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Administration of a live culture of *Lactococcus lactis* DPC 3147 into the bovine mammary gland stimulates the local host immune response, particularly *IL-1β* and *IL-8* gene expression

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Mastitis is one of the most costly diseases to the dairy farming industry. Conventional antibiotic therapy is often unsatisfactory for successful treatment of mastitis and alternative treatments are continually under investigation. We have previously demonstrated, in two separate field trials, that a probiotic culture, *Lactococcus lactis* DPC 3147, was comparable to antibiotic therapy to treat bovine mastitis. To understand the mode of action of this therapeutic, we looked at the detailed immune response of the host to delivery of this live strain directly into the mammary gland of six healthy dairy cows. All animals elicited signs of udder inflammation 7 h post infusion. At this time, clots were visible in the milk of all animals in the investigation. The most pronounced increase in immune gene expression was observed in *Interleukin* (*IL*)*-1β* and *IL-8*, with highest expression corresponding to peaks in somatic cell count. Infusion with a live culture of a *Lc. lactis* leads to a rapid and considerable innate immune response.

Keywords: Mastitis, *Lactococcus lactis*, probiotic treatment.

Mastitis is the most common milk production disease in modern dairy farming. Despite mastitis control programmes, it is estimated to affect up to 30% of dairy cattle in the EU and cost the EU dairy industry approximately €1·55 billion in 2005 (SABRE, 2006). This economic loss is due to increases in veterinary and treatment costs and a decrease in the quality and quantity of milk produced by infected animals. The ability of any individual animal to overcome mastitis is dependent on treatment and that animal's innate immune response. This response begins with the host recognizing the presence of foreign pathogens and is followed by responses at cellular, tissue and organismal level, leading to the eradication of the pathogen. The differential inflammatory responses elicited during intramammary infection correlate with the outcome of the infection, and variations in cytokine production have been described for different pathogens (Bannerman et al. 2004b; Strandberg et al. 2005; Yang et al. 2008).

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Current control methods rely heavily on antibiotics for both therapeutic and prophylactic purposes. This method is not only costly, but is frequently ineffective in chronic subclinical infections, with cure rates for *Staphylococcus aureus* mastitis cases ranging widely from 4 to 92% (Barkema et al. 2006). There are also increasing concerns regarding the overuse of antibiotics in veterinary medicine and the emergence of antimicrobial resistant pathogens (Barkema et al. 2006). This has led to an increased interest in the development of alternative treatments for mastitis (Diarra et al. 2003; Alluwaimi, 2004; Gill et al. 2006a; Kauf et al. 2007). Recently the application of live bacteria as a potential mastitis therapeutic has gained interest. Probiotic bacteria can be used to control several infectious inflammatory and immunologic conditions through antagonism and immunomodulation (Cross, 2002). Commensal bacteria, with a broad spectrum of antimicrobial activity, have previously been isolated from healthy bovine udders and suggested as potential anti-mastitis agents (Al-Qumber & Tagg, 2006). Jiminez et al. (2008) showed that lactobacilli reduce staphylococcal counts in human mastitis milk over a 14-d period, with no clinical signs of mastitis

in the treatment group. However, Greene et al. (1991) investigated the effects of treating bovine subclinical mastitis infections with intramammary infusions of lactobacillus and although an increase in somatic cell counts (SCC) occurred, no increase in intramammary cure rate was observed.

Lactococcus lactis DPC 3147 is a food grade organism that produces the bacteriocin lacticin 3147 (Ryan et al. 1999). This bacteriocin exhibits broad-spectrum antimicrobial inhibition against mastitis-causing pathogens in vitro (Ryan et al. 1998) and when combined with a bismuth-based teat seal, it provides protection against infection with *Streptococcus dysgalactiae* and *Staph. aureus* in dry cows (Ryan et al. 1999; Twomey et al. 2000). Klostermann et al. (2008) recently demonstrated that a resuspended freeze-dried application of *Lc. lactis* is as effective as an antibiotic in curing clinical mastitis cases. Crispie et al. (2008) showed that administration of the lactococcal culture into the mammary glands of uninfected animals elicits an immunomodulatory effect, with substantial recruitment of polymorphonucleocytes (PMN) and lymphocytes to the infused quarters. The aim of this study was to investigate this immunomodulatory effect further, by describing the innate immune response, at the transcriptional level, to a deliberate infusion of *Lc. lactis* DPC 3147 into a healthy mammary gland.

Materials and Methods

Animal selection

For methodology set-up, a preliminary study was performed with a Holstein Friesian in her sixth lactation and a Norwegian Red in her second lactation. The animals were selected based on their low SCC and the healthy appearance of their udders and milk. The follow-up study consisted of four healthy Holstein Friesian cows (Cows H, J, K and L) in their first lactation and were selected using the same selection criteria as above. Quarter milk samples from all cows were collected aseptically for 7 d prior to experimental challenge. The milks were screened for the presence of pathogens by streaking 10 µl onto Aesculin Blood Agar (ABA) plates containing blood agar base No. 2 (Oxoid), supplemented with 7% citrated whole calf blood (v/v) and 0·1% aesculin (v/v) (Sigma, St. Louis MO, USA) and incubating overnight at 37 °C. SCC was performed using a Somacount 300® (Bently Instruments Inc., Chaska MN, USA) somatic cell counter. Infusions and milk and blood sampling were performed under licence from the Irish Department of Agriculture and Food, and the cows' health was subsequently monitored by trained farm staff and veterinary personnel.

Preparation of Lc. lactis *and intramammary challenge*

Lc. lactis DPC 3147, isolated originally from a kefir grain (Ryan et al. 1998), was grown at 30 °C in M17 broth (Difco Laboratories, Detroit MI, USA) supplemented with 0·5% lactose (LM17). Two millilitres of this culture was diluted with 3 ml of sterile Water for Injection B.P.® (Antigen Pharmaceuticals Ltd., Roscrea, Ireland) and this 5-ml suspension (containing 10^8 cfu *Lc. lactis*) was used for challenge. Immediately following the morning milking, one quarter from each animal was infused with this suspension into the teat sinus via the streak canal. The infusions were inoculated to a depth of 17 mm using a syringe with a blunted smoothed tip to prevent injury to the teat. Following infusion the culture was massaged upwards into the quarter. A second quarter from each animal, where possible the contralateral quarter, was selected as the control quarter. To minimize animal handling and conform to animal welfare best practices, no infusion was made in the control quarter.

Milk sampling

Following challenge, 10 ml of milk from each quarter was collected aseptically and 100 µl was plated on LM17 agar plates containing 0·5% lactose to determine *Lc. lactis* counts. One-hundred microlitres was also plated onto ABA plates for total microbiological analysis. Total quarter milk, (or up to a 2-l volume), was then collected from the infused quarter and the control quarter immediately prior to infusion and at 7 h, 24 h, 72 h, 7 d and 14 d post infusion (PI).

Harvesting milk somatic cells for RNA isolation

One millilitre of 0·5 M-EDTA (Sigma-Aldrich, Ireland Ltd., Dublin) was added per litre of milk (Boutinaud et al. 2002) and the milk samples were then centrifuged at 1500 ***g*** at 4 °C for 30 min. The fat layer was removed from each sample using a sterile spatula and the skim milk carefully decanted. The cell pellets were washed twice in phosphate-buffered saline (PBS, Sigma) pH 7·4 with EDTA at a final concentration of 0·5 mM. The washed cell pellets were then resuspended in 1 ml of TriPure isolation reagent (Roche Diagnostics, Bell Lane, East Sussex, UK) and pipetted up and down until fully homogenized.

Blood leucocyte isolation

Blood samples were taken at the same time points as the milk samples. Briefly, 10 ml blood was collected from the tail vein in a sampling tube containing potassium ethylenediaminetetraacetic acid (EDTA K3E 15%, 0·12 ml; BD Vacutainer™ BD Vacutainer Systems, Preanalytical solutions, Belliver industrial Estate, Plymouth, UK) and placed immediately on ice for subsequent RNA extraction. The samples were combined with 40 ml erythrocyte lysis buffer (ELB) from Qiagen (Qiagen House, Crawley, West Sussex, UK) and placed on ice for 15 min. Following centrifugation at 3000 ***g*** the supernatant was decanted and the

Table 1 Primers and conditions used for real-time PCR analyses (T_a is the annealing temperature)

Gene	Primer sequence	Accession no	T_a (°C)
IL-1β	IL1B-591 F: 5′-TGG GTA TCA AGG ACA AGA ATC-3′ IL1B-772 R: 5′-CCA GTT AGG GTA CAG GAC AGA C-3′	NM_174093	51
IL-8	IL8-305 F: 5′-CTA AAC CCC AAG GAA AAG TG-3′ IL8-693 R: 5′-CAA GAT TAA CAA AAA CCG AAA ACA-3′	NM_173925	50
IL-10	IL10-373 F: 5′-CGC TGT CAT CGC TTT CTG-3′ IL10-482 R: 5′-AAC TCA CTC ATG GCT TTG TAG-3′	NM_174088	53
IL-12	IL12-789 F: 5′-GAG CAC CCC GCA TTC CTA CTT C-3′ IL12-974 R: 5′-GAC ACA GAT GCC CAT TCA CTC CAG-3′	U11815	57
TNF-α	TNF α-2394 F: 5′-TAA CAA GCC GGT AGC CCA CG-3′ TNF α-2385 R: 5′-GCA AGG GCT CTT GAT GGC AGA-3′	AF011926	64
NF-κB	NFκB-719 F: 5′-ACC CTA TGA GCC AGA GTT T-3′ NFκB-1216 R: 5′-AAG GCA TTG TTC AGT ATC C-3′	AY849381	54
TLR-2	TLR2-2236 F: 5′-CAT TCC TGG CAA GTG GAT TAT C-3′ TLR2-2433 R: 5′-GGA ATG GCC TTC TTG TCA ATG G-3′	NM_174197	61
TLR-4	TLR4-132 F: 5′-TCT CTA CAA AAT CCC CGA CAA CAT-3′ TLR4-369 R: 5′-AGA AAA GGC TCC CCA GGC TAA ACT-3′	NM_174198	57
CD14	CD14-156 F: 5′-CCT GCG AGC TGG ACG ACG ACG AT-3′ CD14-354 R: 5′-CGA ACG CGC AGA GCC TTG ATT GTG-3′	NM_174008	61
CXCR1	CXCR1-648 F: 5′-CAA TAC AAC GAA ATG GCG GAT GAT-3′ CXCR1-849 R: 5′-CAG GTT GTA GGG CAG CCA GCA GAG-3′	U19947	60
E2D2	E2D2-48 F: 5′-CAG GGG TGG AGT ATT TTT CTT GA-3′ E2D2-339 R: 5′-AGT CCA TTC CCG AGC TAT TCT GTT-3′	XM_582519	57

leucocytes were washed in an additional 20 ml ELB. The cells were then resuspended in 1 ml of TriPure reagent (Roche Diagnostics).

RNA extraction and cDNA synthesis

Total RNA of milk cells and blood cells was extracted using TriPure (Roche Diagnostics) according to the manufacturer's instructions. RNA was quantified using optical density readings at 260 nm and the integrity was analysed following electrophoresis through glyoxyl gels (Ambion). One microgram of RNA was DNAse treated and reverse transcribed to cDNA using the QuantiTect® Reverse Transcription Kit (Qiagen, Crawley, West Sussex, UK) according to manufacturers instructions in a final volume of 20 μl.

Quantification by real-time PCR

Primers were designed for real-time PCR across intron/exon boundaries where possible, to minimize amplification of DNA. The primers were designed using data available in the Genbank database, and accession numbers are given with the primer sequences in Table 1. In addition to the immune genes under investigation a housekeeping gene, a ubiquitin conjugating enzyme (E2D2), was also included for analysis.

Quantitative analysis of the genes of interest was performed in a LightCycler 480 instrument (Roche Diagnostics) using a dilution series of external plasmid DNA standards (Pfaffl, 2001). Plasmid standards were created for each gene by cloning a cDNA PCR product into pCR TOPO (Invitrogen, Life Technologies, Carlsbad CA, USA). Cloning was confirmed by sequencing. One microlitre of each dilution was used per 10 μl LightCycler reaction. The LightCycler 480 SYBR Green I Master kit (Roche Diagnostics) was used for quantification according to the manufacturer's instructions using 0·5 μM forward and reverse primer. Each programme began with initial denaturation at 95 °C for 10 min, followed by 50 cycles of quantification consisting of 5-s denaturation at 95 °C, 10-s annealing and 25-s elongation at 72 °C. Annealing temperatures for each gene are given in Table 1. Melting curve analysis was performed on each product by heating from a temperature 5 °C above the annealing temperature to 95 °C in the continuous fluorescence acquisition mode to ensure specificity of Lightcycler products. For each gene, Lightcycler runs were performed in triplicate.

Statistical analysis

Results for the preliminary study were not included in the statistical analysis to exclude age and breed as a random effect. Gene expression data and SCC data were visually assessed for normality. Expression data and SCC data were then transformed by obtaining the natural log. SCS refers to the transformed variable of the SCC. A hierarchical mixed model (PROC MIXED; SAS Version 9.1, SAS Institute Inc.,

Table 2 Rectal temperature, physical changes and viable *Lactococcus lactis* recovered in (a) infused quarters and (b) control quarters following intramammary *Lc. lactis* DPC 3147 infusion (PI=post infusion)

(a) Infused quarters

	Cow H	Cow J	Cow K	Cow L
Rectal temperature, °C†	39·2	39·8	38·8	N/A‡
Milk presentation†	C2 Clots	C2 Clots	C1 Clots	C2 Clots
Udder presentation†	Slight Swelling	Slight Swelling	Slight Swelling	Slight Swelling
Viable *Lc. lactis* recovered, cfu/ml:				
Pre-infusion	0	0	0	0
7 h PI	>3000	>3000	>3000	>3000
24 h PI	940	2160	2390	1210
48 h PI	80	100	220	40
72 h PI	0	0	0	0

† 7 h PI; ‡ rectal temperature for this animal was not obtained

(b) Control quarters

	Cow H	Cow J	Cow K	Cow L
Milk presentation†	No Clots	No Clots	No Clots	No Clots
Udder presentation†	No Swelling	No Swelling	No Swelling	No Swelling
Viable *L. lactis* recovered, cfu/ml:				
Pre-infusion	0	0	0	0
7 h PI	0	0	0	0
24 h PI	0	0	0	0
48 h PI	0	0	0	0
72 h PI	0	0	0	0

† = 7 h PI

Cary NC, USA) was used to quantify the effect of treatment on SCS and gene expression. The dependent variable was transformed gene expression or SCS. Fixed effects included in the model were time, treatment, and time by treatment interaction. Where significant ($P<0·05$) a covariate, which was the gene expression or SCS for the control and infused quarters prior to the start of the experiment, was included as a fixed effect. This accounted for intra-cow variation. Time relative to the start of experiment was included as a repeated effect within udder quarter, and cow was included as a random effect. The most appropriate covariance structure among records was determined using Akaike information criterion. Least squares means were extracted from the analysis and differences between the control and infused quarters were considered significant at $P<0·05$. For graphical representation (Figs 2, 3 and 4) transformed gene expression data were back-transformed. Fold change was determined as the difference between peak gene expression and pre-infusion levels divided by pre-infusion expression for that gene.

Results

Recovery of viable bacteria from challenged quarters

To establish whether *Lc. lactis* successfully survived following intramammary infusion, milk samples were taken aseptically 7 h, 24 h, 48 h, 72 h and 7 d PI. Viable *Lc. lactis* were recovered at 7 h and 24 h from all cows (H, J, K and L). The bacterium was recovered 48 h PI from Cows H, J, and L and at 72 h PI from Cow K (Table 2). No other bacteria were recovered from the infused quarters throughout the trial. Control quarters remained clear of bacteria for the duration of the trial.

Physical response and milk characteristics

All animals elicited signs of udder inflammation in the infused quarters 7 h PI. These included swollen infused quarters, an elevation in rectal temperature or an elevated SCS i.e. above 12·2 (198 789 cells/ml) SCS (see Table 2 and Fig. 1). At this time, clots were visible in the milk of all four cows. SCS of the animals was recorded as >16·12 (10 000 000 cells/ml) as an accurate estimation could not be made due to the presence of clots. All animals had a self-limiting infection which was completely cleared 7 d PI. Consequently, antibiotic intervention was not required. Statistical analysis of the four Holstein Friesian cows in their first lactation demonstrated that SCS remained at elevated levels until 72 h PI. SCS of the infused quarters were greater than control quarters at 7 ($P<0·01$), 24 ($P<0·001$), 48 ($P<0·001$) and 72 h ($P<0·001$) PI. At 7 d PI, the average SCS for infused quarters was <12·2, so the quarters were considered clear of infection at this time

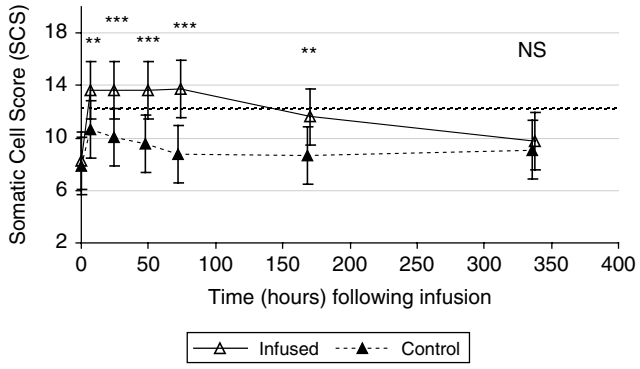

Fig. 1. Average somatic cell score (SCS) ±95% confidence intervals in quarters following infusion with *Lactococcus lactis* DPC 3147 compared with control quarters. *** $P<0{\cdot}001$; ** $P<0{\cdot}01$.

-------- denotes threshold value of 12·2 SCS (198 789 somatic cells/ml), where quarters with values below this were considered healthy and free of infection

(Fig. 1) but was still different from the control quarters ($P<0{\cdot}01$). Similar results were observed in the preliminary study (data not shown).

Cytokine changes in infused quarters

The panel of immune genes investigated consisted of *Toll-like receptor* (*TLR*) *2, TLR4, cluster of differentiation* (*CD*) *14, interleukin* (*IL*)*-1β, IL-8, IL-10, IL-12, tumour necrosis factor* (*TNF*)*-α, nuclear factor-kappa B* (*NF-κB*) and chemokine receptor *CXCR1*. Statistical analysis of the four cows in their first lactation demonstrated that all ten immune genes investigated were significantly upregulated 7 h post challenge. The greatest increase was noticed in *IL-1β*, *IL-8* and *CXCR1* expression, which underwent a 7000-fold, 4400-fold and 2700-fold average increase within 7 h PI respectively ($P<0{\cdot}001$, Fig. 2a, b, c). Expression of all three genes in the infused quarters differed from the control quarters up to 72 h PI ($P<0{\cdot}05$); however, there was no significant difference 7 d PI.

For *TLR2*, the highest levels were detected 7 h PI (average 600-fold increase; $P<0{\cdot}001$; see Fig. 3a) with a second, albeit lesser peak at 72 h PI ($P<0{\cdot}01$). Levels of *TLR2* in the infused quarters were still greater ($P<0{\cdot}05$) than in the control quarters 7 d PI; however, there was no significant difference between the control and infused quarters 14 d (2 weeks) PI. *TLR4* showed a greater fold increase within 7 h of challenge. Expression levels were on average 1000-fold greater than pre-infusion levels ($P<0{\cdot}001$; see Fig. 3b). Expression in the infused quarters was not significantly different from the control quarters 7 d PI.

TNF-α expression was greatest at 7 h PI with, on average, almost a 450-fold increase ($P<0{\cdot}001$) within that time (Fig. 3c). Gene expression levels remained elevated

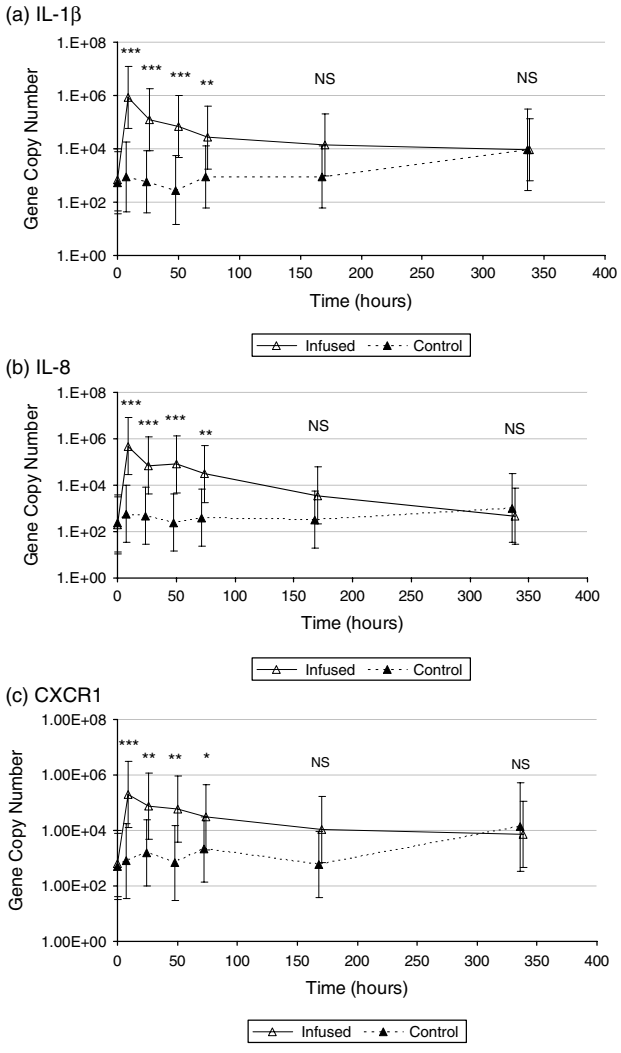

Fig. 2. Gene expression profiles in infused quarters compared with control quarters. (a) IL-1β; (b) IL-8; (c) CXCR1. Values are given as the exponential of transformed data ±95% confidence intervals. *** $P<0{\cdot}001$; ** $P<0{\cdot}01$; * $P<0{\cdot}05$.

until 72 h PI ($P<0{\cdot}01$) when compared with control quarters. The highest levels of *NF-κB* were also observed in all animals 7 h PI ($P<0{\cdot}001$). The fold change in all animals did not vary as much as other genes, with on average a 45-fold increase in RNA levels. Transcript abundance in the infused quarters remained greater ($P<0{\cdot}05$) than in the control quarters until 7 d PI (Fig. 4a). *CD14* was also greater 7 h PI, with an average 500-fold increase ($P<0{\cdot}001$) within that time. Elevated levels ($P<0{\cdot}05$) were still observed 7 d PI; however, there was no significant difference between the control and infused quarters 14 d PI (Fig. 4b). *IL-12* gene expression in the infused quarters was greater than the control quarters at 7, 24 and 48 h PI ($P<0{\cdot}001$). Peak in expression levels occurred at 7 h PI (>300-fold increase). The increase of expression of this gene was short-lived, however, and there was no difference

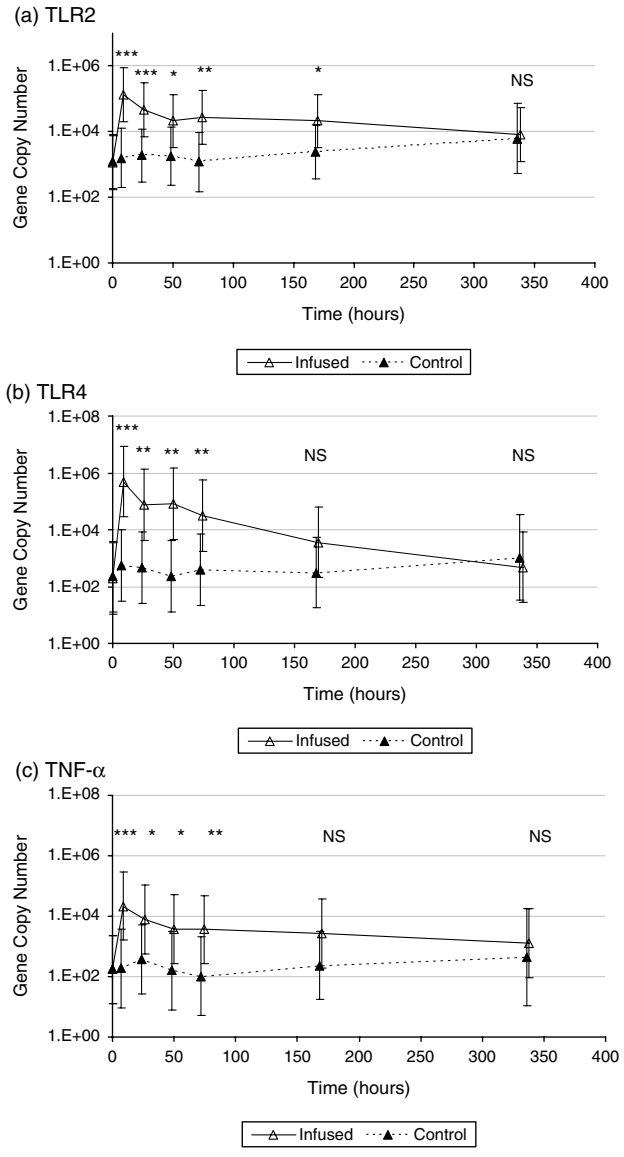

Fig. 3. Gene expression profiles in infused quarters compared with control quarters. (a) TLR2; (b) TLR4; (c) TNF-α. Values are given as the exponential of transformed data ±95% confidence intervals. *** $P<0{\cdot}001$; ** $P<0{\cdot}01$; * $P<0{\cdot}05$.

between the control and infused quarters by 72 h PI (Fig. 4c).

IL-10 expression also peaked at 7 h PI (average 400-fold up-regulation) and infused quarters were greater than control quarters ($P<0{\cdot}001$). Elevated levels remained different ($P<0{\cdot}05$) from control quarters expression until 7 d PI, with no difference 14 d PI (Fig. 4d).

There was no significant difference in expression levels of the housekeeping gene, *E2D2*, throughout the challenge (data not shown). Results from the preliminary study showed similar gene expression profiles, with notable increases in *IL-1β* and *IL-8* observed (data not shown).

Cytokine expression in control quarters and blood

Control quarters acted as internal controls for the infused quarters in each animal. Throughout the trial, there were some slight, non-significant increases in gene expression in control quarters at 7 h and 24 h PI, relative to immediately prior to the start of the experiment; however, these increases were much less pronounced than in the infused quarters. An increase in pro-inflammatory cytokine gene expression was also observed 14 d PI; however, this was not significantly different from pre-infusion levels (Figs 2, 3, 4). No significant changes in gene expression were observed in the blood of any of the animals in this study (data not shown).

Discussion

This study was initiated to determine the effect of a deliberate intramammary infusion with a food-grade bacterium, *Lc. lactis* DPC 3147, in healthy lactating dairy cows. Experimental trials have previously shown that treatment with *Lc. lactis* live culture is effective for cases of clinical and subclinical mastitis (Klostermann et al. 2008). We describe a massive immune response, with an increase in all pro-inflammatory genes investigated. The most significant difference was observed in expression of *IL-1β*, *IL-8* and *CXCR1*, where a 7000-fold, 4400-fold and 2700-fold increase, respectively, was observed within 7 h of infusion. The magnitude of the response is particularly noteworthy as *Lc. lactis* does not colonize within the udder and bacterial counts recovered from milk decrease to zero 72 h PI. All animals experienced an increase in SCC and swollen udder quarters. However, the immune response was short-lived and SCC, as well as expression of most pro-inflammatory genes had returned to pre-infusion levels within 1 week.

As a therapeutic, the immune profile elicited by this Gram-positive bacterium is distinctly different from a pathogen assault. The Gram-positive pathogen *Staph. aureus* fails to upregulate expression of IL-8 and TNF-α at both gene and protein level (Bannerman et al. 2004b; Yang et al. 2008). *Str. uberis* induces a late TNF-α response and a sustained elevated expression of IL-1β protein (Bannerman et al. 2004a). *Str. dysgalactiae* infusion caused a subdued immune response with *IL-8* gene up-regulation typically peaking at a 10–100-fold increase per 30 000 cfu/ml bacteria recovered (data not shown). *Escherichia coli*, a Gram-negative pathogen induces a much more acute response with an increase >50-fold and >100-fold increase of *IL-8* and *TNF-α* gene expression, respectively,within 12 h of challenge, and an increase in abundance of these proteins within 16 h (Bannerman et al. 2004b; Yang et al. 2008). However, the magnitude and speed of the response is still less than that to *Lc. lactis*. Up-regulation of cytokines and chemokines is necessary to mount a successful defence against mammary pathogens. *Lc. lactis* is capable of providing a substantial immune stimulation.

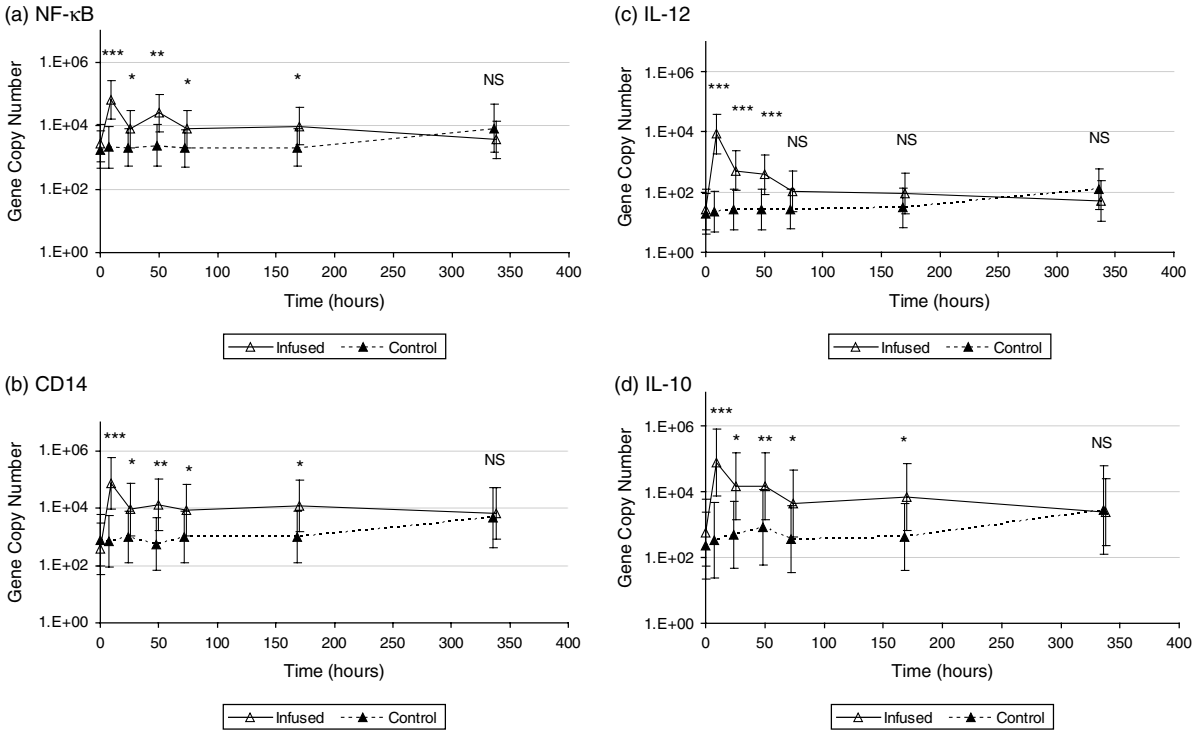

Fig. 4. Gene expression profiles in infused quarters compared to control quarters. (a) NF-κB; (b) CD14; (c) IL-12; (d) IL-10. Values are given as the exponential of transformed data ±95% confidence intervals. *** $P<0{\cdot}001$; ** $P<0{\cdot}01$; * $P<0{\cdot}05$.

As *Lc. lactis* is a Gram-positive bacterium and TLR2 binds to lipotechoic acid, the observed increase in *TLR2* expression was to be expected. However, the increase in *TLR4* and *CD14*, whose gene products are involved in LPS recognition, was of the same magnitude and, in the case of *TLR4* in some animals, greater than the up-regulation of *TLR2*. Ozinsky et al. (2000) proposed that TLRs are recruited to all phagosomes of macrophages to sample the contents, identify the bacteria and initiate the most effective response. Indeed, Goldammer et al. (2004) also observed an increase in both *TLR2* and *TLR4* RNA in mastitic tissue of cows infected by the Gram-positive *Staph. aureus*.

Once a bacterium is recognized through TLR signalling, cells usually secrete TNF-α and IL-1β to induce an acute phase response, activate NF-κB and increase IL-8 protein abundance. Our data set described a massive burst of *IL-1β* and *IL-8* gene expression and a significant up-regulation of *TNF-α* at the first PI sampling time. The up-regulation of *IL-8* gene expression was supported by a concomitant increase in IL-8 protein concentration in a representative milk sample (P Rainard, personal communication), as measured by ELISA (Rainard et al. 2008). In addition the *CXCR1* gene, which codes for an IL-8 receptor on neutrophils, is significantly up-regulated. Further circumstantial evidence that *IL-8* expression is considerably increased is the observation of a large influx of neutrophils to the site of infusion in a comparable study by Crispie et al. (2008). The massive stimulation of *IL-1β* by *Lc. lactis* may be one of the immunomodulatory mechanisms in which the bacterium confers its therapeutic effect. Oviedo-Boyso et al. (2008) has shown administration of the pro-inflammatory cytokines, TNF-α and IL-1β, increases the endocytic activity of the bovine endothelial cells (BEC) for *Staph. aureus* and enhances the ability of BEC to eliminate intracellular *Staph. aureus* and *Staph. epidermidis* in vitro. Wedlock et al. (2008) state that administration of recombinant bovine IL-1β to mammary glands at drying off results in sterile mastitis (i.e. increased SCC) but lowers the incidence of new intramammary infection by *Streptococcus uberis*.

NF-κB expression was also up-regulated following *Lc. lactis* challenge, but the fold change was not as noticeable as for other genes. This may be explained by the relative abundance of cytoplasmic NF-κB protein awaiting activation. *IL-10* was included in the gene panel to describe an anti-inflammatory response in the mammary gland due to the presence of *Lc. lactis*. Peak levels of *IL-10* were observed 7 h PI with an average 400-fold change from pre-infusion levels. As distinct from the majority of the other genes investigated, *IL-10* remained elevated beyond one week PI.

Control quarters exhibited a negligible increase in SCS and expression of a number of pro-inflammatory genes. These increases were most likely due to cross-talk between quarters (Berry & Meaney, 2006). No response was

observed at the systemic level. No infusion was administered to the control quarter. While we cannot rule out the possibility that the process of infusion in this study is the cause of the inflammatory reaction, we believe that it is highly unlikely. Previous and repeated trials by our research team has shown that infusion of sterile water into the control quarter does not cause irritation or inflammation as measured by gene expression, SCC and physical appearance. The immune response to *Lc. lactis* is also dose-dependent with a lower dose of 10^3 cfu eliciting no response (Crispie et al. 2008) and no change in immune gene expression (K Klostermann, unpublished observations).

Treatment with *Lc. lactis* compares very favourably with other therapies recently investigated to treat mastitis. Cytokine therapy has been investigated, but only as a prophylactic treatment and use of some cytokines was found to have serious side-effects, especially at certain times of year (Alluwaimi, 2004; Wedlock et al. 2008; Zecconi et al. 2008). Vaccination strategies have produced varying results and many require repeated dosing or boosters over a series of months (Middleton et al. 2009). LPS treatment was found to give only a transient decrease in bacterial numbers, but not to improve cure rates. Also, repeat dosing might be required, eventually reducing efficacy (Kauf et al. 2007). Lactoferrin has proved effective, but only in combination with antibiotics (Lacasse et al. 2008). Bacteriophage therapy has been hampered by the discovery that phage activity against *Staph. aureus* was inhibited in bovine milk (O'Flaherty et al. 2005; Gill et al. 2006b). However, *Lc. lactis* DPC 3147 may prove to be a successful non-antibiotic treatment for mastitis because of is ability to (a) produce a bacteriocin with broad spectrum antibacterial activity against Gram-positive pathogens (Ryan et al. 1998) and (b) elicit a rapid and substantial innate immune response.

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