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## A model system to investigate the regulation of lactoferrin production by the bovine mammary gland

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### ABSTRACT

Lactoferrin (Lf) is a high value, low concentration protein found in milk. Lf concentrations are found to increase during mastitis. This study was instigated to investigate if the effect of inducing sterile inflammation of the mammary gland, using lipopolysaccharide (LPS), a component of bacterial cell walls, would be a useful physiological model to study the production of Lf. Ten multiparous Friesian-Jersey cross-bred (FxJ) cows (approximately 200 days in milk) with no recent history of mastitis were utilised. All cows were milked on an individual quarter basis, and milk samples were collected one week prior to the treatment date to provide covariate data. Eight cows were infused via the teat canal with 10 ml saline and 10 µg LPS in 10 ml saline into 2 random quarters on each cow. The other two quarters on each cow were not treated. Two cows were untreated controls. All cows were milked twice daily with milk samples collected individually from all 4 quarters for 7 days with additional samples collected at 14 and 21 days. All milk samples were analysed for gross composition (fat, protein, lactose), somatic cell count (SCC), conductivity and Lf concentration. At the first milking after treatment, LPS treatment showed a significant effect on all milk components measured. By the 4th milking after treatment Lf concentrations were approximately 3.5 fold higher in LPS treated quarters compared with saline infused quarters, and remained elevated until 7 days after treatment. SCC remained elevated ( $P < 0.01$ ) in the LPS treated quarters until day 7 also. These results show that LPS can be used to induce the production of Lf in lactating cows and therefore provides a model with which to further investigate the mechanisms that control Lf production.

**Keywords:** lactoferrin; lipopolysaccharide; endotoxin; somatic cell count; conductivity; milk composition.

### INTRODUCTION

The iron-binding whey protein lactoferrin (Lf) is becoming an increasingly popular addition to many nutritional supplements, for both its antibacterial and immunomodulatory properties (Nuijens *et al.*, 1996; Vorland, 1999; Conneely, 2001). Currently, Lf is extracted from bulk milk by dairy companies, both in New Zealand (NZ) and overseas.

Concentrations of Lf in milk change throughout lactation with concentrations higher in colostrum and at drying off than during established lactation (Welty *et al.*, 1976; Gaunt *et al.*, 1980; Rainard *et al.*, 1982; Sanchez *et al.*, 1988; Schanbacher *et al.*, 1993; Rejman *et al.*, 1995; Farr *et al.*, 2002; Turner *et al.*, 2003a). Cow genotype (US Holstein vs NZ Friesian) affects milk Lf concentrations with higher concentrations of Lf found in milk from NZ Friesians. Feed type or the environment that cows are fed in may also have an effect as cows fed total mixed rations had higher concentrations of Lf than pasture fed cows (Turner *et al.*, 2003b).

Little is known about the control mechanisms that regulate Lf concentrations in milk. However, in addition to stage of lactation and cow genotype effects, mastitis, either spontaneous or induced, also elevates milk Lf concentrations (Harmon *et al.*, 1976; Rainard, 1983; Shoshani *et al.*, 2000). Induction of mastitis using *E. coli* is a common research technique to study mastitis. Dramatic elevations in SCC are apparent within hours of infusion and Lf concentrations elevate

within 1-2 days post infusion of the pathogen with concentrations reaching up to 30 times that of normal milk (Rainard, 1983). Lipopolysaccharide (LPS), part of the cell wall of gram negative bacteria (also known as endotoxin) is often used to induce mastitis-like symptoms in dairy cows without causing a bacterial infection (Shuster *et al.*, 1991; Moussaoui *et al.*, 2002; Perkins *et al.*, 2002). Udder oedema, shedding of clots in the milk, elevated rectal temperature and increases in milk SCC are all common responses to LPS and accurately mimic many of the responses that occur during mastitis (Shuster *et al.*, 1991).

In a study investigating the suppression of milk production during endotoxin-induced mastitis (Shuster *et al.*, 1991), milk Lf concentration was also measured. Lactoferrin concentration in the milk from infused quarters increased rapidly and was maximal ( $260 \pm 40$  mg/l) between milkings three and four. Concentrations declined after milking six and were still significantly greater than the uninfused quarters 7 days (14 milkings) after infusion. In the work reported by Shuster *et al.* (1991) cows were Holsteins fed a typical total mixed ration (TMR) diet. As cow strain and feed effects on Lf concentrations have been reported (Turner *et al.*, 2003b) these factors may also affect the changes in Lf concentrations in response to endotoxin challenge.

Little is known of the mechanisms that control the production of Lf and the development of a model system with the ability to induce Lf production as required will be important to developing an understanding of Lf synthesis. The work reported in

this paper was done to determine if the use of LPS (*E. coli* endotoxin) results in elevation of milk Lf concentration in pasture-fed NZ FxJ cows. The study also aimed to examine the variability both between and within animals in their response to the endotoxin challenge. The effect of endotoxin challenge on the milk yield and yield of Lf were also monitored and the effect of the treatment on other milk components was also of interest as it is necessary to ensure that the milk value for commodity products is retained.

## MATERIALS AND METHODS

### Experimental design and animal measurements

In February/March 2003, ten multiparous FxJ cows (approximately 200 days in milk, producing approximately 13.8 l/cow/day in the 2 weeks prior to the treatment period) with no recent (3-4 months) history of mastitis were selected for use. The experiment consisted of a covariate sampling period, then 7 days of intensive monitoring following the treatments. Post-treatment measurements were also collected at days 14 and 21. The cows were managed as a separate herd and were milked daily, at 0700 h and 1500 h throughout the trial period. Milk samples were collected on an individual quarter basis. One week prior to the treatment date, milk samples were collected at a p.m. and following a.m. milking to provide covariate data.

On day 0 of the experimental period, immediately after the a.m. milking, eight cows were infused via the teat canal with 10 ml saline solution and 10 µg LPS in 10 ml saline solution into 2 random quarters on each cow. The other two quarters on each cow were not treated. Two cows were untreated controls. All cows were milked twice daily with milk samples collected individually at p.m. and a.m. milkings from all 4 quarters for 7 days post-infusion with additional samples collected from individual quarters at 14 and 21 days. Milking number one was the first PM milking following the infusions. All milk samples were analysed for gross composition, SCC and Lf concentrations. Milk conductivity was measured at every milking. Milk samples were also collected aseptically for bacteriological analysis one-week prior, and at the milking immediately prior to treatment, and at days 1, 3, 7, 14 and 21 post-treatment to ensure the cows remained clear of pathogens.

### Milk sample analyses

All milk samples were analysed for gross composition (fat, crude and true protein, casein, lactose and total solids using an infrared milk analyser; Fourier Transform Infrared Spectroscopy [FT120]; Foss Electric, Hillerød, Denmark). Only values for fat, crude protein and lactose will be presented in this paper. Somatic cell count was measured using an automated cell counter (Fossomatic 5000; Foss Electric). Lf concentrations were measured using a bovine Lf ELISA quantification kit (Bethyl Laboratories, Inc,

Montgomery, Texas, United States of America) as described by Turner *et al.* (2003b).

Bacteriological assessment of quarter foremilk samples was carried out as described by Lacy-Hulbert *et al.* (2002). Milk conductivity was measured on foremilk from individual quarters using a temperature compensated digital conductivity meter (Milk Checker 2000; Technipharm, Rotorua, NZ). The measurement of milk conductivity using this meter was on an arbitrary linear scale originally derived from micro-siemens. As the individual quarters received different treatments only the raw values from the instrument are reported (as electrical conductivity (EC) units).

### Statistical analyses

Data were analysed using residual maximum likelihood (REML) with cow and quarter within cow as random effects and treatment as a fixed effect. Data collected one week prior to treatment were used as the covariate. SCC and Lf concentration were  $\log_{10}$  transformed to stabilise the variance before statistical analysis. Data from 1 quarter in each of two cows were excluded from the analyses as bacteriological assessment of the milk showed the presence of bacteria in those quarters.

## RESULTS

For the purposes of this paper, results presented are those comparing the saline infused quarters with the LPS infused quarters. However, to provide background information, average Lf concentrations were around 150-160 mg/l in the milk of the 2 untreated control cows, and in the milk from untreated quarters on the treated cows. LPS increased ( $P < 0.001$ ) milk yield, depressed ( $P < 0.001$ ) milk yield and had no effect on milk yield at the respective first, second and subsequent milkings post-infusion (Figure 1A). Marked a.m. versus p.m. differences in milk yield were apparent.

Lf concentrations were elevated ( $P < 0.01$ ) in the milk from quarters treated with LPS for the first 13 milkings (milking #10,  $P = 0.101$ ; Figure 1B). No effect of LPS on Lf concentrations was apparent at the AM milking on day 7 (14<sup>th</sup> milking), or on days 14 and 21 post treatment (milkings 27/28 and 41/42; Figure 1B).

Lf yields were greater ( $P < 0.01$ ) from those quarters treated with LPS (milking #2,  $P = 0.062$ ; Figure 1C). No difference in Lf yields was apparent between the two treatments from the 10<sup>th</sup> milking (5<sup>th</sup> day) post treatment.

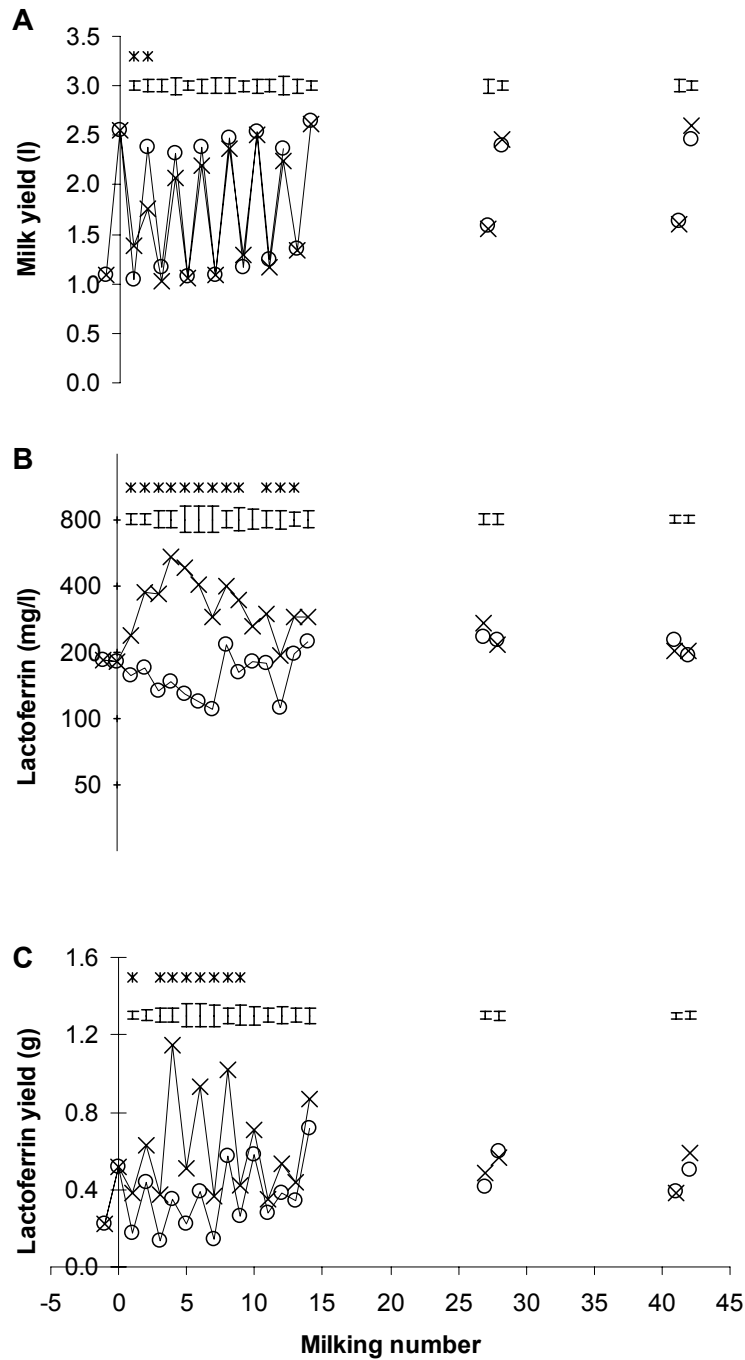
Milk fat concentration was depressed ( $P < 0.01$ ) in the milk of those quarters infused with LPS at the first milking following the infusions (Figure 2A). There was no difference in milk fat concentration of the milk between the two treatments after this 1<sup>st</sup> milking.

For the first 4 milkings following the treatments, milk crude protein concentration was elevated ( $P < 0.05$ ) by the LPS treatment (Figure 2B). There was an inconsistent effect of LPS treatment on milk crude

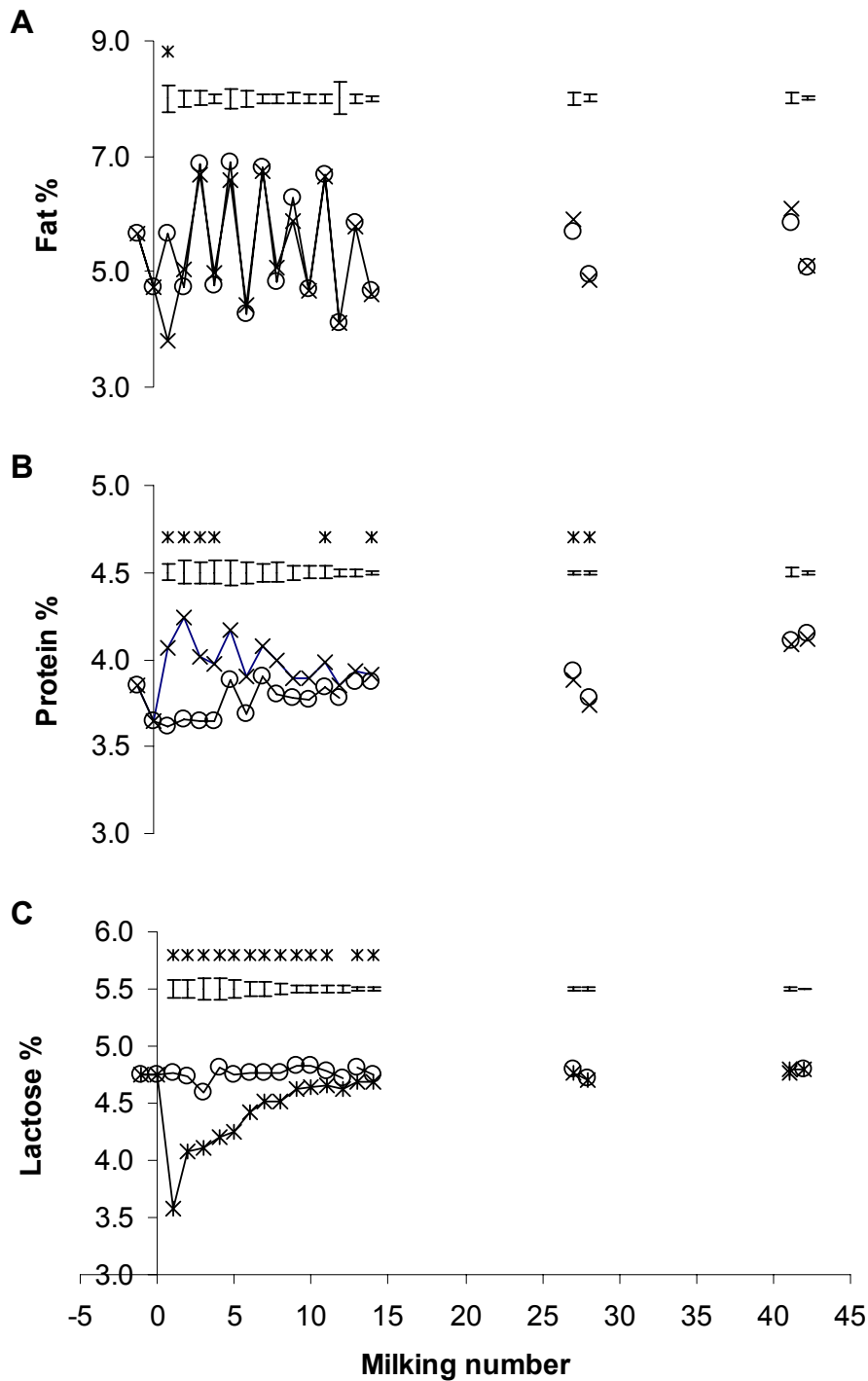
protein concentration for the remainder of the experiment. Milk lactose concentration was depressed ( $P < 0.05$ ), for the first 11 milkings following the LPS treatment, then there was an inconsistent effect of LPS

treatment for the remainder of the experiment (Figure 2C).

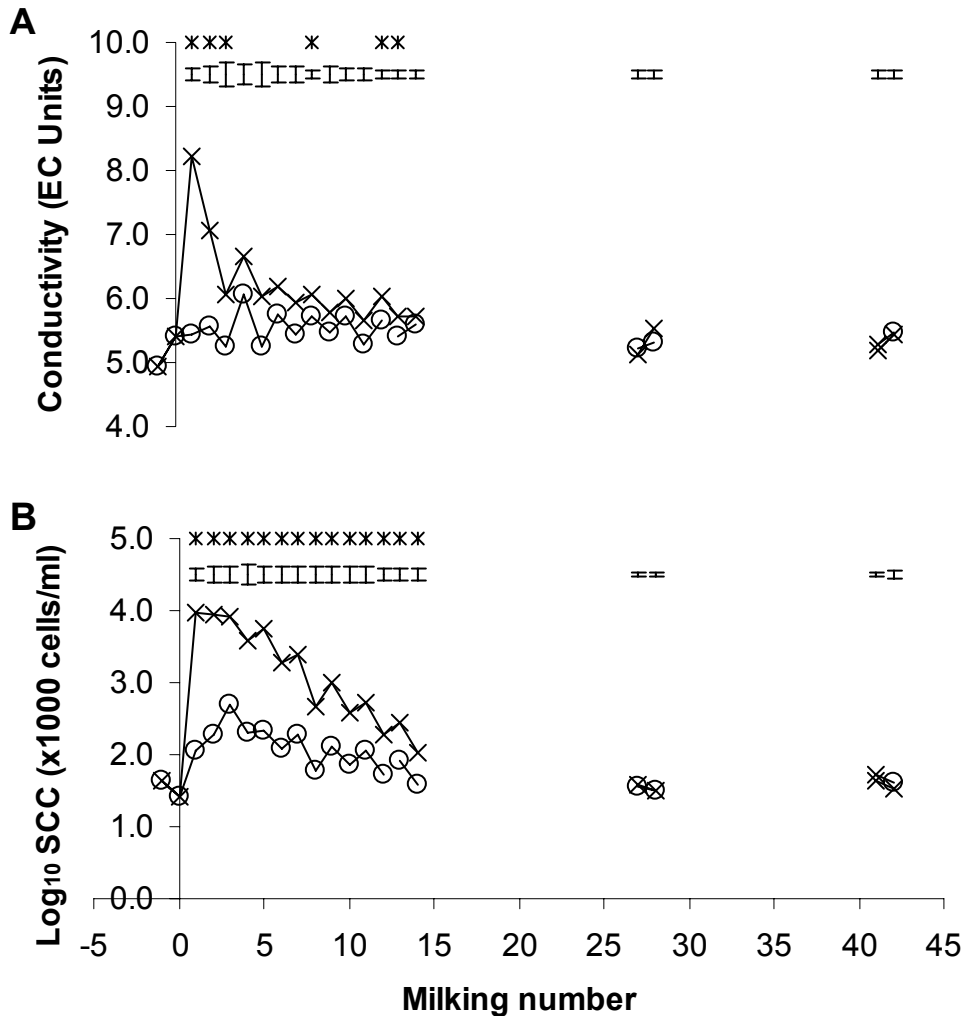
**FIGURE 1:** Effect of intramammary infusion of lipopolysaccharide (LPS) on A: milk yield; B: milk lactoferrin concentrations; C: lactoferrin yields. Eight cows were infused with 10 ml saline (o) and 10 µg LPS in 10 ml saline (x) into 2 random quarters on each cow after milking 0. The other two quarters on each cow were not treated. Samples were collected from individual quarters at subsequent p.m. and a.m. milkings. Data shown are from the saline and LPS infused quarters only. Lf concentrations are plotted on a  $\log_{10}$  scale, the y-axis labels have been back-transformed for ease of interpretation. Vertical bars indicate SED \*  $P < 0.05$



**FIGURE 2:** Effect of intramammary infusion of lipopolysaccharide (LPS) on the gross composition of milk. A: milk fat %; B: milk crude protein %, C: milk lactose %. Eight cows were infused with 10 ml saline solution (○) and 10 μg LPS in 10 ml saline solution (x) into 2 random quarters on each cow after milking 0. The other 2 quarters on each cow were not treated. Samples were collected from individual quarters at subsequent p.m. and a.m. milkings. Data shown are from the saline and LPS infused quarters only. Vertical bars indicate SED. \* P < 0.05



**FIGURE 3:** Effect of intramammary infusion of lipopolysaccharide (LPS) on A: milk conductivity, B: somatic cell count. Eight cows were infused with 10 ml saline solution (○) and 10 µg LPS in 10 ml saline solution (x) into 2 random quarters on each cow after milking 0. The other 2 quarters on each cow were not treated. Samples were collected and measurements taken from individual quarters at subsequent p.m. and a.m. milkings. Data shown are from the saline and LPS infused quarters only. Vertical bars indicate SED. \* P < 0.05



LPS infusion also resulted in elevation of milk conductivity ( $P < 0.05$ ) for the first three milkings following treatment, then there was an inconsistent effect of LPS treatment for the remainder of the experimental period (Figure 3A). Milk SCC was elevated ( $P < 0.01$ ) in LPS infused quarters and remained elevated for the first 7 days following treatments. By days 14 and 21 post treatment, no difference in the SCC of the milk from LPS and saline infused quarters was apparent (Figure 3B).

**DISCUSSION**

The results in this study compare well with other published work where changes in milk composition following infusion of LPS have also been reported. This current study and the work of Shuster *et al.* (1991)

show that the infusion of LPS into the mammary gland of dairy cows results, within 7-8 hours, in a substantial elevation in milk Lf concentrations. Further, both studies show that the elevated concentration of Lf are sustained until approximately 6 days post treatment with concentrations declining during that period, but still remaining significantly higher than the untreated or saline infused quarters.

In the current study, average Lf concentrations were around 150-160 mg/l in the milk of the 2 untreated control cows, and in the milk from untreated quarters on the treated cows. These values are within the same range as those previously reported for pasture-fed NZ Friesian cows (Turner *et al.*, 2003a; Turner *et al.*, 2003b). No differences in Lf concentrations have been found between NZ Friesian and cross-bred cows (Back & Thomson, 2005). Compared with the pre-treatment

values for Lf of around 50 mg/l reported by Shuster *et al.* (1991) who used North American Holstein-Friesians, the Lf concentrations in milk from NZ cows are considerably higher. This difference in the Lf concentration of milk between NZ and overseas Holstein-Friesians has been reported previously (Turner *et al.*, 2003b), suggesting that the difference was a genotype effect and not an environmental effect. The increase in Lf concentration following the LPS infusion reported by Shuster *et al.* (1991) reached a maximal level of about 260 mg/l which was approximately a 4-fold increase. In the current study, Lf concentration peaked around 530 mg/l compared with a concentration of about 150 mg/l in the saline infused quarters. This is an approximately 3.5-fold increase. These results show that the response to LPS in NZ Friesians was similar, despite the final concentrations being about double that seen in the milk of the Holstein-Friesians reported by Shuster *et al.* (1991). The reason for this difference is not known. Determining the cause of this could provide a means with which to further elevate Lf concentrations in bovine milk.

The Lf increase following LPS infusion was accompanied by rapid and sustained increases in both SCC and milk conductivity, both commonly used measures of mammary infection. In agreement with Shuster *et al.* (1991), SCC was significantly elevated for several milkings post infusion. Further, as expected in the current study, milk conductivity was elevated and remained so for the first three milkings post treatment. Milk conductivity is often elevated in cows with induced and spontaneous mastitis (Woolford *et al.*, 1998; Shoshani *et al.*, 2000).

Infusion of LPS into the mammary gland of dairy cows also results in changes in milk yield and in the gross composition of the milk. However, unlike the results reported in this study, Shuster *et al.* (1991) reported an increase in fat % (although this was following an initial decline in the infused quarters). The effect of mastitis on fat concentration in milk is conflicting. Auldish *et al.* (1995) reported lower concentrations of fat in the milk of cows with mastitic infections but Needs and Anderson (1984) reported no difference in the fat concentration of milk from quarters with experimentally induced mastitis compared with control quarters. In contrast to the results of Shuster *et al.* (1991), the initial decline in fat concentration in the current study was not followed by an increase. Similar to the results of Shuster *et al.* (1991) an increase in milk protein and a decrease in lactose concentrations were apparent in the milk from LPS infused quarters. Decreases in lactose concentration in mastitic milk are common also (Auldish *et al.*, 1995). Increases in crude protein concentration following the infusion of LPS could be the result of leakage of serum proteins (such as bovine serum albumin; BSA) into the gland. Increases in BSA have been reported in the milk from cows following LPS infusion (Shuster *et al.*, 1991) and during spontaneous and experimentally induced mastitis (Harmon *et al.*, 1976). Shuster *et al.* (1991) also

reported a rapid increase in urinary lactose following LPS infusion. In the current study, decreases in milk lactose concentration in the milk were also apparent and further suggest that the mammary gland was in a state of 'leakiness' due to the effects of the mammary inflammation. Local inhibition of milk production also occurs following infusion of LPS into cows (Shuster *et al.*, 1991) and could therefore also be responsible for decreases in milk components.

In the current study, the milk yield of the LPS quarters was greater than that of the saline infused quarters at the first milking post treatment (p.m.), but lower at the following a.m. milking. In contrast, Shuster *et al.* (1991) reported a decrease in milk yield in control quarters as well as LPS infused quarters. Although not reported in this paper, the milk yields were not different between the saline and untreated quarters. Depressed milk yields are often associated with mammary inflammation and are an unwanted side-effect of mastitis.

Together these results show that the use of LPS results in a rapid, sustained elevation in milk Lf concentration, which is accompanied by some changes in gross milk composition and milk yield. LPS provides a valid tool to 'switch on' the production of Lf and thus provides a useful model to assist in determining the mechanisms that control the synthesis of Lf.

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