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Effects of increased milking frequency for the first 21 days post partum on selected measures of mammary gland health, milk yield and milk composition

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Somatic cell count (SCC) is a widely used marker of udder health and a predictor of inflammation caused by an immune response. The objective of this study was to determine whether selected measures of mammary gland health as well as milk fatty acid profile were altered by an increase in milking frequency using a unilateral frequent milking (UFM) model. Holstein cows at parturition were assigned to UFM, in which the left udder half of each cow was milked four-times daily (4X) and the right udder half was milked twice daily (2X) for the first 21 days in milk (DIM). Milk yields from each udder half were measured from 1–21 DIM and samples were collected on days 3, 7, 10, 14 and 21 for determination of SCC and milk composition. Flow cytometric analysis with bovine monoclonal antibodies was used to identify milk immune cell populations and milk fatty acid (FA) composition was determined using gas chromatography. Gene expression analysis was used to determine whether there was an alteration in mRNA expression of genes involved in milk fat production including lipoprotein lipase (LPL) and FA-binding protein 3 (FABP3) with ribosomal protein S9 (RPS9) as a house-keeping gene. No difference was detected for milk SCC or cell populations between the udder halves milked 4X as compared with the udder halves milked 2X. In addition, no difference was detected for any FA in milk from the udder half milked 4X as compared with the udder half milked 2X. Overall, using a UFM model, increased milking frequency for the first 21 DIM did not affect selected measures of mammary gland health or milk FA, but was associated with greater milk yield, milk fat percent and yield, and milk protein and lactose yields.

Keywords: Milking frequency, milk immune cell, milk fatty acid profile.

Increasing milking frequency to improve milk production has become an effective management tool for dairy farmers. Increasing milking frequency has been shown to enhance milk yield when cows were milked three (3X) or four (4X) times daily, compared with milking two (2X) times daily (Campos et al. 1994; Erdman & Varner, 1995; Österman & Bertilsson, 2003). Factors associated with intensive management practices, such as increased milking frequency, can affect mammary gland defence mechanisms and may decrease the ability to resist infections. This is especially important during the periparturient period, when dairy cows are most susceptible to infections (Sordillo et al. 2009) and are experiencing oxidative stress (Aitken et al. 2009). An increase in milk production will cause an increase in the metabolic load, possibly weakening defences and negatively affecting the health of the mammary gland (Ingvartsen et al. 2003).

Consisting of several cell types, somatic cell count (SCC) is a widely used marker of udder health. In the healthy mammary gland, predominant SCC types are macrophages followed by lymphocytes, polymorphonuclear cells (PMN) and epithelial cells (Concha et al. 1986). A few reports (Smith et al. 2002; Dahl et al. 2004; Österman et al. 2005) demonstrated a reduction in total SCC with increased milking frequency, suggesting that increased milk production may improve milk quality and udder health contrary to that suggested by Ingvartsen et al. (2003). In contrast, others have reported no effect on SCC due to increased milking frequency (Klei et al. 1997; Hale et al. 2003; Wall & McFadden, 2007).

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Currently, no research has characterized alterations of milk cell populations in response to increased milking frequency from 2X to 4X.

The specific aim of this study was to determine whether selected measures of mammary gland health and milk FA profile were affected by increased milking frequency during the first 21 DIM, using a unilateral frequent milking (UFM) model. Use of the UFM model is important because it eliminates environmental, nutritional and genetic variation in cows and exposes both udder halves to the same systemic factors. Therefore, any treatment differences are expected to be related to intramammary factors.

Materials and Methods

Animals and treatments

Sixteen Holstein cows were assigned at parturition to a UFM scheme; 4-times daily milking (4X; at 5·00, 9·00, 16·00 and 20·00) of the left udder half and 2-times daily milking (2X; at 5·00 and 16·00) of the right udder half for 21 d. Uneven milking intervals were practised because of logistics at the research facility. Milk yields were recorded daily with milk samples collected from udder halves for determination of nutrients, FA profile and immune cell populations at days 3, 7, 10, 14 and 21 post partum. Milk components (fat, protein and lactose via infrared spectroscopy; solids-not-fat and SCC by Fossomatic analysis) were determined by a certified dairy herd improvement association laboratory (Burlington WA 98233, USA). The Fossomatic counter uses flow cytometry to recognize DNA from the cells, the fluorescent light pulses at a different wavelength, and the pulses are counted and displayed. During the course of the experiment, a single milker was responsible for milking times and milking order. Pre-milking procedures included pre-wiping of the teats, forestripping each teat three times, pre-dipping each teat with 0·5% iodine and allowing it sit for at least 20 s, and wiping the teats once more with a dry towel before attaching the milking machine. After milking was completed, sanitation of the teats were followed by a 1% iodine post-dip. All cows were offered water and a common lactation ration that consisted of alfalfa hay, alfalfa baleage, triticale silage, grass hay, corn, barley, canola meal, and commercial concentrate containing mineral/vitamin premix, soybean meal, molasses, beet pulp, wheat middlings and dicalcium phosphate. This total mixed ration contained 17·6% crude protein, 23·2% acid detergent fibre, 37·5% neutral detergent fibre, 3·4% crude fat, and 1·62 Mcal/kg NE_L (on a dry matter basis) for ad-libitum consumption. All procedures involving animals were approved by the University of Idaho Animal Care and Use Committee.

Milk cytometric flow analysis

Milk cells were processed for flow cytometric analysis as previously described (Riollet et al. 2000) with minor modifications. After the milk cells were obtained, the cell pellet was re-suspended in phosphate-buffered saline (PBS) with 0·1% bovine serum albumin (BSA; Sigma Aldrich; St. Louis MO 63103, USA), and 50 µl of the cell suspension was added to 96-well V-bottom plates (Corning; Corning NY 14831, USA). Monoclonal antibodies (mAb) specific to bovine leucocyte differentiation molecules were used to characterize the composition of leucocyte subpopulations from mammary gland secretions. Fifty µl of the appropriate primary mAb (VMRD, Pullman WA 99163, USA) were added to the wells. Plates were incubated on ice for 15–30 min to allow binding of specific mAb, then washed twice with PBS. Appropriate secondary antibodies conjugated with flourescein isothiocyanate (goat anti-mouse IgG1, IgGM, IgG2a, or IgG2b) were added to the wells. After 15–30-min incubation on ice, one wash with PBS and one wash with PBS with 0·1% BSA, the cells were suspended in 200 µl of 2% paraformaldehyde in PBS for flow cytometric analysis using a FACSAria flow cytometer equipped with FACSdiva software (V. 2.0 Becton Dickinson Immunocytometry Systems, San Jose CA 95131, USA) and Flojo (V. 7.5 Tree Star Inc., Ashland OR 97520, USA).

Fatty acid analysis

Milk lipids were extracted using a modified protocol (Clark et al. 1982) of the Folch method (Folch et al. 1957) and methylated using base-catalysed transesterification (Christie, 1982). Fatty acid methyl esters were analysed on a GC (Hewlett-Packard 6890 series with auto injector) fitted with a flame-ionization detector and a 100 m × 0·25 mm (0·2 µm film) capillary column coated with CP-Sil 88 (Chrompack, Middelburg, The Netherlands). After sample injection, the oven temperature was 70 °C for 3 min and then increased to 175 °C at a rate of 3 deg/min and held for 3 min. Oven temperature was then increased to 185 °C at a rate of 1 deg/min and held for 20 min, increased to 215 °C at a rate of 3 deg/min, and then increased to 230 °C at a rate of 10 deg/min and held for 5 min. To quantify FA, response correction factors were determined by the analysis of a butter oil standard with certified values (CRM 164; European Community Bureau of Reference, Brussels, Belgium).

Milk cell isolation for mRNA studies

Total RNA was isolated from milk samples and prepared using a procedure developed by Boutinaud et al. (2002). After the milk cells were obtained, the cell pellet was treated with 600 µl of buffer RLT+(Qiagen, Valencia CA 91355, USA) and β-mercaptoethanol (100:1) and transferred to a Qiashredder spin column (Qiagen) and centrifuged at 8000 ***g*** for 2 min. The liquid collected from the column was stored at − 80 °C.

Gene expression analysis

The RNA isolated was processed using the RNeasy Mini Plus Kit (Qiagen) according to the manufacturer's instructions.

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The quality and concentration of RNA were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland DE 19732, USA). Total RNA was converted into complementary DNA using Applied Biosystems High Capacity Reverse Transcriptase Kit (Applied Biosystems, Foster City CA 94404, USA). Single-stranded cDNA was synthesized by reverse transcription using a PCR Sprint Thermal Cycler (Thermo Electron Corp., Milford MA 02139, USA). The single-stranded cDNA was then used as the template for quantitative PCR to evaluate relative expression of lipoprotein lipase (LPL), fatty acid binding protein 3 (FABP3), and ribosomal protein S9 (RPS9) as a house-keeping gene. Primers for PCR amplification of LPL and RPS9 were designed by Primer Express (Version 1.5, Applied Biosystems) generating the following primers: bovine LPL forward primer: GGAAGAAAGAACAGCA-TATGAATTCTATG, and reverse primer: GGGTTAATACT-CCGGAAAATCCACAT; bovine RPS9 forward primer: GGCGGCTCGTCCGTATC, and reverse primer: AATCTTC-AGGCCCAGGATCTAATC. Bovine FABP3 gene expression assay was designed by Applied Biosystems (assay #Bt03213817_g1). Gene expression of LPL, FABP3, and RPS9 was measured using the 7500 Fast Real-Time PCR System (Applied Biosystems).

Statistical analysis

Statistical analyses were performed using the PROC MIXED procedure of SAS (V. 9.2 SAS Inst. Inc., Cary NC 27511, USA). The model used was: $Y_{ij}=\mu+c_i+D_j+e_{ij}$ where Y_{ij} was the dependent variable for cow *i,* at DIM *j* measuring the difference between the responses of 4X milking of the left udder half and 2X milking of the right udder half. The overall mean was given by μ and measured the main effect of 2X *v.* 4X milking, c_i was the random effect due to cow$_i$, D_j the fixed effect of DIM on the treatment differences, and e_{ij} was the experimental error where $e_{ij} \sim N(\theta, \sigma^2)$. DIM were recorded as days 3, 7, 10, 14 and 21 post partum. Repeated measures analysis was used to account for the effect of DIM within each individual cow, assuming compound symmetry variance covariance structure. Significant effects were declared at $P<0{\cdot}05$. Data are presented as means ± SEM. Gene expression data were statistically analysed using original ΔCt values as responses (normalized with RPS9) and significance was declared at $P<0{\cdot}05$. For gene expression data, least squares means (± SEM) are reported as fold change ($2^{-\Delta\Delta Ct}$) of 4X milking relative to 2X. Correlation analysis was used to assess any association between milk fat percentage and mRNA expression of genes involved in milk lipid uptake and synthesis.

Results

All 16 Holstein cows assigned to the UFM scheme completed the experiment without clinical disease. As expected, SCC started at greater levels during the first days in lactation and decreased with time (data not shown); however, milking frequency did not affect overall milk SCC or SCC linear score ($P>0{\cdot}9$ for both; Fig. 1). No effect of milking frequency was detected on percentages of milk total leucocytes (45·5 *v.* 40·5% ± 0·52 for 4X *v.* 2X, respectively; Fig. 2a), granulocytes (12·7 *v.* 8·8% ± 0·89 for 4X *v.* 2X, respectively; Fig. 2b), mononuclear cells (30·0 *v.* 29·3% ± 0·83 for 4X *v.* 2X, respectively; Fig. 2c), cluster of differentiation (CD) 4 to CD8 ratio (2·90 *v.* 2·75 ± 0·29 for 4X *v.* 2X, respectively; Fig. 2d), T cells (14·72 *v.* 14·10 ± 0·58 for 4X *v.* 2X, respectively; Fig. 2e) or B cells (2·86 *v.* 2·53 ± 0·13 for 4X *v.* 2X, respectively; Fig. 2f) for the udder halves milked 4X as compared with that of udder halves milked 2X ($P \geqslant 0{\cdot}46$ for all). Further, cell population quantities (percent of milk cells × SCC/100) were statistically analysed to determine whether there was a treatment effect on the absolute number of leucocytes, granulocytes and mononuclear cells of milk somatic cells. No difference in any of cell populations tested, however, was detected ($P>0{\cdot}29$ for all).

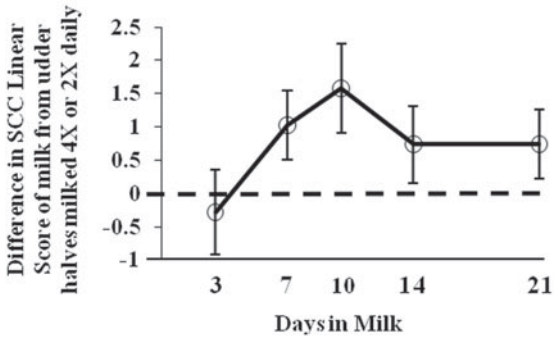

Fig. 1. The difference in somatic cell count linear score in milk between udder halves milked four-times daily (4X) and udder halves milked two-times daily (2X) during the first 21 days in milk. Data are presented as means ± SEM.

Several components of milk were greater during the first 21 DIM for the udder halves milked 4X as compared with those for the udder halves milked 2X including milk yield (13·3 and 9·91 ± 0·55 kg/d, $P<0{\cdot}001$), milk protein yield (0·42 and 0·31 ± 0·006 kg/d, $P<0{\cdot}001$; Fig. 3a), milk lactose yield (0·58 and 0·42 ± 0·004 kg/d, $P<0{\cdot}001$; Fig. 3b), milk fat yield (0·60 and 0·39 ± 0·01 kg/d, $P<0{\cdot}001$; Fig. 3c), and milk fat percent (4·96 and 4·57 ± 0·28%, $P<0{\cdot}02$; Fig. 3d). Milk protein percentage did not differ between treatments (3·50 and 3·53 ± 0·17% for 4X *v.* 2X, respectively, $P=0{\cdot}15$). Similarly, lactose percentage did not differ in milk from udder halves milked 4X *v.* 2X (4·42 and 4·32 ± 0·04%, $P=0{\cdot}07$).

Even though milk fat percentage and yield were greater in milk from udder halves milked 4X *v.* 2X, no difference was observed in gene expression of LPL or FABP3 in cells obtained from milk. However, a weak correlation ($r=0{\cdot}29$; $P=0{\cdot}04$) between FABP3 and milk fat percent on days 7, 14 and 21 was detected. Lastly, no difference was detected in milk total saturated FA (52·32 and 52·37 ± 0·16%, respectively, $P=0{\cdot}79$); monounsaturated FA (33·29 and 33·23 ± 0·16%, respectively, $P=0{\cdot}79$); polyunsaturated FA (5·32 and 5·35 ± 0·01, respectively, $P=0{\cdot}68$), products of the

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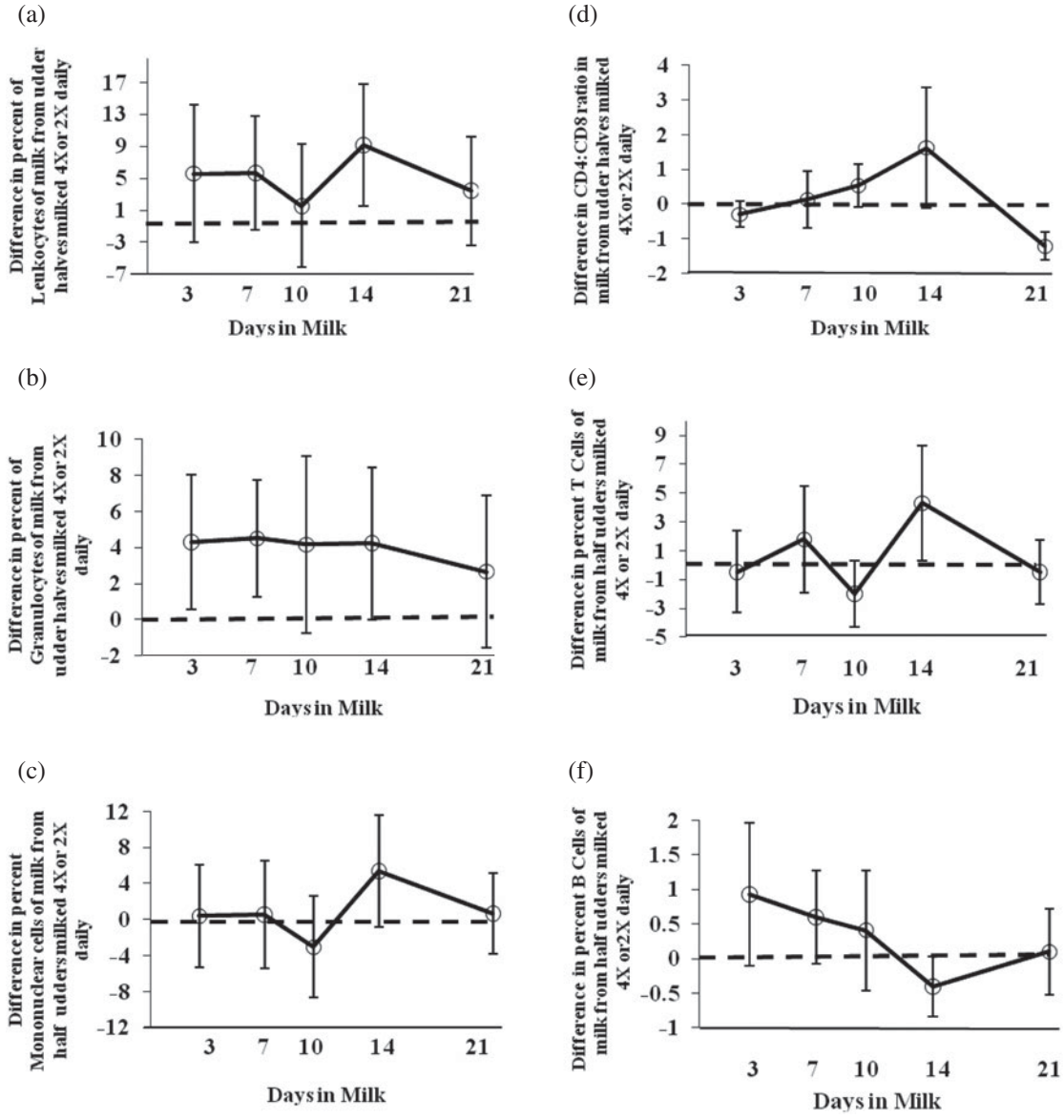

Fig. 2. The difference in flow cytometric analysis of somatic cells between milk samples obtained from udder halves milked four times daily (4X) and udder halves milked two-times daily (2X) during the first 21 days in milk: **(a)**: Total leucocyte cells as a percentage of total cells **(b)**: Granulocyte cells as a percentage of total cells **(c)**: Total mononuclear cell as a percentage of total cells **(d)**: Ratio of CD4 to CD8 stained cells **(e)**: Total T cells as a percentage of total cells **(f)**: Total B cells as a percentage of total cells. Data are presented as means ± SEM.

Δ^9-desaturase enzyme (27·38 and 27·42 ± 0·77%, respectively, $P = 0·75$) or products of de-novo synthesis (17·32 and 17·46 ± 0·28%, respectively, $P = 0·25$) obtained from the udder halves milked 4X as compared with that from udder halves milked 2X.

Discussion

Although the usefulness of increased milking frequency in improving milk yield has been reported, to our knowledge, no research has been conducted characterizing mammary or milk cell populations exposed to increased milking frequency during the periparturient period, in a UFM model. It is possible that an increase in the metabolic load associated with increased milk production, weakens defences and negatively affects the health of the mammary gland (Ingvartsen et al. 2003).

Previous research reported no increase in milk total SCC when milking frequency increased from 2X to 4X daily using a UFM model (Wall & McFadden, 2007), in agreement with the present study. This is also similar to results from Hale et al. (2003) and Smith et al. (2002) who compared SCC from cows milked 4X with those milked 2X and 3X daily.

At the onset of an inflammatory reaction, proportions of cells can change as they are recruited to the site of the

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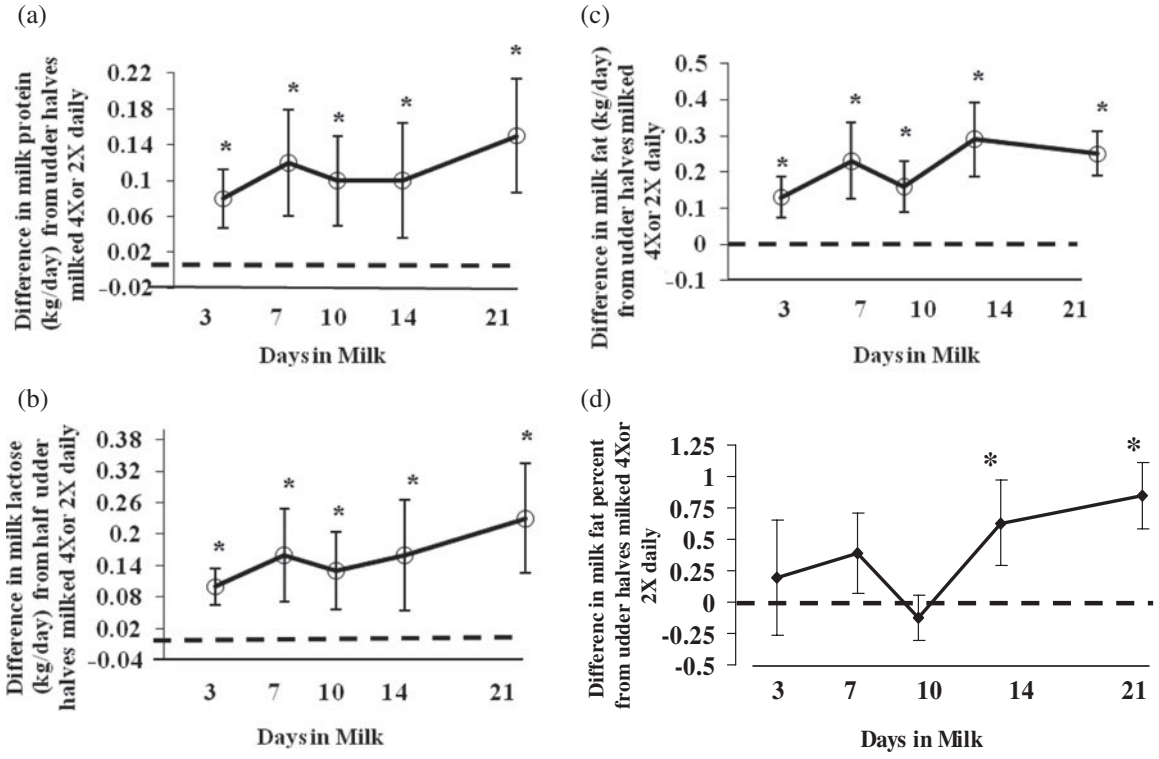

Fig. 3. The difference in milk composition between udder half milked four-times daily (4X) and udder half milked two-times daily (2X) during the first 21 days in milk: **(a)**: Milk protein yield (kg/d) **(b)**: Milk lactose yield (kg/d) **(c)**: Milk fat yield (kg/d) **(d)**: Milk fat percentage. Data are presented as means ± SEM. Asterisks indicate differences ($P < 0{\cdot}05$) observed at specific time points.

inflammation. In the current study, milk cell populations were consistent with those in a healthy mammary gland. PMN leucocytes and macrophages are the phagocytic cells and comprise an important line of defence against pathogens. Macrophages are similar to neutrophils in their phagocytic capabilities and are capable of ingesting bacteria, debris and accumulated milk components. Macrophages together with epithelial cells initiate the inflammatory response needed to eliminate the invading pathogen (Sordillo & Nickerson, 1988). In the healthy mammary gland, macrophages constitute 35–79% of cells (Lindmark-Mansson et al. 2006). This value is consistent with the proportion of leucocytes in the total cell population reported in the current study except for mononuclear cells for which the number was lower than in the healthy mammary gland.

Neutrophil granulocytes are found in mammary tissues and milk secretion during early inflammation and can constitute >90% of total mammary gland leucocytes (Paape et al. 2002; Sordillo & Babiuk, 1991; Sordillo et al. 1997). Recruitment of neutrophils to the site of infection is a normal defence response and is effective in eliminating many pathogens known to cause mastitis (Selsted et al. 1993). In the healthy mammary gland, granulocytes constitute 3–26% of the total number of cells (Lindmark-Mansson et al. 2006) consistent with the proportion of granulocytes in the total cell population reported in the current study.

Generation of specific immunity involves both lymphocytes and antigen-presenting cells. Lymphocytes recognize antigens through membrane receptors specific for infectious pathogens (Sordillo et al. 1997). In the healthy mammary gland, lymphocytes constitute 16–28% of total cells (Lindmark-Mansson et al. 2006) consistent with the proportion of T cells in the total cell population reported in the current study. The proportion of B cells reported in the current study was lower than the proportion of lymphocytes in the total cell population found in the healthy mammary gland (Park et al. 1992). CD8+ cells have been shown (Ismail et al. 1996) to be the predominant phenotype in healthy mammary gland tissue and secretions; with the ratio of CD4 to CD8 less than one. Lindmark-Mansson et al. (2006) reported a CD4 to CD8 ratio of 0·60–3·29 in the health mammary gland consistent with the CD4 to CD8 ratio reported in the current study.

The results reported here for SCC, SCC linear score, and milk cell populations imply that increased milking frequency had no adverse or beneficial effect on selected measures of mammary gland health under our experimental conditions.

Research (Allen et al. 1986; Barnes et al. 1990; Smith et al. 2002) demonstrated a decrease in milk fat percentage when milking frequency was increased. Additionally, Klei et al. (1997) reported a lower milk protein content in cows milked 3X compared with that of cows milked 2X. Results for milk protein percent and yield in the present study are in

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agreement with those of others (Allen et al. 1986; DePeters et al. 1985; Campos et al. 1994; Wall & McFadden, 2007) who found no effect on milk protein percent but increased milk protein yield in cows or udder halves milked more frequently. Results for lactose percentage are similar to those reported by Patton et al. (2006) who detected no difference in lactose concentration when milking frequency increased from 1X to 3X. Increased milk fat yield in the present study is similar to previous studies (Campos et al. 1994; Erdman & Varner, 1995; Klei et al. 1997) where fat yield from cows milked 3X was greater than that for cows milked 2X. Overall, increased milk yield associated with 4X milking may explain the increases in milk protein, lactose and fat yields.

In the present study, milk fat percent was increased with the 4X daily milking *v*. 2X daily, which is contradictory to some reports (Hale et al. 2003; Dahl et al. 2004; Patton et al. 2006). Uneven milking intervals were practised because of logistics at the research facility, which may have affected the milk fat percentage via alteration in size of milk fat globules (Wiking et al. 2004). Others (DePeters et al. 1985; Wiking et al. 2006; Wall & McFadden, 2007) reported no effect of increased milking frequency on milk fat percentage. This discrepancy may be related to the varying length of each experiment: DePeters et al. (1985) compared 2X and 3X milking over the entire lactation whereas Wiking et al. (2006) compared 2X and 4X milkings in mid and late lactation for 2 weeks, and Wall & McFadden (2007) compared 2X and 4X daily milkings during the first 21 DIM.

Other factors may have been involved in the increased milk fat content in the udder halves milked 4X compared with that of cows milked 2X. Specifically, genes involved in the regulation of milk fat synthesis could have contributed to an increase in milk fat concentration. In particular, genes such as LPL and FABP3 that encode for enzymes involved in the uptake and transport of FA could be important regulators of milk fat synthesis. The major contribution to milk lipid from plasma originates from hydrolysis of the triacylglycerol (TAG) components of VLDL and chylomicrons, catalysed by mammary LPL (Clegg et al. 2001). Fatty acid binding protein is involved in the intracellular transport of FA as well as providing FA for stearoyl-CoA desaturase, which can then contribute FA to other enzymes involved in TAG synthesis (Bionaz & Loor, 2008). In the present study, the increase in milk fat percent detected between udder halves milked 4X compared with 2X daily was not reflective of any detectable difference in mRNA expression of LPL or FABP3. There was approximately a 0·5-fold increase in LPL and a 0·6-fold increase in FABP3 (on day 7) but no significance was detected for either gene. The lack of response to increased milking frequency in mRNA expression of either LPL or FABP3 may not reflect enzyme activity if some form of post-translational modification is important (Ollier et al. 2009). It is possible that there may have been an up-regulation of other genes in milk cells that were not tested. For instance, Ollier et al. (2009) observed changes in milk composition without any effect on mammary mRNA expression from cells collected in milk of key enzymes involved in lactose, protein and lipid metabolism. These synthetic mechanisms could involve translational or post-translational regulation, affecting enzyme quantity or activity. Additional research needs to be conducted to fully understand the mechanisms responsible for the alteration in milk fat percentage in response to increased milking frequency.

Klei et al. (1997) reported that increasing milking frequency from 2X to 3X may increase the ratio of de-novo synthesized to preformed FA in milk fat, particularly during early and mid lactation. In the mammary gland of ruminants, short- and medium-chain saturated FA are the major products of de-novo lipogenesis, whereas plasma lipids contribute to longer-chain and monounsaturated FA (Clegg et al. 2001). Results reported here are similar to previous studies (Svennersten-Sjaunja et al. 2002; Wiking et al. 2006) in which increased milking frequency did not alter milk FA composition. These results indicate that de-novo synthesis or use of preformed FA in milk fat was not differently affected by increased milking frequency.

Milk yield results were similar to those reported by Wall & McFadden (2007) showing an increase in milk yield when milking frequency was increased during the first 21 DIM using a UFM model. Others (Hale et al. 2003; Österman & Bertilsson, 2003) have also shown this effect in cows milked with greater milking frequency.

Conclusion

Previous research indicated that increased milking frequency during early lactation increased milk production, and may alter mammary gland health and milk composition. Overall, the present study shows for the first time, that increased milking frequency from 2X to 4X using a UFM model for the first 21 d post partum did not affect selected measures of mammary gland health, such as milk immune cell populations or SCC. Further, increased milking frequency did not alter milk FA profile. The present study confirms that increasing milking frequency resulted in greater milk yield, milk fat percent and yield, milk protein yield and milk lactose yield. Increased milking frequency, however, did not affect milk protein and lactose percentages.

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References

Aitken SL, Karcher EL, Rezamand P, Gandy JC, VandeHaar MJ, Capuco AV & Sordillo LM 2009 Evaluation of antioxidant and pro-inflammatory gene expression in bovine mammary tissue during the periparturient period. *Journal of Dairy Science* **92** 589–598

https://doi.org/10.1017/S0022029911000410 Published online by Cambridge University Press

Allen DB, DePeters EJ & Laben RC 1986 Three times a day milking: Effects on milk production, reproduction efficiency, and udder health. *Journal of Dairy Science* **69** 1441–1446

Amos HE, Kiser T & Loewenstein M 1985 Influence of milking frequency on productive and reproductive efficiencies of dairy cows. *Journal of Dairy Science* **68** 732–739

Barnes MA, Pearson RE & Lukes-Wilson AJ 1990 Effects of milking frequency and selection for milk yield on productive efficiency of Holstein cows. *Journal of Dairy Science* **73** 1603–1611

Bionaz M & Loor JJ 2008 Gene networks driving bovine milk fat synthesis during the lactation cycle. *BMC Genomics* **9** 366

Boutinaud M, Rulquin H, Keisler DH, Djiane J & Jammes H 2002 Use of somatic cells from goat milk for dynamic studies of gene expression in the mammary gland. *Journal of Animal Science* **80** 1258–1269

Campos MS, Wilcox CJ, Head HH, Webb DW & Haven J 1994 Effects on production of milking three times daily on first lactation Holsteins and Jerseys in Florida. *Journal of Dairy Science* **77** 770–773

Christie WW 1982 A simple procedure for rapid transmethylation of glycerolipids and cholesteryl esters. *Journal of Lipid Research* **23** 1072–1075

Clark RM, Ferris AM, Fey M, Brown PB, Hundrieser KE & Jensen RG 1982 Changes in the lipids of human milk from 2 to 16 weeks post partum. *Journal of Pediatric Gastroenterology and Nutrition* **1** 311–315

Clegg RA, Barber MC, Pooley L, Ernens I, Larondelle Y & Travers MT 2001 Milk fat synthesis and secretion: molecular and cellular aspects. *Livestock Production Science* **70** 3–14

Concha C, Holmberg O & Aström G 1986 Cells found in non-infected and Staphylococcus-infected bovine mammary quarters and their ability to phagocytose fluorescent microspheres. *Zentralbl Veterinarmed B*. **33** 371–378

Dahl GE, Wallace RL, Shanks RD & Lueking D 2004 Hot Topic: Effect of frequent milking in early lactation on milk yield and udder health. *Journal of Dairy Science* **87** 882–885

Delamaire E & Guinard-Flament J 2006 Longer milking intervals alter mammary epithelial permeablility and the udder's ability to extract nutrients. *Journal of Dairy Science* **89** 2007–2016

DePeters EJ, Smith NE & Acedo-Rico J 1985 Three or two times daily milking of older cows, and first lactation cows for entire lactation. *Journal of Dairy Science* **68** 123–132

Erdman RA & Varner M 1995 Fixed yield responses to increased milking frequency. *Journal of Dairy Science* **78** 1199–1203

Folch J, Lees M & Sloan Stanley GH 1957 A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry* **226** 497–509

Gisi DD, DePeters EJ & Pelissier CL 1986 Three times daily milking of cows in California dairy herds. *Journal of Dairy Science* **69** 863–868

Hale SA, Capuco AV & Erdman RA 2003 Milk yield and mammary growth effects due to increased milking frequency during early lactation. *Journal of Dairy Science* **86** 2061–2071

Ingvartsen KL, Dewhurst RJ & Friggens NC 2003 On the relationship between lactational performance and health: Is it yield or metabolic imbalance that cause production disease in dairy cattle? A position paper. *Livestock Production Science* **83** 277–308

Ismail HI, Hashimoto Y, Kon Y, Okada K, Davis WC & Iwanaga T 1996 Lymphocyte subpopulations in the mammary gland of the goat. *Veterinary Immunology and Immunophathology* **52** 201–212

Jensen DL & Eberhart RJ 1981 Total and differential cell counts in secretions of the nonlactating bovine mammary gland. *American Journal of Veterinary Research* **42** 743–747

Klei LR, Lynch JM, Barbano DM, Oltenacu PA, Lednor AJ & Bandler DK 1997 Influence of milking three times a day on milk quality. *Journal of Dairy Science* **80** 427–436

Lee CS, Wooding FBP & Kemp P 1980 Identification, properties and differential counts of cell populations using electron microscopy of dry cow secretions, colostrum, and milk from normal cows. *Journal of Dairy Research* **47** 39–50

Lindmark-Månsson H, Bränning C, Aldén G & Paulsson M 2006 Relationship between somatic cell count, individual leukocyte populations and milk components in bovine udder quarter milk. *International Dairy Journal* **16** 717–727

Ollier S, Leroux C, de la Foye A, Bernard L, Rouel J & Chilliard Y 2009 Whole intact rapeseeds or sunflower oil in high-forage or high-concentrate diets affects milk yield, milk composition, and mammary gene expression profile in goats. *Journal of Dairy Science* **92** 5544–5560

Österman S & Bertilsson J 2003 Extended calving interval in combination with milking two or three times per day: effects on milk production and milk composition. *Livestock Production Science* **82** 139–149

Österman S, Östensson K, Svennersten-Sjauna K & Bertilsson J 2005 How does extended lactation in combination with different milking frequencies affect somatic cell counts in dairy cows? *Livestock Production Science* **96** 225–232

Paape M, Mehrzad J, Zhao X, Detilleux J & Burvenich C 2002 Defence of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. *Journal of Mammary Gland Biology and Neoplasia* **7** 109–121

Park YH, Fox LK, Hamilton MJ & Davis WC 1992 Bovine mononuclear leukocyte subpopulations in peripheral blood and mammary gland secretions during lactation. *Journal of Dairy Science* **75** 998–1006

Patton J, Kenny DA, Mee JF, O'Mara FP, Wathes DC, Cook M & Murphy JJ 2006 Effect of milking frequency and diet on milk production, energy balance, and reproduction in dairy cows. *Journal of Dairy Science* **89** 1478–1487

Riollet C, Rainard P & Poutrel B 2000 Kinetics of cells and the cytokines during immune-mediated inflammation in the mammary gland of cows systemically immunized with *Staphylococcus aureus* α-toxin. *Inflammation Research* **49** 486–496

Selsted ME, Tang YQ, Morris WL, McGuire PA, Nonotny MJ, Smith W, Henschen AH & Cullor HS 1993 Purification, primary structures, and antibacterial activities of beta-defensin, a new family of antimicrobial proteins from bovine neutrophils. *Journal of Biological Chemistry* **268** 6641–6648

Smith JW, Ely LO, Graves WM & Gilson WD 2002 Effect of milking frequency on DHI performance measures. *Journal of Dairy Science* **85** 3526–3533

Sordillo LM & Nickerson SC 1988 Quantification and immunoglobulin classification of plasma cells in nonlactating bovine mammary tissue. *Journal of Dairy Science* **71** 84–91

Sordillo LM & Babiuk LA 1991 Modulation of mammary neutrophil function during the periparturient period following in vitro exposure to recombinant bovine interferon-gamma. *Veterinary Immunology and Immunopathology* **27** 393–402

Sordillo LM, Shafer-Weaver K & DeRosa D 1997 Immunobiology of the mammary gland. *Journal of Dairy Science* **80** 1851–1865

Sordillo LM, Contreras GA & Aitken SL 2009 Metabolic factors affecting the inflammatory response of periparturient dairy cows. *Animal Health Research Review* **10** 53–63

Stelwagen K 2001 Effect of milking frequency on mammary functioning and shape of the lactation curve. *Journal of Dairy Science* **84** (E. Suppl.) E204–E211

Svennersten-Sjaunja K, Persson S & Wiktorsson H 2002 The effect of milking interval on milk yield, milk composition, and raw milk quality. In *Proceedings International Symposium: The First North American Conference on Robotic Milking*, pp. V-43–V-48. Toronto ON, Canada

Wall EH & McFadden TB 2007 The milk yield response to frequent milking in early lactation of dairy cows is locally regulated. *Journal of Dairy Science* **90** 716–720

Wiking L, Stagsted J, Björck L & Nielsen JH 2004 Milk fat globule size is affected by fat production in dairy cows. *International Dairy Journal* **14** 909–913

Wiking L, Nielsen JH, Båvius AK, Edvardsson A & Svennersten-Sjaunja K 2006 Impact of milking frequencies on the level of free fatty acids in milk, fat globule size, and fatty acid composition. *Journal of Dairy Science* **89** 1004–1009

Wiktorsson H, Svennersten-Sjaunja K & Salomonsson M 2000 Short or irregular milking intervals in dairy cows—Effects on milk quality, milk composition, and cow performance. In *Proceedings International Symposium on Robotic Milking, Lelystad*, pp. 128–129. The Netherlands