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Bovine subclinical mastitis reduces milk yield and economic return

J.L. Gonçalves[a,1], C. Kamphuis[b], C.M.M.R. Martins[a], J.R. Barreiro[a], T. Tomazi[a], A.H. Gameiro[a], H. Hogeveen[b,c], M.V. dos Santos[a,*]

[a] Department of Animal Nutrition and Production, School of Veterinary Medicine and Animal Sciences, University of São Paulo, Duque de Caxias Norte, 225, 13635900 Pirassununga, São Paulo, Brazil
[b] Business Economics Group, Wageningen University, Hollandseweg 1, 6706kN Wageningen, The Netherlands
[c] Department of Farm Animal Health, Utrecht University, Yalelaan 7, 3584 CN Utrecht, The Netherlands

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ABSTRACT

The effect of different pathogens was studied by evaluating the contralateral (healthy and infected) mammary quarters of 146 lactating cows. The impact of SM on economic return (quarter milk yield × milk price) was determined by applying milk payment estimates on milk collected from healthy vs. infected glands. Cows were considered infected when they had at least 2 out of 3 weekly composite SCC results > 200 × 10^3 cells/mL and a microbiological culture (**MC**) positive result from composite foremilk samples, collected in the third week of sampling. Infected cows were evaluated a second time within 15 days and had milk yield measured at the quarter level and foremilk samples collected by aseptic technique for analysis of MC, milk composition and SCC. Of the 611-composite milk samples, 397 (65%) were culture-negative, and 214 (35%) were culture-positive and the most frequent isolated bacteria were *Corynebacterium* spp. (7.9%), coagulase negative staphylococci (5.8%), *Staphylococcus aureus* (5.3%), *Streptococcus uberis* (4.6%), *Streptococcus agalactiae* (3.9%), other environmental streptococci (2.4%), Gram-negative isolates (2.4%), *Enterococcus* spp. (1.4%) and *Streptococcus dysgalactiae* (0.7%). A total of 55 pairs of healthy contralateral quarters (control) were compared, and no difference was observed between them when evaluating SCC, milk yield, fat and protein concentration and economic return. A total of 124 pairs of healthy had lower SCC (274.9 × 10^3 cells/mL) than infected contralateral quarters (SCC of 1038.5 × 10^3 cells/mL). At the quarter level, IMI caused by minor pathogens had no effect on SCC, milk yield and economic return. Subclinical mastitis caused by contagious and environmental pathogens increased SCC and decreased milk yield when compared with healthy contralateral quarters. Moreover, quarters infected by contagious pathogens had increased concentrations of milk protein and fat when compared with healthy contralateral quarters. Therefore, the milk economic return was lower in quarters with SM caused by environmental pathogens (US$ 0.18/quarter.milking) and contagious (US$ 0.22/quarter.milking) when compared with healthy contralateral quarters. The milk losses ranged from 0.07 kg/quarter.milking to 1.4 kg/quarter.milking and the economic losses ranged from US$ 0.02–0.4/quarter.milking according to the pathogen causing SM.

1. Introduction

Mastitis is one of the most common diseases of dairy cattle, present in both clinical and subclinical form. Subclinical mastitis (SM) is an non-symptomatic form of intramammary inflammation that affects 20–50% of cows in given herds, making this the most frequent form of mastitis (Forsback et al., 2009). The vast majority of mastitis is of bacterial origin, accounting for more than 90% of all mastitis diagnoses. Bacterial pathogens that cause mastitis are generally classified as either contagious or environmental, based upon their primary reservoir and route of transmission (Fox and Gay, 1993; Smith and Hogan, 1993). Bacterial infections cause damage to milk secretory epithelia of the mammary gland and affect the yield of total milk and milk components (Le Roux et al., 2003). This damage can even result in a permanent loss of the capacity to synthesize milk by the mammary tissue (Auldist et al., 1995). Since the dairy industry demands high quality milk (with low SCC and high fat and protein concentrations) for producing dairy products, the economic losses due to SM are a result of the quality deterioration and the reduced milk production (Halasa et al., 2007; Forsback et al., 2010).

Milk quality payment programs (**MQPP**) are strategies of dairy companies to motivate farmers to produce high quality milk (Botaro

* Corresponding author.
E-mail address: mveiga@usp.br (M.V. dos Santos).
[1] Present address: Business Economics Group, Wageningen University, Hollandseweg 1, 6706kN Wageningen, The Netherlands.

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et al., 2013) and previous studies suggested its effectiveness in influencing milk quality (Nightingale et al., 2008). Considering that, we believed that the MQPP could be used in the present study for simulating the milk price with the aim to determine the effect caused by SM pathogens on economic return (quarter milk yield × milk price). Furthermore, the contribution of a single infected mammary gland may overestimate the effect of mastitis at the cow level SCC (Bezman et al., 2015). On the other hand, when composite milk samples were evaluated, a single quarter with high SCC is often masked by the dilution effect from healthy quarters (Forsback et al., 2009; Blum et al., 2014).

Different methods have been used to evaluate the effect of intramammary infection (**IMI**) on milk yield (Hagnestam-Nielsen et al., 2009; Halasa et al., 2009; Tesfaye et al., 2010; van Asseldonk et al., 2010). The most commonly used method was based on SCC analyses for evaluation of the IMI at the herd, cow, or at the quarter level (Dürr et al., 2008; Hand et al., 2012; Bezman et al., 2015), or even between identical twin cows (Pearson et al., 2013). Coulon et al. (2002) compared concentrations of components from quarter milk samples of healthy and subclinically infected quarters from the same cows' udder but they only evaluated milk yield at the cow level. Forsback et al. (2009) compared milk yield of quarters among cows with different levels of SCC ($< 100 \times 10^3$cells/mL vs. $> 100 \times 10^3$cells/mL). Bezman et al. (2015) compared healthy quarters with quarters infected by coagulase negative staphylococci, *Streptococcus dysgalactiae* or quarters after infection by *Escherichia coli*. There are a few studies that have compared healthy mammary quarters vs. their contralateral quarters infected by *Corynebacterium* spp., a minor pathogen, of the same cow (LeVan et al., 1985; Gonçalves et al., 2016). In another study, milk samples were not analyzed at pathogen-specific level nor at cluster level but concentrations of fat, Na, Cl, and IGF-1 of fractionated milk (cisternal milk, quartiles of alveolar milk and residual milk) were higher, whereas those of lactose were lower when compared infected and contralateral healthy quarter (Bruckmaier et al., 2004). However, to the best of our knowledge, no study has reported the effect of SM caused by major pathogens on SCC, milk yield and composition by comparing healthy and infected contralateral mammary quarters. This approach could minimize confounding factors at both cow and herd level (such as the cow's immune status at the time of infection, management systems or environmental challenge) (Gonçalves et al., 2016).

The measurements at the mammary quarter level may be used to more accurately evaluate the impact of IMI on milk yield and composition of dairy cows. Considering the negative effect that IMI caused by specific groups of bacteria (contagious or environmental) have on quarter milk yield and composition (Coulon et al., 2002; Le Roux et al., 2003; Leitner et al., 2006; Forsback et al., 2009; Bezman et al., 2015), we hypothesized that, the methodology of complete and individual quarter milking allows the estimation of the production losses caused by IMI caused by major pathogens. Therefore, the aims of the present study were to: (1) evaluate the effect of SM on milk yield and composition by comparison of contralateral mammary quarters within cow and, (2) determine the effect of SM pathogens at quarter level on economic return (quarter milk yield × milk price).

2. Material and methods

2.1. Dairy herds and selection of cows

Ethics approval was obtained through the Ethical Committee on the Use of Animals of the School of Veterinary Medicine and Animal Science (University of São Paulo, Brazil, protocol number 3020/2013) before the commencement of the study. Lactating Holstein cows (n = 650) with average parity of 2.3 (SEM 0.03) and 191.9 (SEM 3.3) days in milk, from seven Brazilian dairy herds (located in the Midwest area of São Paulo State) and with no history of clinical mastitis within the preceding month were used in this study. The study covered a nine-month period (February to October 2014), in which quarter and composite milk samples of all enrolled cows were collected and analyzed for milk yield, concentrations of milk fat and protein, SCC, and microbiological culture (**MC**). To be selected for the study, herds were required to have cow identification and data recording systems, and had to apply a mastitis control program consistent with those established by the National Mastitis Council (NMC; http://www.nmconline.org). This included consistent use of pre- and postmilking teat dipping, application of dry cow therapy, periodic milking machine maintenance, and proper milking and intramammary treatment procedures. All lactating cows were housed in free-stall barn facilities. Cows were milked in parlors twice a day. The milking routine was similar on all farms. In all herds, cows were fed a total mixed ration (**TMR**) composed of corn silage, grain concentrate, and minerals. Water was available *ad libitum*. All farms were conventional milk producers with mean milk yield of 22.3 (SEM 0.2) Kg/cow.day before the sampling period.

2.2. Milk sampling and quarter milking

First, composite milk samples (pool of the four quarters) were collected from each lactating cow once a week for three consecutive weeks for measuring the milk composition (concentration of milk protein and fat) and SCC (Step1). Milk yield (Kg/cow.day) data was measured using the herd recording system. Information on parity and days in milk was also collected from the database of farms enrolled in the present study (Supplementary Table S1). During the third week of sampling, composite foremilk samples were collected using aseptic technique, following National Mastitis Council guidelines (Oliver et al., 2004). Before milking, teat ends were scrubbed with 70% ethanol and the first three squirts of milk were discarded. A total of 40 mL of composite milk (about 10 mL from each mammary quarter) from cows were collected in sterile tubes. Cows were defined as subclinically infected on the basis of at least 2 out of 3 weekly SCC results $> 200 \times 10^3$ cells/mL, measured on composite milk samples collected weekly, as well as having a positive MC result from composite foremilk samples, collected in the third week. Cows meeting criteria for SCC and having at least 3 measurements were selected and sampled a second time within 15 days and had quarter foremilk samples collected aseptically for MC as previously described (Step 2).

Individual quarter milk samples (Step 2) representative of the whole milking were collected from milk meters (MM6 DeLaval, Campinas, Brazil) for analyses of milk composition and SCC. Milk yields were measured in Kg at the quarter level during a morning milking. The measurement of milk yield was done by milking mammary quarters individually, using a bucket milking system, which was connected to the milking machine vacuum line. The equipment included a pulsator and a cluster of four liners connected to individual silicone tubes equipped with valves for vacuum release. Each teatcup was connected to a separate milk meter to estimate milk yield by quarter, which then drained into a common bucket. The milk meters were supported by a vertical steel bar connected to two horizontal steel bars welded to a platform cart transport (capacity 150 kg), and the stand center had a bucket with a capacity of 50 L. The system allowed the milk to flow separately from each mammary quarter to a milk meter and then into a bucket. After milking, quarter milk samples (40 mL) from the milk meter were collected into plastic tubes containing the antimicrobial Bronopol (2-bromo-2-nitropropane-1,3-diol) as preservative (0.05 g/100 mL milk), according to International Dairy Federation guidelines (IDF-FIL, 1995). Samples were kept refrigerated (4–7 °C) until they were transported to the laboratory for milk composition and SCC analysis.

2.3. Microbiological and milk composition analysis

Microbiological cultures of milk samples were performed according to National Mastitis Council guidelines (Oliver et al., 2004) with inclusion of acetoin test. Briefly, 10 μL of milk were inoculated on blood

agar plates with 5% defibrinated bovine blood. Inverted plates were incubated aerobically at 37 °C for 48 h and observed every 24 h for colony characteristics (shape, size, number, and color), hemolytic ability (presence and type). Gram stain, potassium hydroxide test (KOH) and catalase tests were performed to determine the morphology and differentiation between genera. Specific microbiology procedures are given in Supplementary Tables S2 and S3 according to Murray et al. (2003). All Gram-negative isolates were identified using Enterex® kit (Cefar Diagnósticos, São Pauo, Brazil). Concentrations of milk fat, protein and total solids were determined by infrared absorption, using a milk analyzer (Bentley 2000®, Bentley Instruments Inc., Chaska, MN, USA). The SCC was determined by flow cytometry using a high-capacity somatic cell counter (Somacount300®, Bentley Instruments Inc., Chaska, MN, USA).

2.4. Subclinical mastitis definition

Infected quarters were categorized according to the isolated bacteria into minor, contagious, environmental, and miscellaneous pathogen groups. Mammary quarters were considered to have IMI when milk samples showed an isolation of > 10 colonies (1000 cfu/mL) of minor pathogens (*Corynebacterium* spp. or coagulase negative staphylococci, **CNS**); > 3 colonies (300 cfu/mL) of environmental pathogens (environmental streptococci or Gram-negative); ≥ 1 colony (100 cfu/mL) of contagious pathogens (*Staphylococcus aureus* or *Streptococcus agalactiae*) and other pathogens as described by Dohoo et al. (2011). Non-*aureus* coagulase positive staphylococci (**CPS**), *Enterococcus* spp., *Nocardia* spp., *Prototheca* spp., *Trueperella pyogenes* and yeast were considered miscellaneous pathogens. Mammary quarters were considered healthy when they had no growth of bacteria after 48-h incubation of milk. On the other hand, quarters were considered subclinically infected when milk samples showed an isolation of significant bacterial colony numbers and SCC > 100 × 10^3 cells/mL.

2.5. Experimental design and statistical analysis

The effect of SM was analyzed by applying linear mixed models with the SAS® program (version 9.3; SAS Institute Inc., Cary, NC, USA) after testing for residual normality and homogeneity of variance. Milk yield, concentrations of milk fat and protein, SCC and economic return from healthy quarters vs. infected contralateral quarters within cow were evaluated per type of SM-causing pathogens and following categorization of the mastitis pathogens into one of four groups (minor, n = 45; environmental, n = 43; contagious, n = 27; and miscellaneous, n = 9). Specifically, the effects of SM on all tested variables were evaluated by first splitting the anterior and posterior contralateral mammary quarters in halves and then by calculating the difference of all variables evaluated between healthy vs. infected contralateral quarter and between right healthy quarters vs. left healthy contralateral quarters within cow. For all statistical analyses, significance was declared at P ≤ 0.05 and trends at P ≤ 0.1. The following statistical model was used:

$$Y_{ijklmn} = \mu + H_{i(\text{random})} + Q_j(C_k) + D_l + P_m + M_n + [(M_n \times Q_j(C_k)) + e_{ijklmn}$$

where Y_{ijklmn} was the dependent variable; μ is the overall mean; H_i was the herd (i = 1–7) that was considered as random effect; $Q_j(C_k)$ was the fixed effect of contralateral quarter (j = 1–2, front and rear quarters splitting in halves) nested within cow k; D_l was the days in milk (l = 62–483) as covariate in the model; P_m was the parity (m = 1–6) as covariate in the model; M_n was the presence or absence of subclinical mastitis (n = 1–5, negative, contagious, environmental, minor or miscellaneous pathogens; or n = 15, the SM-causing pathogens); $M_n \times Q_j(C_k)$ was the interaction between the fixed effects of contralateral quarter and infection status; and e_{ijklmn} was the random error term.

We also compared the mean differences of each tested variable (milk yield, concentrations of milk fat and protein, SCC and economic return) between two sets of data (Set A–Set B): (A) 55 pairs of healthy contralateral quarters and (B) 124 pairs of contralateral quarters (healthy vs. infected) within cow distributed by pathogen category (minor, n = 45; environmental, n = 43; contagious, n = 27; and miscellaneous, n = 9). The mean differences between these two sets were referred to as deltas (Δ). The deltas were calculated using the same dataset and linear mixed models as described previously, providing similar results. We did not describe the results on deltas in our results and discussion section but it was presented as a table to further illustrate the approach of contralateral quarters comparison.

Heterogeneity of variances was removed from all SCC data by converting SCC values into linear scores (LS) by the formula described hereafter (Schukken et al., 2003):

$$LS_{SCC} = Log_2\left(\frac{SCC}{100}\right) + 3$$

After that, SCC was presented as geometric mean for the results discussion.

2.6. Economic calculation of milk price and returns

At the quarter level, the milk price (**MP**) per liter was simulated using the MQPP for milk protein and fat from a commercial Brazilian dairy processing company. First, an average milk price base was calculated as the mean Brazilian milk price expressed per L/month using data from the past 20 years (IEA, 2015). Milk yields were converted to L/quarter.milking through the density of milk that was calculated by Fleischmann's formula (Fleischmann, 1896). The monthly milk prices were corrected using the following formula:

$$MP_{\text{corrected},\ t} = MP_{\text{nominal},\ t} \times \left(\frac{INPCJanuary_{2015}}{INPC t}\right)$$

where, $MP_{\text{corrected},\ t}$ was the milk price per liter in month t corrected to January 2015; INPC was the National Consumer Price Index from the Brazilian Institute of Geography and Statistics (**IBGE**) in 2015; $MP_{\text{nominal},\ t}$ was the milk price per liter in month t; $INPC_{January\_2015}$ was the index for January 2015; and $INPC_t$ was the index for month t.

The Brazilian base milk price ($MP_{\text{corrected,t}}$) was set at US$ 0.3/L (R$ 0.94/L), based on price data over the previous 20 years. After these preliminary calculations, we simulated the milk quality payment at quarter level using the concentrations of milk fat and protein at the quarter level that were considered for calculating bonus tracks and neutrality according to Supplementary Table S4. The final milk price (MP_f), considering the milk quality payment at quarter level, was calculated as the sum of the Brazilian base milk price and each adjustment due to quality premiums or penalties in milk price. Additionally, the economic return per milking at the quarter level was calculated using:

$$R_i = MP_{fi} \times MY_i$$

where: R_i was the economic return per milking from mammary quarter i (US$/quarter.milking); MP_{fi} and MY_i were the final milk price (US $/Kg) and milk yield (Kg/quarter.milking) from the mammary quarter i, respectively. The MP_{fi} and Ri were calculated in Brazilian currency (Real; R$) and were converted to US$ dollar (1 US$ ≡ 3.05 R$).

3. Results

3.1. Cow level results

A total of 1915 composite milk samples were collected during three weeks of sampling (week 1, n = 650; week 2, n = 654; week 3, n = 611) (Step 1). During the step 1 of milk sample collection, the percentage of composite milk samples with SCC < 200 × 10^3 cells/mL ranged from 22.7% to 69.1% across the seven farms (Supplementary Table S1). The MC results of composite milk samples collected during the third week (Step 1) are summarized in Table 1. All data of this manuscript are presented as means ± SEM. Of the 611 composite milk

Table 1
Bacteriological culturing results from analysis of composite milk samples (CMS, n = 611) and quarter milk samples (QMS, n = 584) from 7 dairy herds.

Microorganisms	No. isolates		Absolute frequency CMS (%)	Absolute frequency QMS (%)
	CMS	QMS		
No.	611	584	100	100
Negative culture	397	375	64.98	64.21
Positive culture	214	209	35.02	35.79
Minor pathogens	100	80	16.37	13.70
CNS[a]	72	34	11.78	5.82
Corynebacterium spp.	28	46	4.58	7.88
Environmental pathogens	50	59	8.18	10.10
Environmental Streptococci	47	45	7.69	7.71
Gram negative isolates	3	14	0.49	2.40
Contagious pathogens	41	54	6.71	9.25
Staphylococcus aureus	22	31	3.60	5.31
Streptococcus agalactiae	19	23	3.11	3.94
Miscellaneous pathogens	13	13	2.13	2.23
CPS[b]	6	1	0.98	0.17
Enterococcus spp.	3	8	0.49	1.37
Nocardia spp.	0	0	0.00	0.00
Prototheca spp.	1	0	0.16	0.00
Trueperella pyogenes	0	2	0.00	0.34
Yeast	3	2	0.49	0.34
Mixed culture (2 pathogens)	8	3	1.31	0.51
Contamination	2	0	0.33	0.00

[a] Coagulase negative staphylococci.
[b] Non-*aureus* coagulase positive stapholococci.

samples, 397 (64.9%) were culture-negative, and 214 (35%) were culture-positive. The most frequent of these MC positive composite sample results were minor pathogens (n = 100; 16.4%), followed by environmental pathogens (n = 50; 8.2%) and contagious pathogens (n = 41; 6.7%). Thirteen composite milk samples had bacterial growth of miscellaneous pathogens (n = 13; 2%). Mixed culture (presence of 2 pathogens in the same culture) and contaminated samples (more than 2 pathogens in the same culture) represented 1.6% of all composite milk samples.

3.2. Mammary quarter level analysis

3.2.1. Bacteriological culturing results

A total of 146 out of 214 lactating cows were considered as having a subclinical IMI, however 68 cows were culture-positive but did not met the criteria of have SCC > 200,000 cells/mL in 2 out of the 3 samples. Of all quarters sampled, 209 (35.8%) were culture-positive. Minor pathogens were isolated from 80 quarters (13.7%), environmental pathogens from 59 quarters (10.1%) and contagious from 54 quarters (9.2%). Miscellaneous pathogens were isolated in 13 quarters milk samples (2.2%) (Table 1). The most frequently isolated bacteria at the quarter level were *Corynebacterium* spp. (7.9%), followed by CNS (5.8%), *Staphylococcus aureus* (5.3%), *Streptococcus uberis* (4.6%), *Streptococcus agalactiae* (3.9%), other environmental streptococci (2.4%), Gram-negative isolates (2.4%), *Enterococcus* spp. (1.4%) and *Streptococcus dysgalactiae* (0.7%). Mixed culture (2 pathogens) represented 0.5% of all quarter milk samples submitted to MC (Table 1). Table 2 summarizes descriptive data from the 146 dairy cows that were selected for mammary quarter analysis according to the IMI status (step 2).

3.2.2. Comparison between healthy contralateral and infected quarters following categorization of the mastitis pathogens groups

From the 584 quarter milk samples, 55 pairs of healthy contralateral quarters were selected (control), and 124 pairs of healthy vs. infected contralateral quarters were selected and distributed according to the pathogen category (Table 3). As expected, no differences between healthy contralateral quarters were observed for the variables evaluated. There was no effect of SM caused by minor pathogens on milk yield, and concentration of milk protein and fat when compared with their healthy contralateral quarters. In addition, no significant difference of SCC, expressed as geometric mean (P = 0.1) was observed between healthy (208.8 × 10^3cells/mL) and contralateral quarters infected by minor pathogens (505.7 × 10^3cells/mL) (Table 3).

Healthy quarters had lower geometric mean SCC (207.2 × 10^3 cells/mL) than contralateral quarters infected by environmental pathogens (1278.7 × 10^3 cells/mL). Thus, healthy quarters had higher milk yield (3.6 kg/quarter.milking) when compared with contralateral quarter infected by environmental pathogens (3.1 kg/quarter.milking). We observed no effect of IMI caused by environmental pathogens on concentration of milk protein and fat when compared with healthy contralateral quarter (Table 3).

Healthy quarters had lower geometric mean SCC (250.9 × 10^3 cells/mL) than contralateral quarters infected by contagious pathogens (1623.4 × 10^3 cells/mL). Therefore, healthy quarters had higher milk yield (3.5 kg/quarter.milking) than contralateral quarters infected by contagious pathogens (2.8 kg/quarter.milking). Concentration of milk protein and fat was lower in healthy quarters than contralateral counterparts that were infected by contagious pathogens (Table 3).

There was no effect of SM caused by miscellaneous pathogens on milk yield, concentration of milk protein and fat, when compared with healthy contralateral quarters. However, healthy quarters had lower geometric mean SCC (171.3 × 10^3 cells/mL) than contralateral infected by miscellaneous pathogens (846.3 × 10^3 cells/mL) (Table 3).

The milk economic return was not reduced when healthy quarters were compared to contralateral quarters infected by minor pathogens. On the other hand, the economic returns were lower in quarters with SM caused by environmental (US$ 0.18/quarter.milking) and contagious pathogens (US$ 0.22/quarter.milking) when compared with healthy contralateral quarters. Mammary quarters with subclinical mastitis caused by miscellaneous pathogens tended (P = 0.1) to reduce the milk economic return (US$0.3/quarter.milking) when compared with healthy contralateral quarters (Table 3).

3.2.3. Comparison between healthy contralateral and infected quarters per type of SM-causing pathogens

An evaluation at pathogen-level was performed but we did not find reliable results due to the reduced number of isolates for some pathogens. Therefore, we presented data at pathogen-specific level only for pathogens with greater sample size (e.g., at least 10 observations). The milk losses ranged from 0.07 kg/quarter.milking to 1.4 kg/quarter.milking according to the pathogen causing SM (Fig. 1). The economic losses ranged from US$ 0.02–0.43/quarter.milking being higher in SM cases caused by *Enterococcus* spp. (US$ 0.43/quarter.milking) and *Staphylococcus aureus* (US$ 0.26/quarter.milking; Fig. 2).

4. Discussion

The purpose of this study was to determine the effect of IMI by various pathogen groups on milk yield and composition using comparison of infected vs. healthy contralateral quarters within cow. Additionally, we determined the economic return (quarter milk yield × milk price) at the quarter level using one simulation of the milk price by MQPP. Mammary quarters with SM caused by contagious and environmental pathogens increased SCC and decreased milk yield when compared with healthy contralateral quarters. Moreover, IMI caused by contagious pathogens increased concentrations of milk total protein and fat. Overall, the economic return, calculated as quarter milk yield × milk price, was lower in quarters with SM caused by environmental and contagious pathogens when compared to healthy contralateral quarters.

Pathogen isolations from selected herds had predominance of minor

Table 2
Descriptive data of dairy cows (n = 146) that were selected for mammary quarter analysis: parity, days in milk, components of milk and somatic cell count on the cow level according to intramammary infection causing pathogen.

Variables[a]	Minor[b]	Environment[c]	Contagious[d]	Miscellaneous[e]
No.	49	47	38	12
Days in milk	221 ± 171	175 ± 139	183 ± 123	228 ± 139
Parity	2.1 ± 1.2	1.9 ± 1.2	2.5 ± 1.2	2.0 ± 1.1
Milk yield[f]	24.3 ± 10.2	23.1 ± 9	17.5 ± 9.9	19.8 ± 7.6
Protein%	3.4 ± 0.5	3.3 ± 0.4	3.5 ± 0.5	3.7 ± 0.6
Fat%	3.6 ± 1	3.7 ± 0.9	4.0 ± 1	4.8 ± 1.1
SCC[g]	862.9 ± 265.6	730.4 ± 286.8	1058.2 ± 289.5	819.2 ± 395.9

[a] Variables were represented in average and standard error mean (±).
[b] *Corynebacterium* spp. and coagulase negative staphylococci.
[c] Enterobacteriaceae and environmental *Streptococcus*.
[d] *Staphylococcus aureus* and *Streptococcus agalactiae*.
[e] *Enterococcus* spp., *Nocardia* spp., non-*aureus* coagulase positive staphylococci, *Trueperella pyogenes* and yeast.
[f] L/day.
[g] Geometric mean somatic cell count (× 10^3cells/mL).

Table 3
Effect of pathogens groups causing subclinical mastitis on milk yield, composition and economic return using difference between 55 pairs of healthy contralateral quarters and 124 pairs of healthy-infected contralateral quarters distributed by groups of pathogens causing subclinical mastitis.

Variables*	Pairwise contralateral comparison					Residual Error	P-value
	Healthy[1]	Minor[2]	Environment[3]	Contagious[4]	Miscellaneous[5]		
No. Pairs	55	45	43	27	9	-	-
Milk yield[6] H	$3.95^{A,*}$	3.54^{A}	3.64^{A}	3.51^{A}	3.92^{A}	1.9839	0.0376
Milk yield[6] I	3.79^{A}	3.31^{A}	3.08^{B}	2.78^{B}	2.85^{A}		
Δ† Milk yield[6] losses	0.19^{a}	0.23^{ab}	0.61^{b}	0.70^{b}	1.04^{b}		
SCC[7] H	87.08^{A}	208.80^{A}	207.24^{B}	250.86^{B}	171.28^{B}	2789.10	0.0306
SCC[7] I	94.65^{A}	505.73^{A}	1278.71^{A}	1623.43^{A}	846.28^{A}		
Δ SCC[7]	-7.06^{a}	-150.00^{a}	-747.46^{b}	-1335.63^{b}	-705.10^{b}		
Concentration of milk components (g/100 g)							
Protein H	3.38^{A}	3.34^{A}	3.41^{A}	3.47^{B}	3.21^{A}	0.0328	0.0149
Protein I	3.37^{A}	3.36^{A}	3.45^{A}	3.59^{A}	3.27^{A}		
Δ Protein	0.01^{a}	-0.02^{a}	-0.05^{a}	-0.11^{b}	-0.06^{a}		
Fat H	3.56^{A}	3.34^{A}	3.49^{A}	3.47^{B}	3.21^{A}	0.3175	0.0161
Fat I	3.50^{A}	3.36^{A}	3.58^{A}	3.59^{A}	3.27^{A}		
Δ Fat	0.07^{a}	0.05^{a}	-0.10^{b}	-0.12^{b}	-0.07^{ab}		
Economic approach							
Economic return[8] H	1.2179^{A}	1.1256^{A}	1.1659^{A}	1.1291^{A}	1.2117^{A}	0.2010	0.0091
Economic return[8] I	1.2513^{A}	1.0498^{A}	0.9951^{B}	0.9027^{B}	0.9027^{A}		
Δ Economic losses[8]	-0.0429^{a}	0.0735^{a}	0.1790^{b}	0.2221^{b}	0.2984^{a}		

*Variables were represented in average and †Δ represents the adjust values of healthy quarter minus infected; H = represents the healthy quarters; I = represents the infected quarters, except for group Healthy[1], whose comparison was made between healthy contralateral quarters. Values per variable within a columns with different capital letters represents the difference between healthy quarter and their contralateral ($P < 0.05$). Values per variable within a row with different lowercase letters differ significantly at $P < 0.05$.
[1] Right healthy quarters were subtracted from left healthy contralateral quarter.
[2] *Corynebacterium* spp. and CNS.
[3] Enterobacteriaceae and environmental Streptococci.
[4] *Staphylococcus aureus* and *Streptococcus agalactiae*.
[5] *Enterococcus* spp., *Nocardia* spp., non-*aureus* coagulase positive staphylococci, *Trueperella pyogenes* and yeast.
[6] kg/quarter.milking.
[7] Geometric mean somatic cell count (× 10^3cells/mL).
[8] Economic return (quarter milk yield × milk price) = US$/quarter.milking.

pathogens, along with considerable contagious and environmental pathogens. These results are consistent with what we find in other dairy herds in Brazil and similar to reports on causes of SM in other studies. Coulon et al. (2002) evaluated the frequency of isolates causing SM in three herds in France by analyzing 501 quarters samples and reported higher isolation of CNS (13.1%) and *Staphylococcus aureus* (11.1%) than what was found in the current study; but lower isolation of *Corynebacterium* spp. (6.7%) and *Streptococcus uberis* (1.4%) causing SM. Interestingly, there was a lower isolation of *Streptococcus agalactiae* (3.9%) from milk samples evaluated at quarter level in the present study. There has been a recent trend of decreasing isolations of *Staphylococcus aureus* and *Streptococcus agalactiae* from SM due to the adoption of mastitis control, along with an increase in the relative frequency of CNS and environmental streptococci (Makovec and Ruegg, 2003; Taponen and Pyörälä, 2009; Tomazi et al., 2015). The frequency of Gram-negative pathogens isolated from mammary quarters with SM (2.4%) was similar to previous study (< 1%) (Coulon et al., 2002; Koskinen et al., 2010). Non-*aureus* coagulase positive staphylococci was not a frequently isolated pathogen in Brazilian farms, and because of the low incidence of this pathogen, we decided to include it into the miscellaneous group.

Our findings allowed evaluating the effect of IMI caused by various pathogen groups, using comparisons of contralateral quarters within cow. However, Hamann and Reichmuth (1990) described a possible compensatory yield of milk between quarters within an udder. Wever and Emanuelson (1989) found no evidence of the interdependence of udder quarters during their investigations of differential cell counts of milk cells. Contradictory results concerning the compensatory effect

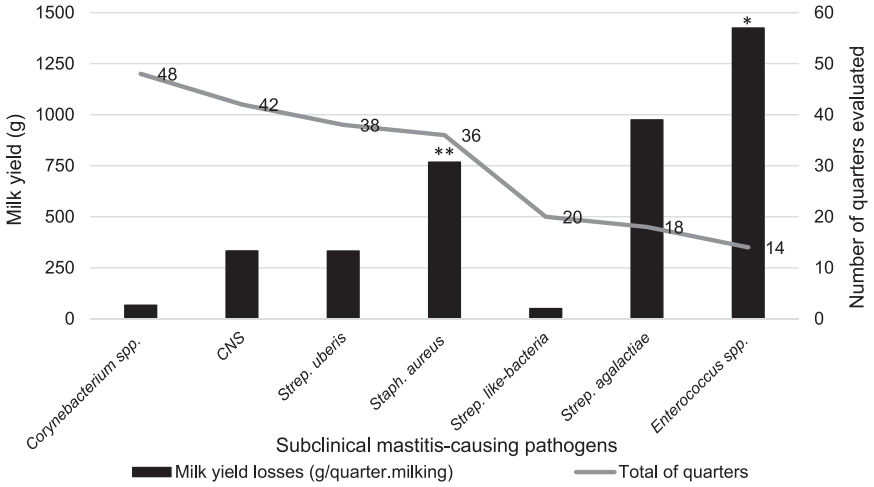

Fig. 1. Milk yield estimated by comparison between pairs of contralateral mammary quarters (healthy minus infected) Asterisk (*) represents significant difference ($P < 0.05$) and two asterisk (**) represents trend ($P < 0.10$).

between quarters have been previously reported by Merle et al. (2007). Coulon et al. (2002) reported that milk quarter evaluations by comparison of healthy controls in the same udder have advantages once optimized for individual animal effects (e.g., animal's genetic, physiological and nutritional characteristics). At least two studies have shown the validity of comparing contralateral quarters within cows. In a previous study, we compared sixty healthy contralateral quarters within cow using methods similar to those used here and, as in the present study, there was no difference in SCC, milk yield and composition (fat content, protein, casein, lactose, total solids and solids nonfat) between healthy contralateral quarters (Gonçalves et al., 2016). Berglund et al. (2007) compared healthy pairs of front and rear quarters with SCC $< 100 \times 10^3$ cells/mL and also did not observe any difference in milk yield.

In the present study, subclinical quarter IMI with minor pathogens had no significant effect on milk yield and composition (Table 3). This is in agreement with recent results from other studies that evaluated natural IMI (Gonçalves et al., 2016; Tomazi et al., 2015; Bobbo et al., 2017), in which *Corynebacterium bovis* and *Staphylococcus chromogenes* were most frequent minor pathogens causing SM. The impact of subclinical IMI by CNS and *Corynebacterium bovis* on milk yield and composition remain controversial (Rainard and Poutrel, 1982; LeVan et al., 1985). Some studies reported a significant negative effect of mastitis caused by CNS on milk yield (Grohn et al., 2004; Leitner et al., 2006). An interesting result of our study was that despite we did not observed statistical difference of milk yield between infected quarters by minor pathogens vs. healthy contralateral ones, Paixão et al. (2017) suggested the immune response to IMI in a single mammary gland quarter altered milk composition and SCC of unaffected mammary glands. In contrast, a recent study (Piepers et al., 2013) found a higher daily milk yield from heifers with subclinical CNS IMI (2.0 kg/d), as compared to non-infected heifers. It has been suggested that this might be attributed to a protective effect of the current CNS infection against a subsequent infection caused by a major pathogen (Piepers et al., 2013).

Mammary quarters infected by environmental or miscellaneous pathogens had similar concentration of milk protein and fat when compared to the healthy contralateral quarters (Table 3). However, milk protein concentration was higher in quarters with SM caused by contagious pathogen groups when compared to their healthy contralateral quarters. Similar to our results, Coulon et al. (2002) reported that quarters infected by *Staphylococcus aureus* had decreased milk lactose content and casein:protein ratio, when compared to their healthy contralateral quarters. Milk protein concentration is increased in quarters with IMI because inflammation in the gland increases permeability of the blood-milk barrier, leading to an increase in milk Na^+ and Cl^- and a concurrent efflux of lactose and K^+ into the bloodstream

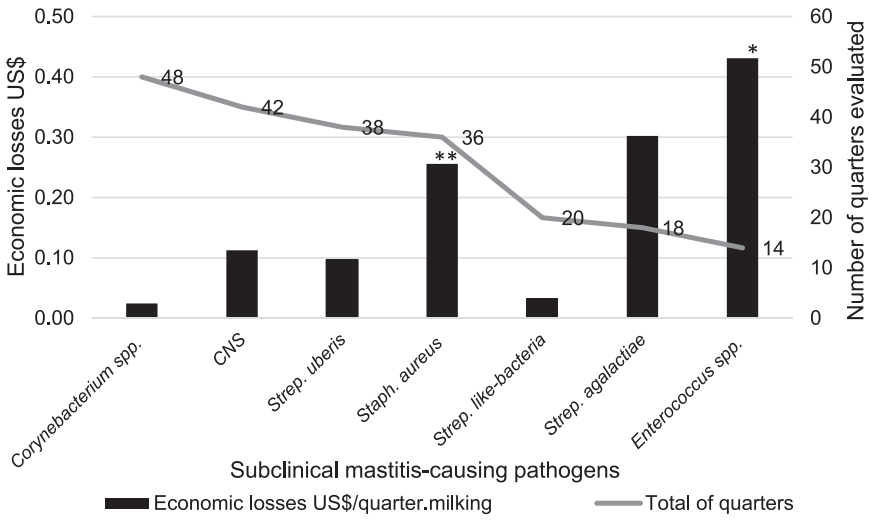

Fig. 2. Economic losses estimated by comparison between pairs of contralateral mammary quarters (healthy minus infected) using milk price simulation Asterisk (*) represents significant difference ($P < 0.05$) and two asterisk (**) represents trend ($P < 0.10$).

(Bansal et al., 2005). Lactose has a major osmotic regulatory function in milk and is a very stable component in milk (Forsback et al., 2010a). Associated with increased SCC, there is influx of whey proteins like bovine serum albumin and immunoglobulins. Additional changes in milk proteins include decreased casein synthesis by secretory cells and an increase in proteolytic enzymes in mastitis (Urech et al., 1999). The multiple impacts of mastitis on milk proteins concentrations makes payment on the basis of protein alone less than ideal, because casein levels are key to industrial yield (Auldist and Hubble, 1998). Moreover, mastitic milk has high concentration of proteolytic enzymes (i.e. plasmin) and the payment considering both milk protein and SCC levels would appear more useful.

In the present study, the concentration of milk fat was higher in quarters with SM caused by contagious pathogen groups than in their healthy contralateral quarters (Table 3). There are contradictory reports on the concentration of milk fat of mastitic milk (Kitchen, 1981; Auldist et al., 1995). Leukocytes have lipolytic enzymes produced in response to the IMI. Lipolytic enzymes cause damage to the membrane of milk fat globules, exposing it to the degradation by lipoprotein lipase in the milk, which leads to higher levels of free fatty acids in milk. Therefore, this high concentration of milk fat could be explained by a reduction in milk yield rather than by a decreased fat synthesis, suggesting only an apparent increase in the concentration of fat (Bansal et al., 2005).

Mammary quarters with SM caused by environmental or contagious pathogens reduced the milk yield by a total of 0.61 and 0.70 kg/quarter.milking, respectively (Table 3). Few studies have evaluated the effect of SM-causing pathogens on milk yield and composition at mammary quarter level (Coulon et al., 2002; Leitner et al., 2006; Bezman et al., 2015). Leitner et al. (2006) reported that mammary quarters infected by *Streptococcus dysgalactiae*, *Staphylococcus aureus*, *Staphylococcus chromogenes* and *Escherichia coli* had significantly higher SCC than in uninfected quarters. Their results indicated that quarters with IMI decreased the milk lactose content and increased the proteolysis of casein. Bezman et al. (2015) compared healthy quarters vs. quarters infected by CNS, *Streptococcus dysgalactiae* or quarters after infection by *Escherichia coli* and reported that the occurrence of IMI significantly affected SCC and milk lactose content ($g\,L^{-1}$). According to Bezman et al. (2015), quarter milk yield decreased by 20% in *Streptococcus dysgalactiae* and by 50% after infection by *Escherichia coli.*

To our knowledge, no previous experimental studies used the MQPP for simulating the milk price at the mammary quarter level with the aim to determine the effect of SM pathogens on economic return (milk yield × milk price). Regarding the pathogen groups evaluated at the quarter level in the current study, contagious and environmental bacteria reduced the economic return. Overall, considering the frequency of contagious (9.2%, 54/584) and environmental pathogens (10.1%, 59/584) causing SM described in the present study, farms would have a reduction of US$ 712.8 from their profit per month when they had contagious cases [(− 0.22 × $\%_{\text{contagious IMI quarters}}$) × two milking/day] and US$ 637.2 per month when they had environmental cases [(− 0.18 × $\%_{\text{environmental IMI quarters}}$) × two milking/day]. Extrapolating these data to one year, the farm's economic returns would be reduced by a total of US$ 8553.6 (contagious IMI) and US$ 7646.4 (environmental IMI) whether it was considered the percentage (average 10% per month) of IMI caused by both agents during the year. In the present study, the milk yield of mammary quarters was assessed from the point of a single milking per day, which is a limitation. We tried to establish an organized and controlled experimental design but some factors may have influenced the results of the present study. For that reason, it is noteworthy that factors as a sample size, absence of duplicate milk sampling for microbiological analysis and the possibility a potential carry-over effect of previous clinical mastitis may be considered as other limitations. The sample size was relatively small compared to larger studies using routinely collected data (Makovec and Ruegg, 2003), but relatively large compared with other studies at the quarter level (Bezman et al., 2015; Gonçalves et al., 2016; Tomazi et al., 2015). We chose for a within cow approach to be able to make a better effect estimation because we compared within cow contralateral quarters, so we automatically corrected for cow and time effect. According to Dohoo et al. (2011), triplicate or duplicate milk samples provided the best combination of sensitivity and specificity for IMI diagnosis, but compared with a single sample, provided only a modest improvement of specificity and little or no improvement of sensitivity. Although the benefits of duplicate samples are there, with a limited budget, it is better to have more animals with single samples than fewer animals with duplicate samples. Although the cows we have selected had not had clinical mastitis during the three weeks (step 1), some of our cows might have had clinical mastitis before we started sampling, especially for cows with *Staphylococcus aureus* IMI. This might have led to an overestimation of the production effect of *Staphylococcus aureus*.

In the current study, quarters infected with minor pathogens were found to have moderately increased SCC, but no effect on milk yield and economic return was observed (Table 3). Subclinical mastitis caused by contagious and environmental pathogens increased SCC and decreased milk yield when compared to healthy quarters. In general, the economic return was lower in quarters with SM caused by environmental and contagious pathogens when compared to their healthy contralateral quarters.

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Conflict of interest statement

The author(s) declare(s) that there is no conflict of interest regarding the publication of this article.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.livsci.2018.01.016.

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