MARCEL GOMES PAIXÃO

MILK COMPOSITION AND HEALTH STATUS FROM MAMMARY GLAND QUARTERS ADJACENT TO GLANDS AFFECTED WITH NATURALLY OCCURRING CLINICAL MASTITIS BEFORE AND AFTER TREATMENT

LAVRAS-MG
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LAVRAS-MG
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COMPOSIÇÃO DO LEITE E ESTADO DE SAÚDE DE GLÂNDULAS MAMÁRIAS ADJACENTES À GLANDULAS NATURALMENTE AFETADAS COM MASTITE CLÍNICA ANTES E APÓS O TRATAMENTO

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do programa de Pós-Graduação em Ciência dos Alimentos, área de concentração em Leite e Produtos Lácteos, para a obtenção do título de "Doutor".

APROVADA em 29 de Setembro de 2017

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Orientador

LAVRAS-MG
2017

ACWKNOLEDGMENTS

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To every thing there is a season, and a time to every purpose under the heaven:

A time to be born, and a time to die; a time to plant, and a time to pluck up that which is planted;

A time to kill, and a time to heal; a time to break down, and a time to build up;

A time to weep, and a time to laugh; a time to mourn, and a time to dance;

A time to cast away stones, and a time to gather stones together; a time to embrace, and a time to refrain from embracing;

A time to get, and a time to lose; a time to keep, and a time to cast away;

A time to rend, and a time to sew; a time to keep silence, and a time to speak;

A time to love, and a time to hate; a time of war, and a time of peace.

What profit hath he that worketh in that wherein he laboureth?

I have seen the travail, which God hath given to the sons of men to be exercised in it.

He hath made every thing beautiful in his time: also he hath set the world in their heart, so that no man can find out the work that God maketh from the beginning to the end.

I know that there is no good in them, but for a man to rejoice, and to do good in his life.

And also that every man should eat and drink, and enjoy the good of all his labour, it is the gift of God.

Ecclesiastes 3:1-13 King James Version (KJV)

Thanks for your friendship my faithful friend Shiro Okami (in memoriam)

"A dog has no use for fancy cars, big homes, or designer clothes. A water log stick will do just fine. A dog doesn't care if you are rich or poor, clever or dull, smart or dumb. Give him your heart and he'll give you his. How many people can you say that about? How many people can make you feel rare and pure and special? How many people can make you feel extraordinary?"

John Grogan - Marley & Me biography

RESUMO GERAL

Objetivou-se, em nosso primeiro estudo (artigo 1), comparar a composição do leite [gordura, proteínas totais (PT), lactose, sólidos desengordurados (SD), cloretos] e o estado de saúde [contagem de células somáticas (CCS), contagem diferencial de leucócitos, e, lactato desidrogenase] de amostras de leite coletadas de quartos mamários sadios, provenientes de animais com apenas um quarto mamário apresentando mastite clínica com amostras de leite de quartos de animais totalmente sadios. Delineou-se o estudo como caso-controle prospectivo; combinou-se o número de lactações e dias em lactação (DL) entre os animais, controles e casos. Casos foram definidos como vacas (n = 59), que apresentaram mastite clínica em apenas um quarto mamário e, controles (n = 59), vacas com ausência de mastite clínica em todos os quartos mamários. Amostras de leites individuais foram coletadas de todas as glândulas mamárias adjacentes às afetadas (casos) e das mesmas glândulas provenientes dos animais controles. No total, 170 quartos mamários foram inscritas por grupo. O leite de quartos mamários, adjacentes aos casos, apresentaram menores concentrações de PT, lactose e SD, e maiores concentrações de gordura e cloretos. Os resultados das análises de CCS, contagem total de leucócitos, e os valores absolutos de neutrófilos, linfócitos e macrófagos das amostras de leite dos quartos mamários adjacentes ao quarto afetado apresentaram maiores médias comparadas às amostras de leite controle. Objetivou-se, em nosso segundo estudo (artigo 2), descrever as variáveis referentes às vacas e quartos mamários e possíveis associações com a composição do leite e a saúde das glândulas mamárias de quartos adjacentes aos naturalmente afetados (n = 170), antes do tratamento de mastite clínica. Variáveis explicativas referentes às vacas (número de lactações; produção de leite; DL; severidade da mastite clínica do quarto afetado) e quartos (infecção intramamária nos quartos adjacentes; casos prévios de mastite clínica nos quartos adjacentes) apresentaram uma associação ou, uma tendência de associação, com várias análises de composição do leite e do estado de saúde do úbere. As análises microbiológicas do leite dos quartos adjacentes apresentaram uma associação com a maioria dos resultados das análises do estado de saúde do úbere; no entanto, não foram encontradas associações entre as análises microbiológicas e a composição do leite. Objetivou-se, em nosso terceiro estudo (artigo 3), comparar a composição do leite (gordura, PT, lactose, SD, cloretos) e o estado de saúde (CCS, contagem diferencial de leucócitos) de quartos mamários saudáveis (n = 147) adjacentes a um quarto previamente tratado contra mastite clínica, com amostras de leite de quartos de animais totalmente sadios (n = 147). Casos (n = 50) foram definidos como vacas que previamente apresentaram um único quarto afetado com mastite clínica e tratados de acordo com o protocolo da fazenda, e, controles (n = 50), como vacas ausentes de mastite clínica em todos os quartos. Os DL e os números de lactações foram combinados entre os grupos de vacas, e posição dos quartos, entre os grupos. Em média, 24 dias após o início do tratamento de mastite no quarto afetado, a composição do leite (gordura, lactose, SD, e cloretos) e alguns parâmetros relacionados ao estado de saúde (contagem de linfócitos e macrófagos) dos quartos adjacentes retornaram aos mesmos níveis dos quartos controle; no entanto, a PT, a CCS, e os neutrófilos apresentaram resultados superiores.

Palavras-chave: Composição do leite. Contagem de células somáticas. Qualidade do leite. Mastite.

GENERAL ABSTRACT

The objective of our first study (article 1) was to compare milk composition [fat, total protein (TP), lactose, solids non-fat (SNF), chloride] and health status [somatic cell count (SCC), differential leukocytes 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 (DIM). 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. Quarter milk samples were collected from all mammary glands adjacent to clinically affected quarters of cases and from the same mammary glands of controls. A total of 170 quarters were enrolled per group. Milk obtained from adjacent quarters of cases contained lesser concentration of TP, lactose and SNF, but had greater concentration of fat and chloride. The SCC, total leukocyte count and absolute numbers of neutrophils, lymphocytes, and macrophages were all increased in milk obtained from adjacent quarters of case cows as compared to milk obtained from quarters of control cows. The objective of our second study (article 2) was to describe cow and quarter variables and possible associations with milk composition and health status of quarters adjacent to a naturally occurring clinical mastitis gland (n = 170) before mastitis treatment. Cow (parity category, milk yield, DIM, severity of clinical mastitis of case quarter) and quarter (intramammary infection of adjacent quarters; previous cases of clinical mastitis on adjacent quarters) explanatory variables were associated or tended toward an association with several milk composition and udder health status traits. Microbiological analyses of adjacent quarters milk samples had an association with the majority of udder health status outcomes analyzed; however, no associations were found between microbiological analyses and milk composition traits. The objectives of our third study (article 3) was to compare milk composition (fat, TP, lactose, SNF, chloride) and health status (SCC, differential leukocytes) of healthy mammary glands (n = 147) adjacent to a gland previously treated for clinical mastitis with milk samples of healthy mammary glands of healthy cows (n = 147). Cases (n = 50) were defined as cows that previously had a single quarter infected with clinical mastitis treated according to an on-farm protocol and controls (n = 50) were defined as cows that had not suffered clinical mastitis. Cows group were matched by DIM and parity and quarters between cows groups were matched by position. On average 24 days after beginning treatment of the case quarter, milk composition (fat, lactose, SNF, and chloride) and some health status (lymphocyte and macrophage count) of adjacent quarters returned to similar levels as milk from control quarters, while TP, SCC, and neutrophils remained greater.

Keywords: Milk composition. Somatic cell count. Milk quality. Mastitis.

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FIRST PART
1 INTRODUÇÃO

A melhoria da qualidade do leite tornou-se um desafio para todos os setores relacionados à produção primária do leite. Esta melhoria, juntamente com o aumento da produtividade do leite dos rebanhos, é peça chave para a permanência efetiva dos produtores na cadeia produtiva do leite, pois tais fatores promovem um aumento do retorno econômico aos produtores, possibilitando, assim, novos investimentos e adequações referentes aos parâmetros normativos da qualidade do leite no Brasil, parâmetros esses, que, felizmente estão cada vez mais exigentes. Para a indústria, a melhoria da qualidade primária do leite é fundamental, e todos os esforços com esse objetivo devem ser direcionados aos consumidores.

Os principais critérios utilizados pela indústria laticinista para averiguar a qualidade do leite produzido nas fazendas são: a composição química (gordura, proteínas, lactose, extrato seco desengordurado), integridade físico-química (ausência de neutralizantes ou redutores de acidez, reconstituintes da densidade, resíduos de antibióticos, inibidores do crescimento microbiano), aspectos microbiológicos (contagem total de microrganismos e ausência de microrganismos patogênicos específicos relacionados à saúde pública) e a saúde da glândula mamária dos animais [aferidos pela contagem de células somáticas (CCS) do leite dos tanques de refrigeração a granel]. Dentre os diversos fatores que interferem negativamente na qualidade do leite produzido nos sistemas produtivos, destaca-se a saúde da glândula mamária dos animais. Tal parâmetro é de ordem sanitária, ou seja, envolve diretamente a saúde dos animais e, indiretamente, a saúde de todos envolvidos no sistema produtivo, tais como produtores, manipuladores, e consumidores, pois, diversos agentes infecciosos da glândula mamária bovina também são considerados zoonóticos. A debilidade

da saúde da glândula mamária dos animais ocasiona alterações visíveis (formação de grumos, secreções purulentas e sanguinolentas no leite, além de inchaço, dor e vermelhidão na glândula mamária dos animais, e também alterações sistêmicas na fisiologia dos animais, tais como depressão, falta de apetite, febre) ou invisíveis (aumento na contagem de células somáticas e concentração de enzimas como a lactato desidrogenase, N-acetyl-beta-D-glucosaminidase, proteínas da fase aguda; alteração dos componentes majoritários e cloretos) no leite produzido. Tais quadros de alterações visíveis no leite (e/ou nos animais) devido à debilidade na saúde da glândula são denominados mastites clínicas, e as alterações invisíveis no leite (e nos animais) são denominadas mastites subclínicas. Tais fenômenos que acometem a glândula mamária são de ordem inflamatória, em geral, devido a um microrganismo infectante (contagioso, oportunista, ou ainda, proveniente do ambiente) e de caráter multifatorial, ou seja, envolvem os animais, o ambiente, os microrganismos infecciosos, além de fatores sociais, econômicos, culturais e religiosos.

Tais fenômenos inflamatórios constituem atualmente um dos problemas sanitários mais importantes na cadeia produtiva do leite no Brasil e no mundo. A preocupação é tão grande, que é necessária uma grande demanda por parte de todos agentes envolvidos, visando buscar alternativas que reduzam os prejuízos relacionados à ocorrência dessa doença, pois, quando tais casos não são controlados, prejudicam a qualidade do leite produzido, diminuem a receita dos produtores [devido principalmente à queda de produção que se verifica nos rebanhos endemicamente acometidos, e, ainda, penalizações monetárias por parte das indústrias laticinistas, a produtores, que, muitas vezes, não atingem níveis adequados desse parâmetro (CCS)] e, ainda, prejuízos às indústrias e consumidores, relacionados ao menor rendimento e qualidade dos derivados lácteos processados.

As perdas relacionadas à mastite clínica para os produtores rurais incluem gastos com medicamentos, descarte do leite de animais tratados, gastos com assistência técnica, reflexos na reprodução dos animais, penalização monetária ao produtor por parte dos laticínios, e redução da produção de leite dos animais afetados durante todo período restante de lactação, além da necessidade de descarte de animais cronicamente infectados e maior necessidade de reposição do rebanho. Os efeitos da elevação da CCS do leite devido aos quadros de mastite subclínica sobre o rendimento e qualidade do leite e produtos lácteos incluem diminuição da velocidade do processo de coagulação do leite e menor rendimento na fabricação de queijos, devido, respectivamente, a uma menor concentração de caseína e maior concentração de proteínas solúveis, além de uma menor retenção de gordura pela coalhada formada; aumento no teor de umidade em queijos, devido a uma menor capacidade da coalhada por expulsar o soro, e redução da qualidade sensorial e vida de prateleira de queijos e de outros produtos, tais como iogurtes e leites UHT, devido a uma aceleração do desenvolvimento de defeitos como a rancidez e sabor amargo, causados respectivamente pelos processos de lipólise e proteólise.

É importante ressaltar que, tanto os quadros clínicos, quanto os casos subclínicos de mastite são prejudiciais a todos os elos envolvidos na produção e processamento do leite e derivados lácteos; porém, pesquisas recentes apontam para outros fatores aquém destes previamente citados (presença direta de mastite clínica ou subclínica), e que podem estar relacionados ao aumento da CCS no leite, ocasionando, consequentemente, os mesmos efeitos detrimentais anteriormente citados sobre a composição do leite e reflexos nos produtos lácteos processados. Tais fatores estão relacionados ao leite de quartos mamários de animais denominados sadios (sem alterações visíveis no leite e ausência de microrganismos nas análises microbiológicas) e, que, consequentemente, levantaram dúvidas quanto ao funcionamento do sistema imune dos animais ao

nível de glândula mamária. Os quartos da glândula mamária bovina têm sido considerados como entidades fisiológicas distintas, ou seja, independentes, e, portanto, o uso de um ou mais quartos como tratamentos controles geralmente é aceito em experimentos científicos. No entanto, as evidências de uma nova teoria, denominada de interdependência entre os quartos mamários, e seus reflexos nos aspectos produtivos e na composição do leite produzido tem se acumulado recentemente, revelando que quartos sadios reagem (alteração nos níveis de CCS, lactose, gordura, condutividade elétrica, e N-acetyl-beta-D-glucosaminidase) quando os quartos vizinhos são infectados com mastite subclínica, quando comparados ao leite de quartos de animais totalmente sadios (ausência de mastite subclínica em todos os quartos mamários).

No entanto, a literatura ainda necessita de estudos que abordem a temática da teoria da interdependência dos quartos mamários em relação aos casos clínicos da doença e suas alterações na composição do leite produzido em quartos sadios adjacentes ao infectado, principalmente em relação à concentração dos teores de gordura, proteínas, lactose, cloretos, e nos requisitos referentes à saúde das glândulas mamárias como a contagem de células somáticas, concentração individual de leucócitos (neutrófilos, linfócitos e macrófagos) e de enzimas como o lactato desidrogenase, fatores estes, que impactam diretamente a qualidade e o rendimento dos derivados lácteos.

Diante do exposto, essa pesquisa teve como objetivo principal promover uma elucidação da teoria da interdependência dos quartos mamários e averiguar o reflexo na composição e qualidade do leite produzido nos quartos mamários adjacentes ao quarto infectado com mastite clínica, visando, ainda, caracterizar tal impacto de acordo com aspectos intrínsecos relacionados aos animais.

2 REFERENCIAL TEÓRICO

2.1 Qualidade do leite

A preocupação das indústrias e profissionais do campo com relação à qualidade primária do leite tornou-se evidente nos últimos anos, a fim de se obter bons resultados econômicos (VIEIRA, 2010), pois, de maneira geral, as indústrias de grande porte passaram a estabelecer novas condições para a aquisição do leite cru refrigerado, com remuneração aos produtores que produzem leite com qualidade e em maiores volumes, visando tanto o maior rendimento dos produtos produzidos, quanto à diminuição dos custos de transportes e otimização das rotas de captação.

Além disso, mudanças nos padrões de distribuição dos produtos lácteos, diferentes formulações dos produtos, padrões internacionais visando à exportação, e uma maior expectativa dos consumidores em relação à qualidade dos produtos processados resultaram em uma demanda por produtos com um padrão de identidade e qualidade excelente, tanto no início, quanto no final da vida de prateleira desses produtos. Para a produção de derivados lácteos que possuam um padrão de qualidade superior, as indústrias processadoras necessitam de um leite cru também de alta qualidade; e para isso, este é definido basicamente em relação a sua composição adequada (por exemplo, níveis de gordura e proteínas dentro dos padrões normais); ausência de odores e *off flavors* não característicos; ausência de resíduos de drogas utilizados na cadeia produtiva (ou pelo menos, abaixo dos padrões detectáveis), de adição intencional ou não de água, ou de outros adulterantes; com uma baixa contagem total de microrganismos; e com uma CCS baixa. Visando garantir a qualidade do leite cru para posterior processamento de produtos lácteos, as indústrias de laticínios monitoram constantemente o leite recebido, tanto ao nível de plataforma na

própria indústria, quanto ao nível de produtores rurais envolvidos no processo (MURPHY et al., 2016). Dentre esses aspectos utilizados pela indústria para aferir a qualidade do leite cru recebido, os mais importantes devido aos potenciais efeitos negativos sobre os produtos processados são a CCS e as contagens bacterianas totais do leite (MURPHY et al. 2016). Neste trabalho (e nesta revisão) enfatizaremos apenas o efeito detrimental da CCS do leite sobre a composição do mesmo e sobre os produtos lácteos processados.

2.1.1 Contagem de células somáticas do leite

No Brasil, a Instrução Normativa nº 7 de 2016 estabeleceu novos limites e datas dos parâmetros relacionados à saúde da glândula mamária dos animais (CCS) e, atualmente, na região sudeste, o limite ainda é de 500 mil células/mL para leite cru refrigerado proveniente de leite de tanques de refrigeração a granel (BRASIL, 2016). Apesar de todos os avanços relacionados ao monitoramento da saúde da glândula mamária e qualidade primária do leite, a CCS do leite ainda é o método "gold standard" para averiguar a saúde geral dessas glândulas dos animais em lactação em um rebanho, devido a sua alta sensibilidade e especificidade (NYMAN et al., 2016). A contagem de células somáticas no leite pode ser realizada diretamente pela contagem microscópica direta, ou por sistemas automatizados que utilizam a metodologia de citometria de fluxo (contadores eletrônicos) (INTERNATIONAL DAIRY FEDERATION - IDF, 2006). Os contadores eletrônicos, como o Bentley Somacount (Bentley Instruments, Chaska, MN), Fossomatic 5000/FC (Eden Prairie, MN) e o Delta SomaScope (Advanced Instruments, Norwood, MA) são os equipamentos mais utilizados por laboratórios credenciados relacionados aos programas de qualidade do leite dos produtores em todo mundo. A metodologia de contagem por microscopia direta é pouco utilizada, no entanto, é o método designado

como referência e também utilizado para a calibração de contadores eletrônicos (MURPHY et al., 2016).

Nos Estados Unidos da América, a Portaria de leite pasteurizado de 2013, que é o documento que regula as normas técnicas de produção e beneficiamento do leite nesse país, requer que a contagem de células somáticas em tanques de refrigeração a granel seja menor que 750.000 células/mL. Os laticínios realizam os testes e fornecem ao Estado e às agências reguladoras do leite a granel os resultados mensais de CCS para avaliação da qualidade. Na maioria dos programas de regulamentação, as amostras de leite utilizadas para determinar a CCS são coletadas de uma a três vezes por mês (FOOD AND DRUG ADMINISTRATION – FDA, 2013). No entanto, a maioria dos produtores de leite norte-americanos, assim como os grandes produtores de leite brasileiros, busca reduzir as médias de CCS do leite dos tanques a granéis, pois incentivos monetários relativos à qualidade do leite denominados "*premium*" são oferecidos por cooperativas ou outros compradores de leite cru. Nos EUA, maiores incentivos monetários (bonificações) são oferecidos a produtores que atingem metas geralmente variando entre 100.000 e 350.000 células/mL para a CCS do leite dos tanques a granéis (MURPHY et al., 2016). Na região sudeste do Brasil, as maiores bonificações relativas à CCS do leite de tanques a graneis são oferecidas a produtores que atingem médias abaixo de 200.000 células/mL (PAIXÃO et al., 2014).

O leite, naturalmente, sempre contém certa quantidade de células somáticas. Estas são compostas principalmente por vários tipos de células de defesa e certa concentração de células de escamação do tecido animal, e suas proporções dependem do estado de saúde do úbere. Em uma glândula mamária lactante saudável, a maior proporção das células somáticas é constituída por leucócitos (glóbulos brancos) (ÖSTENSSON; HAGELTORN; ÅSTRÖM, 1988). De acordo com Harmon (1994), as células somáticas são formadas

principalmente por macrófagos e linfócitos, e, apenas uma pequena fracção é composta por neutrófilos e células epiteliais provenientes da escamação natural do tecido mamário. A quantidade dessas células somáticas no leite de animais individuais totalmente saudáveis é, usualmente, menor que 200.000 células/mL de leite (BRANLEY, 1992). O aumento da CCS constitui uma parte importante da resposta imune do animal, e, é, portanto, um indicador amplamente utilizado para monitorar a saúde da glândula mamária e indicar a presença de mastite subclínica. De acordo com Reneau (1986), uma glândula mamária saudável apresenta de 50.000 a 200.000 células/mL; níveis acima de 283.000 células/mL, a glândula é considerada infectada (mastite subclínica) e, nos casos clínicos, a contagem de células somáticas chega a milhões de células por mL. Porém, o nível ideal de CCS em amostras de leite de quartos de animais sadios (sem considerar efeitos como dias em lactação, número de lactações, estações do ano, dieta), ainda é muito discutido na literatura, e outros autores defendem níveis ainda menores de CCS do leite proveniente de quartos de animais sadios: valores menores que 155.000 células/mL de leite (DJABRI et al., 2002); valores menores que 100.000 células/mL de leite (BEZMAN et al., 2015; FORSBÄCK et al., 2011; SCHWARZ et al., 2010).

2.1.2 Proporções individuais de leucócitos do leite

De acordo com Burvenich, Guidry e Paape (1995), as proporções de leucócitos polimorfo nucleares (neutrófilos), macrófagos e linfócitos, no leite de animais saudáveis, são de, aproximadamente, 12%, 60% e 28% do total de células somáticas, respectivamente. No entanto, existe uma grande variação dos dados disponíveis na literatura referente ao tipo de leucócito predominante em leite de animais saudáveis. Diversos autores relatam a predominância de macrófagos (KITCHEN, 1981; LEITNER et al., 2000), linfócitos (LEITNER et

al., 2000; SCHWARZ et al., 2011a, 2011b), ou, ainda, de neutrófilos (KOESS; HAMANN, 2008; PILLA et al., 2012, 2013) em leite de animais saudáveis. Dentre os leucócitos associados à CCS, a concentração de neutrófilos é a fração dessas células que possuem maior variação (KOESS; HAMANN, 2008; MERLE; SCHRÖDER; HAMANN, 2007). Tais diferenças encontradas nas concentrações de leucócitos disponíveis na literatura podem estar associadas a diferentes metodologias utilizadas entre estudos (contagem de células diferenciais por microscopia ou por citometria de fluxo) e ainda devido a diferenças nos delineamentos experimentais (raça dos animais, dias em lactação, número de lactações, casos prévios de mastite).

Independentemente das diferenças encontradas nas proporções de leucócitos no leite entre esses estudos, existe uma conclusão geral entre os pesquisadores, que é o aumento substancial na concentração de neutrófilos no leite em casos de mastite clínica ou subclínica, e essas infecções resultam em um rápido acúmulo de grandes quantidades de células somáticas no úbere, predominantemente de neutrófilos (HARMON, 1994; ÖSTENSSON; HAGELTORN; ÅSTRÖM, 1988). Os neutrófilos presentes no leite estão associados a uma maior concentração de proteinases ativas, como as catepsinas B, D, G e elastase (LEROUX et al., 2002), que juntamente com o sistema enzimático plasmina podem alterar a fração proteica do leite. A estimativa da proporção dos leucócitos no leite é importante, pois fornece informações mais detalhadas sobre a saúde das glândulas mamárias dos animais a níveis de glândulas mamárias, pois ocorrem variações nas proporções de leucócitos (aumento na proporção de neutrófilos em relação aos demais leucócitos) antes mesmo do aumento das células somáticas (PILLA et al., 2012), mesmo quando a CCS no leite encontram-se em níveis muito baixos (9.000 células/mL) (SCHWARZ et al., 2011a, 2011b), indicando que a glândula mamária pode estar em uma fase inicial de mastite clínica ou subclínica.

Tecnologias que determinam a contagem (número de células) e proporções dos diferentes leucócitos no leite (macrófagos, linfócitos e neutrófilos) em nível de fazenda já estão disponíveis no mercado internacional, como, por exemplo, o sistema eletrônico QScout MLD. Esse sistema, desenvolvido por uma empresa norte-americana, denominada Advanced Animal Diagnostics (MORRISVILLE, NC, EUA), realiza os testes baseados na tecnologia de citometria de imagem, e apresenta uma velocidade do teste relativamente rápida (em torno de 8 minutos para o leite dos quatro quartos do animal), possibilitando a identificação de casos subclínicos de mastite baseados nos níveis de neutrófilos do leite em quartos individuais. Além disso, o sistema possui conectividade com a internet, o que permite ao veterinário ou encarregado técnico da fazenda, a verificação imediata dos resultados dos testes dos animais diretamente no site da empresa (http://www.qscoutlab.com/dairy/qstats-dairy/).

2.1.3 Variação natural da contagem de células somáticas do leite

Dentre os fatores que influenciam a CCS do leite de animais individuais, podemos citar a raça, o número de lactações, as estações do ano, o estresse, os dias em lactação, entre outros. Todos esses fatores estão associados a variações na CCS do leite de animais livres de infecção na glândula mamária. De acordo com Laevens et al. (1997) e Schutz et al. (1990), os dias em lactação possuem uma associação positiva com a CCS do leite. No início da lactação, os valores de CCS também encontram-se aumentados, devido à presença de imunoglobulinas provenientes do colostro e, consequentemente, de células de defesa. O aumento de CCS ao final da lactação está associado à maior descamação natural do epitélio da glândula mamária (MONARDES, 1994), ou ainda, devido a casos prévios de mastites que ocorreram durante a lactação. De acordo com Harmon

(1994) e Neijenhuis (2004), a associação positiva entre o número de lactações dos animais e a CCS do leite está relacionada a exposições das glândulas mamárias em lactações anteriores a infecções por patógenos ambientais, ou, ainda, devido ao efeito negativo do próprio processo de ordenha mecânica sobre a integridade dos tetos dos animais. O conhecimento prévio de todos os fatores que influenciam a CCS do leite é importante para o correto diagnóstico de possíveis infecções na glândula mamária (RIBAS, 1994).

2.1.4 Efeito das células somáticas na composição do leite e derivados lácteos

Quando fatores como raça, número de lactações, dias em lactação, e dietas dos animais são controlados, diferenças na composição do leite são geralmente atribuídas à CCS. Variações nos teores de gordura, proteínas totais, concentrações elevadas de plasminas e outras enzimas, e uma redução nos teores de lactose e sólidos totais em leites com alta CCS são relatadas na literatura (AULDIST; HUBBLE, 1998; HORTET; SEEGERS, 1998). Muller (2002) descreve que a extensão do aumento da CCS e as mudanças na composição do leite estão diretamente relacionadas com a superfície do tecido mamário atingido pela reação inflamatória e, de acordo com Cullen (1966), a magnitude das respostas imunes do animal frente à infecção e, consequentemente, da elevação da CCS, estão associados com a severidade dos casos clínicos, e, ainda, de acordo com Bobbo et al. (2017), com a etiologia dos processos infecciosos. As células somáticas do leite apresentam uma grande variedade de enzimas proteolíticas e lipolíticas, nas quais são liberadas durante o mecanismo de morte intracelular dos microrganismos causadores da doença, e que podem contribuir de forma significativa para a proteólise e a lipólise dos constituintes do leite (PHILPOT; NICKERSON, 1991; SANTOS; MA; BARBANO, 2003). As concentrações de muitas enzimas, ou mesmo, a sua atividade enzimática no leite,

encontram-se elevadas durante quadros de mastite (ANDREWS et al., 1991; FOX; MORRISSEY, 1981; KITCHEN, 1981). As enzimas de maior importância para a indústria de laticínios são as que apresentam atividades proteolíticas, pois o aumento da proteólise no leite e derivados apresenta um impacto negativo sobre a qualidade e propriedades tecnológicas dos mesmos.

No complexo enzimático denominado plasmina, composto por várias frações (ativadores do plasminogênio, inibidores dos ativadores de plasminogênio, inibidores da plasmina, plasminogênio, e plasmina), encontra-se uma enzima proteolítica alcalina (plasmina), que é responsável pela hidrólise das caseínas (α_{s1} caseína, α_{s2} caseína, e principalmente β caseína) e este é o fator mais importante referente à proteólise natural do leite (FOX; McSWEENEY, 2003; ISMAIL; NIELSEN, 2010). A plasmina é naturalmente encontrada no leite, e sua forma ativa é produzida a partir do zimógeno denominado plasminogênio. A conversão do plasminogênio em plasmina ocorre pela ação específica de ativadores do plasminogênio, os quais também são proteases (LÄHTEENMÄKI; KUUSELA; KORHONEN, 2001), e, no leite de animais, que apresentam quadros clínicos ou subclínicos de mastite, há uma maior concentração de ativadores do plasminogênio, denominados uroquinase (u-PA), que são associados às células somáticas (BASTIAN; BROWN, 1996; ISMAIL; NIELSEN, 2010). Esses ativadores aumentam a concentração de plasmina no leite, iniciando o processo de quebra das caseínas (ASLAM; HURLEY, 1997; DATTA; DEETH, 2001). Tal processo enzimático contribui para uma maior susceptibilidade a defeitos em derivados lácteos como a gelificação do leite UHT (BASTIAN; BROWN, 1996; RECIO et al., 1996), alteração nas propriedades coagulativas do leite e de maturação em queijos (BOBBO et al., 2016; CONSIDINE et al., 2004; LE MARÉCHAL et al., 2011; MURPHY et al., 2016).

De acordo com Kitchen (1981), devido à ação da plasmina, ocorre redução das proteínas sintetizadas na glândula mamária (α e β caseína, α-lactoalbumina, e β-lactoglobulina), e ocorre um aumento das proteínas de origem sanguínea (albumina sérica e imunoglobulinas) em virtude do aumento de permeabilidade vascular secundário ao processo inflamatório, e também aumento da ɣ caseína, uma proteína solúvel oriunda da lise da β caseína pela plasmina. De acordo com esse autor, os níveis de proteína total do leite têm pouca variação, mas a concentração de cada tipo de proteína varia acentuadamente devido à ação da plasmina. Barry e Donnelly (1981) relatam que 90% da atividade de proteinase no leite com alta CCS estão associadas à plasmina. Entretanto, DerHam e Andrews (1982) relatam que apenas um terço da atividade de proteinase no leite é devido à plasmina. Somers et al. (2003) também afirmam que a plasmina não é a única enzima responsável pela degradação de caseína no leite de vacas com alta CCS, e os autores concluíram que proteinases provenientes diretamente das células somáticas e também provenientes do sangue podem desempenhar um papel significativo na hidrólise da caseína do leite.

A plasmina possui uma maior velocidade de reação em uma faixa de pH entre 7,5 a 8,0, e uma temperatura ótima de 37 °C; portanto, grande parte dos danos causados à fração caseínica do leite ocorrem ainda no úbere do animal, anteriormente à ordenha. Uma vez que a CCS é elevada no leite de um animal, o aumento da atividade proteolítica pode permanecer elevado, mesmo após o decréscimo da CCS do leite (SAEMAN et al., 1988). Outro fator relevante relacionado ao sistema enzimático da plasmina, é que tanto a plasmina, quanto os ativadores de plasminogênio, possuem certa atividade em temperaturas de refrigeração (2ºC a 5ºC), mediando à conversão de plasminogênio à plasmina e a proteólise da caseína do leite, respectivamente. Além disso, nessa faixa de temperatura de refrigeração, a fração β caseína do leite torna-se mais solúvel,

permitindo maior acesso a ação da plasmina, resultando em maior proteólise durante a refrigeração do leite por longos períodos (20 a 21 dias de prateleira); este problema é ainda mais grave no caso do armazenamento do leite UAT a temperaturas ambientes (22 a 25°C) e por períodos ainda mais longos (6 a 9 meses de vida de prateleira), pois esta faixa de temperatura é próxima da atividade ideal da enzima (ISMAIL; NIELSEN, 2010). Outro problema relacionado a esse sistema enzimático é a sua resistência a altas temperaturas, pois a plasmina, o plasminogênio, e principalmente os ativadores do plasminogênio são termo resistentes e não sofrem desnaturações em tratamentos térmicos convencionais (High temperature and Short time - HTST) (72°C/15 segundos) (DULLEY, 1972) e, em alguns tratamentos térmicos UAT (Ultra Alta Temperatura) (138°C/2 segundos) em torno de 20% a 40% da atividade de plasmina ainda permanece ativa; porém, tratamentos térmicos acima de 147°C resultam em inativação completa do sistema (DATTA; DEETH, 2001; ISMAIL; NIELSEN, 2010). De acordo com Prado et al. (2006), a concentração de plasmina no leite pode aumentar após o processo de pasteurização convencional (72°C/15 segundos), pois todos os inibidores dos ativadores de plasminogênio são inativados neste binômio tempo/temperatura, facilitando assim, a ação dos componentes que são termo-resistentes, como os ativadores de plasminogênio e a posterior conversão do plasminogênio a plasmina.

O aumento da atividade e a concentração de plasmina no leite com alta CCS também ocasionam alterações nas membranas dos glóbulos de gordura do leite, pois estes são formados por glicolicoproteínas, e a ação da plasmina facilita a lipólise da gordura do leite, gerando ácidos graxos livres (AGL), que podem contribuir para defeitos sensoriais em leites pasteurizados e derivados lácteos durante a vida de prateleira (LI et al., 2014). Defeitos sensoriais devidos à alta concentração de CCS e enzimas lipolíticas em leite pasteurizado (SANTOS; MA; BARBANO, 2003), iogurtes (FERNANDES; OLIVEIRA;

LIMA, 2007) e, especialmente, derivados lácteos com altos teores de gordura, como a manteiga (McDANIEL; SATHER; LINDSAY, 1969) são relatados na literatura. Estudos prévios relatam maiores concentrações de AGL no leite de vacas com mastite (BACHMAN et al., 1988). Porém, a associação entre a concentração total de gorduras no leite e a CCS revelam resultados divergentes. Kitchen (1981) e Auldist et al. (1995) relatam uma menor concentração de gordura no leite de vacas com mastite subclínica (alta CCS), enquanto Rogers, Mitchell e Bartley (1989) não observaram um efeito claro da CCS na gordura do leite. Por outro lado, Mitchell et al. (1986) relatam um aumento na concentração de gordura devido à alta CCS. Azzara e Dimmick (1985) verificaram uma diminuição no teor de gordura em leites com alta CCS, e os autores defendem que essa diminuição é proveniente de uma síntese reduzida de leite na glândula mamária, como resultado de danos epiteliais e/ou devido à ação de enzimas lipolíticas dos leucócitos. Schultz (1977) e Forsbäck et al. (2009) afirmam que teores de gordura superiores relatados em leites com alta CCS são associados a uma drástica queda de produção de leite no úbere do animal, sugerindo um aumento ilusório na proporção do teor de gordura em relação aos demais componentes. Apesar dos resultados contrastantes, a gordura do leite é o componente que mais sofre variações, e essas variações encontradas na literatura também podem estar associadas a diferenças entre rebanhos estudados, incluindo dietas, raças, dias em lactação dos animais, número de lactações dos animais e especialmente a fração do leite utilizada nas análises.

O leite de quartos mamários de animais em lactação livres de mastite clínica ou subclínica não possui uma grande variação no teor de lactose, e análises desse componente do leite realizadas no mesmo animal, e, em diferentes dias, também possuem uma variação mínima, quando comparados a análises dos demais componentes do leite em diferentes dias no mesmo animal (FORSBÄCK et al., 2010). No entanto, a concentração de lactose no leite é inversamente

proporcional à concentração de leucócitos (CCS), cloretos e sódio do mesmo, pois durante casos de mastite, esses componentes provenientes do sangue, juntamente com outras enzimas, migram para o leite devido a danos celulares, e ocorre uma alteração no balanço de osmolalidade da fração solúvel do leite, e, para manter estes níveis normais, as células alveolares produtoras de leite reduzem o fluxo de lactose para o lúmen da célula (CHAVEZ et al., 2004; McMANAMAN; NEVILLE, 2003). Além disso, o potássio, mineral predominante no leite, também decresce devido ao dano celular (SCHÄELLIBAUM, 2000). Um leite com alta CCS e, consequentemente, com concentrações reduzidas de lactose, é detrimental para a fabricação de produtos lácteos que utilizam o processo de acidificação a partir de culturas lácteas (SCHALLIBAUM, 2001). Além disso, uma diminuição na lactose ocasionará uma redução nos sólidos desengordurados totais do leite, e, visando aumentar a firmeza e a viscosidade de iogurtes e queijos de alta umidade feitos a partir de coagulação ácida ou mista (como os queijos *Petit Suisse e o Quark*, por exemplo), as indústrias de laticínios utilizam-se de processos como a concentração do leite ou adição de matéria seca (de origem láctea) visando aumentar os níveis de sólidos desengordurados nestes produtos (LUCEY, 2004), portanto, quanto menores esses sólidos no leite de origem, maior será o custo para indústria láctea.

Santos, Ma e Barbano (2003) relatam que a alta CCS no leite, ocasiona a diminuição da multiplicação dos microrganismos utilizados nas culturas "*starters*" em produtos lácteos, devido ao alto nível de substâncias antimicrobianas, sobretudo, a lactoferrina e lactoperoxidase presentes nesses leites. De acordo com Cansian (2005), em queijos do tipo Mussarela, a alta CCS do leite tem impacto direto nas etapas de "acidificação" e "filagem" da massa, pois não ocorrerá, ou ocorrerá de forma mais lenta, a conversão da galactose à glucose e, em seguida, a ácido lático pela cultura "*starter"*, que iria diminuir o

pH do meio e reagir gradualmente com o fosfoparacaseinato de cálcio, removendo este da massa e tornando-a cada vez mais desmineralizada, capaz de ser moldada em água a temperaturas elevadas. Esse problema (alta CCS do leite) torna a massa do queijo pouco elástica e de difícil filagem, necessitando maiores volumes de água e de temperaturas mais altas, o que reflete em maiores perdas de sólidos e problemas relacionados à textura, derretimento e elasticidade desses queijos. De acordo com Murphy et al. (2016), a CCS do leite cru acima de 100.000 células/mL está associado a efeitos diretos na redução do rendimento de queijos, e níveis mais elevados (CCS acima de 400.000 células/mL) estão associados a defeitos de textura e/ou sabor em queijos e outros produtos (leites UAT, iogurtes, leite em pó, manteigas). No entanto, um estudo recente revelou que animais com uma CCS do leite muito baixa (menor que 19.000 células/mL) estão associados a uma menor firmeza da coalhada na produção de queijos, quando comparados a animais que apresentavam uma CCS do leite em níveis normais (38.000 a 71.000 células/mL) (BOBBO et al., 2016). Diante de tais resultados inesperados, os autores especulam que tais animais que possuíam a CCS do leite muito reduzida podem apresentar uma imunodeficiência contra uma possível invasão de microrganismos, pois, os leucócitos do leite, quando em níveis normais, auxiliam na resposta inflamatória contra possíveis invasões de patógenos, e, além disso, esses animais também apresentaram uma menor produção de leite, menor porcentagem de caseínas, e menor concentração de lactose no leite, quando comparados aos animais com CCS do leite na faixa entre 38.000 a 71.000 células/mL (BOBBO et al., 2016).

2.2 Mastite

A mastite é definida como uma reação inflamatória da glândula mamária (INTERNATIONAL DAIRY FEDERATION - IDF, 1987) e é geralmente

provocada por um microrganismo patogênico ao adentrar o úbere do animal através do canal do teto, supera os mecanismos de defesa e começa a se multiplicar nos canais do úbere, produzindo toxinas que são prejudiciais para a glândula mamaria e para a qualidade do leite produzido. O tecido mamário é, então danificado, causando aumento da permeabilidade vascular, alterando, assim, a composição do leite, ocorrendo um extravasamento dos componentes do sangue para o leite, como soro proteínas, enzimas e sais; diminuindo a síntese de caseínas e lactose e a diminuição da qualidade da gordura (HARMON, 1994).

De acordo com Hamann (2010), a mastite clínica ocorre na maioria das vacas leiteiras ao menos uma vez por ano (ou ao menos uma vez em uma lactação). De acordo com Pol e Ruegg (2007), a mastite é a enfermidade que mais acomete vacas leiteiras e, dentre as despesas veterinárias, representam os maiores gastos, e, é a razão mais frequente do uso de antibacterianos em rebanhos comerciais. As perdas econômicas devido à mastite incluem reduções na produção de leite, aumento do custo de produção, depreciação da qualidade do leite, redução da longevidade dos animais, aumento dos custos com mão de obra e tratamentos, e, além disso, possibilidade de transmissão para outros animais (PINZÓN-SÁNCHEZ; RUEGG, 2011) e diminuição na taxa de fertilidade desses (FUENZALIDA; FRICKE; RUEGG, 2015). É considerada a doença que provoca os maiores prejuízos à pecuária leiteira no Brasil, e, em grande parte, do mundo (ANDRADE et al., 2007) e, também, o fator que mais contribui para as perdas econômicas da cadeia produtiva do leite (JANZEN, 1970; LARANJA; MACHADO, 1994; LESCOURRET; COULON, 1994; SHOOK, 1989).

Laranja e Machado (1994) afirmam que, no Brasil, não se tem estimativa de prejuízos econômicos causados pela mastite, no entanto, diversos estudos, apontam uma alta prevalência da doença. A prevalência da mastite varia muito entre rebanhos leiteiros, e resultados de literaturas prévias citam índices

de mastite subclínica entre 20,69% e 72,46% e entre 0,60% e 17,50%, para a mastite clínica (BRANT; FIGUEIREDO, 1994; BRITO et al., 1999; BUENO et al., 2002; LARANJA; MACHADO, 1994; MOTA; PINHEIRO JUNIOR; SILVA, 2004; PARDO; STURION; BASILE, 1999).

Prejuízos anuais da ordem de US$ 225.504,000, devido à mastite, são relatados por órgãos norte americanos (NATIONAL MASTITIS COUNCIL - NMC, 1996). Lopes et al. (2011), estudando a influência da CCS sobre o impacto econômico da mastite em rebanhos bovinos leiteiros, concluiu que as despesas com prevenção dessa doença representaram, no máximo, 10,8% do impacto econômico total da mastite, e demonstrou vantagens em investir nessa prática, pois irá contribuir significativamente para diminuição da CCS no tanque e, consequentemente, para reduzir o impacto econômico da mastite. No mesmo trabalho, os autores relataram que as perdas por descarte de leite de animais em tratamento tiveram as maiores representatividades, com percentuais de até 51,7%.

Devido à variedade de fatores que determinam a ocorrência da mastite, sua incidência depende da exposição do animal aos patógenos, da eficácia dos mecanismos de defesa do úbere, e da presença de fatores de riscos presentes no ambiente, assim como as interações entre esses fatores (OVIEDO-BOYSO et al., 2007; SURIYASATHAPORN et al., 2000). A extensão das alterações ocasionadas no animal pela doença é determinada pela gravidade da infecção (HARMON, 1994; INTERNATIONAL DAIRY FEDERATION - IDF, 1987; PYÖRÄLÄ, 2003), e as alterações relativas às variações na composição do leite são dependentes dos patógenos envolvidos (contagiosos, ambientais, oportunistas) e ainda das respostas imunes dos animais (níveis de CCS do leite) frente a esses diferentes grupos de patógenos (BOBBO et al., 2017).

2.2.1 Mastite: Diferentes aspectos e definições

De acordo com Nielsen (2009), a mastite pode ser classificada em clínica ou subclínica. De acordo com Pinzón-Sánchez e Ruegg (2011), a mastite clínica pode, ainda, ser classificada de acordo com sua gravidade: Leve - quando apenas o leite é identificado como anormal; moderada - quando o leite é classificado como anormal acompanhado de inchaço ou vermelhidão da glândula mamária; grave - quando o animal exibe sinais de doença sistémica, tal como depressão, anorexia, desidratação ou febre. A classificação da gravidade da doença é importante para o estabelecimento de protocolos de tratamento eficazes, e, ainda, auxiliar os criadores na identificação de animais com um risco mais elevado de desenvolvimento de bacteremia e de necessidade de uma terapia suporte (WENZ et al., 2001). A duração da infecção ainda classifica a mastite clínica como aguda ou crônica, nas quais um início súbito define os casos agudos e os crônicos são caracterizados por um processo inflamatório que duram meses, resultando em desenvolvimento de tecido fibroso no úbere (JAIN, 1979; INTERNATIONAL DAIRY FEDERATION - IDF, 1987).

Quando os sinais visíveis de infeção não são bem definidos, a doença é denominada como mastite subclínica, e esta é a forma mais comum da doença (AKERS, 2002). A mastite subclínica pode ser diagnosticada pela presença de patógenos na cultura bacteriológica do leite, no entanto, essa prática ainda não é rotineiramente empregada nas fazendas produtoras de leite. O método padrão atual mais confiável para detecção da mastite subclínica é a CCS do leite de animais individuais (leite de conjunto) ou do leite proveniente de quartos individuais. Outros parâmetros, como a condutividade eléctrica do leite, Califórnia mastite teste (CMT), os níveis de lactose, enzimas como a lactato desidrogenase, níveis de proteínas de fase aguda no leite (haptoglobina e proteína amilóide sérica A), têm sido propostos como indicadores de mastite

subclínica (ÅKERSTEDT; PERSSON WALLER; STERNESJÖ, 2007; HAMANN, 2005; PYÖRÄLÄ, 2003).

A mastite subclínica caracteriza-se pela alta incidência, com índices variando de 44,8% a 97,0% e a redução na produção do leite varia entre 25,4% e 43,0% (BRANT; FIGUEIREDO, 1994). Busanello et al. (2017), em estudo recente sobre os índices de prevalência e incidência de novos casos mensais de mastite subclínica baseados na variação de CCS do leite de animais individuais (infectados ≥ 200.000 células/mL de leite; não infectados < 200.000 células/mL de leite) relativo ao período de 2011 a 2015 em 517 fazendas produtoras de leite, com diferentes tamanhos de rebanhos, provenientes de cinco regiões do Brasil, encontraram índices de 46,4% e 0,17, respectivamente, para prevalência e indecência de novos casos mensais. Os autores descrevem que poucas variações foram encontradas para a prevalência da mastite subclínica durante o período estudado nas diferentes regiões, porém, menores prevalências foram encontradas para rebanhos que realizavam a análise de CCS de animais individuais com maiores frequências. Os autores enfatizam que, tanto os índices relativos à prevalência, quanto os índices relativos à incidência mensal da doença, não melhoraram (reduziram) ao longo do período estudado (em todas as categorias de rebanhos e regiões em estudo), e, concluíram, que, ainda existe a necessidade de implantação de programas referentes à melhoria da qualidade do leite no Brasil.

2.2.2 Etiologia da mastite

Mais de 140 tipos diferentes de microrganismos podem causar a mastite, e a infecção é dependente da interação entre a vaca, o meio ambiente e os microrganismos (RIBEIRO et al., 2006). A maioria dos agentes causadores são bactérias dos gêneros *Staphylococcus*, *Streptococos* e agentes patogênicos gram-

negativos (MAKOVEC; RUEGG, 2003). Ranjan et al. (2006) afirmam que, embora haja grande diversidade entre os patógenos causadores de mastite em bovinos, como *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis* e *Escherichia coli*, esses são responsáveis por cerca de 80% dos casos. Individualmente, *Staphylococcus aureus* destaca-se como um dos microrganismos mais frequentemente encontrados nas infecções intramamárias de bovinos em todos os continentes, e também é aquele que isoladamente determina as maiores perdas na pecuária leiteira (SCHLEGELOVÁ et al., 2003; VASUDEVAN et al., 2003; ZSCHÖCK et al., 2000). No Brasil, o *Staphylococcus aureus* ainda é considerado como o principal agente causal da mastite bovina, com taxas de isolamento entre rebanhos que variam entre 8,3% e 49,23% (BRITO et al. 1999; COSTA et al., 1995; COSTA, 2008; COSTA et al., 2012; DONATELE; MOTTA; FOLLY, 2002; LAFFRANCHI; MULLER; FREITAS, 2001; LANGONI et al., 1991; MORETTI et al., 1998).

Os agentes etiológicos da mastite podem ser classificados em dois grupos: contagiosos e ambientais definidos pela fonte de infecção. Quando o microrganismo é encontrado no úbere e sua transmissão ocorre durante o processo de ordenha, o patógeno é denominado contagioso; e quando o animal é infectado por microrganismos provenientes do meio ambiente, o patógeno é intitulado ambiental (COSTA, 1998; COSTA et al., 2000). Os patógenos predominantes nas infecções contagiosas são *Staphylococcus aureus*, *Streptococcus agalactiae*, *Corynebacterium bovis*, *Streptococcus dysgalactiae* e *Mycoplasma* sp. (PEDRINI; MARGATHO, 2003). A mastite contagiosa caracteriza-se por grande número de infecções subclínicas, com episódios esporádicos de mastite clínica (EBERHART et al., 1987). Coliformes (*Escherichia coli*, *Klebsiella spp.* e *Enterobacter aerogenes*), Streptococcus *spp.* (*Streptococcus uberis*, *Streptococcus dysgalactiae* e *Streptococcus bovis*) e

Enterococos *spp.* (*Enterococcus faecium* e *Enterococcus faecalis*) são classificados como patógenos ambientais (HOGAN; SMITH, 1987; NATIONAL MASTITIS COUNCIL - NMC, 1999). Além disso, de acordo com Smith e Hogan (1995), ainda existem os patógenos oportunistas que habitam a pele dos animais, e, são, basicamente, bactérias estafilococos coagulase negativos. Além desses patógenos, também podem ocorrer mastites causadas por leveduras, algas e vírus; porém, a causada por esse último patógeno é extremamente rara (NASCIF JUNIOR, 2001).

Atualmente, muitas propriedades leiteiras de alta produção nos Estados Unidos da América têm conseguido êxito no controle dos patógenos contagiosos da mastite (MAKOVEC; RUEGG, 2003) e, a etiologia das mastites clínicas nas propriedades estão mais voltadas para grupos mais diversificados de organismos, principalmente os oportunistas e ambientais gram-negativos e gram-positivos (LAGO et al., 2011; OLIVEIRA; HULLAND; RUEGG, 2013; PINZÓN-SÁNCHEZ; RUEGG, 2011; SCHUKKEN et al., 2012; SMITH; TODHUNTER; SCHOENBERGER, 1985) e, as pesquisas atuais indicam poucas opções terapêuticas contra esses organismos (OLIVEIRA; HULLAND; RUEGG, 2013).

Além disso, um aumento na taxa de casos de mastite clínica ou subclínica que resultam em ausência de crescimento de microrganismos nos testes microbiológicos também têm sido verificado nestes rebanhos norte-americanos. De acordo com Makovec e Ruegg (2003), a proporção das amostras de leites de quarto individuais submetidas aos laboratórios credenciados no estado de Wisconsin (EUA), na qual resultaram em não crescimento de microrganismos, aumentou de 22,6% no ano de 1994, para 49,7% em 2001. Pesquisas na Finlândia revelam uma taxa maior ainda: em torno de 83,5% dos casos de mastite sem crescimento microbiano (MYLLYS et al., 1998). Este aumento de casos de mastite clínica resultando em ausência de crescimento de microrganismos nos testes microbiológicos está associado a uma drástica

redução de incidências de mastites ocasionadas por *Staphylococcus aureus* e *Streptococcus agalactiae*, devido a adequação destes rebanhos às práticas modernas de manejo. Em estudo realizado no Brasil, Souza et al. (2009), ao analisar 3.749 amostras de leite de 2.657 animais oriundos de 24 rebanhos leiteiros localizados nos estados do Rio de Janeiro e Minas Gerais, verificou que dessas amostras, somente 30,3% não apresentaram crescimento bacteriano e em 2.614 amostras de leite (69,7%) foi identificada a presença de pelo menos um patógeno da mastite.

Em fazendas de alta produção em Wisconsin (EUA), os principais agentes causadores de mastite são patógenos gram-negativos (FUENZALIDA; FRICKE; RUEGG, 2015; OLIVEIRA; HULLAND; RUEGG, 2013); e de acordo com Ruegg (2011) e Smith, Todhunter e Schoenberger (1985), estes microrganismos tendem a ocasionar uma inflamação intramamária de menor duração, e os animais acometidos apresentam uma taxa de cura espontânea elevada, quando comparados a quartos de animais acometidos com microrganismos gram-positivos; deste modo, ocasionando uma maior proporção de amostras de leite provenientes de animais infectados sem uma consistente identificação de microrganismos. De acordo com Oliveira e Ruegg (2014), animais diagnosticados com casos de mastite clínicas e que não apresentam crescimento de microrganismos nas amostras de leite também possuem respostas aos tratamentos de mastite similares aos animais diagnosticados com mastite clínica causada por patógenos gram-negativos. No entanto, é importante observar que a magnitude destas respostas imunes está associada com a severidade dos casos (CULLEN, 1966), e a taxa de não crescimento de microrganismos em amostras de leites de animais infectados também varia entre fazendas em uma mesma região (PINZÓN-SÁNCHEZ; RUEGG, 2011).

De acordo com Carvalho (2001), as bactérias *Streptococcus agalactiae* e *Staphylococcus aureus* ainda são os agentes etiológicos causadores de mastite

mais comum no Brasil, devido a novas infecções que ocorrem durante os procedimentos de ordenha entre os animais. Barkema et al. (1998) relatam que rebanhos com baixos índices de CCS no leite de tanques apresentaram maiores incidências de mastites clínicas ocasionadas por *Escherichia coli* e *Streptococcus dysgalactiae* e, rebanhos com uma CCS alta do leite de tanques apresentaram uma maior incidência de mastite clínica causada por patógenos causadores de mastite contagiosas, como *Staphylococcus aureus*, enfatizando a importância do controle das mastites contagiosas e a importância da CCS do leite do tanque como ferramenta essencial para o monitoramento da saúde geral do rebanho. Philpot (2002) ressalta que as infecções contagiosas tendem a ser subclínicas, de longa duração e acompanhadas por elevação significativa na CCS do rebanho, enquanto que a mastite ambiental se faz presente principalmente na forma clínica, tem curta duração e pouco impacto na CCS do rebanho. Isso ocorre, provavelmente, em função do leite de animais acometidos com mastite clínica ser descartado, fato este, que não ocorre quando os animais são acometidos com mastite subclínica.

Os agentes causadores de mastite podem ser classificados ainda em patógenos maiores ou menores. Os patógenos maiores provocam grandes mudanças na composição do leite, incluindo grande aumento na CCS, e são os responsáveis pelo maior impacto econômico da doença. Estes patógenos incluem *Staphylococcus aureus*, *Streptococcus agalactiae* e coliformes (HARMON, 1994). Ao contrário, os patógenos menores, como estafilococos coagulase-negativo, não estão associados a mudanças drásticas na composição e na produção de leite, e quando comparadas as amostras de leite proveniente de quartos contralaterais saudáveis, apenas um aumento na CCS dos quartos infectados é observado (TOMAZI et al., 2015). Porém, casos de mastites subclínicas causados por patógenos menores como *Corynebacterium bovis* estão associados a decréscimos nos teores de lactose, sólidos desengordurados, e

aumento na CCS do leite dos quartos acometidos, quando comparados a amostras de leites de quartos contralaterais sadios (GONÇALVES et al., 2016).

2.2.3 Fatores contra a mastite inerentes aos animais

De acordo com Oliveira (2012), depois que o úbere do animal é infectado, a chance de cura depende de seu sistema imunológico, características de virulência do organismo patogénico, e a eficácia do protocolo de tratamento. O desenvolvimento de resistência antimicrobiana em patógenos causadores de mastite é um dos fatores que reduz a probabilidade de cura. Conseguir uma cura para um caso de mastite é altamente dependente da imunidade do hospedeiro e das características específicas do organismo infeccioso; assim, os testes de susceptibilidade aos antimicrobianos realizados *in vitro* nem sempre são eficientes na predição de desfechos clínicos nos animais acometidos (APPARAO; OLIVEIRA; RUEGG, 2009; HOE; RUEGG, 2005).

Apesar da importância da determinação da sensibilidade de patógenos causadores de mastite a antimicrobianos como ferramenta para monitorar o desenvolvimento da resistência antimicrobiana (POL; RUEGG, 2007), a maioria dos responsáveis tratam a mastite clínica com base nos sinais clínicos, não realizando a análise microbiológica do leite, assim, muitos tratamentos são realizados independentemente da etiologia da doença (HOE; RUEGG, 2006). Estudos recentes relatam que entre 18 a 46% das amostras de leite obtidos a partir de animais que apresentam mastite clínica, não apresentam resultados positivos na cultura de microrganismos (LAGO et al., 2011; OLDE RIEKERINK et al., 2008; PINZÓN-SÁNCHEZ; RUEGG, 2011), o que dificulta a escolha e uso dos antimicrobianos (OLIVEIRA; RUEGG, 2014). De acordo com Oliveira e Ruegg (2014), resultados negativos na cultura de microrganismos podem ocorrer quando o animal ainda está infectado, porém, a

quantidade de colônias eliminadas no leite é inferior ao limite de detecção do método microbiológico utilizado, sendo que, em alguns casos, a terapia antimicrobiana é a mais indicada; porém, em outros, uma cura espontânea é observada.

De acordo com Carneiro, Domingues e Vaz (2009), os microrganismos causadores de mastite apresentam vários fatores de virulência que facilitam o estabelecimento da infecção na glândula mamária dos animais. Alguns patógenos conseguem evadir às defesas do animal através de adesão a células epiteliais e produção de cápsulas, o que dificulta a ação destrutiva dos neutrófilos, e ainda destroem ou inativam os leucócitos devido à produção de toxinas. Carvalho-Castro et al. (2016), em estudo relacionado a epidemiologia molecular de *Streptococcus agalactiae* isolados de casos clínicos e subclínicos de mastite de animais proveniente de 36 rebanhos brasileiros, revelou que a maioria das estirpes isoladas possuíam genes de virulência do tipo fbsB ("*fibrinogen-binding protein B"*) (relacionadas a adesão), hylB ("*Hemolysin secretion protein precursor"*) e cfb ("*CAMP-factor"*) (relacionados a danos nos tecidos), e PI-2b ("*Pilus Island"*) (relacionados a adesão e invasão). De acordo com esses autores, a identificação dos genes de virulência em microrganismos específicos causadores de mastite são importantes ferramentas para o planejamento, implementação, prevenção e tratamentos contra tais microrganismos, e os resultados obtidos podem facilitar futuros estudos destinados ao desenvolvimento de vacinas.

De acordo com Sordillo e Streicher (2002), a prevenção do estabelecimento da mastite nos organismos hospedeiros ocorre quando as respostas imunológicas desses respondem adequadamente frente aos diferentes fatores de virulência do organismo infectante. De acordo com Carneiro, Domingues e Vaz (2009), o sistema imune da glândula mamária bovina, assim como em outros sistemas, é dividida em inata (ou inespecífica) e adaptativa

(também conhecida como específica ou adquirida), e de acordo com Sordillo et al. (1997), esses fatores de proteção inatos e adaptativos agem em conjunto para promover uma proteção adequada à glândula mamária contra a mastite.

Nos estágios iniciais da mastite a imunidade inata é predominante, e é atribuída a fatores como barreiras relacionadas à anatomia dos tetos (esfíncter do teto), presença de substâncias naturalmente encontradas no leite (lactoferrina, sistema lactoperoxidase, lisozima e complemento), na queratina presente no canal do teto (ácidos graxos de cadeia intermediária como o mirístico, palmitolêico e linoleico), ou, ainda, no epitélio da porção distal da roseta de *Furstenberg* (parte interior do esfíncter mamário), que possuem ação antimicrobiana e células de defesa do animal (macrófagos, neutrófilos e células semelhantes às células exterminadoras naturais - "*natural killer-like cells*") (CARNEIRO; DOMINGUES; VAZ, 2009; GIRAUDO, 1996; SORDILLO et al., 1997).

Os macrófagos são células capazes de fagocitar e ingerir microrganismos, fragmentos celulares e componentes do leite que estão acumulados (SORDILLO; NICKERSON, 1988) e sua principal função é iniciar a resposta imune pela identificação dos patógenos invasores, liberação de componentes quimioatrativos e recrutação (diapedese) dos neutrófilos ao local de infeção (OVIEDO-BOYSO et al., 2007; PAAPE et al., 2002). Uma função secundária dos macrófagos é a remoção dos neutrófilos que sofreram apoptose devido ao evento de fagocitose, sendo esta função um requisito importante para a resolução da inflamação, e, também, para a própria proteção dos tecidos (BRATTON; HENSON, 2011), pois os neutrófilos, durante o processo de eliminação dos agentes infecciosos, produzem metabólitos reativos ao oxigênio e liberam enzimas durante o processo de degranulação (liberação do conteúdo intracelular) (GIRAUDO, 1996). De acordo com Lotz et al. (2004), alguns grupos de bactérias alteram essa apoptose normal dos neutrófilos pelos

macrófagos e geram o acúmulo de metabólitos que podem causar danos aos tecidos e quadros de inflamações. De acordo Schwarz et al. (2011b), uma alta concentração de neutrófilos do leite é um sinal claro da fase inicial da mastite, pois essas células, que foram recrutadas pelos macrófagos, são a primeira linha de defesa contra os microrganismos invasores com o intuito de prevenir uma infecção generalizada.

De acordo com Rainard e Riollet (2006), a imunidade inata dos animais não aumenta em intensidade devido a repetidos casos de mastite pelos mesmos microrganismos infectantes no animal, porém, estão, sempre presentes, ou, são ativadas rapidamente devido a numerosos estímulos no local da infecção. Diferentemente do sistema inato, o sistema imune específico possui a capacidade de reconhecer o patógeno, e promover uma eliminação seletiva, e esses fatores de reconhecimento são mediados por anticorpos, macrófagos e linfócitos (CARNEIRO; DOMINGUES; VAZ, 2009) e, essas respostas imunes, podem ser aumentadas quando há uma exposição repetida pelo mesmo microrganismo, devido à memória imunológica de certos linfócitos à presença de anticorpos patógeno-específicos (SORDILLO et al., 1997). De acordo com Nickerson (1989) e Sordillo et al. (1997), os linfócitos controlam, estimulam, e suprimem respostas imunes em receptores de membranas específicos contra os microrganismos invasores.

O esfíncter muscular mantém o canal do teto fechado entre as ordenhas, impedindo a penetração bacteriana, sendo esse, portanto, a primeira linha de defesa anatômica contra os patógenos invasores (GIRAUDO, 1996; SORDILLO et al., 1997). Pesquisas apontam que a posição do quarto mamário (anterior ou posterior) também é um fator predominante para o estabelecimento da mastite, e quartos mamários anteriores possuem uma maior incidência de mastite clínica quando comparados aos quartos posteriores. Guarín e Ruegg (2016), em um estudo caso-controle relatam que quartos mamários anteriores possuem o dobro

de chances de estarem afetados com mastite clínica quando comparados aos quartos posteriores. De acordo Neijenhuis (2004), a maior incidência de casos clínicos de mastite em quartos anteriores está associada a menor produção de leite desses, quando comparados aos quartos posteriores, e devido a essa menor taxa de produção, uma sobre-ordenha ocorre nesses quartos devido aos equipamentos de ordenha, ocasionando maiores lesões aos esfíncteres dos tetos e predispondo a doença. De acordo com Guarín, Paixão e Ruegg (2017), o diâmetro exterior central das glândulas mamárias também é outro fator associado à mastite subclínica (CCS superior a 150.000 células/mL), e quanto maior o diâmetro exterior central dos tetos, menor a CCS do leite (tendência de associação); no entanto, quando analisados somente os tetos anteriores dos animais, os diâmetros exteriores dos ápices dos tetos foram associados positivamente com a CCS do leite desses quartos, ou seja, tetos com ápices de diâmetros exteriores maiores resultam em leite com CCS superior. Os autores também relatam outros fatores anatômicos dos tetos associados à CCS, como o escore de hiperqueratose do esfíncter do teto (sem anel, anel suave ou leve, anel áspero, anel muito áspero), e quartos classificados com o escore de hiperqueratose como "muito áspero" resultaram em maiores valores de CCS no leite, quando comparados a esfíncteres totalmente sem anéis (GUARÍN; PAIXÃO; RUEGG, 2017).

Cardozo et al. (2015), em estudo relativo aos fatores de risco para ocorrência de novos casos e de casos crônicos de mastite subclínica (animais saudáveis: CCS do leite <200.000 células/mL em duas análises consecutivas mensais; animais identificados com um novo caso de mastite subclínica: CCS do leite do mês anterior <200.000 células/mL e CCS do leite do mês posterior >200.000 células/mL; animais cronicamente infectados: duas análises mensais consecutivas com CCS do leite >200.000 células/mL) em 30 rebanhos localizados no sul do Brasil (Santa Catarina) e abrangendo o leite de conjunto de

1700 animais das raças Holandesa, Jersey e cruzados (Holandesa e Jersey), identificaram vários fatores de risco para as variáveis em estudo. Animais com alto número de lactações (>3), com maiores danos no esfíncter do canal teto (escore de hiperqueratose entre 3 e 4), com uma altura do úbere muito baixa em relação ao jarrete, com tetos muito sujos, e proveniente de fazendas onde a linha de ordenha não era obedecida (animais com mastite clínica não eram ordenhados por último), possuíram uma associação positiva com a ocorrência de novos casos de mastite subclínica. Fatores como a manutenção esporádica (não periódica) dos equipamentos de ordenha, animais com uma fase mais avançada da lactação (mais que 100 dias em lactação), e animais com a altura dos úberes baixa (no jarrete ou abaixo do jarrete) foram identificados como fatores de risco para casos crônicos de mastite subclínica.

Além dos fatores inerentes aos animais, a incidência da mastite (clínica ou subclínica) é influenciada pelo manejo, fatores ambientais tais como habitação dos animais, equipamentos de ordenha, regime de alimentação, qualidade higiênica dos alimentos e da água, sanidade dos animais, implementação de medidas preventivas e práticas gerais relacionadas com a terapia na secagem dos animais (BARKEMA et al., 1999; COENTRÃO et al., 2008; ELBERS et al., 1998; NYMAN et al., 2007; PEELER et al., 2000; SCHREINER; RUEGG, 2003; SOUZA et al., 2005) e ainda fatores relacionados aos aspectos socioeconômicos e da disponibilidade de assistência técnica a esses produtores (PAIXÃO et al., 2015).

2.2.4 Lactato desidrogenase e saúde da glândula mamária

Durante o processo inflamatório na glândula mamária, certas enzimas hidrolíticas são liberadas (KALANTARI, SHAHABEDDIN; FOROUSHANI, 2013), e a lactato desidrogenase (LDH) (EC 1.1.1.27) é uma enzima

citoplasmática hidrolítica não-lisossomal liberada de neutrófilos, macrófagos, células epiteliais do úbere e células intersticiais danificadas durante este processo inflamatório (OLISZEWSKI et al., 2002) e que foi proposta como biomarcador para a verificação da saúde da glândula mamária. Estudos demonstram que a atividade dessa enzima aumenta significativamente no leite de quartos infectados com mastite subclínica e, sua atividade apresenta uma alta correlação positiva com a CCS do leite, especialmente em quartos infectados (CHAGUNDA et al., 2006; HISS et al., 2007). No entanto, Nyman et al. (2014) encontraram uma associação positiva entre a LDH e CCS do leite, mesmo quando apenas amostras de leite de animais saudáveis foram analisadas; e Kalantari, Shahabeddin e Foroushani (2013) não encontraram uma associação entre o LDH do soro sanguíneo e a CCS do leite, porém, uma associação positiva foi encontrada entre a LDH do leite e o mesmo limiar de CCS do leite, demostrando a alta associação positiva entre essas diferentes metodologias desenvolvidas para a verificação da saúde do úbere e, consequentemente, da qualidade do leite produzido.

Atualmente, existem diversas metodologias disponíveis no mercado para a verificação da concentração do LDH no leite. Em nível de laboratório, existem diversos kits comerciais para atividade cinética do LDH [Desidrogenase Lática LDH UV - Bioclin, Belo Horizonte, Minas Gerais, Brasil; AXON LDH IFCC KIT - Axon Lab, Baden, Suíça (utilizado por WALL et al., 2015); CytoTox 96® Non-Radioactive Cytotoxicity Assay - Promega, Madison, Winsconsin, EUA (utilizado por WENZ et al., 2010; e PAIXÃO et al., 2017)]. Nesses kits comerciais, o LDH do leite catalisa a redução do Piruvato com o NADH, obtendo-se Lactato e NAD^+, e a concentração catalítica é determinada a partir da velocidade de decomposição do NADH, calculado a partir da redução de absortividade pelo espectrofotômetro. Larsen (2005) desenvolveu uma metodologia para avaliar o LDH do leite baseada em ensaios com fluorescência,

e que não utiliza pré-tratamentos no leite. Posteriormente, essa tecnologia foi adaptada a sistemas de monitoramento automáticos de rebanhos em nível de fazendas (Herd Navigator) (DeLaval, Tumba, Suiça) e apresentando resultados com valores adequados de sensibilidade (80-82%) e especificidade (98%) (MAZERIS, 2010; RUTTEN et al., 2013; VREEBURG, 2010). Outro método de leitura direta do LDH do leite em nível de fazenda é a leitura com o analisador DT 60 II (Ortho-Clinical Diagnostics, Johnson & Johnson, Neckargemund, Alemanha) utilizado por Hiss et al. (2007).

2.3 Os quartos mamários – Independentes ou interdependentes?

Estudos com animais de produção demandam o uso de um grande número de animais para a obtenção de dados estatisticamente significativos, pois estas populações apresentam uma variação genética considerável. Portanto, sempre que possível, utiliza-se tratamentos controles intra-animal, uma vez que estes reduzem a variação genética e diminuem o número de repetições necessárias. Os quartos da glândula mamária bovina têm sido considerados como entidades fisiológicas distintas e, portanto, o uso de um ou mais quartos como tratamentos controles, geralmente, é aceito em experimentos científicos (CHANG; WINTER; NORCROSS, 1981; GRÖNLUND; JOHANNISSON; PERSSON WALLER, 2006; LUTZOW et al., 2008; RINALDI et al., 2010).

De acordo com Nickerson e Akers (2011), em vacas leiteiras, cada lado simétrico do úbere é considerado independente e tem seu próprio sistema vascular, suprimento nervoso e aparelho suspensor. O úbere é dividido em duas metades distintas, separadas pelos ligamentos de suspensão medial, que fornecem a maior parte do suporte que mantém o úbere anexado à parede ventral do animal. Curiosamente, as duas metades do úbere podem ser facilmente dissecadas por um corte longitudinal ao ligamento suspensor medial; porém não

existem barreiras anatómicas evidentes entre os quartos anteriores e posteriores e, entre ambos os lados do úbere apenas um septo fino de tecido conjuntivo está presente. Além disso, não há conexões diretas entre esses quartos. Isto é facilmente demonstrado utilizando-se de injeções com corantes nos quartos. Este fato é, por vezes, uma vantagem em algumas situações experimentais, sendo que, um lado do úbere poderá servir como tratamento controle.

Ainda de acordo com Nickerson e Akers (2011), cada um dos quartos mamários funciona como uma glândula separada dentro do úbere e tem a sua própria secreção de leite no tecido parenquimal. O parênquima é composto de alvéolos, dutos, e de tecido conjuntivo, na qual suporta e protege os tecidos que sintetizam o leite. Os milhões de alvéolos presentes são unidades produtoras de leite do úbere, e são formados por estruturas globulares microscópicas com 50 a 250 mm de diâmetro, dependendo do volume de leite acumulado. Os componentes precursores do leite são absorvidos a partir de vasos sanguíneos capilares adjacentes aos alvéolos por células epiteliais mamárias e são convertidos em proteínas, lactose e gordura. Estes componentes são liberados com os demais componentes do leite para o lúmen, ou no interior do alvéolo, para o acúmulo de leite entre as ordenhas.

Apesar das evidências anatômicas claras que comprovam a teoria de independência dos quartos mamários, Jensen et al. (2013) em pesquisa relacionada a indução de mastite com *Staphylococcus aureus* e *Escherichia coli* em um único quarto de animais completamente sadios, demonstraram que quartos da glândula mamária de bovinos são interdependentes durante a infecção por mastite, pois ocorreram mudanças profundas na expressão do RNA mensageiro de quartos vizinhos ao infectado com *S. aureus* e, especialmente nos infectados com *E. coli*. A comparação dos quartos controle (sadios) de animais infectados com *S. aureus* revelou a indução de alterações na expressão do RNA mensageiro nos quartos vizinhos sadios, 24 horas após a infecção induzida. Os

quartos controles permaneceram bacteriologicamente estéreis durante o período de estudo e, portanto, as diferenças observadas não ocorreram devidas a infecções. No entanto, os detalhes da resposta transcricional (expressão do RNA mensageiro) induzida por esses dois agentes patogênicos de mastite apresentaram-se distintas e, os autores sugeriram que essa alteração na transcrição dos quartos não infectados tem o intuito de evitar ou limitar a propagação da infecção para esses quartos.

Mitterhuemer et al. (2010), em um experimento utilizando-se de tecnologia de "*microarray*", com objetivo de comparar o transcriptoma (expressão de RNA mensageiro) de quartos de bovinos não infectados e infectados com *Escherichia coli*, relataram que foram identificados um total de 476 genes diferencialmente expressos (DEG), confirmando que a infecção por *E. coli* altera a resposta transcricional de quartos vizinhos saudáveis a uma glândula mamária infectada. Em outro estudo, Whelehan et al. (2011) relataram que a infecção em quartos por *S. aureus* induz a uma resposta em quartos vizinhos, pois, a expressão das proteínas de fase aguda no soro, haptoglobina e proteína amilóide sérica A, e a Beta Defensina 105A, também encontraram-se elevadas em quartos controle não infectados, em animais infectados com uma estirpe de baixa virulência de *S. aureus*, quando comparados aos tecidos de animais totalmente saudáveis.

Em contraste, estudos anteriores investigando a resposta da glândula mamária de bovinos à infecção utilizando-se de controle intra-animal, não identificaram grandes quantidades de genes semelhantes entre os quartos sadios e os infectados (BUITENHUIS et al., 2011; LUTZOW et al., 2008). Porém, nesses estudos, os animais foram infectados apenas uma vez, enquanto que no estudo conduzido por Jensen et al. (2013), os animais foram inoculados três vezes ao longo de 24 horas, o que pode ter resultado em um sinal que

desencadeou uma maior resposta imune nos quartos mamários não infectados (controles).

Em estudo conduzido por Petzl et al. (2008), os autores observaram que a CCS do leite dos quartos saudáveis (controles) aumentaram com o avançar do quadro infeccioso das bactérias inoculadas (*E. coli* e *S. aureus*) em um quarto individual. Bansal et al. (2005) e Merle, Schröder e Hamann (2007) também relataram valores de CCS superiores no leite de quartos não infectados de animais naturalmente infectados, quando comparados a animais totalmente saudáveis. Estudos prévios também citam uma interdependência entre CCS de quartos mamários em um mesmo animal (BARKEMA et al., 1997; BERRY; MEANEY, 2006). Tais autores atribuem este fato a diferentes variações na produção de leite dos animais, diferentes respostas imunes entre animais, e possíveis infecções cruzadas entre os quartos de um mesmo animal, e esses autores não mencionam uma interdependência fisiológica dos quartos mamários.

Bansal et al. (2005), em estudo no qual os autores classificaram os quartos de animais em dois grupos distintos: grupo 1 - úberes saudáveis (todos os quatro quartos com CCS abaixo de 100.000 células/mL e ausência de microrganismos); e grupo 2 - úberes com quartos infectados (pelo menos um quarto com CCS acima de 100.000 células/mL e/ou presença de microrganismos), encontraram diferenças significativas entre esses dois grupos, para todas as variáveis analisadas (lactose, gordura, condutividade elétrica, contagem de células somáticas e NAGase), com exceção dos teores de proteínas e pH, indicando que quartos saudáveis de úberes com sinais subclínicos de mastite funcionam em um nível metabólico diferente dos quartos de animais totalmente saudáveis, revelando um contraste com a teoria da independência dos quartos, citada por outros autores.

Evidências relacionadas à teoria da interdependência do sistema imune de glândulas mamárias em um mesmo animal e, consequentemente, na qualidade

do leite produzido, podem ser observadas em vários estudos, e não apenas em animais em lactação. Quesnell et al. (2012), estudando a sobrevivência bacteriana e a resposta inflamatória imediata após inoculação em apenas um quarto com *Escherichia coli* em animais no final da lactação (antes da secagem), observaram que os níveis relativos a interleucina anti-inflamatória 10 (IL-10) foram aproximadamente 3.5 vezes mais elevadas após 12 horas da inoculação e em todos os quartos (tanto no inoculado, quando nos quartos contralaterais não inoculados denominados controles). Bouchard et al. (1999) estudando a produção de óxido nítrico em mastite induzida por *Escherichia coli* identificou picos de CCS e óxido nítrico no mesmo intervalo de tempo em ambos os quartos (tratamento e controle) (1 quarto inoculado com o microrganismo e os demais quartos adjacentes inoculados com solução salina). Maiores concentrações de óxido nítrico e CCS foram observados nos quartos inoculados com *Escherichia coli*, porém picos de CCS e de óxido nítrico também foram observados nos quartos contralaterais; no entanto, esses autores não compararam estatisticamente os resultados relativos aos quartos controles antes e após a infusão.

As evidências sobre a teoria da interdependência entre os quartos mamários nos aspectos produtivos e na composição do leite vêm se acumulando recentemente, mostrando que quartos sadios reagem quando os quartos adjacentes são infectados com mastite subclínica. No entanto, até o momento, não foram encontradas pesquisas que abordem o quadro clínico da doença e seus reflexos na composição e na qualidade do leite em quartos mamários sadios adjacentes ao quarto acometido, tanto antes, quanto após o tratamento dos clinicamente afetados. Devido ao maior grau de inflação da glândula mamária em casos clínicos da doença (quando comparados aos quadros subclínicos), hipotetizamos com este estudo que o impacto na composição e qualidade do leite

nos quartos vizinhos ao não infectado, será maior que os impactos previamente relatados em quartos adjacentes aos casos subclínicos da doença.

3 CONCLUSÃO

Considerando os aspectos apresentados, bem como a importância do tema, o presente estudo teve como objetivo verificar a prevalência de diferentes patógenos em vacas leiteiras com mastite clínica em apenas um quarto mamário e, comparar a composição e a qualidade do leite (saúde da glândula mamária) produzido nos quartos saudáveis adjacentes ao quarto acometido antes e após o tratamento de mastite com o leite proveniente de glândulas mamárias de animais totalmente saudáveis (ausência de sinais clínicos de mastite), verificando-se se existe uma interdependência entre os quartos mamários dos animais acometidos, a nível de composição do leite e saúde das glândulas mamárias. Além disso, serão verificados se existem fatores de riscos relativos entre as variáveis estudadas (composição do leite e saúde das glândulas mamárias), a predominância dos diferentes agentes etiológicos causadores de mastite, e os fatores inerentes aos animais.

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SECOND PART - ARTICLES

ARTICLE 1 **Milk composition and health status from mammary gland quarters adjacent to glands affected with naturally occurring clinical mastitis**

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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, SNF, chloride) and health status (SCC, differential leukocytes 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 DIM. 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, SNF, SCC, 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 SCC (≥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 lesser concentration of total protein, lactose and SNF, but had greater concentration of fat and chloride. The SCC, total leukocyte count and absolute numbers of neutrophils, lymphocytes, and macrophages were all increased in milk obtained from adjacent quarters of case cows as compared to milk obtained from quarters of control cows. The relative proportion of neutrophils was increased while the proportion of macrophages was decreased in milk obtained from cases. Approximately 30% of milk samples obtained from adjacent quarters of cases had SCC ≥150,000 cells/mL as compared to 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 two 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-beta-D-glucosaminidase were observed among milk sample obtained from cows free of subclinical mastitis in all quarters as compared to milk obtained from cows that had at least one quarter with subclinical mastitis (Bansal et al., 2005). Hamann et al. (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) as compared to 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 non-infected glands and proposed that the mammary gland cannot be considered as four isolated entities. Similarly, Jensen et al. (2013) studied transcriptional response after experimentally induced intramammary infections. 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).

While 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 mammary gland will help to clarify immune mechanisms after a clinical intramammary infection.

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 impact of unseen signs of infection (SCC, LDH, leukocytes, 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 impact 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, lactose, chloride, solids non-fat) and health status (SCC, differential leukocyte count and lactate dehydrogenase) 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, 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 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 - 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 days 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, California). 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) total protein (**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 leukocytes count (**TLC**) of 50,000 cells/mL, and 7) lactate dehydrogenase (**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. non-preserved 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 one week, until it was used for microbiological analysis at the UW 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 (-18ºC) and samples preserved with bronopol were stored under refrigeration (4ºC) 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 UW Milk Quality Laboratory. One day after 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 were plated onto one quarter plate of MacConkey agar, and aerobically incubated at 37ºC 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 & Lactate Dehydrogenase

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

An aliquot (1.9 mL) of each thawed aseptic milk samples were also transferred to Eppendorf tubes in order to proceed to LDH analysis. Three centrifugations were performed at 14,000 × *g* at 4°C for 4 min (1st centrifugation) and then 30 minutes (subsequent centrifugations). After each centrifugation the fat layer was removed and skimmed 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, Wisconsin). 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; 10.0; 12.5 nmole of B-NADH/well) (Sigma-Aldrich, N0786) (Sigma-Aldrich, Saint Louis, Missouri) 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 Leukocytes 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, North Carolina).

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, Wisconsin]. Milk total protein, fat, lactose and solids non-fat 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, Hillerød, Denmark).

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, while base-10 logarithms were used to transform SCC, TLC, absolute values of

individual leukocyte populations [neutrophils (**neu**); lymphocytes (**lym**); macrophages (**mac**)] and LDH. An arcsine transformation was used to normal the proportion of leukocytes.

To test the null hypothesis that parity (1st, 2nd or ≥3th), and pregnancy status (yes or no), did not differ between cows assigned to be cases or controls, 2 separate Chi-squared 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 (as compared to no or non-significant growth) did not differ between milk samples collected from adjacent quarters of cases or controls a Chi-squared analyses 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 quarters milk samples (**AQMS**) or control quarters milk samples (**CQMS**)], 14 separate multivariate linear regression analyses were performed with PROC GLM. Outcome variables were: 1) fat, 2) TP, 3) lactose^2, 4) SNF, 5) chloride, 6) Log_{10}SCC, 7) Log_{10}(TLC+1), 8) Log_{10}(Neu+1), 9) $ARCSIN\sqrt[2]{Neu\ \%/_{100}}$, 10) Log_{10}(Lym+1), 11) $ARCSIN\sqrt[2]{Lym\ \%/_{100}}$, 12) Log_{10}(Mac+1), 13) $ARCSIN\sqrt[2]{Mac\ \%/_{100}}$, 14) Log_{10}LDH.

Initial models included:

Individual outcome variable = μ + $PREGSTATUS_{ij}$ + $PARITY_j$ + $QTYPE_j$ + $QPOS_{ij}$ + $MICSTATUS_{ij}$ + DIM_j + MY_j + $COWQUARTER_{ij}$ + ε_{ij}

Where outcome variable = each response variable [fat, TP, $lactose^2$, SNF, chloride, $Log_{10}SCC$, $Log_{10}(TLC+1)$, $Log_{10}(Neu+1)$, $ARCSIN\sqrt[2]{Neu\ \%/_{100}}$, $Log_{10}(Lym+1)$, $ARCSIN\sqrt[2]{Lym\ \%/_{100}}$, $Log_{10}(Mac+1)$, $ARCSIN\sqrt[2]{Mac\ \%/_{100}}$, $Log_{10}LDH$] of the *i*th quarter, and the *j*th cow; μ = regression constant term; $PREGSTATUS_{ij}$ = pregnancy status (yes or no); $PARITY_j$ = parity (1^{st}, 2^{nd}, or $\geq 3^{rd}$) ; $QTYPE_j$ = 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 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 were not associated with

composition outcomes and were 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 SCC ≥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 analyses that report a SCC mean of 155,000 cells/mL in quarters subclinically infected with CNS (Djabri et al., 2002).

The model included:

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

Where:

SCC ≥150,000 (outcome variable) = Quarters within cow with SCC ≥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 (1^{st}, 2^{nd}, or $\geq 3^{rd}$) (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 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. 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 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 variable, quarter within cow 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, there were no differences 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 as compared to rear quarters (95% C.I. 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 ($P \leq 0.001$) as compared to cows assigned to control (Table 1). For fat, TP and SCC statistical analyses, four cows did not have any DHI previous analyses, reducing the number of enrolled cows to 114.

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

Variables		Cow type (Enrolled)[1]				
		Case		Control		*P* - value
Categorical		n = 59	%	n = 59	%	
Parity	1	22	37.30	22	37.30	1.000
	2	20	33.90	20	33.90	[2]
	≥3	17	28.80	17	28.80	
Pregnancy Status	No	44	74.60	39	66.10	0.310
	Yes	15	25.40	20	33.90	[2]
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						
Days in milk		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 (cells/mL)[3]		5.4[a]	± 0.01	4.7[b]	± 0.08	≤ 0.001

[a-b]Means within a line with different superscripts differs in ANOVA test ($P \leq 0.05$).
[1]Case – primiparous or multiparous cows, with four functional quarters, presenting mild or moderate clinical mastitis (Pinzón-Sánchez and Ruegg, 2011) after at least 13 days after the previous mastitis event; Control – Four functional quarters; within 30 days in milk 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.
[2]Two tailed χ^2 test between enrolled cases and control cows.
[3]Data recovered from the last Dairy Herd Improvement Association analyzes for 114 enrolled cows. This data is from composite milk samples collected for routine DHI analysis.

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 non-contaminated samples, the majority (65%) had no growth of microorganisms while environmental *Streptococci* spp. were the most frequently isolated

pathogens. Other etiological agents included coagulase-negative Staphylococci spp. (CNS), *Klebsiella* spp. and other microorganisms (Table 2).

Adjacent and Control Quarters. Milk samples from 7 quarters of control cows were missed 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 non contaminated samples, most (88%) resulted in no growth of microorganisms. CNS were the most frequently recovered organism follows by environmental *Streptococci* spp. and other microorganisms. Of the 131 non contaminated samples from control quarters, almost all (96.2%) resulted in no growth of microorganisms while environmental *Streptococci* spp., CNS, and yeast were recovered from a few quarters. There was a tendency of greater frequency of contaminated milk samples in control quarters (n = 39; 22.9%) as compared to adjacent quarters (n = 27; 15.9%) ($P = 0.09$) (Table 2).

Results from chi-square analysis indicated that milk samples obtained from quarters adjacent to cases were about 3 times as likely to result in microbial growth (95% C.I., 1.21 - 9.49) ($P = 0.01$) [n = 274 (340 total samples less 66 contaminated samples)] as compared to milk samples obtained from control quarters . No *Mycoplasma* spp. were recovered from any pooled milk samples (Table 2).

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.

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

[1]No *Mycoplasma* spp. was recovered from any pooled milk samples.
[2]The proportion of "No Growth", "Gram positive", "Gram negative", and "Yeast", does not include contaminated samples.
[3]Includes "No Growth" and "Non-significant growth".
[4]Two tailed χ^2 test between frequencies of "No Growth" and "Growth" (Gram positive, Gram negative and Yeast) in adjacent and control quarters.
[5]Two tailed χ^2 test between frequencies of "Contaminated" and "non-contaminated" (No growth and growth) samples in adjacent and control quarters.

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 as compared to CQMS ($P = 0.006$) (Table 3). Control quarter milk samples had greater mean TP, lactose and SNF as compared to 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 as compared to 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 as compared to 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) as compared to 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% range 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) as compared to 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%) as compared to 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) as compared to 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%), as compared to CQMS (12.4%) ($P = 0.043$). Adjacent quarter milk samples were 3.7 times more likely to have mean SCC greater than 150,000 cells/mL (1.72 - 7.98) as compared to CQMS ($P = 0.043$).

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

	Type of milk samples		
Multivariate analyses	Adjacent to case n =170	Control quarters n =170	*P*-value[1]
Fat (%)	3.07 ± 0.13	2.54 ± 0.13	0.006
Total Protein (%)	3.07 ± 0.04	3.25 ± 0.04	0.006
Lactose[2] (%)	4.65 ± 0.68	4.83 ± 0.68	0.008
Solids non-fat (%)	8.68 ± 0.07	9.00 ± 0.07	0.002
Chloride[3] (mg/L)	1042.74 ± 40.79	931.79 ± 41.13	0.057
Log_{10}SCC (cells/mL)	5.02 ± 0.06	4.57 ± 0.06	≤ 0.001
Log_{10}(TLC +1) (cells/mL)	5.07 ± 0.06	4.64 ± 0.06	≤ 0.001
Log_{10} (Neu +1) (cells/mL)	4.82 ± 0.07	4.33 ± 0.07	≤ 0.001
Neutrophils (%)[4]	58.36 ± 1.28	51.70 ± 1.28	≤ 0.001
Log_{10} (Lym +1) (cells/mL)	4.36 ± 0.07	3.87 ± 0.07	≤ 0.001
Lymphocytes (%)[4]	20.70 ± 0.88	21.42 ± 0.88	0.400
Log_{10} (Mac +1) (cells/mL)	4.27 ± 0.08	3.93 ± 0.08	0.002
Macrophages (%)[4]	20.20 ± 1.47	27.60 ± 1.47	≤ 0.001
Log_{10}LDH (U/L)[3,5]	1.47 ± 0.03	1.36 ± 0.03	0.020
Quarters with SCC ≥ 150,000 cells/mL of milk (%)[6]	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, days in milk, parity and quarter within cow fixed effect.
[2]*P*-values based on the squared values of lactose. LSD and SEM were back-transformed.
[3]Total number of quarters analyzed = 290.
[4]*P*-values based on the formulae ($ARCSIN\ \sqrt{Leukocytes\ \%/_{100}}$). LSD and SEM were back-transformed.
[5]LDH = Lactate dehydrogenase.
[6]*P*-value based on a multivariate logistic regression.

DISCUSSION

Clinical mastitis has a direct impact 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 impacts 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 impact 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/d and with a similar composition but greater SCC as compared to typical Wisconsin dairy farms (USDA-NASS, 2015). The herd used digested manure solids as bedding, and similar to other larger WI 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 as compared to sampling in stationary parlors. We reported a greater percentage of clinical mastitis cases with no growth of microorganisms as compared to Fuenzalida et al. (2015) (38.7%) and Pinzón-Sánchez and Ruegg (2011) (42%) but a lesser percentage as compared to 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 *Sta. aureus* and *Str. 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 intramammary infection 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 intramammary infections by opportunistic organisms and have similar post-treatment outcomes as compared to 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. While our samples were frozen for up to 7 days 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 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 *Streptococci* spp. were the primary Gram-positive microorganisms. Among Gram-negative microorganisms, a greater proportion of *Klebsiella* spp. was found as compared to 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, which a greater incidence was found in front quarters, as compared to rear quarters (Neijenhuis, 2004; Guarín and Ruegg, 2016).

Quarter Type Milk Samples Comparison

As compared to milk obtained from healthy cows, milk from mammary gland quarters adjacent to a single affected with clinical mastitis quarter were shown to have altered composition (fat, TP, lactose, solids non-fat, chloride) and health status (SCC, leukocytes, 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 independence 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 infers that the changes we observed were mediated at a systemic (rather than local) level. Irrespective of 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 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 total solids concentrations in milk with greater SCC were reported by Auldist and Hubble (1998) and Hortet and Seggers (1998). Adjacent quarters in our study had a greater SCC as compared to 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 as compared to 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) this suggests 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 as compared to 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 as compared to 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%) as compared to 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, that 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, 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 which use acidification with starter cultures process (Schallibaum, 2001).

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

A more detailed estimation of quarter health status can be determined by examining cell differentiation, because of 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, 2011b). Overall, the values for Log_{10}(TLC +1) were similar to LogSCC and milk from adjacent quarters had greater total leukocytes and greater numbers and proportions of individual cell types (with the exception of lymphocytes) as compared to 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). While the absolute number of all types of leukocytes was greater in AQMS, the proportion of neutrophils was lesser as compared to 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%) while a greater proportion of macrophages (28%) as compared to 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.

As compared to our results, Pilla et al. (2012) reported a greater concentration of lymphocytes in healthy quarters. However, the percentage of lymphocytes in non mastitic 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 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 non-lysosomal hydrolytic enzyme liberated from neutrophils, macrophages, damaged udder epithelial cells and interstitial cells during 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.

While 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 impact on whole udder milk nor differences between initial and recurrent cases and this type of research is needed to fully understand the impact 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, as compared to 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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ARTICLE 2 ***Short communication*: Cow and quarter effects associated to milk composition and udder health status from quarters adjacent to a naturally occurring clinical mastitis gland**

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***Short communication*: Cow and quarter effects associated to milk composition and udder health status from quarters adjacent to a naturally occurring clinical mastitis gland**

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ABSTRACT

Apparently healthy adjacent quarters from cows with a single gland affected with clinical mastitis have its milk composition and udder health status altered due to interdependence within quarters. The objective of this study was to describe cow and quarter variables and possible associations with milk composition and health status of quarters adjacent to a naturally occurring clinical mastitis gland. Milk samples (foremilk) of apparently healthy mammary glands adjacent to a single infected clinical mastitis gland from 59 cows were analyzed for fat, total protein, lactose, chloride, solids non-fat, SCC, differential leukocytes count, lactate dehydrogenase, and microbiological analyses. Multivariate linear models were used to assess possible associations between

previous cited foremilk samples analyses with cow (parity, pregnancy status, milk yield, DIM, severity of clinical mastitis and microbiological analyses of case quarter milk sample) and quarter (microbiological analyses of adjacent quarters; previous mastitis event in adjacent quarters prior to the study within the current lactation, adjacent quarter position, adjacent quarter position related to the case quarter) explanatory variables. Our results suggests that cow (parity category, milk yield, DIM, severity of clinical mastitis of case quarter) and quarter (intramammary infection of adjacent quarters; previous cases of clinical mastitis on adjacent quarters) explanatory variables were associated or tended toward an association with several milk composition and udder health status traits. After controlling for parity, milk yield of the cows, and previous cases of clinical mastitis of adjacent quarters, microbiological analyses of adjacent quarters milk samples had an association with the majority of udder health status outcomes analyzed; however, no associations were found between microbiological analyses and milk composition traits. Our results suggest that adjacent quarters milk compositional traits were not affected by microbiological analyses of these quarters, and it indicate that the main source of variation within these traits as compared to healthy milk samples in our previous study is mainly associated to the interdependency within quarters, thus, consolidating this theory.

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

Short communication

Milk composition and udder health status traits of adjacent quarters milk samples (**AQMS**) is altered throughout the udder of the cow when a single quarter is infected with clinical mastitis (Paixao et al., 2017). Previous studies has investigated cow effects (DIM, parity, milk yield, microbiological analyses)

and its association with composition and udder health status traits of composite or quarter milk samples (Schwarz et al., 2011; Nyman et al., 2014; Bezman et al., 2015). However, no previous studies have described cow and quarter effects of apparently healthy adjacent quarters from cows presenting a single quarter infected with clinical mastitis (visual abnormalities of milk and udder). The objective of this study was to assess possible associations within cows and quarters explanatory variables and composition and udder health status outcomes from 170 AQMS.

The study was conducted on a commercial Wisconsin dairy farm as previously described by Paixão et al. (2017). The experiment was designed as a cross sectional study and cows that had experienced a single quarter affected with 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) before administration of any treatment were eligible to participate. Cows which had a previous mastitis event were eligible to participate at least 13 days after the end of the last mastitis event. Cow variables [parity, average daily milk yield for the previous 7 d of the cow (**MY)**, DIM, position of the clinically infected quarter, and severity of the clinical mastitis) were randomly assigned for the study and information about enrolled cows were obtained from herd management software (Dairy Comp 305, Valley Agricultural Software, Tulare, CA). Prior to the study, a sample size was calculated, and based on the most limiting variable (fat) at least 170 AQMS were required (Paixão et al., 2017). Farm visits, collection, storage, and analyses of composition (fat, total protein, lactose, chloride, SNF), udder health status (SCC, differential leukocytes count, lactate dehydrogenase), and microbiological analyses of AQMS and case quarters milk samples were performed as previously described by Paixão et al. (2017).

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, while SCC, TLC, absolute value of individual leukocyte counts [neutrophils (**Neu**); lymphocytes (**Lym**); macrophages (**Mac**)] and LDH were transformed using base-10 logarithms.

The null hypotheses that composition and udder health status of AQMS was not associated with the selected cow and quarter explanatory variables were tested using 11 separate multivariate linear regression performed with PROC GLM. Outcomes variables were: 1) fat, 2) TP, 3) lactose^2, 4) SNF, 5) chloride, 6) $\log_{10}$SCC, 7) $\log_{10}$(TLC+1), 8) $\log_{10}$(Neu+1), 9) $\log_{10}$(Lym+1), 10) $\log_{10}$(Mac+1), and 11) $\log_{10}$LDH.

Initial models included the following:

Variable of interest = μ + PREGSTATUS$_j$ + PARITY$_j$ + SEVERITY$_j$ + ADJMAST$_{ij}$ + MICSTATUS$_{ij}$ + CASEMICSTATUS$_j$ + QPOS$_{ij}$ + QPOSCASE$_{ij}$ + DIM$_j$ + MY$_j$ + COWQUARTER$_{ij}$ + ε_{ij}.

Where:

Variable of interest = each outcome variable [fat, TP, lactose^2, SNF, chloride, $\log_{10}$SCC, $\log_{10}$(TLC+1),$\log_{10}$(Neu+1), $\log_{10}$(Lym+1), $\log_{10}$(Mac+1), $\log_{10}$LDH] of the *i*th quarter and *j*th cow; μ = regression constant term; PREGSTATUS$_{ij}$ = pregnancy status (yes or no); PARITY$_j$ = parity (1st or $\geq$ 2nd); SEVERITY$_j$ = severity of clinical mastitis (mild or moderate); ADJMAST$_{ij}$ = previous case of clinical mastitis on adjacent quarter in the current lactation (yes or no); MICSTATUS$_{ij}$ = microbiological analyses of the adjacent quarters foremilk samples (growth or no growth of microorganism); CASEMICSTATUS$_j$ = microbiological analyses of the case quarter foremilk sample (growth or no growth microorganisms); QPOS$_{ij}$ = quarter position (left front, right front, left rear, right rear); QPOSCASE$_{ij}$ = position of the adjacent quarter related to the

case quarter (same side as the affected; alternate side and adjacent to the affected; alternate side and crossed from the affected quarter); DIM_j = days in milk of the cow (continuous); MY_j = average daily milk yield for the previous 7 d of the cow (continuous); $COWQUARTER_{ij}$ = fixed effect to account for the correlation between quarters within cow; and ε_{ij} = residual variation.

All pairs of outcome and explanatory variables were first offered to univariate analysis with PROC GLM, and cow or quarter variables unconditionally associated with an outcome with a *P*-value ≤ 0.25 were then offered to multivariate linear regression modeling using forward and backward stepwise selection with PROC MIXED. Confounding effects, first order interactions and correlations among explanatory variables were screened using forward and backward stepwise selection. The Goodness of fit of each model was compared using Akaike information criterion. The variable quarter within cow was included as a random effect in all multivariate models. The experimental unit of these analyses was adjacent quarter milk sample within cow.

Final regression model for milk fat within AQMS was:

$Fat_{ij} = \mu + MY_j + COWQUARTER_{ij} + \varepsilon_{ij}$.

Final regression model for milk TP within AQMS was:

$TP_{ij} = \mu + PARITY_j + SEVERITY_j + MY_j + DIM_j + COWQUARTER_{ij} + \varepsilon_{ij}$.

Final regression model for milk lactose^2 within AQMS was:

$Lactose^2_{ij} = \mu + PARITY_j + ADJMAST_{ij} + MY_j + COWQUARTER_{ij} + \varepsilon_{ij}$.

Final regression model for milk SNF within AQMS was:

$SNF_{ij} = \mu + PARITY_j + ADJMAST_{ij} + COWQUARTER_{ij} + \varepsilon_{ij}$.

Final regression models for milk chloride, $\log_{10}$SCC, $\log_{10}$(TLC +1), $\log_{10}$(Neu +1), $\log_{10}$(Lym +1), and $\log_{10}$(Mac +1) within AQMS were:

Variable of interest [chloride, $\log_{10}$SCC, $\log_{10}$(TLC +1), $\log_{10}$(Neu +1), $\log_{10}$(Lym +1), or $\log_{10}$(Mac +1)]$_{ij}$ = μ + PARITY$_j$ + ADJMAST$_{ij}$ + MICSTATUS$_{ij}$ + MY$_j$ + COWQUARTER$_{ij}$ + ε$_{ij}$.

Final regression model for milk $\log_{10}$LDH within AQMS was:

$\text{Log}_{10}\text{LDH}_{ij}$ = μ + SEVERITY$_j$ + ADJMAST$_{ij}$ + COWQUARTER$_{ij}$ + ε$_{ij}$.

The explanatory variables QPOS$_{ij}$; QPOSCASE$_{ij}$; PREGSTATUS$_j$; CASEMICSTATUS$_j$; and interaction terms were not associated with any outcome variable. In the models which microbiological status of the AQMS was included [$\log_{10}$SCC, $\log_{10}$(TLC+1), $\log_{10}$(Neu+1), $\log_{10}$(Lym+1), $\log_{10}$(Mac+1)], the number of analyzed quarters were 143, due to quarters that were classified as contaminated and not included in the analyses. In the $\log_{10}$LDH and chloride models, the numbers of analyzed quarters were 131 and 150 respectively, due to contaminated samples and samples data that were lost.

Individual cow MY was the only explanatory variable associated with fat within AQMS (P = 0.001) and not showed in Table 1 (mean and standard deviation = 3.04% ± 1.58). Cows with moderate mastitis in the case quarter tended to had greater means of TP (3.22%) and greater LDH (54.95 U/L) in adjacent quarters as compared to cows with mild mastitis (2.99% and 32.36 U/L for TP and LDH, respectively) (Table 1) (P = 0.052 and 0.009 respectively for TP and LDH).

Milk samples from AQMS had greater mean lactose (4.76%) in first lactation cows as compared to cows with 2 or more lactations (4.46%) (P = 0.003) and tended toward greater SNF mean (8.75%) as compared to cows with 2 or more lactations (8.49%) (P = 0.092) (Table 1). Adjacent quarters from cows with 2 or more lactations were associated with a greater chloride content (1,257.23 mg/L) as compared to first lactation cows (1,002.31 mg/L) (P = 0.006). Cows with 2 or more lactations also had greater mean of SCC (512,861 cells/mL), TLC (398,107 cells/mL), neutrophils (245,470 cells/mL), and

macrophages (61,659 cells/mL) in AQMS as compared to first lactation cows SCC (213,796 cells/mL), TLC (199,526 cells/mL), neutrophils (123,026 cells/mL) and macrophages (26,915 cells/mL) [(P = 0.005, 0.035; 0.05, and 0.009, respectively for $\log_{10}$SCC, $\log_{10}$(TLC+1), $\log_{10}$(Neu+1) and $\log_{10}$(Mac+1)] and tended toward a greater lymphocytes count (67,608 cells/mL) as compared to first lactation cows (36,307 cells/mL) (P = 0.07).

Adjacent quarters which had a previous cases of clinical mastitis in the current lactation had lesser mean lactose and SNF (4.49% and 8.48%, respectively for lactose and SNF) as compared to AQMS without any previous cases of mastitis (4.74% and 8.76%, respectively for lactose and SNF) (P = 0.007 and 0.022, respectively for lactose and SNF). As compared to AQMS without any previous cases of clinical mastitis in the current lactation, AQMS that had a previous cases of mastitis in the current lactation had greater mean SCC (812,830 cells/mL and 134,896 cells/mL for AQMS with and without previous mastitis, respectively, P < 0.001), TLC (588,843 cells/mL and 131,825 cells/mL for AQMS with and without previous mastitis, respectively, P < 0.001), neutrophils (380,189 cells/mL and 79,432 cells/mL for AQMS with and without previous mastitis, respectively, P < 0.001), lymphocytes (97,723 cells/mL and 25,118 cells/mL for AQMS with and without previous mastitis, respectively, P < 0.001), and macrophages (87,096 cells/mL and 19,054 cells/mL for AQMS with and without previous mastitis, respectively, P < 0.001), and a tendency of greater means for chloride (1,218.92 mg/L and 1,040.61 mg/L for AQMS with and without previous mastitis, respectively, P = 0.058) and LDH (53.70 U/L and 33.11 U/L for AQMS with and without previous mastitis, respectively, P = 0.061) (Table 1).

As compared to AQMS with no growth of microorganisms in microbiological analyses, AQMS that had growth of microorganism in microbiological analyses had greater mean SCC (776,247 cells/mL and 144,543

cells/mL for AQMS with and without growth of microorganisms, respectively, $P < 0.001$), TLC (467,735 cells/mL and 169,824 cells/mL for AQMS with and without growth of microorganisms, respectively, $P = 0.005$), neutrophils (301,995 cells/mL and 100,000 cells/mL for AQMS with and without growth of microorganisms, respectively, $P = 0.004$), lymphocytes (74,131 cells/mL and 33,113 cells/mL for AQMS with and without growth of microorganisms, respectively, $P = 0.024$) and macrophages (63,095 cells/mL and 26,302 cells/mL for AQMS with and without growth of microorganisms, respectively, $P = 0.012$). Microbiological status of the AQMS had no associations ($P \leq 0.05$) nor tended toward any associations ($P \leq 0.1$) with composition analyses (fat, TP, lactose, SNF, chloride) and LDH (Table 1).

Table 1. Multivariate linear regression results from composition and udder health status (LSM ± SEM) of foremilk samples from 170 quarters adjacent to a single mammary gland affected with clinical mastitis of 59 from one large commercial Wisconsin dairy herd and cows and quarter variables categories which presented an association or tended toward and association with the outcome variable (continue)

		Outcome variable									
Explanatory variable	Categories	Total protein (%) n = 170	Lactose (%) n = 170	SNF (%) n = 170	Chloride (mg/L) n = 131	Log_{10} SCC[1] n = 143	Log_{10} (TLC+1)[1] n = 143	Log_{10} (Neu+1)[1] n = 143	Log_{10} (Lym+1)[1] n = 143	Log_{10} (Mac+1)[1] n = 143	Log_{10} LDH (U/L) n = 150
Mastitis severity in the case quarter[2]	Mild (n = 125)[3]	2.99[b†] ± 0.06									1.51[b] ± 0.06
	Moderate (n = 45)[3]	3.22[a†] ± 0.10									1.74[a] ± 0.08
Parity category[4]	1 (n = 64)[3]	3.01[b†] ± 0.09	4.76[a] ± 0.88	8.75[a†] ± 0.14	1,002.31[b] ± 88.79	5.33[b] ± 0.14	5.30[b] ± 0.14	5.09[b] ± 0.15	4.56[b†] ± 0.15	4.43[b] ± 0.13	
	>1 (n = 106)[3]	3.20[a†] ± 0.07	4.46[b] ± 0.78	8.49[b†] ± 0.11	1,257.23[a] ± 70.75	5.71[a] ± 0.12	5.60[a] ± 0.12	5.39[a] ± 0.13	4.83[a†] ± 0.12	4.79[a] ± 0.11	
Adjacent quarters with previous clinical mastitis[5]	No (n = 150)[3]		4.74[a] ± 0.67	8.76[a] ± 0.08	1,040.61[b†] ± 56.49	5.13[b] ± 0.09	5.12[b] ± 0.09	4.90[b] ± 0.09	4.40[b] ± 0.09	4.28[b] ± 0.09	1.52[b†] ± 0.05
	Yes (n = 20)[3]		4.49[b] ± 0.92	8.48[b] ± 0.13	1,218.92[a†] ±99.43	5.91[a] ± 0.18	5.77[a] ± 0.17	5.58[a] ± 0.18	4.99[a] ± 0.17	4.94[a] ± 0.16	1.73[a†] ± 0.11
Adjacent quarter microbiological analyses	Growth (n = 17)					5.89[a] ± 0.17	5.67[a] ± 0.16	5.48[a] ± 0.17	4.87[a] ± 0.17	4.80[a] ± 0.16	
	No Growth (n = 126)					5.16[b] ± 0.09	5.23[b] ± 0.09	5.00[b] ± 0.10	4.52[b] ± 0.10	4.42[b] ± 0.09	

[a-b]Means within a column, for an explanatory variable, with different superscripts differ ($P \leq 0.05$) or had a tendency (†) ($P \leq 0.1$).
[1]Cells per mL of milk.

Table 1. (conclusion)

[2]Severity of mastitis categories distribution for LDH: 106 with mild and 44 with moderate mastitis.

[3]Categories distribution for the outcome with highest number of quarters analyzed (total protein, lactose, and SNF).

[4]Parity categories distribution: Chloride (42 for 1; 89 for >1); Log_{10}SCC, Log_{10}(TLC+1) and leukocytes models (48 for 1; 95 for >1).

[5]Adjacent quarters with previous clinical mastitis cases in the current lactation categories distribution: Chloride (115 for no and 16 for yes); Log_{10}SCC, Log_{10}(TLC+1) and leukocytes models (127 for no and 16 for yes); Log_{10}LDH (115 for no and 16 for yes).

Farm characteristics, pre-enrollment characteristics of the cows and microbiological analyses of AQMS and case quarters were described elsewhere (Paixão et al., 2017). The greater LDH content of AQMS from cows presenting moderate clinical mastitis severity as compared to AQMS from mild clinical mastitis cows could be associated to the type of microorganisms affecting the case quarters, because gram-negative microorganism incidence were greater on moderate mastitis case quarters as compared to mild ones (data not showed), and deleterious effects of lipopolysaccharides membrane of these microorganisms may have caused a greater activation of the innate immunity response of these cows (Schukken et al., 2012) and released a greater concentration of LDH to adjacent quarters. Previous studies regarding the association of mastitis severity, milk composition and udder health status of AQMS is until now unavailable. Nyman et al. (2014) found a positive association between TP and LDH in composite milk samples from healthy cows, but no association between TP and SCC, in agreement with our results.

Foremilk samples of adjacent quarters from cows with 2 or more parities had a negative association with lactose, in agreement with Bezman et al. (2015), and this is associated with a greater SCC, as compared to foremilk quarters samples from heifers. Greater SNF in first parity cows is associated to greater lactose content, as compared to cows with 2 or more parities. The positive association between chloride and AQMS from cows with 2 or more parities is a result from blood serum components due to greater SCC on these quarters. Total leukocytes count, neutrophils and macrophages count had and association with parity, while lymphocytes tended toward an association, in disagreement with Pilla et al. (2013) that found no association between parity and leukocytes; whereas, Schwarz et al. (2011) also found an association between parity, macrophages and lymphocytes proportions, but no association with neutrophils. This greater number of neutrophils and macrophages cells in greater parity

AQMS could be associated with exposure of the mammary glands in previous lactations to environment pathogens and milking machines (Harmon, 1994; Neijenhuis, 2004). Our result suggests that cows with 2 or more parities had a greater initiation of immune response system, resolution of inflammation and tissue protection, due to a greater macrophages and neutrophils concentration (Oviedo-Boyso et al., 2007; Bratton and Henson, 2011).

No literature was found regarding the effect of repeated mastitis cases on cow's milk composition and udder health status at AQMS levels. Our results reveals that AQMS with a previous cases of clinical mastitis within the current lactation had a positive association with udder health parameters (SCC, TLC, neutrophils, lymphocytes, macrophages), a tendency of positive association with chloride and LDH, a negative association with lactose and SNF, and no association with fat and TP, as compared to AQMS without a previous cases of clinical mastitis. These changes in lactose, chloride and udder health parameters could be associated to adjustments in osmolarity during previous clinical mastitis cases (McManaman and Neville, 2003).

Quarters with growth of microorganisms in microbiological analyses had an association with greater SCC, TLC, neutrophils, lymphocytes and macrophages, but no associations with composition analyses, as compared to AQMS with no growth of microorganism, in agreement with Tomazi et al. (2015), which also relates a positive association between subclinically infection with CNS and greater quarter milk SCC, but no association with milk composition analyses, as compared to healthy contralateral quarters. No association was found between LDH and microbiological analyses, in disagreement Nyman et al. (2014), however, these authors have not considered an adjacent quarter infected with clinical mastitis and our results suggests that the severity of the clinical mastitis on the case quarter (mild or moderate) has an association with the LDH concentration on adjacent quarters.

Our results reveal that cow and gland explanatory variables have an effect in all analyzed outcomes, and suggest that these factors should be considered in future studies in the same topic. The lack of association between microbiological analyses and composition traits ($P > 0.1$) in AQMS is another evidence for the interdependency within mammary glands within a cow, and reveals that the differences on milk composition between control and case cows related by Paixão et al (2017) were not associated to AQMS microbiological status.

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ARTICLE 3 Milk composition and health status of quarters adjacent to an on-farm protocol treated clinical mastitis gland

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Milk composition and health status of quarters adjacent to an on-farm protocol treated clinical mastitis gland

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ABSTRACT

The theory of interdependency within cows' mammary glands has been established, and quarters adjacent to glands with clinical mastitis may have their composition and health status impaired. The main focus of this complementary study was to compare foremilk composition (fat, total protein, lactose, SNF, and chloride) and health status (SCC, differential leukocytes) of healthy mammary glands adjacent to a gland previously treated for clinical mastitis with foremilk samples of healthy mammary glands of healthy cows. The study was designed as a prospective case control study and enrolled cows (cases and controls) were matched by DIM and parity. Case cows (n = 50) were defined as cows that previously had a single quarter infected with mild or moderate clinical mastitis treated according to an on-farm protocol one day after mastitis identification. Case cows also had to remain in the herd and did not finish their lactation period

within at least 20 d after beginning treatment. Control cows (n = 50) were defined as cows that had not suffered clinical mastitis in the current lactation. Foremilk samples from each quarter of case and control quarters were collected in order to assess concentration of fat, total protein, lactose, SCC, SNF, microbiological analyses, chloride content, and differential leukocytes. Enrolled adjacent quarters (n = 147) were matched according to position with control quarters (n = 147). Multivariate linear regression and logistic regression analyses were performed in order to assess possible associations of milk composition and health status traits between quarter type (case or control). On average, 24 d after beginning treatment of the case quarter, milk composition (fat, lactose, SNF, and chloride) and some health status (lymphocyte and macrophage count) of adjacent quarters returned to similar levels as control quarters, while total protein, SCC, and neutrophils remained greater for adjacent quarters. No effect of quarter position or microbiological results of enrolled quarters were identified. Our results suggest that, at least 20 d (average of 24 d) after an episode of clinical mastitis in a neighboring gland, adjacent quarters are still recovering from this mastitis episode, and we conclude that interdependency of quarters is a physiological systemic two-way immune response route, and each particular milk component has a different behavior after clinical mastitis.

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

INTRODUCTION

Interdependence within quarters of a cow has been previously investigated, and changes in health status and milk composition of quarters adjacent to a subclinically infected quarter were reported (Bansal et al., 2005;

Hamann et al., 2005; Jensen et al., 2013), with greater impact for milk of quarters adjacent to a clinically infected quarter (Paixão et al., 2017).

Evidence for the theory of interdependence within cows' quarters' immune mechanisms can be observed in various studies, and not only in lactating cows. Quesnell et al. (2012), studying bacterial survival and immediate inflammatory response after an intramammary *Escherichia coli* bacterial challenge during late gestation of cows, identified that relative levels of the anti-inflammatory cytokine interleukin 10 were elevated approximately 3.5-fold within 12 h post challenge in all quarters (challenged and contralateral control quarters). Bouchard et al. (1999), studying nitric oxide production in induced *E. coli* mastitis, found peaks of SCC and nitric oxide at the same time on both infused and contralateral control quarters after infusion. The peaks of nitric oxide and SCC were greater in endotoxin-infused quarters as compared to control quarters. A rise in SCC and nitric oxide within contralateral quarters after infusion with saline solution were also observed, but the authors did not compare these outcomes between time periods (before and after infusion). According to van Amersfoort et al. (2003), gram-negative bacterial invasion often leads to a systemic inflammatory response. However, our previous study identified that whether or not a microorganism is recovered from the clinical mastitis gland, milk composition and health status are altered throughout the udder, irrespective of gland position and even for mild cases of clinical mastitis (Paixão et al., 2017). Studies regarding the mechanisms that trigger these immune responses on adjacent quarters during a clinical mastitis episode are not found in the literature.

This new information leads to a range of new questions. It is well known that selecting treatment based on on-farm culture results for cows with clinical signs of mastitis minimizes milk loss, new infections, loss of milk quality premiums, improves animal welfare, and avoids reproductive problems (Lago et

al., 2011; Pinzón-Sánchez and Ruegg, 2011; Fuenzalida et al., 2015). Because it is not known how long milk composition and health status of adjacent quarters remains impaired due to a neighboring infection, when using adjacent quarters in the same cow as the control studies may report an underestimation of variance and erroneous statistical results (McCullagh and Nelder, 1989; Schukken et al., 2003) even after mastitis treatment of the case quarter. The main focus of this complementary study was to investigate, by performing a case-control study in a single herd, if a single mild or moderate case of clinical mastitis in a cow still impairs milk composition (fat, total protein, lactose, solids non-fat, chloride) and health status (SCC, differential leukocytes count) of milk from unaffected adjacent mammary glands at least 20 d after beginning mastitis treatment of the case quarter, as compared to results from healthy mammary glands of healthy cows.

MATERIALS AND METHODS

Eligibility, Inclusion

The study was conducted on a commercial Wisconsin dairy farm and it is complementary data from Paixão et al. (2017). As previously described, the herd has 3,152 lactating Holstein cows housed in a freestall barn, with digested manure solids as bedding, fed a balanced TMR, milked 3 times per day in a 72 stall rotary parlor and with an average daily milk production of 33.6 kg per cow. Milking routine includes observation of foremilk for mastitis detection, pre dipping, drying of the mammary gland quarters and post dipping, respectively. The herd has a quarter level dataset for clinical mastitis, and SCC of individual cows is tested monthly. Average bulk tank milk values for total protein, fat, and SCC were 3.13%, 3.62%, and 301,000 cells/mL, respectively during the study period.

The study, designed as a prospective matched case-control, occurred between November 4 to December 30, 2015. Eligible criteria for cows assigned as cases were as follows: primiparous or multiparous; initially with 4 functional mammary gland quarters; a single quarter with a 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); remained in the herd and had not finished lactation period by at least 20 d after beginning mastitis treatment. Cows assigned as case that had lost the clinically infected quarter during the study period were also eligible to participate, however cows that had a new case of clinical mastitis in any other quarter than the current case were not eligible to participate. If cows had a previous case of mastitis, an interval of at least 13 days after the previous case was required to be included in the study.

Control cows were listed, assigned and selected as previously described by Paixão et al. (2017). The cows assigned as control for this complementary study are distinct from Paixão et al. (2017) control cows. Eligible criteria for control cows were as follows: 4 functional mammary gland quarters; same parity as cases, quarter position matched with case cows; within 30 DIM and same milking group as cases; no history of clinical mastitis within the current lactation. Information about all enrolled cows (parity, DIM, milk yield, pregnancy status, mastitis history, severity of mastitis, affected quarter, mastitis treatment, days of milk out of tank) were collected from a herd management software (Dairy Comp 305, Valley Agricultural Software, Tulare, California).

The College of Agricultural and Life Sciences Animal Care and Use Committee have approved this study, under the protocol number A005251.

Sample Size

In order to provide an excess of 95% confidence and 80% power to detect differences in fat of (0.35%), total protein (**TP**) (0.15%), lactose (0.1%), chloride (8.0 mg/100 mL), SCC (50,000 cells/mL of milk), and total leukocytes count (**TLC**) (50,000 cells/mL of milk) in foremilk samples, power calculations were performed. Based on the most limiting variable (fat), an estimated sample size of a minimum of 170 quarter foremilk samples per group was required (170 samples from quarters adjacent to a single clinically infected quarter and 170 samples from control quarters matched by position within case quarters), totaling 57 cows per group.

Farm Visits and Sample Collection

A total of 68 cows experienced clinical mastitis in a single quarter during the study period and were enrolled (potentially 68 cases and 204 adjacent quarters), but 18 case cows (18 case and 54 adjacent quarters) were not eligible to participate, because 3 case cows were dried off before 20 d after the beginning of the mastitis treatment, 3 were sold, 6 had no matched control cow, 5 had clinical mastitis in an adjacent quarter, and 1 had no information about mastitis severity in the herd management software, totaling 50 enrolled cows and 150 quarters per group (150 adjacent quarters from case and 150 quarters of control cows matched by position).

At least 20 d after beginning mastitis treatment of the cows assigned as case, researchers collected 3 foremilk samples during weekly farm visits from each quarter of case and control cows 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, and chloride (30 mL) and, 3. non-preserved sample used for determination of differential leukocyte count (10 mL). After collection of milk samples, the non-preserved

sample was immediately used to determine differential leukocyte counts using a commercially available test (QScout MLD, Advanced Animal Diagnostics, Morrisville, North Carolina). Aseptic foremilk samples from case cows after mastitis treatment and control cows were stored under refrigeration and along with bronopol preserved foremilk samples were immediately taken to the milk quality laboratory at UW Madison for further analyses. All foremilk samples enrolled were collected after verification of normal appearance of the milk and mammary gland during the milking routine and using the same order and preparation procedures between cows groups.

Clinical mastitis treatment

One day after detection of clinical mastitis in the case cows, mastitis treatment protocols were administered by farm personnel based on the on farm culture system and severity of the case (mild or moderate). The farm clinical mastitis treatment protocol included 3 FDA approved β-lactam drugs for intramammary use in lactating cows (cephapirin, ceftiofur, and amoxicillin). The farm culture system was based on growth on blood agar, MacConkey, and selective medium for *Streptococcus* spp.. Quarter milk samples that resulted in no growth of microorganisms on the on farm culture were not treated by the farm personnel unless at least 3 prior clinical cases resulted in no growth of microorganism within the same quarter and lactation. Farm culture results were not used in our study analysis and researchers did not interfere in any mastitis treatment protocols. Adjacent quarters milk samples from all enrolled cows were included in the statistical analyses, including quarters adjacent to cases that had no growth of microorganism (not treated by the farm personnel).

Microbiological Analysis

One day after the farm visit, frozen foremilk milk samples were thawed at room temperature and used to inoculate agar for identification of pathogens. All microbiological analyses were done according to NMC (1999) protocols and previously described by Paixão et al. (2017). 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). All microbiological analysis was performed at the UW Milk Quality Laboratory.

Determination of Differential Leukocytes Count

Determination of differential leukocytes count (total leukocyte, neutrophil, lymphocyte and macrophage count) (cells/mL) was performed immediately after collection of the non-preserved foremilk samples from enrolled quarters on the farm using a commercially available test based on image cytometry to differentiate cells by morphology (QScout MLD, Advanced Animal Diagnostics, Morrisville, North Carolina) and proportions were calculated as previously described by Paixão et al. (2017).

Assays for Content of Chloride

Chloride content was determined by thawing aseptic frozen milk samples at room temperature one day after the weekly farm visit, using the samples that were previously incubated for microbiological identification. An aliquot of 1.0 mL of thawed aseptic milk samples were used to access chloride content by colormetric titration method performed in a commercial chloride analyzer (Nelson-Jameson M926 Chloride Analyzer System, Nelson-Jameson Inc., Marshfield, Wisconsin) as previously described by Paixão et al. (2017).

Determination of SCC and Milk Composition

Analyses of total protein, fat, lactose and solids non-fat of the bronopol preserved foremilk samples enrolled were performed by a Fourier Transform InfraRed spectroscopy commercial equipment (MilkoScan FT+, FOSS, Hillerød, Denmark) and SCC was measured by flow cytometry (Fossomatic FC, Foss, Hillerød, Denmark) in a commercial DHI laboratory [AgSource Cooperative Resources International (CRI), Verona, Wisconsin] as previously described by Paixão et al. (2017).

Statistical Analysis

In order to achieve normal distribution of data, lactose values were squared; while SCC, TLC and absolute values of neutrophils, lymphocytes, and macrophages were converted using base-10 logarithms. Adjustments were also used for TLC and individual leukocyte population [Log_{10}(absolute value +1)]. The proportion of individual leukocytes populations were transformed to arcsine values to achieve normal distribution. Statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC) at a significance value of $P \leq 0.05$. The results were then back transformed to their original scale for a better understanding.

Two separate χ^2 analyses were performed using PROC FREQ in order to test the null hypothesis that parity (1^{st}, 2^{nd} or $\geq 3^{th}$), and pregnancy status (yes or no) were not associated with cow groups (cases or controls). The experimental unit was cow. Five separate ANOVA tests (one for each outcome) were performed using PROC GLM in order to test the null hypotheses that the continuous variables DIM, average daily milk yield for the previous 7 d (**MY**), last DHI test-day values for milk fat, TP and SCC (Log_{10}) did not have an association with cows assigned as cases or controls. The experimental unit of these analyses was the cow.

A χ^2 analysis were performed using PROC FREQ to test the null hypothesis that the proportion of quarter milk samples with no growth of microorganisms (as compared to microbiological growth) did not differ between quarters adjacent to a case at least 20 d after the beginning of mastitis treatment and control quarters. The experimental unit of these analyses was mammary quarter.

Thirteen multivariate linear regression analyses were performed with PROC GLM in order to test the null hypotheses that milk composition (1. fat; 2. TP; 3. lactose^2; 4. SNF; 5. chloride) and health status [6. Log_{10}SCC; 7. Log_{10}(TLC+1); 8. Log_{10}(Neutrophils+1); 9. Log_{10}(Lymphocytes+1); 10. Log_{10}(Macrophages+1); 11. $ARCSIN\sqrt{Neutrophils\,\%/_{100}}$; 12. $ARCSIN\sqrt{Lymphocytes\,\%/_{100}}$; 13. $ARCSIN\sqrt{Macrophages\,\%/_{100}}$] outcomes were not associated with quarter-type [adjacent to case quarters milk samples within at least 20 d after the beginning of mastitis treatment (**AQMS20**) or control quarters milk samples (**CQMS**)]. The initial models for each of the 13 outcome variables included: each previously cited response variable of the *i*th quarter, and the *j*th cow; a regression constant term (μ); quarter type (AQMS20 or CQMS) of the *j*th cow; DIM of the *j*th cow (continuous); parity (1st, 2nd, or ≥3rd) of the *j*th cow; MY of the *j*th cow (continuous); quarter position (left front, right front, left rear, right rear) of the *i*th quarter from the *j*th cow; microbiological analyses of the enrolled *i*th quarter of *j*th cow (growth or no growth); pregnancy status (yes or no) of the *j*th cow; a fixed effect to account for the correlation between the *i*th quarter within *j*th cow; and a residual variation (ε_{ij}). Parity and DIM variables (matched between case and control cows) were forced into all models.

The 13 final models were achieved as previously reported by Paixão et al. (2017) (all pairs of variables unconditionally associated with an outcome at $P \leq 0.25$ in the univariate analyses screened with PROC GLM were then offered to PROC MIXED multivariate linear regression models by forward and backward stepwise selection including confounding effects, first order interactions terms, and correlations among explanatory variables; Akaike information criterion was used in order to check the Goodness of fit of each model). Explanatory variables that were associated ($P \leq 0.05$) with the outcomes milk composition and health status and that remained in the final models were parity, quarter type, DIM, MY, and the random effect of quarter within cow. Microbiological analyses were not associated with composition outcomes, although they were associated with health status outcomes. However, due to a low proportion of quarters that resulted in growth of microorganisms, this explanatory variable was not included in any final models. The mammary quarter within cow was the experimental unit of these analyses.

A multivariate logistic regression model was performed with PROC GLIMMIX in order to test the null hypothesis that proportion of quarters with SCC ≥150,000 cells/mL (Djabri et al., 2002) was not associated with quarter-type (AQMS20 or CQMS). The initial model included: the response variable (SCC ≥150,000 cells/mL) of the *i*th quarter, and the *j*th cow; a regression constant term (μ); quarter type (AQMS20 or CQMS) of the *j*th cow; DIM of the *j*th cow (continuous); parity (1^{st}, 2^{nd}, or $\geq 3^{rd}$) of the *j*th cow; MY of the *j*th cow (continuous); quarter position (left front, right front, left rear, right rear) of the *i*th quarter from the *j*th cow; microbiological analyses of the enrolled *i*th quarter of the *j*th cow (growth or no growth); pregnancy status (yes or no) of the *j*th cow; a fixed effect to account for the correlation between the *i*th quarter within *j*th cow; and a residual variation (ε_{ij}). The final model were achieved as previously reported by Paixão et al. (2017) (all pairs of variable unconditionally

associated with the outcome at $P \leq 0.25$ in the univariate analyses screened with PROC GLM were then offered to PROC GLIMMIX multivariate logistic regression model by forward and backward stepwise selection including confounding effects, first order interactions terms, and correlations among explanatory variables; comparison of the - 2 Residual Log Pseudo-Likelihood was used in order to check the Goodness of fit of each model). Explanatory variables that were associated ($P \leq 0.05$) with the response variable and that remained in the final models were parity, quarter type, DIM, MY, and the random effect of quarter within cow. Microbiological analyses results were not included in the final models due to insufficient number of quarters with microbiological growth. The experimental unit of these analyses was quarter milk sample within cow.

RESULTS

Pre-enrollment characteristics of the cows

As expected by the matching criteria, parity categories (1^{st}, 2^{nd} or 3^{rd}) did not differ between cows groups ($P = 1.0$) (Table 1). Control cows were around 3 times more likely to be pregnant as compared to case cows ($P = 0.013$). The majority of the case cows had mild inflammation (74%), a greater incidence of front quarters (58%), and 58% of case cows had one or more previous cases of clinical mastitis in their current lactation at least 13 d before the study began, while none of the control cows had a previous case of clinical mastitis in their current lactation, as expected by eligibility criteria (Table 1).

During mastitis treatment of the case cows the mean number of days of milk out of tank was 8.4 (range 3 to 28). Days in milk and MY did not differ between cows groups ($P = 0.8$ and 0.21, respectively, for DIM and MY) (Table 1). The last DHI analyses for fat and total protein from composite milk samples did not differ between cows group ($P = 0.552$ and 0.975, respectively for fat and

total protein); while case cows had a greater composite SCC (407,380 cells/mL) as compared to control cows SCC (39,810 cells/mL) ($P < 0.001$) (Table 1).

Table 1. Descriptive characteristics of pre-enrollment characteristics of case cows (after at least 20 d within the beginning of mastitis treatment) and control cows enrolled in the trial.

Variables		Cow type (Enrolled)[1]				
		Case cows after treatment		Control cows		*P*-value
Categorical		n = 50	%	n = 50	%	
Parity	1	20	40	50	40	1.000
	2	14	28	14	28	
	≥3	16	32	16	32	
Pregnancy status	No	37	74	25	50	0.013
	Yes	13	26	25	50	
Severity (treated quarter)	Mild	37	74			
	Moderate	13	26			
Position (treated quarter)	Front	29	58			
	Rear	21	42			
Previous mastitis in current lactation (any quarter - excluding the current treated quarter for case cows)	0	21	42	50	100	
	≥1	29	58	0	0	
Continuous						
Days of milk out of tank during mastitis treatment (case cows)		8.4 ± 4.3				
Days in milk		203 ± 145		196 ± 125		0.800
Milk yield of cows (kg/d)		37.06 ± 11.67		39.60 ± 8.17		0.210
Fat (%)[3]		3.90 ± 1.02		3.77 ± 0.65		0.552
Total protein (%)[3]		3.15 ± 0.31		3.15 ± 0.33		0.975
Log_{10}SCC (cells/mL)[3]		5.61 ± 0.74		4.60 ± 0.55		< 0.001

[a-b]Means within a line with different superscripts differs by the ANOVA test ($P \leq 0.05$).

[1]Case - primiparous or multiparous cows, initially with four functional quarters, that presented mild or moderate clinical mastitis (Pinzón-Sánchez and Ruegg, 2011) after at least 13 days after the previous mastitis event and remained in the herd and had not finished lactation period within at least 20 d after the beginning of the mastitis treatment; Control - Four functional quarters; within 30 d in milk 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.

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

[3]Data recovered from the last Dairy Herd Improvement Association analyzes for all enrolled cows. This data is from composite milk samples collected for routine DHI analysis.

Microbiological analyses

Treated clinical case quarters. After at least 20 d within the beginning of the mastitis treatment, 87.5% of the non-contaminated case milk samples or non-culled case quarters (n = 32) had no growth of microorganisms, and only 3 different species were isolated in 4 case quarters milk samples (Environmental *Streptococci* spp. = 2; *Pseudomonas* spp. = 1; and yeast = 1) (Table 2). Contaminated samples and culled quarters together represented 36% of the total of outcomes for these enrolled quarters.

Quarters adjacent to a treated quarter. Of the potential total number of adjacent milk samples (n = 150 per group), 3 samples were missed or unusable, for a total of 147 milk samples analyzed. Of the non-contaminated adjacent quarters milk samples collected within at least 20 d of the beginning of mastitis treatment (n = 118), the majority (83.9%) had no growth of microorganisms. Coagulase negative *Staphylococcus* spp. represented 68.4% of the total microorganisms isolated in these samples (n = 13, of a total of 19 isolates) while Environmental *Streptococci* spp. represented 21% (n = 4, from a total of 19 isolates) (Table 2).

Control quarters. Of the potential total number of control quarter milk samples (n = 150), 3 samples were excluded due to matching criteria (the same matched quarters milk samples as the missed or unusable quarters from case cows after mastitis treatment), for a total of 294 milk samples analyzed (n = 147 quarters per group). Of the non-contaminated control quarter milk samples (n = 117), 93.2% had no growth of microorganisms, and 7 and 1 milk samples were identified with gram-positive and gram-negative microorganisms, respectively (Table 2). Coagulase negative *Staphylococcus* spp. represented 62.5% of the total microorganisms identified (n = 5). Control quarters milk samples were 2.61 times more likely to present no growth of microorganisms (n = 109) in

microbiological analyses as compared to adjacent quarters milk samples collected after mastitis treatment (n = 99) ($P = 0.026$).

Table 2. Microbiological results of treated and adjacent quarters of case cows within at least 20 to 39 d after the beginning of the clinical mastitis treatment and quarters of healthy cows matched by parity and DIM of cows from a large commercial dairy herd in Wisconsin.

Microbiological results[1]	Type of milk samples						*P*-value
	Treated quarters		Quarters adjacent to a treated[2]		Control quarters[2]		
	n	%[3]	n	%[3]	n	%[3]	
No growth[4]	28	87.5	99	83.9	109	93.2	0.026[5]
Gram positive							
Coagulase negative *Staphylococcus* spp.			13	11.0	5	4.3	
Environmental *Streptococci* spp.	2	6.3	4	3.4	1	0.9	
Gram positive *Bacillus* spp.					1	0.9	
Gram negative							
Gram negative *Bacillus* spp.			1	0.8	1	0.9	
Pseudomonnas spp.	1	3.1					
Yeast	1	3.1	1	0.8			
Subtotal	32	100.0	118	80.3	117	79.6	
Contaminated samples	10	20.0	29	19.7	30	20.4	
Culled quarters	8	16.0					
Total	50	100.0	147	100.0	147	100.0	

[1]No *Mycoplasma* spp. was recovered from any pool of samples.
[2]6 quarters were excluded from analyses due to lost samples and matching criteria.
[3]The proportion of "No Growth", "Gram positive", "Gram negative", and "Yeast", does not include contaminated samples nor quarters that were lost or culled.
[4]Includes "No growth" and "Non-significant growth".
[5]Two tailed χ^2 test between frequencies of "No Growth" and "Growth" (Gram positive, Gram negative and Yeast) in adjacent and control quarters.

Association of Foremilk Composition and Health Status Between Quarter Types

Due to exclusion criteria (18 cows were not eligible to participate and 6 milk samples were unusable) our sample size (147 quarters per group) was able to detected the following differences: fat of 0.38%, TP of 0.16%, lactose of 0.11%, chloride of 8.6 mg/100 mL, and SCC of 54,000 cells/mL. The number of quarters analyzed in all 5 models of composition and 8 models of health status was 147 quarters per group, for a total of 294 analyzed quarter foremilk samples. Of the total of 5 models analyzed for composition (fat, TP, lactose^2, SNF and chloride), only 1 (TP) had an association with quarter type (P = 0.022), and mean CQMS TP (3.23%) was greater as compared to mean AQMS20 TP (3.11%) (Table 3).

The total of 8 models analyzed for health status [cells/mL of SCC, TLC, neutrophils, lymphocytes, and macrophages; and percent of neutrophils, lymphocytes, and macrophages], 4 models had an association with quarter type (AQMS20 or CQMS) and 1 had a tendency of association (macrophage %) (P = 0.068) (Table 3). Quarters adjacent to case within at least 20 d after the beginning of mastitis treatment had greater mean SCC (57,544 cells/mL), TLC (58,884 cells/mL), neutrophil count (33,113 cells/mL), and neutrophil proportion (57.0%, range 16.6 to 84.1%), as compared to CQMS SCC (35,481 cells/mL), TLC (40,738 cells/mL), neutrophil count (20,893 cells/mL) and neutrophil proportion (53.7%, range 24.5 to 82.3%) (P = 0.014, 0.049, 0.027, and 0.031 for SCC, TLC, neutrophil count and neutrophil proportion, respectively) (Table 3). A tendency of lesser macrophage proportion was associated with AQMS20 (21.9%, range 3.7 to 66.7%) as compared to CQMS macrophage proportion (25.0%, range 1.6 to 64.7%) (P = 0.068). No association was found between proportion of quarters with SCC ≥150,000 and quarter type (P = 0.278).

Table 3. Multivariate linear and logistic regression results for composition and health status (LSM ± SEM) of milk samples from mammary glands adjacent to a single treated clinical mastitis quarter within at least 20 d after the beginning of mastitis treatment and quarters of control cows.

	Type of milk samples		
Multivariate analyses	Quarters adjacent to a treated[1] n= 147	Control quarters n= 147	*P*-value[2]
Fat (%)	2.51 ± 0.17	2.75 ± 0.17	0.299
Total protein (%)	3.11 ± 0.04	3.23 ± 0.04	0.022
Lactose (%)[3]	4.86 ± 0.61	4.82 ± 0.61	0.497
Solids non-fat (%)	8.90 ± 0.06	8.99 ± 0.06	0.326
Chloride (mg/L)	900 ± 30.70	918 ± 30.69	0.676
Log_{10}SCC (cells/mL)	4.76 ± 0.06	4.55 ± 0.06	0.014
Log_{10}(TLC+1) (cells/mL)	4.77 ± 0.06	4.61 ± 0.06	0.049
Log_{10}(Neu+1) (cells/mL)	4.52 ± 0.06	4.32 ± 0.06	0.027
Log_{10}(Lym+1) (cells/mL)	4.00 ± 0.06	3.86 ± 0.06	0.106
Log_{10}(Mac+1) (cells/mL)	4.03 ± 0.07	3.91 ± 0.07	0.219
Neutrophils (%)[4]	57.00	53.70	0.031
Lymphocytes (%)[4]	21.03	21.30	0.903
Macrophages (%)[4]	21.94	25.09	0.068
Quarters with SCC ≥150.000 (%)[5]	22.45	13.60	0.278

[1]Within 20 to 39 d after the beginning of the clinical mastitis treatment (mean = 24 d).
[2]*P*-values related to the "quarter type milk samples" outcome variable. Other explanatory variables included in all models were: DIM (continuous) (matched variable), parity (matched variable), and milk yield (continuous).
[3]*P*-values based on the squared values of lactose. LSD and SEM were back-transformed.
[4]*P*-values based on the formulae ($ARCSIN\ \sqrt[2]{Leukocytes\ \%/_{100}}$).
[5]*P*-value based on a multivariate logistic regression.

DISCUSSION

Characteristics of farm and cows

Our study farm had a greater number of cows but a similar daily milk production per cow and milk components as compared to the average dairy farm in WI (181 cows per herd; 33.75 kg/day, 3.1%, 3.76% for milk production per cow, total protein and fat, respectively) (USDA-NASS, 2016). As previously reported by Paixão et al. (2017), the bulk tank SCC had greater values as compared to the WI average (202,000 cells/mL) (USDA-NASS, 2016) and this

may be associated with the use of digested manure solids as bedding (Rowbotham and Ruegg, 2015).

Cows assigned for control in this study were about 3 times more likely to be pregnant as compared to case cows, and this statistical difference was not previously identified by Paixão et al. (2017). Control cows for this complementary study differ from our previous report, but pregnancy status of all cows was randomly assigned in both studies. We hypothesize that this may be due to the previously reported negative effect on reproduction of clinical mastitis in primiparous case cows (Fuenzalida et al. 2015). In both studies, enrolled cows parity category (1st, 2nd or 3rd) that have the greater concentration of cows were first lactation (40%) (and parity matched between cows group), but cows in the current study also had a greater DIM (20 to 38 more d and also matched between cows group) as compared to our preliminary study, which may also have amplified this effect because these cows had more days to implement reproduction programs and to observe the outcomes. The mean SCC of the last DHI analyses was also greater for case cows (407,380 cells/mL) as compared to control (39,810 cells/mL) and greater as compared to the same case cows before mastitis treatment (251,188 cells/mL) (Paixão et al., 2017). This greater composite SCC is somewhat expected, because the DHI analysis of case cows for this complementary study occurred simultaneously with the initial phase of clinical mastitis infection and in our previous report it was before mastitis event. The days that milk from case cows was withheld from the market (days of milk out of tank) was greater as compared to Lago et al. (2011) (5.2 to 5.9 d).

Microbiological results of case, adjacent and control quarters

Microbiological results of the enrolled case and adjacent quarters before mastitis treatment were described elsewhere (Paixão et al., 2017). Microbiological results of the treated quarters at least 20 d within the beginning

of mastitis treatments revealed that the majority had no microbiological growth and few still infected. Short term clinical mastitis treatment outcomes (incidence of clinical cures, bacteriological cures, new infections, treatment failures, and days to clinical cure) were recorded but it was not discussed in this study, because this explanatory variable is not common between cows groups (case or controls).

According to Middleton and Fox (2001), veterinarians have performed therapeutic dry-off with chlorhexidine or povidone-iodine infusion in quarters chronically infected with *Staphylococcus aureus* in order to cease lactation and to prevent mastitis outbreaks. In our study, *Staphylococcus aureus* was not isolated from any milk samples before (Paixão et al., 2017) or after mastitis treatment, but case quarters that were culled using chlorhexidine represented 16% of the all case quarters enrolled. Previous microbiological results revealed that these quarters had gram-positive microorganisms (n = 2), gram-negative (n = 2), no growth of microorganisms (n = 3) or contamination (n = 1) (data not shown). The indiscriminate culling of quarters in our study herd was not justified by a *Staphylococcus aureus* mastitis outbreak, nor was the drug used FDA approved, however, farm personnel reported that milk from these cows was withheld from the market for a period of time, in order to ensure consumer safety. This practice is of uncertain acceptability, as there is no information available on how long residues from these drugs remain in the milk.

Coagulase negative *Staphylococcus* spp. and environmental Streptococcus spp. were the main microorganisms isolated in both AQMS20 and CQMS (with a greater proportion of both microorganisms for AQMS20 as compared to CQMS). In a recent study with 10 Wisconsin dairy herds, Baumberger et al. (2016) identified that *Staphylococcus* spp. and *Streptococcus spp.* were the main microorganisms isolated in teat skin swabs from cows without clinical signs of mastitis before 2 different premilking preparation

(conventional or a teat scrubber). Coagulase negative *Staphylococcus* spp. are part of the normal teat skin flora and may enter the teat canal (Devriese and De Keyser, 1980) and some species are also found in the environment (White et al., 1989). According to Thorberg et al. (2009) CNS control is complex because of a wide range of species that may cause the infection.

Control quarters milk samples in our study were 2.6 times more likely to present no growth of microorganisms as compared to AQMS20. Factors such as crossed infections (from case quarter to adjacent quarter) and previous cases of clinical mastitis in the current lactation of case cows could be associated to the lesser proportion of no growth results from AQMS20 as compared to CQMS. However, due to the very low proportion of quarters classified as growth of bacteria, microbiological results were not included in the final models for health status and milk composition comparison between quarter types.

The high number of contaminated samples in all milk samples from our study is associated with the type of milking parlor (rotary moving parlor) which is harder to collect aseptic milk samples as compared to stationary parlors (Paixão et al., 2017).

Association of Foremilk Composition and Udder Health Status Between Quarter Types

Our preliminary study revealed that a single quarter with mild or moderate clinical mastitis alters milk composition and health status throughout the udder; and quarters adjacent to case quarters present similar symptoms as a very mild infection, due to physiological interdependence of quarters in a systemic interaction level and regardless of position (Paixão et al., 2017). Control cows assigned for this current study differs from our preliminary study, but CQMS composition and health status results were very similar between studies (fat = 2.54%; total protein = 3.25%; lactose = 4.83%; SNF = 9%;

chloride = 931.8 mg/mL; SCC = 37,153 cells/mL; neutrophil count = 21,378 cells/mL; lymphocyte count = 7,412 cells/mL; macrophage count = 8,510 cells/mL) (Paixão et al., 2017).

We identified that several milk composition traits (fat, lactose, SNF, chloride) of AQMS20 were improved as compared to adjacent quarters milk samples before mastitis treatment (**AQMS**) (Paixão et al., 2017) and had not differed as compared to CQMS (when controlling for DIM and parity of the cows) in this study. In other hand, some health status traits (SCC, TLC, neutrophil proportion and number of cells) and total protein content of AQMS20 still impaired and statistically differ from CQMS, but with a lesser degree of impact as compared to results previously reported for AQMS (Paixão et al., 2017). This degree of impact can be observed by comparing the *P* values of each outcome between studies, because the same explanatory variables were used and quarters from control cows had similar composition and health status results. In contrast with our previous study that reported a SCC mean of 104,712 cells/mL in AQMS, AQMS20 SCC mean was less than 100,000 cells/mL (mean = 57,543 cells/mL) a threshold that has been previously reported as unhealthy by some authors (Forsbäck et al., 2011; Schwarz et al., 2010; Bezman et al., 2015) but it still greater as compared to CQMS SCC (mean = 35,481 cells/mL).

Changes in milk composition of cows from same breed, parity and under same diet are commonly related to SCC changes (Paixão et al., 2017), and our results suggest that each composition trait responds differently after at least 20 d within the beginning of mastitis treatment. Our results showed that AQMS20 chloride content, which is associated with a rapid response when inflammation occurs (McManaman and Neville, 2003) returned to normal levels, but total proteins, SCC and neutrophils still greater as compared to CQMS. Dynamics of clinical mastitis inflammation based on foremilk compositional traits of adjacent quarters are not available in the literature, but our results suggest that after a

clinical mastitis episode, the synthesis of individual milk components in these quarters react in distinct ways, because some traits has a rapidly response and returned to normal levels, while others still altered as compared to healthy herd mates. As previously reported, a systemic (rather than local) effect was also observed in all models, because no outcome was associated with quarter position.

No association was found between quarter type (AQMS20 or CQMS) and fat content. A lower fat content in AQMS20 was observed as compared to our previous report for AQMS (3.07% of fat) (Paixão et al., 2017), in agreement with Forsbäck et al. (2009) which affirms that damages in mammary epithelial cells increases milk fat content due to reduced milk yield. The normal chloride content of AQMS20 as compared to CQMS also confirms that these quarters were not under alertness due to a neighboring infection. Our overall mean foremilk fat was greater as compared to foremilk samples of healthy quarters from Danish Holstein cows milked within a different interval period (12 h between milkings) (Nielsen et al, 2005); however, these authors identified that foremilk samples from healthy quarters cannot predict the entire milking fat content. Milk fat content has a tremendous variation including diet, herd, breed, DIM, parity and milk fraction analyzed. In our study the only variable that differ among quarters groups was a previous case of mastitis that were treated (at least 20 d before enrollment) in AQMS20, but milk from case quarters were not enrolled in the analyses, and the remaining adjacent quarters were matched by position within control cows quarters. The apparent raise in fat content along with an increase in SCC as previously reported by Paixão et al. (2017) for AQMS is detrimental to dairy industry, because SCC has a positive association with concentration of lipolytic enzymes that even after heat treatment still activated and contributes to form free fatty acids in milk and dairy products throughout the shelf life period (Li et al., 2014) and has been associated with

sensory defects in pasteurized milk (Santos et al., 2003), yoghurts (Fernandes et al., 2007) and specially on rich fat dairy products such as butter (McDaniel et al., 1969).

Mean TP variations were +0.04% and -0.02%, respectively, as compared to our previous results for AQMS and CQMS (Paixao et al., 2017), but this rise in AQMS20 TP was not enough to reach the normal levels as compared to CQMS because a negative association between TP and AQMS20 was identified. A previous study reported a greater mean foremilk TP in healthy quarters from healthy cows milked in a 12 h interval (Nielsen, et al., 2005) and the authors identified that foremilk samples differ within milk fraction analyzed (foremilk or entire milk) in both milking interval analyzed (6h or 12h). This fact was also noted by Bansal et al. (2005) which also identified differences in TP concentration between milk fraction analyzed. Total protein is the top rated milk component for grade A milk payment calculus and corresponds for about 65% of the total components income (Jesse and Cropp, 2008). Our sample size had enough power to detect small statistical differences in TP between quarter types, and all milk fractions were taken under the same circumstance, including potential confounders within cows. Considering a hypothetical situation that this difference of 0.12% between AQMS20 and CQMS TP remains constant between milk fraction (foremilk and entire milk), it will represent a loss of 0.078% (0.12% of 65%) in the total components income for dairy farmers even after 20 d within the beginning of clinical mastitis treatment of a mild or moderate single case quarter. Our results suggest that clinical mastitis is far more costly for producers and industry than previously reported.

Our lactose means were not associated with quarter type and AQMS20 that previously was associated with a lesser lactose content (AQMS) (Paixão et al., 2017) returned to normal levels. When cow's quarters are free from clinical or subclinical infections, lactose in milk is very stable and has a very low day-to-

day variation as compared to others milk components (Forback et al. 2010). This characteristic is associated to the regulatory osmotic function of lactose during milk ejection from alveolus (Ling et al., 1961). Lactose content in milk is inversely related to leukocytes and chloride content, because an influx of these blood components due to infections alters the osmolality of the soluble fraction of the milk and in order to maintain the normal osmolality the alveolus reduces the influx of lactose to the lumen (McManaman and Neville, 2003; Chavez et al., 2004). Our previous report found that this equilibrium between lactose and chloride is also altered in AQMS due to interdependency of quarters within a cow (Paixão et al., 2017) and our present results confirms this theory because the equilibrium was restored to normal levels after treatment, because chloride and lactose had no association with quarter type. A previous study found no association between lactose content of quarters from healthy Danish Holstein cows milked in a 12 h interval and milk fraction analyzed (foremilk or entire milk) but it had a lower lactose means as compared to our results (Nielsen et al. 2005). Our lactose results cannot be extrapolated for the entire milking because our milking interval was different (8 h) as compared to the Nielsen et al. (2005) (12 h), but it suggest that no economic loss in lactose content of AQMS20 was observed within the study period. The association between a lesser TP and AQMS20 was not enough to produce a statistically difference between quarters type SNF, but no inference can be made about SNF for the entire milking because milk TP, which represents almost half concentration of SNF, has an association with fraction analyzed (foremilk or entire milk) (Nielsen et al., 2005).

We observed a log reduction of 1-fold (91.18% of reduction) for AQMS20 SCC as compared to our previous reported for AQMS SCC; however, AQMS20 still had an association with a greater SCC as compared to CQMS. Control quarters mean SCC and proportion of quarters with SCC threshold

≥150,000 cells/mL were very similar as previous reported for this quarter type (37,153 cells/mL and 12.35% of quarters with SCC ≥150,000 cells/mL) (Paixão et al., 2017). No association was found between quarter type and SCC threshold ≥150,000 cells/mL, suggesting that the greater proportion of AQMS20 was not behaving as mild infected due to interdependency of quarters as previously reported by Paixão et al. (2017) which reported 30% of AQMS with SCC ≥ 150,000 cells/mL and a SCC mean of 104,712 cells/mL. Somatic cell count is the gold standard method in order to provide information about cow and quarter milk health status due to its greater sensitivity and specificity (Mollenhorst et al. 2010; Nyman et al. 2016). Total leukocyte counts mean values for both quarter types were similar to SCC mean values, and AQM20 also was associated with greater TLC as compared to CQMS. Considering that interdependency of mammary quarters has already been assessed by Paixão et al. (2017), this positive association between AQMS20 and SCC (and TLC) suggests that these quarters still are influenced by the last clinical mastitis episode on the case quarter within the study period.

A log reduction in all individual leukocytes count of AQMS20 were identified as compared to our previous report (AQMS) (Paixão et al., 2017): lymphocytes had the greatest log reduction (0.36; 56% of reduction), followed by neutrophils (0.3; 49% of reduction) and macrophages (0.24; 42.5%), respectively. Individual leukocytes count and proportions of leukocytes of CQMS were very similar between our studies. This log reduction of lymphocytes and macrophages of AQMS20 as compared to our previous study were adequate in order to return these traits to the same levels of healthy milk quarter samples from healthy cows (CQMS). Nevertheless, AQMS20 had an association with greater neutrophil count and proportion, and a tendency for lesser macrophage proportion. As previously reported, this tendency of reduced proportion of macrophages in AQMS20 is associated to an influx of neutrophils

in these quarters (Schwarz et al., 2011) due to interdependency of quarters (Paixão et al., 2017). Considering the time period within the beginning of treatment and our sampling date, this reduction of AQMS20 leukocytes levels is somehow expected. This variation in both proportion and count of leukocytes in AQMS20 are the most important evidence for the theory of interdependence of quarters after mastitis treatment, and suggests that these quarters still suffering an influence of the last clinical mastitis episode on the neighboring gland. The stimulus or suppression of immune response membrane receptors are controlled by lymphocytes (Nickerson, 1989; Sordillo et al., 1997); then macrophages act phagocytizing the invading pathogen, and recruits massively number of neutrophils in order to fight the infection (Oviedo-Boyso et al., 2007). Following this order, neutrophils content in AQM20 will be the latest leukocyte to return to normal levels after clinical mastitis treatment of the case quarter, but studies concerning milk composition and health status of adjacent quarter milk samples after mastitis treatment have not been previously described.

Our sample size was not designed to assess individual associations between clinical mastitis treatment outcomes and milk composition and health status results between quarter types. A greater sample size is required to satisfy the *Bonferroni* adjustment necessary for multiple comparisons tests, and further studies are required in order to fully understand these effects. Nevertheless, we cannot assume that the differences identified in SCC, neutrophils and TP between quarter types were associated to microbiological analyses results, because the proportion of quarters with microbiological growth was very low in both quarter types enrolled.

Our results are a very important find for design of experiments, and indicates that at least 20 d (mean of 24 d, varying from 20 to 39 d) within the beginning of mastitis treatment of the case quarter is not enough time for adjacent quarters milk samples SCC, protein and neutrophils to return to normal

levels (as compared to milk from healthy cows that had no previous clinical mastitis cases in the current lactation in any quarter). In order to answer the question of how long the adjacent quarter milk SCC, neutrophils and SCC still impaired due to a neighboring infection with clinical mastitis, further studies including a longer period after the beginning of mastitis treatment are required; however, this answer could be very difficult (or even impossible) to achieve in a commercial farm and even in a well-controlled experimental trial, because the incidence of clinical and subclinical mastitis in a herd is very difficult to predict due to a shift from subclinical to clinical infections and vice versa (Newbould, 1974). In order to obtain an unbiased data, none of the enrolled control and adjacent to case quarters should present a clinical mastitis case in this entire hypothetical study period, and in addition, all clinical mastitis case quarters should presented the same short term treatment outcome, and a efficacy of 100% of bacteriological cure is impossible to achieve. Our results indicates that the most confident and robust way to achieve powerful statistical analyses in future trials in this topic could only be achieved by changing the design of the experiment, and comparisons between cows that had a previous cases of clinical mastitis in the current lactation with cows that had no previous case should be avoided (if the objective of the study is to obtain a response variable that is not the effect of subsequent cases of clinical mastitis in adjacent quarter milk samples total protein, SCC or neutrophils). Future studies which aims to compare these outcomes (SCC, total protein, neutrophils number of cells and proportions) within adjacent quarters of cows that had a previous clinical mastitis case in the current lactation, should include a numerical explanatory variable (such as days since last mastitis event) and a categorical explanatory variables (such as number of previous mastitis event) in the models. Others explanatory variables (DIM and parity of the cows) and a proper sample size should also be accounted.

It is important to observe that none of the adjacent quarters enrolled in our study had clinical mastitis during the study period (5 of the case cows previously enrolled were excluded due to at least 1 clinical case of mastitis during the study period). However, short-term clinical mastitis treatment outcomes and incidence vary among farms and within cows on the same herd (Lago et al., 2011). The objective of this study was not to identify associations between short clinical mastitis treatment outcomes and milk composition and health status from adjacent and control quarters, but were to investigate in a practical manner (by not interfering in any clinical mastitis treatment protocol of the farm and including all short terms clinical mastitis treatment outcomes) which composition and health status traits of adjacent quarters milk samples still impaired (or not) after at least 20 d within the beginning of mastitis treatment of the case quarter as compare to milk samples from healthy herd mates.

Our results reveals new evidences for the theory of interdependency of quarters within a cow, and quarters adjacent to a treated one also reacts positively (with some limitations for SCC, total protein and neutrophils) in a systemic interaction level and regardless of position after at least 20d within the beginning of mastitis treatment of the case quarter. It is also important to highlight that the objective of our study was not to spread an indiscriminate use of antibiotics for clinical mastitis treatment, because an on-farm culture protocol was already established on the herd before the beginning of the study; but it was to check if (and if yes, which) milk composition and health status traits of neighboring quarters still impaired during this time period. Further studies including milk samples from the entire milking are required in order to fully understand the economic impact of a single clinical mastitis case both before and after clinical mastitis treatment on adjacent quarter milk composition and health status.

CONCLUSIONS

New evidences for the theory of interdependence of quarters were assessed by this prospective, matched case-control study performed with 294 quarters from 100 cows matched by DIM and parity on a single high production dairy herd. We observed that fat, lactose, solids non-fat, chloride, lymphocyte, macrophage count, and proportion of quarters with SCC ≥150,000 cells/mL of milk samples from quarters adjacent to an on-farm protocol treated clinical mastitis gland returned to the same levels and proportions as milk samples from unaffected herd-mates. Generally, at least 20 d within the beginning of mastitis treatment of case quarters, compositional traits and health status from adjacent quarters were improved as compared to before the beginning of treatment, but a lesser total protein, greater SCC and neutrophils (both proportion and count) were observed as compared to milk samples from unaffected herd-mates. As previous reported, we did not observed an effect of microbiological analyses nor quarter position in any tested outcome. Our results suggests that interdependency of quarters is a physiological systemic two-way immune response route, and each particular milk component has a different behavior after at least 20d within the beginning of a clinical mild or moderate mastitis case treatment, but this time period is not enough for SCC, TP, and neutrophils to return exactly to normal levels as of healthy herd-mates.

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APPENDIX

Table 1. Clinical mastitis treatments outcomes of singly affected quarters from 50 cows after at least 20 d within the begging of treatment classified within previous severity of infection and microbiological group from one large commercial Wisconsin dairy herd (continue)

Short terms treatment outcomes[1,2]	Severity of the clinical mastitis	All		Microbiological group[3]							
				Gram-positive[4]		Gram-negative and yeast[5]		No growth		Cont.[6]	
		n	%	n	%	n	%	n	%	n	%
Bacteriological cure	Mild[7]	14	58.3	2	50.0	1	100	11	57.9		
	Moderate[7]	5	62.5			2	66.7	3	75.0		
	All[8]	19	59.4	2	40.0	3	75.0	14	60.9		
Treatment failure	Mild[7]	7	29.2	1	25.0			6	31.6		
	Moderate[7]	3	37.5	1	100	1	100	1	25.0		
	All[8]	10	31.3	2	40.0	1	20.0	7	30.4		
New infection	Mild[7]	3	12.5	1	25.0			2	10.5		
	Moderate[7]										
	All[8]	3	9.4	1	20.0			2	8.7		
Subtotal	Mild[9]	24	48.0	4	8.0	1	2.0	19	38.0		
	Moderate[9]	8	16.0	1	2.0	3	6.0	4	8.0		
	All[9]	32	64.0	5	10.0	4	8.0	23	46.0		
Lost quarter (culled)	Mild[9]	6	12.0	1	2.0	2	4.0	2	4.0	1	2.0
	Moderate[9]	2	4.0	1	2.0			1	2.0		
	All[9]	8	16.0	2	4.0	2	4.0	3	6.0	1	2.0
Contaminated samples	Mild[9]	7	14.0	4	8.0			2	4.0	1	2.0
	Moderate[9]	3	6.0			1	2.0	2	4.0		
	All[9]	10	20.0	4	8.0	1	2.0	4	8.0	1	2.0
Total	Mild[9]	37	74.0								
	Moderate[9]	13	26.0								
	All[9]	50	100	11	22.0	8	16.0	29	58.0	2	4.0

[1]According to Lago et al. (2011);
[2]Short terms clinical mastitis treatment outcomes from 20 to 39 d after the beginning of the clinical mastitis treatment (mean = 24 d);
[3]Microbiological analyses of milk from enrolled case quarter at 24h after clinical mastitis detection;
[4]Includes Coagulase negative *Staphylococcus* spp. (n = 2) and Environmental *Streptococci* spp. (n = 9);
[5]Includes *Escherichia coli* (n = 1), *Enterobacteria* spp. (n = 1), *Klebsiella* spp. (n = 5) and yeast (n = 1);
[6]Cont. = Contaminated milk samples;
[7]Percentages calculated from the subtotal of mild or moderate cases analyzed within column;

Table 1. (conclusion)
[8]Percentages calculated from the subtotal of all mild and moderate cases analyzed within column;
[9]Percentages calculated from the total of cases analyzed (n = 50).