Milk composition and health status from mammary gland quarters adjacent to glands affected with naturally occurring clinical mastitis

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ABSTRACT

Mammary gland quarters have usually been considered to be anatomically and physiologically independent, but some recent research has indicated more interdependence than previously reported. The objective of this study was to compare milk composition (fat, total protein, lactose, solids-not-fat, and chloride) and health status (somatic cell count, differential leukocyte count, and lactate dehydrogenase) of milk samples from unaffected mammary glands of an udder with a single clinically inflamed quarter to results of milk samples from healthy mammary glands of healthy cows. The study was designed as a prospective case control study with case and control cows matched by parity and days in milk. Cases were defined as cows ($n = 59$) experiencing clinical mastitis in a single mammary gland, and controls ($n = 59$) were defined as cows that had not experienced clinical mastitis during the current lactation. Quarter milk samples were collected from all mammary glands adjacent to clinically affected quarters of cases and from the same mammary glands of controls. Samples were used to assess concentration of chloride and lactate dehydrogenase, fat, total protein, solids-not-fat, somatic cell count, and differential leukocyte count. Microbiological analysis was also performed on milk samples obtained from clinically affected mammary glands ($n = 59$). Logistic regression models were used to assess possible associations among quarter somatic cell count ($\geq 150,000$ cells/mL) and quarter type (adjacent to case or control). Multivariate linear models were used to compare milk composition and health status between quarter types. A total of 170 quarters were enrolled per group. Milk obtained from adjacent quarters of cases contained a lesser concentration of total protein, lactose, and solids-not-fat, but had a greater concentration of fat and chloride. The somatic cell count, total leukocyte count, and absolute numbers of neutrophils, lymphocytes, and macrophages were all increased in

milk obtained from adjacent quarters of case cows compared with milk obtained from quarters of control cows. The relative proportion of neutrophils was increased, whereas the proportion of macrophages was decreased in milk obtained from cases. Approximately 30% of milk samples obtained from adjacent quarters of cases had a somatic cell count $\geq 150,000$ cells/mL compared with 12% of milk samples obtained from quarters of control cows. The position of the mammary gland was not associated with any outcomes. In conclusion, our results support previous research that indicates the immune response to intramammary infection in a single mammary gland quarter alters milk composition and health status throughout the udder.

Key words: mastitis, milk composition, somatic cell count, milk quality

INTRODUCTION

The bovine udder is divided into 2 distinct halves separated by suspensory ligaments, and a thin septum of connective tissue divides the front and rear quarters with no direct connections. Each mammary gland quarter is considered anatomically and physiologically independent and has its own vascular and nervous system, and suspensory apparatus (Nickerson and Akers, 2011). The assumption that each quarter is independent has resulted in use of within-udder experiments that include both treated and control quarters of a single cow (Grönlund et al., 2006; Lutzow et al., 2008; Rinaldi et al., 2010).

The theory of independence among mammary gland quarters has been challenged as several studies have demonstrated changes in milk of adjacent healthy quarters when a single quarter is affected with mastitis (Bansal et al., 2005; Hamann et al., 2005; Jensen et al., 2013). Differences in lactose, fat, electrical conductivity, SCC, and *N*-acetyl- β -D-glucosaminidase were observed among milk samples obtained from cows free of subclinical mastitis in all quarters compared with milk obtained from cows that had at least one quarter with subclinical mastitis (Bansal et al., 2005). Hamann et al.

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(2005) reported differences in the proportion of neutrophils and lymphocytes between milk samples collected from quarters of healthy cows (no subclinical mastitis in any glands) compared with milk samples collected from cows with at least one gland affected with subclinical mastitis. They suggested that inflammation in the infected glands influenced responses in the noninfected glands and proposed that the mammary gland cannot be considered as 4 isolated entities. Similarly, Jensen et al. (2013) studied transcriptional response after experimentally induced IMI. They reported profound changes in the expression of mRNA of healthy quarters adjacent to quarters infected with *Staphylococcus aureus* and *Escherichia coli*. They suggested that mastitis pathogens directly affect host mammary cells but also influence adjacent glands with the purpose of reducing subsequent transmission (Jensen et al., 2013).

Although these studies suggest that composition and quality of milk from mammary glands adjacent to affected quarters is likely altered in response to infection in a single quarter, associations between a single quarter affected with clinical mastitis (visual abnormalities of milk and udder) and milk composition in adjacent quarters have not been previously reported. A better understanding of relationships among the mammary gland will help to clarify immune mechanisms after a clinical IMI.

Milk from quarters with visible changes in milk appearance (clinical mastitis) are immediately discarded and cannot be used by processors. Previous cited authors have investigated the effect of unseen signs of infection (SCC, LDH, leukocytes, and microbiological analyses) in milk of adjacent quarters, but researchers have not investigated the influence of a single quarter-case of clinical mastitis on composition of milk from adjacent quarters. The effect of a clinical case of mastitis on composition of milk of adjacent quarters may be greater than that of cows experiencing subclinical signs as the magnitude of inflammation is greater and the etiological agents typically differ. The objective of this study was to compare composition [fat, total protein (TP), lactose, chloride, and SNF] and health status [SCC, differential leukocyte count, and lactate dehydrogenase (LDH)] in milk samples from unaffected mammary glands of an udder with a single clinically inflamed quarter to results of milk samples from healthy mammary glands of healthy cows.

MATERIALS AND METHODS

Eligibility and Inclusion

The study was conducted on a commercial Wisconsin dairy farm that contained 3,152 lactating Holstein

cows. The herd had a milking routine that included observation of foremilk for detection of mastitis, complete records of clinical mastitis at the quarter level, and monthly SCC testing of individual cows. The enrolled farm had a daily average milk production of 33.6 kg per cow. The average bulk tank milk values of SCC, fat, and TP were 301,000 cells/mL, 3.62%, and 3.13%, respectively. The cows were housed in a freestall barn, with digested manure solids as bedding, and fed a balanced TMR. Cows were milked 3 times per day in a 72-stall rotary parlor.

The study was designed as a prospective, matched case-control study. Primiparous and multiparous cows with 4 functional mammary gland quarters were potentially eligible for participation in the study if clinical mastitis was detected in a single quarter during November 4 to December 30, 2015. Cases were defined as cows with a single quarter that experienced mild (occurrence of abnormal milk only) or moderate (occurrence of abnormal milk and swelling, redness, or pain in the udder) clinical signs of mastitis (Pinzón-Sánchez and Ruegg, 2011). Cows that had experienced previous clinical cases were not eligible until at least 13 d after a previous mastitis event. Control cows were matched by parity and were required to be within 30 DIM of cases, were in the same milking group as cases and had no history of clinical mastitis within the current lactation.

Each week, on the day before researchers visited the farm, a list of enrolled cases was obtained and used to identify eligible control cows. Information about enrolled cows (parity, DIM, milk yield, pregnancy status, mastitis history, severity of mastitis, affected quarter) was obtained from herd management software (Dairy Comp 305, Valley Agricultural Software, Tulare, CA). Formal randomization was not used to select controls, but researchers maintained a list of eligible controls and selected the first eligible control for each case as cows entered the rotary parlor during milking.

This research was approved by the College of Agricultural and Life Sciences Animal Care and Use Committee, protocol number A005251.

Sample Size

Prior to the study, power calculations were performed to estimate sample size needed to provide an excess of 95% confidence and 80% power to detect the following differences: (1) fat of 0.35%, (2) TP of 0.15%, (3) lactose of 0.1%, (4) chloride of 8.0 mg/100 mL, (5) SCC of 50,000 cells/mL of milk, (6) total leukocyte count (TLC) of 50,000 cells/mL, and (7) LDH; EC number 1.1.1.27) of 0.9 U/I. Based on the most limiting variable (fat), sample size was estimated to be a minimum of 170 quarters per group.

Farm Visits and Sample Collection

During the data collection period, 68 eligible cows experienced clinical mastitis, but appropriate control cows could not be matched for 9 cases, resulting in a total of 59 case cows matched with 59 control cows (for a total of 177 potential quarter milk samples per group). Individual quarter milk samples (foremilk) were collected from apparently healthy mammary gland quarters of cows experiencing a case of clinical mastitis in a single mammary quarter and the same quarters of matched control cows that did not have clinical mastitis. Before the study began, farm personnel were trained on study procedures and instructed to verify the normal appearance of milk and mammary glands adjacent to each eligible affected gland and then collect 3 foremilk samples from each enrolled quarter in the following order: (1) bronopol preserved sample used to test composition (fat, TP, lactose, SNF) and SCC (30 mL); (2) aseptically collected sample used for microbiology, chloride, and LDH assay (30 mL); and (3) nonpreserved sample used for determination of differential leukocyte count (10 mL). Samples from case cows were collected by farm workers from all unaffected quarters of cases within 24 h after detection of clinical mastitis and before treatments were administered. A sample was also aseptically collected from the affected gland of cases and was frozen on the farm for up to 1 wk, until it was used for microbiological analysis at the University of Wisconsin Milk Quality Laboratory. After collection of milk samples, 1 sample was immediately used to determine differential leukocyte counts. Aseptically collected samples from case cows were frozen (-18°C) and samples preserved with bronopol were stored under refrigeration (4°C) on the farm until they were picked up by researchers during weekly farm visits. Researchers collected milk samples from matched control cows during weekly farm visits, using the same order and preparation procedures followed for cases.

Microbiological Analysis

All microbiological analysis was performed at the University of Wisconsin Milk Quality Laboratory. One day after the weekly farm visit, all frozen quarter milk samples were thawed at room temperature, used to inoculate agar for identification of pathogens, and used for analysis of chloride and LDH. All microbiological analyses were done according to NMC (1999) protocols. In brief, aliquots of 100 μL of each milk sample were inoculated onto one half plate of blood agar and 10 μL was plated onto one quarter plate of MacConkey agar, and aerobically incubated at 37°C for 24 to 48 h. Differentiation between *Staphylococcus* spp. and

Streptococcus spp. was done through colony appearance on blood agar and catalase reactions. All *Staphylococcus* spp. were coagulase negative and were not further speciated. *Streptococcus* spp. were defined as catalase negative; all were negative using the Christie, Atkins, Munch-Petersen test, and individual species were not determined. Gram-negative bacteria were differentiated based on motility, indole, ornithine reactions, oxidase, and growth on triple sugar iron slants. Contamination was defined based on growth of 3 or more different colony types from a single milk sample. Milk samples were screened for *Mycoplasma* spp. using composite samples as previously described (Fuenzalida et al., 2015).

Assays for Content of Chloride and Lactate Dehydrogenase

One day after the weekly farm visit and after microbiological inoculation procedures, 1.0 mL of thawed aseptic milk sample was used to determine chloride concentration using colorimetric titration performed using a commercial chloride analyzer (Nelson-Jameson M926 Chloride Analyzer System, Nelson-Jameson Inc., Marshfield, WI).

An aliquot (1.9 mL) of each thawed aseptic milk sample was also transferred to Eppendorf tubes to proceed to LDH analysis. Three centrifugations were performed at $14,000 \times g$ at 4°C for 4 min (first centrifugation) and then 30 min (subsequent centrifugations). After each centrifugation, the fat layer was removed and skim milk or supernatant was transferred to a new tube. After the final centrifugation, the supernatant was stored at -70°C until further analysis.

Activity of quarter milk LDH was measured using a commercial kit (CytoTox96 Non-Radioactive Cytotoxicity Assay, Promega Corp., Madison, WI). The method was modified from Lauzon et al. (2006) and Wenz et al. (2010). Prepared samples were thawed at room temperature and diluted with PBS + 1% BSA based on the total neutrophil count in the milk fraction. Samples with $\geq 600,000$ cells/mL were diluted in 1:30, whereas samples below that threshold were diluted in 1:3. Then 50 μL of the diluted sample was added to the plate wells. A within-plate control was established by diluting 1 μL of LDH control with 5 mL of PBS + 1% BSA, and adding 50 μL of the mixture to separate wells. A between-plates control was established by creating a single pool of 20 random different samples and diluting in 1:30 PBS + 1% BSA and adding 50 μL to separate wells. A standard curve was established with different concentrations of β -NADH (0, 2.5, 5, 7.5, and 10.0; 12.5 nmol of β -NADH/well; N0786, Sigma-Aldrich, St. Louis, MO) diluted in PBS + 1% BSA. After that, 50 μL of substrate mix was added to each well and plates

were incubated 15 min in the dark before reading absorbance at 490 nm. All samples, standard curves and controls were done in triplicate.

Determination of Differential Leukocyte Count

Determination of differential leukocytes count (total leukocyte, neutrophils, lymphocytes, and macrophages count; cells/mL) was performed using a commercially available on-farm test based on image cytometry to differentiate cells by morphology and proportions were calculated using the integrated software (each specific leukocyte cell count is divided by the total cell count and the result is multiplied by 100; QScout MLD, Advanced Animal Diagnostics, Morrisville, NC).

Determination of SCC and Milk Components

Bronopol preserved milk samples from enrolled quarters were submitted for analysis in a commercial DHI laboratory [AgSource Cooperative Resources International (CRI), Verona, WI]. Milk TP, fat, lactose, and SNF were analyzed by a Fourier Transform InfraRed spectroscopy commercial equipment (MilkoScan FT+, Foss, Hillerød, Denmark) and SCC was measured by flow cytometry (Fossomatic FC, Foss).

Statistical Analysis

Statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC) and statistical significance was defined at $P \leq 0.05$. To achieve normality, lactose values were transformed using their squared value, whereas base-10 logarithms were used to transform SCC, TLC, absolute values of individual leukocyte populations [neutrophils (**Neu**); lymphocytes (**Lym**); and macrophages (**Mac**)], and LDH. An arc-sine transformation was used to normalize the proportion of leukocytes.

To test the null hypothesis that parity (1st, 2nd, or ≥ 3 th) and pregnancy status (yes or no) did not differ between cows assigned to be cases or controls, 2 separate χ^2 analyses were performed using PROC FREQ. The experimental unit was cow. The hypothesis that continuous variables DIM, average daily milk yield for the previous 7 d (**MY**), previous DHI test-day values for milk fat, TP, and SCC ($\log_{10}$) did not differ between case and control cows was tested using 5 separate ANOVA tests (one for each outcome variable) with PROC GLM. The experimental unit of these analyses was the cow.

To test the null hypothesis that the proportion of samples with microbiological growth (compared with no or nonsignificant growth) did not differ between

milk samples collected from adjacent quarters of cases or controls a χ^2 analysis was performed using PROC FREQ. The experimental unit was mammary quarter.

To test the null hypotheses that milk composition and health status was not associated with quarter-type [adjacent to case quarter milk samples (**AQMS**) or control quarter milk samples (**CQMS**)], 14 separate multivariate linear regression analyses were performed with PROC GLM. Outcome variables were

- (1) fat,
- (2) TP,
- (3) lactose²,
- (4) SNF,
- (5) chloride,
- (6) $\log_{10}\text{SCC}$,
- (7) $\log_{10}(\text{TLC}+1)$,
- (8) $\log_{10}(\text{Neu}+1)$,
- (9) $\text{ARCSIN } \sqrt[3]{\text{Neu \%} / 100}$,
- (10) $\log_{10}(\text{Lym}+1)$,
- (11) $\text{ARCSIN } \sqrt[3]{\text{Lym \%} / 100}$,
- (12) $\log_{10}(\text{Mac}+1)$,
- (13) $\text{ARCSIN } \sqrt[3]{\text{Mac \%} / 100}$, and
- (14) $\log_{10} \text{LDH}$.

Initial models included the following:

$$\begin{aligned} \text{Individual outcome variable} = & \mu + \text{PREGSTATUS}_{ij} \\ & + \text{PARITY}_j + \text{QTYPE}_j + \text{QPOS}_{ij} + \text{MICSTATUS}_{ij} \\ & + \text{DIM}_j + \text{MY}_j + \text{COWQUARTER}_{ij} + \varepsilon_{ij}, \end{aligned}$$

where outcome variable = each response variable [fat, TP, lactose², SNF, chloride, $\log_{10}\text{SCC}$, $\log_{10}(\text{TLC}+1)$, $\log_{10}(\text{Neu}+1)$, $\text{ARCSIN } \sqrt[3]{\text{Neu \%} / 100}$, $\log_{10}(\text{Lym}+1)$, $\text{ARCSIN } \sqrt[3]{\text{Lym \%} / 100}$, $\log_{10}(\text{Mac}+1)$, $\text{ARCSIN } \sqrt[3]{\text{Mac \%} / 100}$, and $\log_{10} \text{LDH}$] of the i th quarter and the j th cow; μ = the regression constant term; PREGSTATUS_{ij} = pregnancy status (yes or no); PARITY_j = parity (1st, 2nd, or ≥ 3 rd); QTYPE_j = quarter type (adjacent to case or control), QPOS_{ij} = quarter position (left front, right front, left rear, right rear); MICSTATUS_{ij} = microbiological analyses of the adjacent and control quarters milk samples (growth or no growth); DIM_j = days in milk of the cow (continuous); MY_j = milk yield of the cow (continuous); COWQUARTER_{ij} = fixed effect to account for the correlation between quarters within cow; and ε_{ij} = residual variation. Matching variables (parity and DIM) were forced into all models.

All pairs of outcomes and explanatory variables were first screened using univariate analysis with PROC GLM, and variables unconditionally associated with

an outcome at $P \leq 0.25$ were offered to multivariate linear regression models using forward and backward stepwise selection with PROC MIXED. Confounding effects, first-order interactions terms and correlations among explanatory variables were also checked using forward and backward stepwise selection. The goodness of fit of each model was checked by comparing the Akaike information criterion. The explanatory variables quarter position, pregnancy, and first order interactions terms were not associated with any outcome variable in any models and were not included in the final models. Microbiological status of the enrolled milk samples was not associated with composition outcomes and was not included in the final models. Microbiological status was not included in the final models for health status due to an insufficient number of microbiologically positive quarters of control cows. Fixed effects remaining in the final models of milk composition and health status included parity, quarter type, DIM, and milk yield. The experimental unit of these analyses was mammary quarter within cow.

To test the null hypothesis that proportion of quarters with $\text{SCC} \geq 150,000$ cells/mL was not associated with quarter type (adjacent to case or control), a multivariate logistic regression model was performed with PROC GLIMMIX. This SCC threshold is based on a meta-analysis that reported a SCC mean of 155,000 cells/mL in quarters subclinically infected with CNS (Djabri et al., 2002).

The model included

$$\begin{aligned} \text{SCC} \geq 150,000 = & \mu + \text{PREGSTATUS}_{ij} + \text{PARITY}_j \\ & + \text{QTYPE}_j + \text{QPOS}_{ij} + \text{MICSTATUS}_{ij} + \text{DIM}_j \\ & + \text{MY}_j + \text{COWQUARTER}_{ij} + \varepsilon_{ij}, \end{aligned}$$

where $\text{SCC} \geq 150,000$ (outcome variable) = quarters within cow with $\text{SCC} \geq 150,000$ of the i th quarter, and the j th cow; μ = regression constant term; PREGSTATUS_{ij} = pregnancy status (yes or no); PARITY_j = parity (1st, 2nd, or ≥ 3 rd; matching variable); QTYPE_j = quarter type (adjacent to cases or control); QPOS_{ij} = quarter position (left front, right front, left rear, right rear); MICSTATUS_{ij} = microbiological analyses of the adjacent and control quarter milk samples (growth or no growth); DIM_j = days in milk of the cow (continuous); MY_j = milk yield of the cow (continuous); COWQUARTER_{ij} = fixed effect to account for the correlation between quarters within cow; and ε_{ij} = residual variation. Confounding effects, first-order interaction terms, and correlations among explanatory variables were also screened using forward and backward stepwise selection. The goodness of fit of each tested model was

checked by comparing the -2 residual log likelihood pseudo-likelihood. The explanatory variables quarter position, pregnancy, and all first-order interaction terms were not associated with the outcome variable and were not included in the final model. Microbiological status was not included in the final models due to insufficient number of quarters with microbiological growth. The quarter within cow variable was included as a random effect in all univariate and multivariate models and the experimental unit of this analysis was quarter milk sample within cow. The final model included the fixed effects of parity, quarter type, DIM, and milk yield.

RESULTS

Characteristics of the Cows

Of enrolled cows, most were in first or second lactation and about one-third were pregnant (Table 1). As expected based on matching criteria, no differences were present in parity or DIM, nor were there differences in MY or milk composition (Table 1). Most cases of mastitis occurring in case cows were mild ($P \leq 0.001$) and cases were 3.2 times more likely to occur in front compared with rear quarters (95% CI 1.53–7.00). Among cows assigned as cases, about 50% had experienced a previous case of mastitis in the current lactation. The mean number of days since the previous clinical mastitis event was 78 d.

Composite SCC obtained from the previous test-day DHI report was greater for cows assigned to cases ($P \leq 0.001$) compared with cows assigned to control (Table 1). For fat, TP, and SCC statistical analyses, 4 cows did not have any previous DHI analyses, reducing the number of enrolled cows to 114.

Microbiological Analyses

Clinical Cases. Of 59 cows that had mild or moderate clinical mastitis in a single quarter, 8 (13.6%) samples were contaminated (Table 2). Of the noncontaminated samples, the majority (65%) had no growth of microorganisms, whereas environmental *Streptococcus* spp. were the most frequently isolated pathogens. Other etiological agents included CNS, *Klebsiella* spp., and other microorganisms (Table 2).

Adjacent and Control Quarters. Milk samples from 7 quarters of control cows were missing or unusable, so samples from the same quarters of 7 cases were excluded. Thus, of the potential total number of milk samples ($n = 177$ per group), 170 quarters per group were analyzed for a total of 340 milk samples.

Of the 143 noncontaminated samples, most (88%) resulted in no growth of microorganisms. Coagulase-

Table 1. Descriptive characteristics of pre-enrollment characteristics of case and control cows enrolled in the trial

Variable	Category	Cow type (enrolled) ¹				<i>P</i> -value
		Case		Control		
		n = 59	%	n = 59	%	
Parity	1	22	37.30	22	37.30	1.000 ²
	2	20	33.90	20	33.90	
	≥3	17	28.80	17	28.80	
Pregnancy status	No	44	74.60	39	66.10	0.310 ²
	Yes	15	25.40	20	33.90	
Severity of case	Mild	44	74.60			
	Moderate	15	25.40			
Position of affected quarter	Front	38	64.40			
	Rear	21	35.60			
Previous mastitis in current lactation (any quarter)	0	31	53.00	59	100.00	
	≥1	28	47.00			
Continuous						
DIM		178	±17.8	175	±17.1	0.890
Milk yield of cows (kg/d)		36.4	±1.4	36.9	±1.3	0.810
Fat ³ (%)		3.7	±0.10	3.8	±0.09	0.870
Total protein ³ (%)		3.2	±0.05	3.1	±0.04	0.460
Log ₁₀ SCC ³ (cells/mL)		5.4 ^a	±0.01	4.7 ^b	±0.08	≤0.001

^{a,b}Means within a row with different superscripts differ by the ANOVA test ($P \leq 0.05$).

¹Case = primiparous or multiparous cows, with 4 functional quarters, presenting mild or moderate clinical mastitis (Pinzón-Sánchez and Ruegg, 2011) after at least 13 d after the previous mastitis event; control = 4 functional quarters, within 30 DIM of the cow with a case of clinical mastitis, same parity as the case cow, no clinical mastitis event in any quarters during current lactation.

²Two-tailed χ^2 test between enrolled cases and control cows.

³Data recovered from the last DHIA analysis for 114 enrolled cows. These data are from composite milk samples collected for routine DHI analysis.

negative staphylococci were the most frequently recovered organisms followed by environmental *Streptococcus* spp. and other microorganisms. Of the 131 noncontami-

nated samples from control quarters, almost all (96.2%) resulted in no growth of microorganisms whereas environmental *Streptococcus* spp., CNS, and yeast were

Table 2. Microbiological results of quarter milk samples collected from clinical cases, quarters adjacent to clinical cases, and quarters of healthy cows matched by parity and DIM of cows from a large commercial dairy herd in Wisconsin

Microbiological result ¹	Type of milk samples						<i>P</i> -value
	Clinical case		Quarters adjacent to case		Control quarters		
	n	% ²	n	% ²	n	% ²	
No growth ³	33	64.7	126	88.1	126	96.2	0.01 ⁴
Gram-positive							
Environmental <i>Streptococcus</i> spp.	8	15.7	2	1.4	2	1.5	
CNS	3	5.9	12	8.4	2	1.5	
Gram-negative							
<i>Klebsiella</i> spp.	3	5.9					
<i>Escherichia coli</i>	1	2.0					
<i>Pasteurella</i> spp.	1	2.0	1	0.7	1	0.8	
<i>Enterobacteria</i> spp.	1	2.0					
<i>Serratia</i> spp.			1	0.7			
Yeast	1	2.0	1	0.7			
Subtotal	51	100.0	143	100	131	100	0.09 ⁵
Contaminated samples	8	13.6	27	15.9	39	22.9	
Total	59		170		170		

¹No *Mycoplasma* spp. were recovered from any pooled milk samples.

²The proportion of no growth, gram-positive, gram-negative, and yeast does not include contaminated samples.

³Includes no growth and nonsignificant growth.

⁴Two-tailed χ^2 test between frequencies of no growth and growth (gram-positive, gram-negative, and yeast) in adjacent and control quarters.

⁵Two-tailed χ^2 test between frequencies of contaminated and noncontaminated (no growth and growth) samples in adjacent and control quarters.

recovered from a few quarters. There was a tendency of greater frequency of contaminated milk samples in control quarters ($n = 39$; 22.9%) compared with adjacent quarters ($n = 27$; 15.9%; $P = 0.09$; Table 2).

Results from χ^2 analysis indicated that milk samples obtained from quarters adjacent to cases were about 3 times as likely to result in microbial growth (95% CI, 1.21–9.49, $P = 0.01$, $n = 274$; 340 total samples minus 66 contaminated samples) compared with milk samples obtained from control quarters. No *Mycoplasma* spp. were recovered from any pooled milk samples (Table 2).

Association of Composition and Health Status of Milk Between Quarter Types

Of 340 total milk samples, 50 samples were not able to be analyzed for LDH and chloride, reducing the total number of samples for these analyses to 290. The total number of milk samples used in all other models was 340. From the 15 models tested for the hypotheses of association between quarter type milk samples and the outcome variable, 14 demonstrated an association with quarter type and 1 (chloride) approached statistical significance ($P = 0.057$; Table 3). Mean values of fat were greater for AQMS compared with CQMS ($P = 0.006$; Table 3). Control quarter milk samples had greater mean TP, lactose, and SNF compared with milk obtained from AQMS (Table 3; $P \leq 0.008$).

Milk samples from quarters adjacent to a clinical case tended to have ($P = 0.057$) greater chloride compared with CQMS (Table 3). Both SCC (104,712 cells/mL) and TLC (117,490 cells/mL) of adjacent quarter milk samples were greater than values of CQMS SCC (37,153 cells/mL) and TLC (43,652 cells/mL) ($P \leq 0.001$; Table 3). In AQMS, there was greater mean concentration (66,070 cells/mL) and proportion (mean 58.36%, range 19.05 to 81.69%) of neutrophils compared with CQMS (21,380 cells/mL; mean 51.7%, range from 21.43 to 92.86%; $P \leq 0.001$). Concentration of lymphocytes was greater in AQMS (22,209 cells/mL) compared with CQMS (7,415 cells/mL; $P \leq 0.001$), but relative percentage of lymphocyte did not differ among quarter types (mean 20.7%, ranging from 5.04 to 56%, and mean 21.42%, ranging from 4.62 to 51.72% for AQMS and CQMS, respectively; $P = 0.4$).

The concentration of macrophages was greater in AQMS (18,621 cells/mL) compared with CQMS (8,512 cells/mL; $P = 0.002$), but CQMS had a greater relative percentage of macrophages (mean 27.6%, range 3.36 to 71.43%) compared with AQMS (mean 20.20%, range from 0.55 to 54.55%; $P \leq 0.001$). Adjacent quarter milk samples had greater mean LDH (30.5 U/L) compared with CQMS (22.9 U/L; $P = 0.02$). A greater proportion of quarters with SCC $\geq 150,000$ cells/mL was associated with AQMS (30.0%) compared with CQMS (12.4%; $P = 0.043$). Adjacent quarter milk samples were 3.7 times

Table 3. Multivariate linear and logistic regression results for composition and milk quality (LSM $\pm$ SEM) of milk samples from mammary glands adjacent to a single affected mastitis quarter and quarters of control cows

Multivariate analysis	Type of milk samples		<i>P</i> -value ¹
	Adjacent to case <i>n</i> = 170	Control quarters <i>n</i> = 170	
Fat (%)	3.07 $\pm$ 0.13	2.54 $\pm$ 0.13	0.006
Total protein (%)	3.07 $\pm$ 0.04	3.25 $\pm$ 0.04	0.006
Lactose ² (%)	4.65 $\pm$ 0.68	4.83 $\pm$ 0.68	0.008
SNF (%)	8.68 $\pm$ 0.07	9.00 $\pm$ 0.07	0.002
Chloride ³ (mg/L)	1,042.74 $\pm$ 40.79	931.79 $\pm$ 41.13	0.057
Log ₁₀ SCC (cells/mL)	5.02 $\pm$ 0.06	4.57 $\pm$ 0.06	≤ 0.001
Log ₁₀ (TLC+1) (cells/mL)	5.07 $\pm$ 0.06	4.64 $\pm$ 0.06	≤ 0.001
Log ₁₀ (Neu+1) (cells/mL)	4.82 $\pm$ 0.07	4.33 $\pm$ 0.07	≤ 0.001
Neutrophils ⁴ (%)	58.36 $\pm$ 1.28	51.70 $\pm$ 1.28	≤ 0.001
Log ₁₀ (Lym+1) (cells/mL)	4.36 $\pm$ 0.07	3.87 $\pm$ 0.07	≤ 0.001
Lymphocytes ⁴ (%)	20.70 $\pm$ 0.88	21.42 $\pm$ 0.88	0.400
Log ₁₀ (Mac+1) (cells/mL)	4.27 $\pm$ 0.08	3.93 $\pm$ 0.08	0.002
Macrophages ⁴ (%)	20.20 $\pm$ 1.47	27.60 $\pm$ 1.47	≤ 0.001
Log ₁₀ LDH ⁵ (U/L)	1.47 $\pm$ 0.03	1.36 $\pm$ 0.03	0.020
Quarters with SCC $\geq 150,000$ cells/mL of milk ⁵ (%)	30.00	12.35	0.043

¹*P*-values related to the “quarter type milk samples” outcome variable. Others explanatory variables in all models were milk yield of the cow, DIM, parity, and quarter within cow fixed effect.

²*P*-values based on the squared values of lactose. The LSD and SEM were back-transformed.

³Total number of quarters analyzed = 290.

⁴*P*-values based on the formula $\text{ARCSIN } \sqrt{\text{Leukocytes \%} / 100}$. The LSD and SEM were back-transformed.

⁵*P*-value based on a multivariate logistic regression.

more likely to have mean SCC greater than 150,000 cells/mL (1.72–7.98) compared with CQMS ($P = 0.043$).

DISCUSSION

Clinical mastitis has a direct effect on dairy farm income, mainly due to discarded milk, milk yield, and quality losses, costs with treatments (Seegers et al., 2003; Gröhn et al., 2004; Pinzón-Sánchez and Ruegg, 2011), and losses in reproduction performance (Fuenzalida et al., 2015). Economic effects of clinical mastitis for processors are also described in the literature and are mainly associated with alteration of compositional characteristics of dairy products via an increase in bulk milk SCC (Le Maréchal et al., 2011; Murphy et al., 2016). The focus of this study was to investigate the effect of clinical mastitis on composition and health status of adjacent quarters, and elucidate if a single clinically inflamed quarter (with visible changes on milk) has an effect on milk composition and health status of the healthy adjacent quarters, by performing a case-control study in a single herd.

Characteristics of the Farm and Animals

The farm enrolled in this study contained more cows but produced a similar amount of milk per cow per day and with a similar composition but greater SCC compared with typical Wisconsin dairy farms (USDA-NASS, 2015). The herd used digested manure solids as bedding, and similar to other larger Wisconsin herds that use manure solids, the bulk tank SCC was greater than similar herds that use sand (Rowbotham and Ruegg, 2015).

Distributions of mastitis severity scores in the enrolled case cows were similar to Pinzón-Sánchez and Ruegg (2011) and Fuenzalida et al. (2015) who reported about 70% mild and 30% moderate mastitis in Wisconsin dairy herds, indicating that the presentation of clinical mastitis and cases was typical of large herds in this region. The greater proportion of contamination found in our samples is likely associated with sampling in a moving rotary milking parlor, making it harder to collect aseptic milk samples compared with sampling in stationary parlors. We reported a greater percentage of clinical mastitis cases with no growth of microorganisms compared with Fuenzalida et al. (2015; 38.7%) and Pinzón-Sánchez and Ruegg (2011; 42%) but a lesser percentage compared with results from Finland (83.5%; Myllys et al., 1998).

According to Makovec and Ruegg (2003), the proportion of clinical and subclinical milk samples submitted to the Wisconsin Veterinary Diagnostic Laboratory that

result in no microbial growth increased from 22.6% in 1994 to 49.7% in 2001. This increase occurred concomitant with a dramatic decrease in recovery of *Staphylococcus aureus* and *Streptococcus agalactiae* as herds expanded and adopted modern management procedures. In large dairy farms in Wisconsin, gram-negative pathogens are the predominant organisms recovered from milk samples collected from cows experiencing clinical mastitis (Oliveira et al., 2013; Fuenzalida et al., 2015). The digested manure solids used in this herd as bedding are associated with greater exposure to gram-negative pathogens, which are expected to have a shorter duration of IMI and greater rate of spontaneous cure (Smith et al., 1985; Ruegg, 2011), thus resulting in a greater proportion of bacteriologically negative samples. Bacteriologically negative clinical cases in herds that have controlled *Sta. aureus* and *Str. agalactiae* are often a result of a successful inflammatory response to IMI by opportunistic organisms and have similar posttreatment outcomes compared with clinical mastitis caused by gram-negative pathogens (Oliveira and Ruegg, 2014). The magnitude of the immune response is well-known to be associated with severity of clinical mastitis (Cullen, 1966). Fuenzalida et al. (2015) noted that in contrast to microbiologically positive cases of clinical mastitis, microbiologically negative mild and moderate clinical cases did not result in reduced reproductive performance, and attributed the lack of effect to a localized and successful immune response. Pinzón-Sánchez and Ruegg (2011) observed variation of microbiological results of clinical mastitis among farms in the same region, but a greater proportion of no growth (55%) was identified on farms that recorded a greater proportion of cows with mild cases (72%), similar to our data. Although our samples were frozen for up to 7 d before culturing, we think that it is unlikely that this process affected our results, as the farm cultured fresh milk samples within hours of collection and recorded a similar proportion of culture-negative results (data not shown).

Similar to studies conducted in other large herds in this region (Pinzón-Sánchez and Ruegg, 2011; Oliveira et al., 2013; Fuenzalida et al., 2015), environmental *Streptococcus* spp. were the primary gram-positive microorganisms. Among gram-negative microorganisms, a greater proportion of *Klebsiella* spp. was found compared with previous studies (Pinzón-Sánchez and Ruegg, 2011; Oliveira et al., 2013; Fuenzalida et al., 2015). The greater incidence of these environmental pathogens causing clinical mastitis in our study is typical of herds using manure-based bedding (Rowbotham, 2015; Rowbotham and Ruegg, 2016). The position of the quarter affected with clinical mastitis in our study was in agreement with the literature, in which a greater

incidence was found in front quarters compared with rear quarters (Neijenhuis, 2004; Guarín and Ruegg, 2016).

Quarter Type Milk Sample Comparison

Compared with milk obtained from healthy cows, milk from mammary gland quarters adjacent to a single quarter affected with clinical mastitis were shown to have altered composition (fat, TP, lactose, SNF, and chloride) and health status (SCC, leukocytes, and LDH). This finding is a potentially important consideration for design of experiments, in agreement with McCullagh and Nelder (1989) and Schukken et al. (2003), who reported that incorrect assumptions of interdependence result in underestimation of variance and erroneous statistical results.

Interdependence of SCC among quarters has been described by Barkema et al. (1997) and Berry and Meaney (2006) and was attributed to variation of milk yield and immune competency among cows. When considering quarters within a cow, these authors concluded that position of the unaffected quarters influenced the SCC in unaffected quarters but did not mention physiological interdependence. In contrast, we noted that position of the unaffected quarters to the affected gland was not associated with any outcomes, which implies that the changes we observed were mediated at a systemic (rather than local) level. Irrespective of the anatomical location, our study confirms that clinical mastitis occurring in a single gland has an influence on composition and health status of the apparently healthy glands. Our results support the lack of independence among quarters, and this issue should be considered in the design of future studies.

When differences in breed, parity, DIM, and diet are controlled, differences in milk composition are generally associated with SCC. Variations in fat and TP, increased concentrations of plasmin and other enzymes, and a reduced mean in lactose and TS concentrations in milk with greater SCC were reported by Auldist and Hubble (1998) and Hortet and Seegers (1998). Adjacent quarters in our study had a greater SCC compared with CQMS but most were bacteriologically negative. In agreement with our results, Bansal et al. (2005) reported a positive association between SCC and healthy quarters from unhealthy cows compared with healthy quarters from healthy cows. The mean SCC of AQMS in our study was greater than 100,000 cells/mL, a threshold that some authors consider unhealthy (Schwarz et al., 2010; Forsbäck et al., 2011; Bezman et al., 2015) and was almost 3 times greater than the mean SCC of CQMS (about 37,000 cells/mL), suggesting a mild inflammatory response, most likely

as a result of the greater inflammation occurring in the clinically affected quarter.

Bansal et al. (2005) did not identify differences in fat content of foremilk collected from healthy quarters of healthy cows compared with healthy quarters from unhealthy cows, but did find lesser fat content in milk strippings collected from healthy quarters of cows with mastitis. An increase in fat concentration has been associated with reduced milk yield (Forsbäck et al., 2009) due to damage of mammary epithelial cells. We observed large differences in fat content of milk samples based on quarter type. Increased fat content may be a result of a mild inflammatory process occurring in the glands adjacent to the clinically affected quarters. Other indicators of mild inflammation in AQMS include increased chloride and LDH. These changes are associated with inflammation (McManaman and Neville, 2003) or disruption of mammary cells (Oliszewski et al., 2002).

The lesser TP content we observed in AQMS compared with CQMS differs from Bansal et al. (2005) who reported no association between TP in all milk fractions and health status. However, Bansal et al. (2005) defined udder health using a SCC threshold of 100,000 cells/mL and did not include animals that experienced clinical mastitis. Thus the degree of inflammation occurring in the affected quarters of animals enrolled in our study was much greater than they observed and probably accounted for the large difference that we observed. Bruckmaier et al. (2004) reported greater TP content in healthy quarters from unhealthy cows (3.44–3.64%) compared with our AQMS TP values, but included cross-bred cattle in the experiment (in contrast to the Holsteins enrolled in our study). Milk with greater SCC is associated with increased activity of proteolytic heat-stable enzymes, which are responsible for milk casein degradation, reduced shelf life of pasteurized and heat-treated dairy products, and altered coagulation properties of milk and ripening process of cheeses (Considine et al., 2004; Le Maréchal et al., 2011; Murphy et al., 2016). The lesser concentration of TP in AQMS and greater SCC is indicative that milk from apparently healthy gland of cows with clinically affected quarters is of potentially lower value for processing.

In agreement with our results, Bansal et al. (2005) reported that lactose was reduced in milk obtained from apparently healthy quarters of cows with increased SCC. When inflammation occurs, tight junction cells and mammary cell membranes are disrupted and paracellular routes open, leading to an influx of blood constituents (such as chloride, leukocytes, and enzymes) into the lumen, and consequently, the osmotic pressure increases, and to adjust osmolarity, the lactose concentration is reduced in milk (Holt, 1985;

McManaman and Neville, 2003). A reduced content of lactose in high SCC milk is potentially detrimental to dairy products that use the process of acidification with starter cultures (Schallibaum, 2001).

Decreased lactose and protein in AQMS resulted in decreased SNF for these quarters compared with CQMS. Together with fat, SNF in milk is important for cheese processors and has a direct effect on cheese yield (Johnson and Law, 2010). To increase firmness and viscosity of yogurts and acid coagulated soft cheeses, the dairy industry uses concentration process in milk or DM addition to raise the content of SNF (Lucey, 2004), and the lower its original content in milk, the more costly is this process. Greater chloride content in AQMS quarters compared with CQMS is associated with a greater influx of ions from blood into milk in this higher SCC milk (Chavez et al., 2004). Decreased SNF is also a factor that increases chloride percentage in milk (Hastings and Peterson, 1940).

A more detailed estimation of quarter health status can be determined by examining cell differentiation, because variations in leukocyte proportions occur earlier than the overall rise of somatic cells (Pilla et al., 2012) even at low levels (9,000 cells/mL; Schwarz et al., 2011a,b). Overall, the values for $\log_{10}(\text{TLC} + 1)$ were similar to $\log\text{SCC}$ and milk from adjacent quarters had greater total leukocytes and greater numbers and proportions of individual cell types (with the exception of lymphocytes) compared with CQMS ($P \leq 0.01$). Comparisons among total and individual leukocyte counts between healthy adjacent quarters of cows with a single clinically affected cow (with visible changes in milk) have not been previously described, and our results indicate that both the proportion of cell types (as previously described by Schwarz et al., 2011b; Pilla et al., 2012, 2013) and number of individual leukocytes were greater in those quarters (AQMS), suggesting that the specific immune system of the cow is activated when clinical mastitis occurs, even in mild or moderate cases.

Neutrophils were the primary type of leukocyte collected from both AQMS and CQMS, in agreement with Koess and Hamann (2008) and Pilla et al. (2012, 2013). Although the absolute number of all types of leukocytes was greater in AQMS, the proportion of neutrophils was lesser compared with Pilla et al. (2012) and ranged from 52% (CQMS) to 58% (AQMS). In AQMS, the remainder of the leukocytes were evenly distributed between lymphocytes (21%) and macrophages (20%), whereas a greater proportion of macrophages (28%) compared with lymphocytes (21%) was noted in CQMS. According to Schwarz et al. (2011a), an influx of neutrophils in the adjacent quarters to an unhealthy quarter results in a reduced proportion of macrophages,

but wide variation in the proportion of macrophages has been previously described.

Compared with our results, Pilla et al. (2012) reported a greater concentration of lymphocytes in healthy quarters. However, the percentage of lymphocytes in nonmastitic milk has been reported to be widely variable (Merle et al., 2007; Koess and Hamann, 2008). Our results indicate that immune system of the udder is well primed to fight infection. Macrophages are phagocytic cells, capable of ingesting microorganisms, cellular fragments, and accumulated milk components (Sordillo and Nickerson, 1988) and their main task is to initiate the immune response by identifying invading pathogens, releasing chemoattractants, and inducing neutrophils into the site of infection (Oviedo-Boyso et al., 2007). A secondary task of macrophages is to remove neutrophils under apoptosis due to a phagocytosis event, which is a key requisite for the resolution of inflammation as well as tissue protection (Bratton and Henson, 2011).

The concentration of LDH in foremilk samples has been positively associated with milk SCC (Hiss et al., 2007), and similarly, we observed increased LDH in AQMS. Lactate dehydrogenase is a nonlysosomal hydrolytic enzyme liberated from neutrophils, macrophages, damaged udder epithelial cells, and interstitial cells during the inflammatory process (Oliszewski et al., 2002). Kalantari et al. (2013) found no association between blood serum LDH and milk SCC, but a positive association was found between milk LDH and the same milk SCC threshold and Nyman et al. (2014) found a positive association between LDH and SCC even when only samples from healthy cows were analyzed. This finding suggests that the immune system of the adjacent quarters is likely activated at the udder level, and likely serves to reduce the chance of additional infections in adjacent glands.

Although our study indicates that differences occur in composition and health status of foremilk samples collected from apparently unaffected glands adjacent to clinically affected mammary gland quarters, our study did not examine the effect on whole udder milk or differences between initial and recurrent cases, and this type of research is needed to fully understand the effect of mastitis on composition and quality of milk.

CONCLUSIONS

Interdependence between milk from mammary glands adjacent to a gland experiencing mild or moderate clinical mastitis was studied in 340 quarters from 118 cows in a single high producing herd. After correcting for DIM and parity, differences in quarter milk composition and health status was found in adjacent quarters,

compared with milk samples collected from unaffected herd-mates. Generally, milk collected from quarters of unaffected cows had more protein and lactose, less fat and chloride and lesser SCC, leukocytes and reduced LDH. These changes in milk composition and health status were not associated with microbiological analyses of the enrolled quarters nor quarter position. Our results suggest that a single quarter with clinical mastitis affected the overall immune status of the udder, altering milk composition and health status in adjacent quarters.

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