernatant). Mean values for untreated quarters are also shown. Groups with different letters (italicised) differ significantly from each other ($P<0{\cdot}05$).

of the values. Differences were considered significant at $P<0{\cdot}05$.

Results

Intramammary immunological effect of infusing Lc. lactis *DPC3147 or an antibiotic*

In all experiments, infection-free animals were used and bacteriology monitored throughout the experiment at every milking. Cows' health status was monitored throughout by trained personnel. Results confirmed that all animals remained pathogen-free throughout the investigations, and none suffered any teat damage (data not shown). The effect of *Lc. lactis* DPC3147 on the mammary immune system was investigated by analysing leucocyte numbers and phenotypes in milk. As controls, one quarter in each of three cows was infused with a lactating-cow antibiotic (Multimast L.C.) and one quarter was left untreated. A third quarter in each cow was infused with the diluted *Lc. lactis* DPC3147 culture. Milk samples were collected pre- and post-infusion and analysed for SCC and leucocyte subpopulation. Figure 1 presents the average PMN values in milk samples from the three cows 24 h prior to treatment (day −1), immediately prior to treatment (day 0) and after treatment (days 1, 2, 5 and 7). Actual values were calculated using the percentage of positive cells from flow cytometry analysis and SCC. Overall, PMN levels in all the control quarters remained unchanged during the trial. The probiotic-infused quarters experienced a dramatic 12 000-fold increase in neutrophils over the first 24-h period from an overall average value of 83·6 cells/ml immediately prior to infusion to 1×10^6 cells/ml 24 h after treatment (Fig. 1) and continued to rise to a peak mean value of $1{\cdot}8\times10^6$ cells/ml 48 h post-infusion (>20 000-fold increase). In contrast, the antibiotic generated significantly less ($P<0{\cdot}05$) recruitment of PMN to the udder with a less pronounced increase in PMN levels in milk in the first 24 h post-infusion (from $1{\cdot}65\times10^2$ cells/ml to $7{\cdot}96\times10^3$ cells/ml). When analysed by day, it can be seen from Fig. 1 that the mean numbers of PMN recruited to the *Lc. lactis*-treated quarters on days 1 and 2 were similar but that the numbers of PMN recruited on day 1 and day 2 differed significantly from pre-infusion and from day 5 and day 7 post-infusion ($P<0{\cdot}05$).

Effects of heat-killed cells and cell-free supernatant

To determine whether viable *Lc. lactis* DPC3147 were required to produce the immune response generated above,

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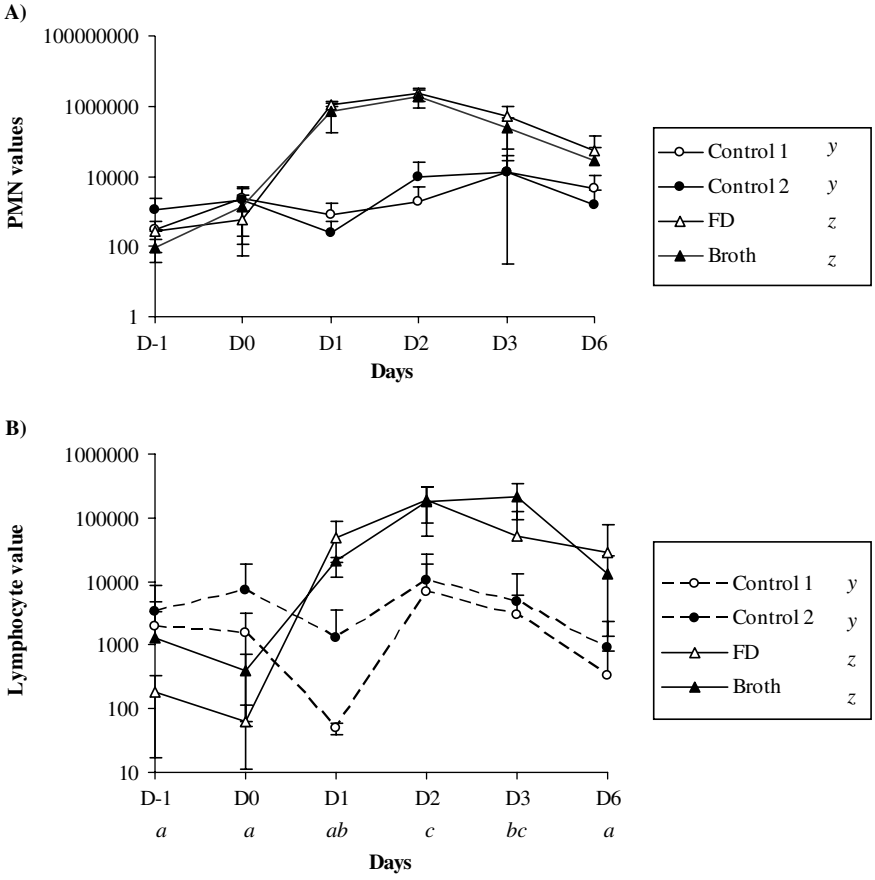

Fig. 4. Mean (A) PMN and (B) lymphocyte values recruited in cows before and after treatment with a 16 h culture of *Lc. lactis* DPC3147 (Broth) or a suspension in sterile water of freeze-dried *Lc. lactis* DPC3147 (FD). Mean values for untreated control quarters are also shown. Groups with different letters differ significantly from each other ($P<0{\cdot}05$).

the effect of infusing heat-killed *Lc. lactis* DPC3147 cells was also studied. Teats were randomly allocated to treatment and infused with live or heat-killed lactococci, cell-free supernatant or untreated controls. Milk samples were collected pre- and post-infusion and PMN and lymphocytes were enumerated as already described. Figure 2 shows the average PMN and lymphocyte values in milk samples from the three cows prior to, and after treatment. The live culture, as in the previous experiment, had immunostimulant activity, inducing a significant recruitment of PMN ($P<0{\cdot}001$) and lymphocytes ($P<0{\cdot}001$) to the live culture-treated quarters (Fig. 2). Average PMN and lymphocyte numbers increased respectively from $1{\cdot}85\times 10^4$ cells/ml and $8{\cdot}69\times10^2$ immediately prior to infusion to $1{\cdot}45\times10^6$ cells/ml and $3{\cdot}8\times10^3$ respectively at 24 h post-infusion (Fig. 2). PMN levels in the live culture-treated quarters peaked at 48 h post-infusion, with levels reaching $1{\cdot}86\times10^6$ cells/ml, a 100-fold increase on the levels pre-infusion. While the cell-free supernatant appeared to generate high levels of PMN on day 3, the levels recruited were significantly lower ($P<0{\cdot}05$) than those recruited in response to infusion with the live culture, and even at their highest level on day 3, were still only 27-fold higher than the pre-infusion levels. The heat-killed cells also elicited a weaker influx of PMN and lymphocytes in each of the cows, with average PMN numbers rising almost 10-fold from $1{\cdot}25\times10^4$ immediately prior to infusion to $1{\cdot}19\times10^5$ cells/ml 24 h post-infusion, and reaching their highest level on day 3 (35-fold increase on pre-infusion levels) 24 h post-infusion. The levels recruited on day 3 in response to the heat-killed culture however, while high, remained significantly lower than in the live culture treated quarters ($P<0{\cdot}05$), as was the case in the cell-free supernatant treated quarters. Overall PMN and lymphocyte values were also compared with respect to day post-infusion. As can be seen in Fig. 2, the highest mean values of PMN in the live-culture treated quarters, were obtained on day 1 and day 2 post-infusion. Levels of PMN present in these quarters on these days differed significantly from the values obtained pre-infusion (day −1 and day 0) and days 3 and 7 post-infusion ($P<0{\cdot}05$). The highest levels of lymphocytes in the live-culture-treated quarters was obtained on day 3 post-infusion and this level was significantly higher than the levels pre-infusion (day −1 and day 0) and day 2 and day 7 post-infusion ($P<0{\cdot}05$).

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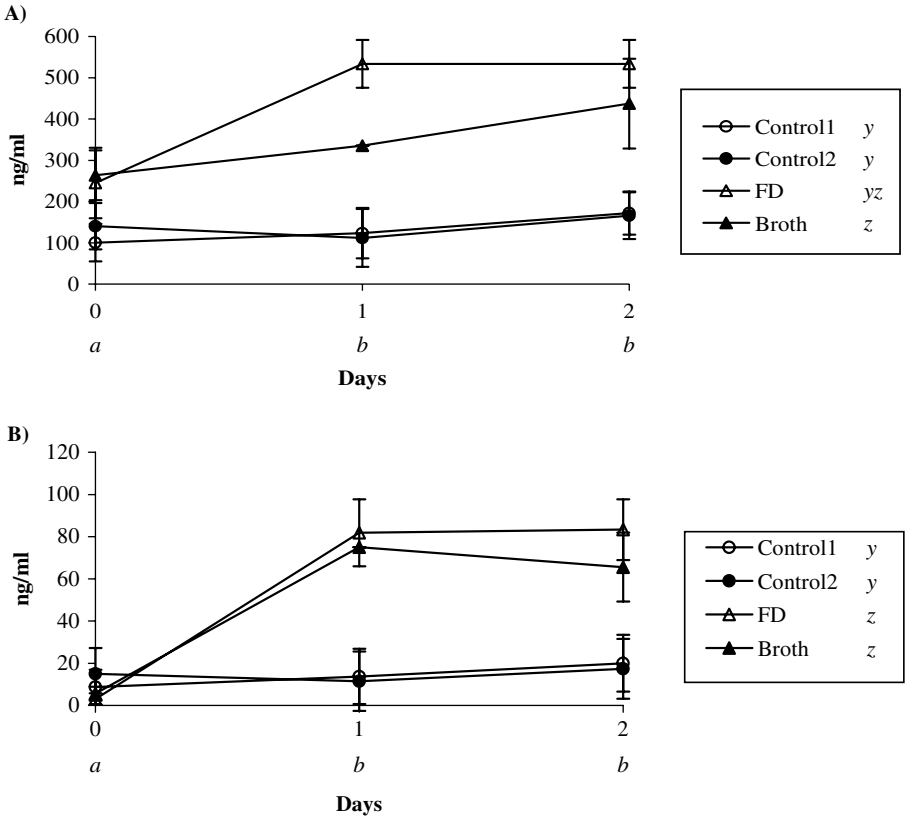

Fig. 5. Mean (A) haptoglobin and (B) milk amyloid A concentrations in response to treatment with a 16 h culture of *Lc. lactis* DPC3147 (Broth), or a freeze-dried preparation of the same bacterium (FD). Mean values for untreated control quarters are also shown. Groups with different letters (italicised) differ significantly from each other ($P<0{\cdot}05$).

The results of analysis of APP concentration are shown in Fig. 3. As can be seen, the live culture triggered substantial increases in both Hp and MAA concentrations. The heat-killed cells induced slightly higher levels of the APP than occurred in the cell-free supernatant-treated quarters (Fig. 3A and B). Live cells, however, triggered the greatest response. In fact, the mean Hp and MAA concentration in the live culture-treated quarters was significantly higher than the levels observed in any of the other treatment or control groups ($P<0{\cdot}001$) (Fig. 3).

Effect of freeze-dried cells

A 16-h broth culture and a suspension of freeze-dried cells were prepared and enumerated as described above. The broth culture consisted of $3{\cdot}1\times10^8$ cfu/ml and the resuspended freeze-dried preparation consisted of $4{\cdot}6\times10^8$ cfu/ml. Figure 4 shows the average PMN and lymphocyte numbers recruited in response to treatment with the 16-h culture (broth), the resuspended freeze-dried preparation (FD) or the untreated controls. The freeze-dried and broth culture preparations both stimulated an immune response. Recruitment of PMN was most significant on day 2 post-infusion for both treatments. The broth culture and freeze-dried preparations also stimulated recruitment of lymphocytes. On day 2 post-infusion, the levels of lymphocytes recruited were significantly higher ($P<0{\cdot}001$) than the levels on day 1 post-treatment or the levels pre-treatment (day 0 and day −1). The broth culture elicited an immune response for a more prolonged period with highest levels of lymphocytes achieved on day 3 post-infusion.

Analysis of the levels of APP (Fig. 5) indicated that both the freeze-dried preparation and the overnight culture could induce the acute phase response. The highest mean levels of both Hp and MAA were observed in the quarters treated with the freeze-dried preparation (Fig. 5). This indicates that the immunogenic factor(s) is not lost in the washing or freeze-drying processes and that this powder is suitable for use as a treatment. Thus, both 'fresh' and freeze-dried preparations of *Lc. lactis* DPC3147 are capable of eliciting an immune response in the mammary gland. Mean MAA concentrations in the freeze-dried and broth-culture treated groups were significantly higher than those in control quarters ($P<0{\cdot}001$). The mean Hp concentration of the broth treated quarters was intermediate between the freeze-dried treated and control groups.

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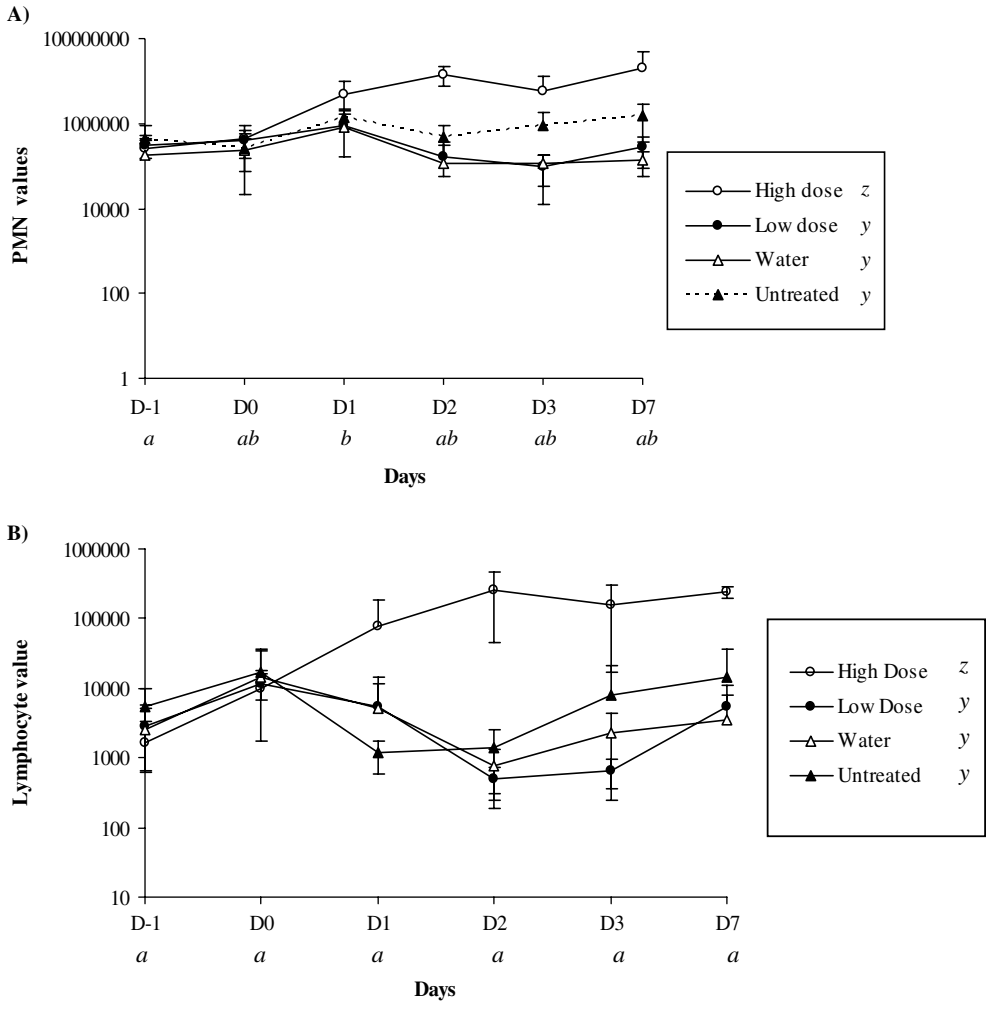

Fig. 6. Mean (A) PMN and (B) lymphocyte concentrations in response to treatment with a high or low level dose of lactococci or sterile water. Mean values for untreated quarters are also shown. Groups with different letters differ significantly from each other ($P<0{\cdot}05$).

Response to high or low levels of lactococci

With pathogenic bacteria it is known that relatively low levels ($\sim 10^3$ cfu/ml) can elicit infection and a substantial immune response (Twomey et al. 2000). In all previous experiments we had used high levels of the lactococcal culture. To see whether a low level would elicit a similar response, infusion mixtures containing either a high or low level of lactocoocci were prepared as described in Materials and Methods. The low-level and high-level suspensions were enumerated by plating on LM17. The results indicated that the high level consisted of $2{\cdot}4 \times 10^8$ cfu/ml and the low level consisted of $9{\cdot}8 \times 10^2$ cfu/ml. Figure 6 shows the mean PMN and lymphocyte numbers in response to treatment with both the low or high doses of lactococci. While the high dose generated a significant immune response, with increases in PMN and lymphocyte numbers in the mammary gland, PMN and lymphocyte numbers following the infusion of the low dose levels remained relatively unchanged compared with control values (Fig. 6). This implies that a dose of $>10^3$ cfu/ml per treatment is required in order to exert an effect. These results are supported by the results of the APP analysis, which shows that levels of Hp and MAA did not change following treatment with the low dose of lactococci, whereas the high dose induced significant ($P<0{\cdot}001$) increases in levels of both proteins (Fig. 7).

Discussion

The intramammary immune system's ability to eliminate infections naturally depends on a rapid and competent response to pathogens (Burvenich et al. 1994). Indeed, impairment of the immune response is associated with increased susceptibility to mastitic infection (Burvenich et al. 1994). The primary phagocytic cells of the bovine mammary gland, PMN and macrophages, comprise the first

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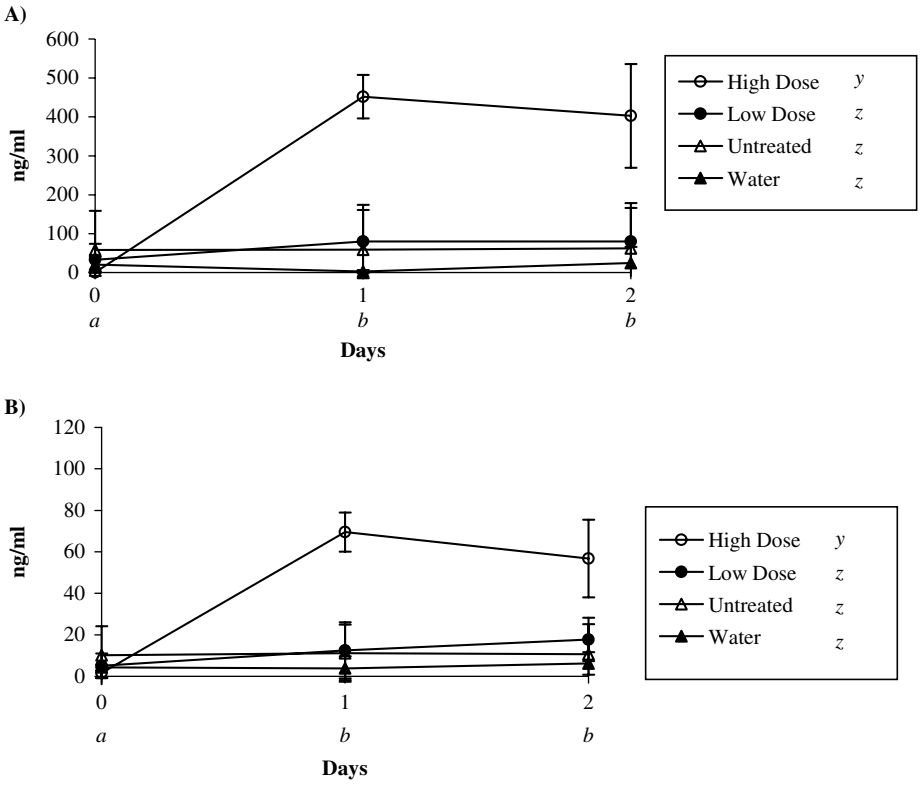

Fig. 7. Mean (A) haptoglobin and (B) milk amyloid A concentrations in response to treatment with a high or low level dose of lactococci or sterile water. Mean values for untreated quarters are also shown. Groups with different letters (italicised) differ significantly from each other ($P<0{\cdot}05$).

line of defence against invading bacteria. Usually, the mammary immune response is very effective, and mammary quarters come into contact with pathogens far more often than infection occurs (Burton & Erskine, 2003). However, when the inflammatory events that should lead to rapid neutrophil recruitment into the gland fail to occur or occur too slowly, health of the gland is affected and mastitis develops. Some pathogens, most notably *Staph. aureus*, have the ability to evade the immune system by surviving intracellularly in PMN or invading mammary epithelial cells (reviewed in Barkema et al. 2006). The evasive tactics employed by *Staph. aureus* are also believed to influence cure rate by antibiotics and it is regarded as a notoriously difficult pathogen to treat (reviewed in Barkema et al. 2006).

Given the poor cure rates for many antibiotics, and the problems associated with antibiotic resistance, their use in prophylactic preventative mastitis treatments may be restricted in the future, and for this reason there is a growing need for effective natural alternatives. Attempts have been made previously to find such treatments with varying degrees of success. Studies have demonstrated that infusion of lactoferrin and casein hydrolysates were both effective at eliminating pathogens in infected cows (Kai et al. 2002; Shamay et al. 2003; Silanikove et al. 2005). Both studies proposed that the increased rate of cure was at least partly due to induction of the innate immune response in the mammary gland.

The results of the immunological assays presented here support this theory, as intramammary treatment with *Lc. lactis* DPC3147 triggered the influx of neutrophils, thus providing the mammary gland with an enhanced mechanism for eliminating mastitis pathogens. In all the experiments described in this paper, infusion of the culture led to a local immune response, characterized by a short-lived elevation in SCC, and not to a systemic response. Hp and MAA, both major acute phase proteins in cattle (Pyörälä et al. 2003), were also expressed in the mammary gland following infusion of *Lc. lactis*. These proteins are non-specific innate immune components, secreted by the mammary gland (Eckersall et al. 2006), which play a variety of roles in the restoration of homeostasis and restraint of microbial growth (Gronlund et al. 2006). As the initial experiments involved infusion of *Lc. lactis* together with its supernatant, it was deemed possible that the immunostimulation observed could be due to factors released into the supernatant (e.g., free lacticin, lactic acid) or to factors associated with the culture itself (e.g., bound lacticin, and muramyl peptides in the Gram-positive cell wall) or a combination of cell-associated and released molecules. The fact that the cell-free supernatant did not elicit a significant immune response however, suggested

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that the major immunogenic factor(s) involved must be cell-associated. This was supported by the fact that the washed, freeze-dried culture, from which the supernatant had been removed, induced as significant a response post-infusion as the live 'broth' culture. When the live 'broth' culture was compared with the freeze-dried culture, a slightly more prolonged effect was observed with the broth culture, with highest levels of PMN being observed on day 3. The difference in numbers of PMN recruited by the broth culture in comparison with the freeze-dried culture on day 3 was not significant. It is interesting to note however, that the cell-free supernatant and dead cells also elicited the highest levels of PMN on day 3 post-infusion. These combined results imply that perhaps a supernatant-associated factor may contribute to the immunogenic response, albeit in a less-pronounced fashion and at a more delayed stage. Regardless, the most significant and quickest response occurred when live lactococci were present. Interestingly, the effect of infusion of *Lactobacillus plantarum* and other lactic acid bacteria demonstrated that *Lc. lactis* could elicit a stronger and more rapid immune response than other lactic acid bacteria (data not shown), implying this effect is limited to lactococci.

It also appears that not only must lactococci be present, the *Lc. lactis* culture must also be viable to induce an adequate immune response, as a heat-killed culture did not elicit significant recruitment of PMN to the mammary gland. It is also possible, however, that the factor(s) responsible for eliciting the immune response in the udder is heat labile, or utilized rapidly or destroyed when the cells are killed by boiling. Other methods of killing the cells, for instance by u.v. irradiation, should therefore be investigated before a conclusion is drawn. As *Escherichia coli* was amongst the organisms eliminated by this treatment (Klostermann et al. 2008), we can conclude that the mechanism probably does not solely involve direct killing by the bacteriocin. Instead, as proven by the immunoassays, *Lc. lactis* infusion acts as an immunostimulant that induces a prompt recruitment of neutrophils to the mammary gland. Whether this immunostimulation is bacteriocin-related or not is unknown. We do know, however, that large amounts of the bacteriocin are attached to the bacterial cell surface (RP Ross, unpublished results), and this bound lacticin may stimulate the immune system. Regardless, it is clear that infusion of *Lc. lactis*, an organism used in the food industry, can stimulate the bovine immune system.

Immunological responses vary substantially from host to host even within species, and it must be noted that the population of animals studied in this paper was small. A probiotic treatment consisting of *Lactobacillus acidophilus* and *Lb. casei* was investigated previously as a 'natural' alternative to antibiotic therapy for mastitis (Greene et al. 1991). This treatment was used to treat subclinical mastitis but was unsuccessful, as the treatment increased SCC with no effect on the infection rate. Similarly, preliminary experiments to examine the cure rate of treatments consisting of *Lb. plantarum* and *Lb. helveticus* were also unsuccessful (data not shown). In contrast, treatment with the organism used in this paper, *Lc. lactis* DPC3147, was as effective as an antibiotic in curing clinical mastitis cases (Klostermann et al. 2008). As the therapy outlined involved the use of an organism that does not persist in the udder (Klostermann et al. 2008), the risk of harmful residues appearing in the milk following treatment is reduced. Instead, the immune system is activated upon infusion with live *Lc. lactis*, resulting in recruitment of neutrophils and lymphocytes to the gland, along with APP which further promote a rapid inflammatory response. In this respect, it is interesting that the host response to the lactococcal culture is acute (PMN increased 100-fold from pre-infusion levels) when compared with the heat-killed culture (35-fold increase in PMN), suggesting a probiotic-like effect in a local infection situation. Preliminary studies have indicated that infusion of lactococci also induces release of cytokines such as IL8 and TNF-α (M Daly and L Giblin, personal communication). In conclusion, the mechanism by which the live culture can provide host protection against mastitis infection may be associated with its ability to elicit a rapid immune response, inducing substantial recruitment of PMN, lymphocytes and localised production of APP, which together can subsequently clear the gland of the infecting pathogen.

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