JULIANO LEONEL GONÇALVES

Impact of subclinical mastitis on milk yield and economic return of dairy cows

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Impact of subclinical mastitis on milk yield and economic return of dairy cows

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CERTIFICADO

Certificamos que o Projeto intitulado "Impacto da mastite subclínica sobre custos de produção e qualidade do leite em rebanhos leiteiros", protocolado sob o nº 3020/2013, utilizando 2000 (duas mil) vacas, sob a responsabilidade do Prof. Dr. Marcos Veiga dos Santos, foi aprovado em reunião de 26/6/2013 e está de acordo com os princípios éticos de experimentação animal da Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo.

We certify that the Research "Impact of subclinical mastitis on cost of production and milk quality in dairy herds", protocol number 3020/2013, utilizing 2000 (two thousand) cows, under the responsibility Prof. Dr. Marcos Veiga dos Santos, was approved in the meeting of day 6/26/2013 and agree with Ethical Principles in Animal Research adopted by Ethic Committee in the Use of Animals of the School of Veterinary Medicine and Animal Science of University of São Paulo.

São Paulo, 16 de dezembro de 2013.

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DEDICATION

I dedicate this study to the two most important people in my life.
Those who always gave me affection and love, who were
by my side at all times and they taught me respect,
humility, discipline and commitment.
They are my pride and example.
I dedicate this thesis to my dear parents.

Dauri Gonçalves e Maria Aparecida Leonel Gonçalves.

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EPIGRAPH

"The Lord is my light and my salvation; whom shall I fear? The Lord is the stronghold[a] of my life; of whom shall I be afraid? (De Davi, Sl 27,1).

RESUMO

GONÇALVES, J. L. **Impacto da mastite subclínica sobre a produção de leite e retorno econômico de vacas leiteiras.** 2017. 149 f. Tese (Doutorado em Ciências) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2017.

Os objetivos gerais da tese foram avaliar: (i) os efeitos da mastite subclínica (MS) causada por patógenos primários sobre a CCS, contagem diferencial de células e produção de leite; (ii) perdas de produção de leite ocasionadas pela MS, em nível de vacas e quartos mamários; e (iii) o impacto econômico da MS causado por patógenos primários. A tese foi estruturada em quatro estudos. *No estudo 1*, amostras de leite de quartos mamários (n = 302) foram submetidas a cultura microbiológica (CM) e contagem diferencial de leucócitos (MLD). Quartos com resultados cultura-positiva apresentaram 102/156 (65,4%) amostras de leite MLD-positivas, e 28/135 (20,7%) das amostras de leite MLD-negativas tiveram CM-positivas. Quando a CM foi considerada o padrão-ouro para o diagnóstico da mastite, o diagnóstico por meio da MLD apresentou sensibilidade (*Se*) de 65,4% ($IC_{95\%}$ = 57,4 a 72,8%) e especificidade (*Sp*) de 79,3% ($IC_{95\%}$ = 71,4% a 85,7%). Em conclusão, o uso da MLD em vacas com CCS mensal > 200,000 células/mL para triagem de quartos identificou os mais prováveis de ser cultura-positivos. *No estudo 2*, o efeito de diferentes tipos de patógenos foi estudado avaliando pares de quartos mamários contralaterais (sadios e infectados) de 146 vacas em lactação. O impacto da MS sobre o retorno econômico (produção de leite × preço do leite) foi determinado pela aplicação de estimativas de pagamento do leite de quartos sadios e infectados. As perdas de leite variaram de 0,07 Kg/quarto.ordenha a 2,9 Kg/quarto.ordenha de acordo com o patógeno causador de MS. As perdas econômicas foram maiores em casos de MS causados por *Enterococcus* spp. (US$ 0,43/quarto.ordenha), *Strep. dysgalactiae* (US$ 0,74/quarto.ordenha) e *E. coli* (US$ 0,98/quarto.ordenha). Além disso, houve uma tendência de *Staph. aureus* e *Citrobacter* spp. ocasionar perdas de US$ 0,26 e 0,29/quarto.ordenha, respectivamente. Em geral, o retorno econômico foi menor em quartos com MS causada por patógenos ambientais e contagiosos (US$ 0,18 e 0,22/quarto.ordenha, respectivamente) quando comparados com os quartos contralaterais sadios. *No estudo 3*, um total de 146 das 650 vacas em lactação foram selecionadas de sete rebanhos por apresentar amostras compostas de leite com alta CCS (> 200.000 células/mL) e isolamento de patógeno primário causador de MS. Destas vacas

selecionadas, 1.436 amostras de leite de quartos foram coletadas durante três amostragens sucessivas com intervalos de 15-20 dias. A produção de leite em nível de quartos mamários foi mensurada por meio de ordenha completa e individual. Os isolados bacterianos foram identificados por CM, MALDI-TOF MS e sequenciamento parcial do gene 16S rRNA. As perdas de leite e os retornos econômicos variaram de acordo com o tipo de patógeno causador da mastite: - 0,24 a -0,87 kg/quarto.ordenha (*Streptococcus* ambientais) e -1,57 a -1,69 kg/quarto.ordenha (*Staph. aureus*). Em geral, os quartos mamários que apresentaram cura da MS causada por *Staph. aureus* e *Streptococcus* ambientais apresentaram aumento no retorno econômico de aproximadamente 0,47 e 0,69 US$/quarto.ordenha, respectivamente. *No estudo 4*, registros do controle leiteiro (n = 1.200.002) foram obtidos da associação Paranaense do gado Holandês, os quais incluíram dados de 92.560 vacas Holandesas em lactação de 781 rebanhos, de janeiro de 2010 a dezembro de 2015. Uma regressão segmentada foi ajustada para estimar o ponto de corte na escala Log_{10}CCS em que a produção de leite começou a ser afetada pela MS: 0.90 (~ 7.963 células/mL). Como conclusão, vacas de primeira cria apresentaram redução de 1,37 a 2,28 kg/vaca/dia na produção de leite para cada aumento de uma unidade Log_{10}CCS acima do ponto de corte, enquanto vacas com duas ou mais crias apresentaram perdas de 2,36 a 4,20 kg/vaca/dia. Em geral, os resultados desta tese indicaram que as perdas de leite dependem do tipo de patógeno que causa SM. Os patógenos primários mostraram maiores efeitos sobre a qualidade do leite do que quando foram observados pela abordagem com base nos resultados de cultura negativa ou positivos. A metodologia de avaliação do efeito da mastite subclínica sobre a produção de leite interfere na estimativa das perdas de leite e deve incluir fatores como DIM e número de paridade.

Palavras-chave: Mastite. Subclínica. Leucócitos. Perdas de produção. Retorno econômico.

ABSTRACT

GONÇALVES, J. L. **Impact of subclinical mastitis on milk yield and economic return of dairy cows.** 2017. 149 f. Tese (Doutorado em Ciências) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2017.

The general objectives of the present thesis were to evaluate: (i) the effects of subclinical mastitis (SM) caused by major pathogens on SCC, milk leukocyte differentials (MLD) and milk yield; (ii) milk yield losses caused by SM at the cow and quarter level; and (iii) the economic impact of SM caused by major pathogens. The thesis was structured in four studies. *In study 1*, quarter milk samples (n = 302) from 78 cows with SCC >200,000 cells/mL were analyzed by milk leukocyte differential (MLD) methodology and by microbiological culture (MC). Quarters with positive-culture results were obtained from 102/156 (65.4%) of MLD-positive milk samples, while 28/135 (20.7%) of MLD-negative milk samples were MC-positive. When MC was considered the gold standard for mastitis diagnosis, the sensitivity (*Se*) of the MLD was 65.4% ($IC_{95\%}$ = 57.4 to 72.8%) and the specificity (*Sp*) was 79.3% ($IC_{95\%}$ = 71.4% to 85.7%). In conclusion, the use of the MLD on cows with monthly composite SCC > 200×10^3cells/mL for screening at quarter level identified quarters more likely to be culture-positive. *In study 2*, the effect of different pathogens was evaluated by comparison of 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 versus infected glands. The milk losses ranged from 0.07 Kg/quarter.milking to 2.9 Kg/quarter.milking, and varied according to the pathogen causing SM. Economic losses were higher for SM caused by *Enterococcus* spp. (US$ 0.43/quarter.milking), *Strep. dysgalactiae* (US$ 0.74/quarter.milking) and *E. coli* (US$ 0.98/quarter.milking). Additionally, there was a trend for *Staph. aureus* and *Citrobacter* spp. to induce economic losses of US$ 0.26 and 0.29/quarter.milking, respectively. In general, the economic return was lower in quarters with SM caused by environmental and contagious pathogens (US$ 0.18 and 0.22/quarter.milking, respectively) when compared to their healthy contralateral quarters. *In study 3*, a total of 146 out of 650 lactating cows were selected from seven dairy herds for having composite milk SCC > 200,000 cells/mL in combination with the isolation of a major mastitis pathogen. From these selected cows, 1,436 quarter milk samples

were collected during three successive sampling occasions at intervals of 15-20 days. Quarter milk yield was measured by milking the mammary quarters individually using three successive milk samplings over time. Bacterial isolates were identified by microbiological culture, MALDI-TOF MS and partial sequencing of the 16S rRNA gene. Milk losses and economic returns varied according to the type of mastitis-causing pathogen: 0.24 to -0.87 kg/quarter.milking for environmental streptococci, and -1.57 to -1.69 kg/quarter.milking for *Staph. aureus*. Overall, mammary quarters that were cured from SM caused by *Staph. aureus* and environmental streptococci exhibited an increase in economic return of approximately 0.47 and 0.69 US$/quarter.milking, respectively. *In study 4*, test day records (*n* = 1,200,002) were obtained from the Paraná State Holstein Association, which included data from 92,560 lactating cows, from 781 herds, from January 2010 to December 2015. A segmented regression was fitted to estimate the cut-off point of Log_{10}SCC scale where milk yield started to be affected by mastitis: 0.90 (~7,963 cells/mL). In conclusion, first lactation cows have a reduction of 1.37 to 2.28 kg/cow/d of milk yield for each increase of one unit of Log_{10}SCC over the cutoff point, whereas second and later lactation cows are expected to have milk yield losses of 2.36 to 4.20 kg/cow/d for each unit increase of Log_{10}SCC over the cutoff point. Overall, the results of this thesis indicated that milk losses depend on the type of pathogen causing SM. Major pathogens have showed greater effects on milk quality than when it was observed using the approach of culture results of negative or positive. The methodology for evaluation of subclinical mastitis effect on milk yield interferes in the estimation of milk losses, and should include factors such as DIM and number of parity.

Keywords: Mastitis. Subclinical. Leukocytes. Milk loss. Economic return.

FIGURES LIST

Figure 1 - Production and consumption of milk in millions of liters, Agribusiness Projections – Brazil 2012/13 to 2022/23 24

Figure 2 - Milk production evolution, regarding the major Brazilian regions (1985-2015) 26

Figure 3 - Inspected milk production (federal, state and municipal), 2011 to 2015, per Brazilian region..... 29

Figure 4 - Front and rear pairs of contralateral mammary quarters..... 32

Figure 5 - Flowchart explaining the use of QScout® Farm Lab integrated to the herd routine 39

Figure 6 - Effect of stage of lactation (DIM1 = 4 to 100, DIM2 = 101 to 200 and DIM3 = 201 to 431) on milk macrophages%, lymphocytes% and phagocytes% 48

Figure 7 - Sensitivity and specificity evaluated at different threshold setting of QScout® MLD..... 49

Figure 8 - Flowchart of steps used for milk sampling..... 65

Figure 9 - Milk yield estimated by comparison between pairs of contralateral mammary quarters (healthy minus infected) 76

Figure 10 - Economic losses estimated by comparison between pairs of contralateral mammary quarters (healthy minus infected) using milk price simulation 77

Figure 11 - Identification of species of subclinical mastitis-causing bacteria based on ribosomal protein set, characterized by MALDI-TOF MS..... 100

Figure 12 - Effect of chronic subclinical mastitis caused by specific pathogens on (A) milk yield (Kg/quarter.milking), (B) linear score SCC and (C) economic return (US$/quarter.milking) versus healthy quarters using the comparison from assay 2: dynamic of the same quarter during three milk samplings distributed in categories of negative quarters ($N_1N_2N_3$) versus positive quarters ($P_1P_2P_3$), plotted as a line-graph, when $P < 0.05$ 106

Figure 13 - Effect of non-chronic subclinical mastitis caused by specific pathogens on somatic cell count ($\times 10^3$cells/mL) versus healthy quarters using the comparison from assay 3: dynamic of the same quarter during three milk samplings distributed in categories o of negative quarters ($N_1N_2N_3$) versus positive-negative-negative quarters ($P_1N_2N_3$), plotted as a line-graph, when $P < 0.05$ 107

Figure 14 - Effect of non-chronic subclinical mastitis caused by specific pathogens on somatic cell count ($\times 10^3$cells/mL) versus healthy quarters using the comparison from assay 3: dynamic of the same quarter during three milk samplings distributed in categories of negative quarters ($N_1N_2N_3$) versus positive-positive-negative quarters ($P_1P_2N_3$), plotted as a line-graph, when $P < 0.05$..... 108

Figure 15 - Somatic cell count mean per parity number and stage of lactation (n = 1,200,002 test day records)..... 120

Figure 16 - Milk yield mean per parity number and stage of lactation (n = 1,200,002 test day records)..... 120

Figure 17 - Estimates and standard error of the cutoff point (c) where milk losses associated with $Log_{10}SCC$ start for the Holstein cows in Brazilian herds from 2010 to 2015 (Parity 1 = solid line and Parities > 1 = dotted line)..... 124

Figure 18 - Estimates and standard error of the linear regression coefficients of expected milk loss per unit increase in $Log_{10}SCC$ (b2) for the Holstein cows in southeast Brazil from 2010 to 2015 (Parity 1 = solid line and Parities > 1 = dotted line). 126

Figure 19 - The expected daily milk losses: (A) and (B) milk losses% distributed in different groups of SCC according to parity 1 and group of parities > 1, respectively..... 128

TABLES LIST

Table 1 - Frequency of mastitis pathogen identification by microbiological culture of mammary quarter foremilk samples (n = 294) from two herds in North Carolina..... 45

Table 2 - Mean values for individual cell populations and combinations of cell populations from quarter milk samples considering the mastitis definition (n = 291)..... 47

Table 3 - Mean values for individual cell populations and combinations of cell populations from quarter milk samples considering the category of pathogens subclinical mastitis-causing (n = 291)..... 51

Table 4 - Data from all lactating cows (Step 1a) collected weekly on dairy farms (n = 7): number of lactation, days in milk, components of milk and somatic cell count at the cow level..... 63

Table 5 - Summary of steps used for identification of bacteria in the genus *Staphylococcus* spp..... 66

Table 6 - Summary of steps used for identification of bacteria in the genus *Streptococcus* spp. and *Enterococcus s*pp..... 66

Table 7 - The concentrations of milk fat and protein distributed in bonus tracks, neutrality and penalty..... 70

Table 8 - Bacteriological culturing results from analysis of composite milk samples (CMS, n = 611) and quarter milk samples (QMS, n = 584) from 7 dairy herds 72

Table 9 - 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 73

Table 10 - Effect of mastitis on milk yield, composition and economic return using difference between contralateral mammary quarters (n = 179 pairs) distributed by groups of pathogens causing subclinical mastitis..... 75

Table 11 - Total of steps performed in the current study and the performed variables..... 92

Table 12 - Classification of mammary quarters according to microbiological culture results..... 92

Table 13 - Assays and approaches used in statistical models for evaluating effect of mastitis..... 93

Table 14 - Microbiological culture results of mammary quarter milk samples (Step 2: sampling 1, n = 584; sampling 2, n = 470; and sampling 3, n = 382) from 7 dairy herds..... 97

Table 15 - Identification of the species that cause subclinical mastitis by MALDI-TOF MS of the quarter milk samples from three sampling periods (herds, n = 7) 98

Table 16 - Divergent results between microbiological cultures and MALDI-TOF MS, confirmed by 16S rRNA gene sequencing 99

Table 17 - Effect of chronic subclinical mastitis (PPP, n = 114) on milk yield, somatic cell count, and economic return versus non-chronic subclinical mastitis (PNN, n = 135; PNP, n = 39; and PPN, n = 66) 102

Table 18 - Effect of chronic subclinical mastitis caused by major pathogens (PPP, n=54) on milk yield, somatic cell count, and economic return versus non-chronic subclinical mastitis caused by major pathogens (PNN, n=66; PNP, n=15; and PPN, n=15) 103

Table 19 - Effect of chronic subclinical mastitis caused by environmental streptococci (PPP, n=24) on milk yield, somatic cell count, and economic return versus non-chronic subclinical mastitis caused by environmental streptococci (PNN, n=12; PNP, n=6; and PPN, n=9) 104

Table 20 - Effect of chronic subclinical mastitis caused by *Staph. aureus* (PPP, n=27) on milk yield, somatic cell count, and economic return versus non-chronic subclinical mastitis caused by Staph. aureus (PNN, n=3; and PNP, n=3) 105

Table 21 - Counts and descriptive statistics of test day records included in the study of milk losses associated with somatic cell counts in Brazilian dairy herds (2010 to 2015) 121

Table 22 - Results of the final model estimating milk losses of Holstein cows at parities 1 and greater than 1 at the stages of lactation (1, 2, 3…20), according to the SCC cutoff 100,000 cells/mL 129

ABBREVIATION LIST

APCBRH	Holstein breeders association of Parana
BMT	Bulk milk tank
CAMP	Christie, Atkins, and Munch-Peterson
CFU	Colony-forming unit
CMS	Composite milk samples
CMT	California mastitis test
CNS	Coagulase negative staphylococci
CONAB	National supply company
CPS	Coagulase positive staphylococci
DHIA	Dairy herd improvement association
DIM	Days in milk
DNA	Deoxyribonucleic acid
EML	Expected milk loss
EOC	Effective operational cost
FAO	Food and agriculture organization
GPD	Gross domestic product
IBGE	Brazilian Institute of Geography and Statistics
IDF	International Dairy Federation
IMI	Intramammary infection
INPC	National consumer price index
MALDI-TOF MS	Matrix assisted laser desorption ionization-time of flight mass spectrometry
MAPA	Brazilian ministry of agriculture, livestock and food supply
MC	Microbiological culture
MLD	Milk leukocyte differential
MP	Milk price
MQPP	Milk quality payment programs
MY	Milk yield
NL	Number of lactation
NMC	National mastitis council

OECD	Organization for economic cooperation and development
PCR	Polymerase chain reaction
PMNL	Polymorphonuclear leucocyte
QMS	Quarter milk samples
SCC	Somatic cell count
SCS	Somatic cell score
SD	Standard deviation
SEBRAE	Expert in micro enterprises and small businesses in Brazil
SEM	Standard error
SM	Subclinical mastitis
TC	Total cost
TLC	Total leukocyte count
TOC	Total operational cost
TMR	Total mixed ration
USDA	United States department of agriculture

SUMMARY

1 INTRODUCTION 23

1.1 BRAZILIAN MILK PRODUCTION 23

1.1.1 Milk production and dairy producer profile per Brazilian region 25

1.1.2 Dairy exports and imports 27

1.1.3 Inspected production and farm-gate prices 28

1.1.4 Milk production costs 29

1.2 THESIS JUSTIFICATION 30

1.3 HYPOTHESIS 33

1.4 GENERAL OBJECTIVES 34

1.5 SPECIFIC OBJECTIVES 34

2 USING MILK LEUKOCYTE DIFFERENTIALS FOR DIAGNOSIS OF SUBCLINICAL BOVINE MASTITIS 36

2.1 ABSTRACT 36

2.2 INTRODUCTION 37

2.3 MATERIALS AND METHODS 38

2.3.1 Animals and herds 38

2.3.2 Milk sampling 39

2.3.3 Microbiologic analysis 40

2.3.4 Somatic cell count 41

2.3.5 Milk leukocyte differential 41

2.3.6 Subclinical mastitis definition 42

2.3.7 Experimental design and statistical analysis 43

2.4 RESULTS 44

2.4.1 Microbiologic analysis 44

2.4.2 Somatic cell count and milk leukocyte differential 46

2.4.3 Performance of categorical analysis on instrument readout (negative or healthy vs. positive or infected) 48

2.5 DISCUSSION 51

2.5.1 Microbiologic Analysis 52

2.5.2 Somatic cell count and milk leukocyte differential 52

2.5.3 Performance of categorical analysis on readout (negative or healthy vs. positive or infected). 54

2.5.4 Comparison of MLD results for quarters following categorization by mastitis pathogen groups (minor, environmental, contagious and miscellaneous)..... 55
2.6 CONCLUSION..... 55
3 BOVINE SUBCLINICAL MASTITIS REDUCES MILK YIELD AND ALTERS COMPOSITION AT CONTRALATERAL MAMMARY QUARTER LEVEL WITHIN COW..... 58
3.1 ABSTRACT..... 58
3.2 INTRODUCTION..... 59
3.3 MATERIAL AND METHODS..... 61
3.3.1 Dairy herds and selection of cows..... 61
3.3.2 Milk sampling and quarter milking..... 62
3.3.3 Microbiological and milk composition analysis..... 65
3.3.4 Subclinical mastitis definition..... 67
3.3.5 Experimental design and statistical analysis..... 67
3.3.6 Economic calculation of milk price and returns..... 69
3.4 RESULTS..... 71
3.4.1 Cow level results..... 71
3.4.2 Mammary quarter level analysis..... 71
3.5 DISCUSSION..... 77
3.6 CONCLUSION..... 81
4 CHRONIC SUBCLINICAL MASTITIS REDUCES MILK YIELD AND ECONOMIC RETURN: AN EVALUATION USING SUCCESSIVE MILK SAMPLING OVER TIME..... 83
4.1 ABSTRACT..... 83
4.2 INTRODUCTION..... 84
4.3 MATERIAL AND METHODS..... 86
4.3.1 Ethics approval..... 86
4.3.2 Dairy herds and cow selection..... 86
4.3.3 Milk sampling and quarter milking..... 87
4.3.4 Microbiological and milk composition analyses..... 87
4.3.5 Bacterial species identification by MALDI-TOF MS..... 88
4.3.6 16S rRNA gene sequence analyses..... 89
4.3.7 Mastitis characterization and experimental design..... 90
4.3.8 Economic return..... 93
4.3.9 Statistical analyses..... 94
4.4 RESULTS..... 95

4.4.1 Effect of subclinical mastitis on milk production and composition by comparison of multiple samplings of mammary quarters 95
4.4.2 Identification by MALDI-TOF MS and sequencing of the 16S rRNA gene 95
4.4.3 Assay 1: effect of chronic subclinical mastitis on milk yield, somatic cell count, and economic return versus non-chronic subclinical mastitis. 100
4.4.4 Assay 2: effect of chronic subclinical mastitis caused by specific pathogens on milk yield, somatic cell count, and economic returns versus healthy quarters. 105
4.4.5 Assay 3: effect of non-chronic subclinical mastitis caused by specific pathogens versus healthy quarters on milk yield, somatic cell count, and economic return. 107
4.5 DISCUSSION 108
4.6 CONCLUSION 114
5 MILK LOSSES ASSOCIATED WITH SOMATIC CELL COUNTS PER PARITY AND STAGE OF LACTATION: A COW-LEVEL ANALYSIS. 116
5.1 ABSTRACT 116
5.2 INTRODUCTION 116
5.3 MATERIAL AND METHODS 118
5.3.1 Data 118
5.3.2 Statistical analysis 119
5.3.3 Post-analysis calculations 123
5.4 RESULTS 124
5.4.1 Cutoff point 124
5.4.2 Regression on days in milk, on parity and on Log_{10}SCC 125
5.4.3 Estimated losses 126
5.5 DISCUSSION 129
5.6 CONCLUSION 133
6 FINAL CONSIDERATIONS 135
REFERENCES 138
APPENDIX 147

Chapter 1

Thesis introduction

1 INTRODUCTION

1.1 BRAZILIAN MILK PRODUCTION

Brazil has displayed continuous growth of bovine milk production. During the last 20 years, production has more than doubled (103.1%), from 15.1 billion in 1991 to 30.7 billion liters of milk in 2010. According to the United States Department of Agriculture (USDA), Brazil was the sixth largest milk producer in the world in 2015, with an average of 35 billion liters, behind only the European Union, the United States, India, China and Russia (IBGE, 2015).

In the period from 1990 to 1995 the average annual growth of milk production was 2.6%, between 1995 and 2000 it was 3.7% per year. From 2000 to 2005, it grew 4.5% and, between 2005 and 2010, 4.6% (BRASIL, 2013). From 2013 on, Brazilian milk production has been expected to increase at an annual rate of 1.9% (Figure 1), which will correspond to the production of 41.3 billion liters of raw milk at the end of 2023 (Agribusiness Projections - Brazil 2012/13 to 2022/23), 20.7% higher than the production in 2013 (BRASIL, 2013).

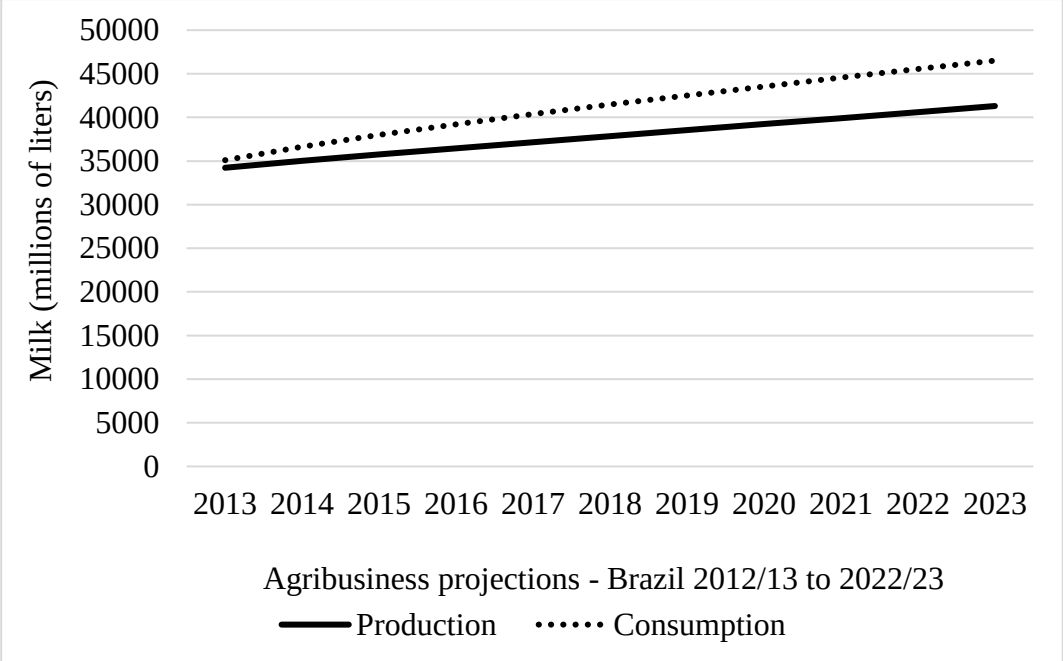
Since 2015 the Brazilian economy has been experiencing difficulties due to an economic crisis. Dairy farming registered an increase in production costs, as well as a reduction in the number of milked cows and milk production. There was also a drop in the price of milk paid to the producer, as well as a contraction in product acquisition by industries and exports of dairy products (IBGE, 2015). Regarding demand, a continued decline in Gross Domestic Product (GDP) by 3.8% in 2016/2017 is expected, with the loss of income and employment. While inflation remains at high levels, leading to reduction in dairy product consumption, this decline will lead to decreased production and encourage producers to reduce their costs (CONAB, 2016).

Even in the face of the economic crisis that began in 2015, the projections made by the Organization for Economic Cooperation and Development and Food and Agriculture Organization (OECD / FAO), Agricultural Outlook 2015-2024, indicate that Brazil has produced the following quantities of primary dairy commodities in 2016: 761.21 tons of cheese; 596.63 tons of whole milk powder; 163.89 thousand tons of skimmed milk powder; and 87.49 thousand tons of butter. Between 2016 and 2024, cheese production is expected to increase by

13.9% (+ 1.6% per year), reaching 867.15 thousand tons at the end of the period; total milk powder by 25.5% (+ 2.9% per year), reaching 749.03 thousand tons; skimmed milk powder by 6.9% (+ 0.8% per year), reaching 175.16 thousand tons; and butter by 7.0% (+ 0.8% per year), reaching 93.6 thousand tons (BRASIL, 2013).

According to the Brazilian Institute of Geography and Statistics (IBGE), the Brazilian population estimate for 2026 is 219 million. In order to supply the domestic market, milk production should be at least 37 billion liters, maintaining the same level of current consumption, which is approximately of 170 liters/inhabitant/year (ZOCCAL, 2016). Consumption should grow at an annual rate of 1.9%, consistent with the country's milk production. However, placing consumption at a level slightly above national production (Figure 1), a larger volume of milk may be required via imports, which would be close to 1.0 billion liters of milk by the year 2023, unless specific public policies for the dairy sector are implemented (BRASIL, 2013). The availability of milk per capita in Brazil is still low when compared to that of developed countries, where the purchasing power of the population is higher. The average consumption in these developed countries reaches 220 liters/inhabitant/year. To meet growing dairy consumption and population growth, the volume produced in Brazil in 2026 should reach the level of 48 billion liters (ZOCCAL, 2016).

Figure 1 - Production and consumption of milk in millions of liters, Agribusiness Projections – Brazil 2012/13 to 2022/23



Source: BRASIL, *Ministério da Agricultura, Pecuária e Abastecimento* - MAPA (Ministry of Agriculture, Livestock and Food Supply), Agribusiness Projections – Brazil 2012/2013 to 2022/23

1.1.1 Milk production and dairy producer profile per Brazilian region

In Figure 2, the milk production per region in Brazil, over the last 20 years, is described (IBGE, 2015). The South region is the first largest milk producing among regions since 2014, when it surpassed the Southeastern region for the first time, and was responsible for 35.2% of the Brazilian milk production in 2015. The Southeast region, in the second position, represented 34.0% of the total production. Minas Gerais (MG) is the leading milk producing State in the country, with 9.14 billion liters/year, representing 76.8% of the production in the Southeast region and 26.1% of the total national production. Paraná State (PR) surpassed Rio Grande do Sul (RS) and reached the second national rank. These two southern States together represent 75.2% of the regional production and 26.5% of the country's milk production. The fourth largest milk producer was the State of Goiás (GO), with 73.3% of production in the Midwest region and 10.1% of the total national production.

Milk production was a significant economic activity in 5,500 municipalities of Brazil in 2015. The largest was by Castro (PR), which reached 250.00 million liters, followed by the cities of Patos de Minas (MG), with 149.65 million liters, and Carambeí (PR), with 140.00 million liters (IBGE, 2015).

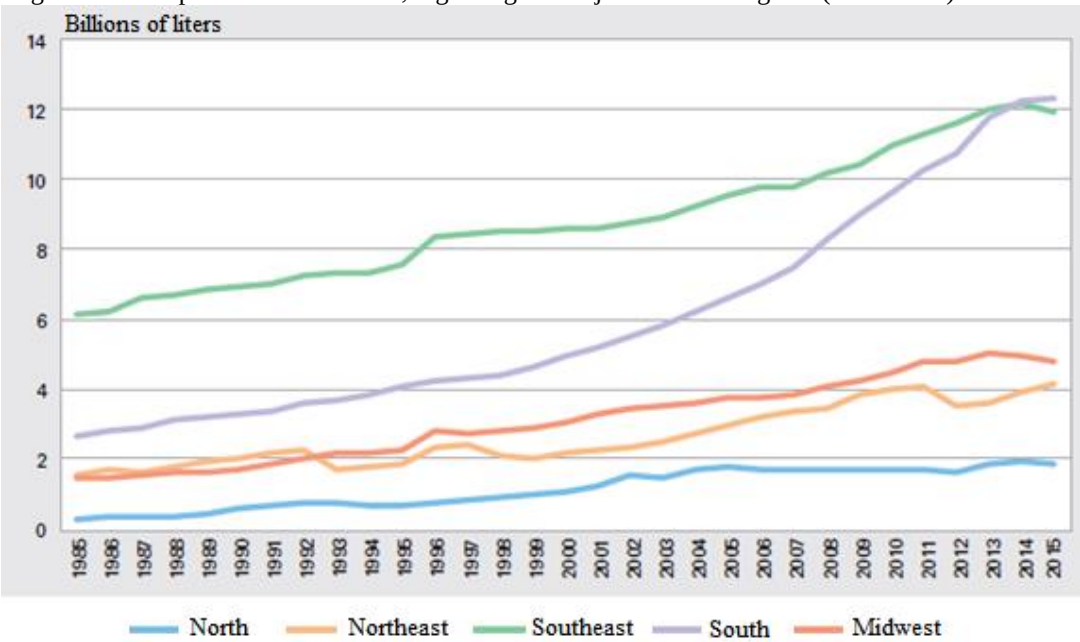
In 2015, milk production consisted of 35 billion liters, representing a 0.4% decrease when compared to the previous year (IBGE, 2015). Low prices, rising production costs and weakening domestic demand due to the economic crisis led to a decline in output in all regions of the country in 2015: North region (-13.1%); Northeast region (- 5.4%); Southeast region (- 0.7%); South region (- 0.9%); and Central-West region (-9.2%) (CONAB, 2016). As a result, among the twenty-seven States and the Federal District, only six increased their production in 2015: Rio Grande do Sul (+ 1.7%), São Paulo (+ 3.3%), Santa Catarina (+ 0.4 %), Rio de Janeiro (+ 5.5%), Pernambuco (+ 6.1%) and Acre (+ 5.0%) (BRASIL, 2013).

In 1996, milk production was present in 37.2% of all Brazilian agricultural establishments. In 2006, this number decreased nacionally to 25.8%, especially in the Southern region, which presented a greater reduction in the number of rural establishments. Between 1996 and 2006, there was a 2.9% decrease per year, which meant 46,900 fewer dairy farmers per year in Brazil (SEBRAE, 2013).

A total of 55% of the country’s dairy farms is maintained by confinement, in which lactating cows receive feed in troughs; 25% in semi-confinement, where the cows graze during

a period of the day, but spend most of the time in paddocks; and 20% in pastures. The most common breed in systems of milk production is Holstein (74%) followed by Girolando (31%) (SEBRAE, 2013).

Figure 2 - Milk production evolution, regarding the major Brazilian regions (1985-2015)



Source: IBGE, Research Directory, Agricultural Coordination, Municipal Livestock Survey (1985-2015)

Regarding the total number of dairy farms in Brazil, 29.8% consist of a herd of up to nine heads of lactating cows. Dairy farms with 10 to 99 lactating cows represent 59.7% of the total, while those with more than 100 heads of lactating cows correspond to 10.5%. Although a large number of farms appear in the stratum of herds composed of one to nine heads, these represent only 4.9% of the commercialized milk in the country. Only 34.9% of the total number of establishments in this stratum produce milk for sale, which means that a larger proportion of the milk is for the household's own consumption. The average milk production at these dairy farms is only 6.8 L/day. In terms of milk volume, the herds with 10 to 99 heads of lactating cows are responsible for most of the milk produced in the country (56.7%), and more than 75% of these establishments sell milk for the market. The farms that have 100 or more heads of lactating cows represent 38.4% of the milk produced in the country, and present the highest number of properties that commercialize the milk (87.1%), showing that this group are more market-oriented (SEBRAE, 2013).

With regard to herd productivity, milk production increased by 55.4% between the years 2000 and 2010, a result not only of the increase in herd productivity (21.3%), but also of the significant increment in the number of milked cows (28.2%) (SEBRAE, 2013). In 2015, a total of 21.75 million of dairy cows were milked, representing a decrease of 5.5% when compared to 2014. Regarding the total number of cattle in the country (n = 212.4 million), 10.1% were dairy cows. The region with the largest number of dairy cows was the Southeast, with 34.3% of the total. The reduction in the number of dairy cows was observed in all major regions of the country, mainly Northeast (-9.5%) and North (-6.7%). The increase in production costs, coupled with the low farm-gate price of milk, discouraged many producers from investing in production, leading several of them to dry their cows. According to the US Department of Agriculture (USDA), Brazil retained the third largest dairy herd, behind India and the European Union (IBGE, 2015).

Data from 2015 indicated that the average productivity in Brazil was 1,609 liters/cow/year, a 5.5% increase when compared to 2014. The South region presented the highest national productivity, 2,900 liters/cow/year, representing an increase of 3.9% when compared to the previous year's result. The States of the South region occupied the first three positions in terms of milk productivity - Rio Grande do Sul (RS) obtained the best indicator (3,073 liters/cow/year), followed by Paraná (PR) (2,840 liters/cow/year) and Santa Catarina (SC) (2.755 liters/cow/year). The city with the highest milk yield (liters/cow/year) was Araras (SP), where one of the largest dairy farms in the country is located, followed by Castro (PR) and Vila Flores (RS) (IBGE, 2015).

1.1.2 **Dairy exports and imports**

The vast majority of milk produced in Brazil is used for domestic consumption; and only 0.5% of the total production is exported as powdered milk, condensed milk, sour cream and butters. In addition, only 3% of all domestic consumption of milk and milk products comes from imports. In 2015, the primary origins of dairy imports were: Argentina (43.3% of the total imported value); Uruguay (43.2% of the total imported value); and the United States (4.0% of the total imported value). Fourteen other countries completed the remaining imported values. Among the 24 types of dairy products imported in 2015, whole milk powder accounted for

40.2% of the total value of imports, followed by skimmed milk powder (17.2%); and whey powder (6.7%) (CONAB, 2016).

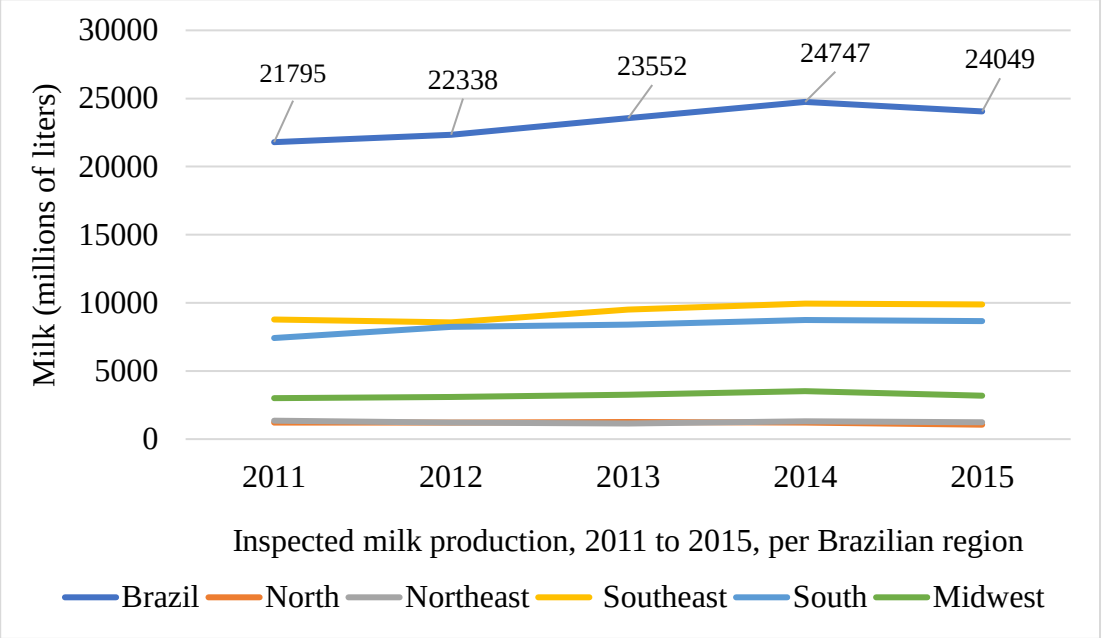
Exports in 2015 were directed to 42 countries, mainly Venezuela, which accounted for 77.8% of the total exported value. Subsequently, the remaining dairy exports were absorbed by the Saudi Arabian market (4.2% of annual exported value) and Angola (3.6% of annual exported value). Among the twenty-six types of dairy products exported in 2015, whole milk powder accounted for 76.8% of the total exported value in the year; followed by other products such as condensed milk, representing 13.5% of the annually exported value; and milk creams, which represented 4.1% of the total annual value exported (CONAB, 2016).

With the exception of the years 2005, 2007 and 2008, the trade balance of dairy products showed a deficit in the last decade. In 2015, exports declined by 8.1%, when compared to the previous year, to US$ 305.5 million, and imports decreased by 25.1% to US$ 402.1 million. As a result, the trade deficit reached US$ 96.6 million, a decrease of 9.0% when compared to the previous year's deficit of US$ 106.2 million. Currently, the Brazilian domestic market remains protected from subsidized imports of powdered milk by imposing *anti-dumping* measures on imports from the European Union (+ 14.8%) and New Zealand (+ 3.9%), valid until 02/05/2018, added to the rates of the Common External Tariff (CONAB, 2016).

1.1.3 **Inspected production and farm-gate prices**

In 2015, the national average farm-gate milk price was R$ 0.99/L, resulting in a production value of R$ 34.71 billion. The highest average price was found in the Northeast (R$ 1.18/L), while the lowest was in the North of the Brazil (R$ 0.87/L). According to IBGE (2015), national milk production under federal, state and municipal inspection declined by 2.8%, when compared to the previous year (CONAB, 2016). The difference between the total milk produced in Brazil (35 billion liters) and the amount of raw milk purchased by dairy industries under sanitary inspection (24.05 billion liters) reflects the national production of non-inspected milk (IBGE, 2015). Therefore, the production of inspected milk represented 68.7% of the total produced in Brazil. Figure 3 shows data regarding inspected (federal, state and municipal) milk production, from 2011 to 2015, per Brazilian region. The average monthly production under inspection in 2015 stood at 2.0 billion liters.

Figure 3 - Inspected milk production (federal, state and municipal), 2011 to 2015, per Brazilian region



Source: CONAB, *Companhia Nacional de Abastecimento* (National Supply Company), 2016

1.1.4 **Milk production costs**

Generally, milk production costs are divided in effective operational cost (EOC), total operational costs (TOC) and total costs (TC). The effective operational cost consists of the sum of all expenses disbursed by farmers. Total operational costs is a sum of effective operational cost with depreciations and familiar labor. The milk production costs, based on March/2016, surveyed by the National Supply Company (CONAB-Companhia Nacional de Abastecimento) in cities of the States of Minas Gerais (Ibiá and Pompéu), Rio Grande do Sul (Ijuí and Passo Fundo) and São Paulo (Guaratinguetá and Mococa), showed that the arithmetic mean of the variable cost in these six municipalities in the South and Southeast regions was of R$ 0.94/L; the operating cost was of R$ 1.12/L; and the total cost was of R$ 1.41/L. The total cost ranged from a minimum of R$ 1.23/L in Ijuí to a maximum of R$ 1.65/L in Guaratinguetá. The comparison of the monthly average real gross prices paid to the producer between April/2015 and March/2016, corrected by the IGP-M for March/2016, reveals that the farm-gate prices were sufficient to cover the average variable costs in the six surveyed counties. Regarding the comparison with operating costs, the prices covered these costs only in the cities of Ijuí and

Mococa. The average farm-gate prices did not cover the total costs of production in any of the six municipalities, jeopardizing the continuity of production (CONAB, 2016).

Given the current scenario of milk production in Brazil, one possible way to achieve the target of producing 40.3 billion liters of milk in 2023 will be the increase of cow productivity, associated with reducing production costs, training the medium milk producing properties (10 to 99 lactating cows) to a maximum. This should necessarily include strict sanitary management measures and reduction of mastitis and improvement programs of the Brazilian milk quality.

1.2 THESIS JUSTIFICATION

Bovine mastitis, defined as ‘inflammation of the mammary gland’, can have an infectious or noninfectious etiology. Microorganisms as diverse as bacteria, mycoplasma, yeasts and algae have been implicated as causes of bovine mastitis; however, bacterial infections are the most common causes of mammary gland inflammation (BRADLEY, 2002).

Mastitis is a common disease in dairy herds in many different countries. It can be challenging to deal with, as it is caused by a wide range of different pathogens (GRÖHN et al., 2004). Classically, mastitis pathogens have been classified as either contagious or environmental. In essence, the contagious pathogens can be considered as microorganisms that have adapted to survive within the host, in particular within the mammary gland. They are capable of establishing subclinical infections, which typically manifest as an elevation in the somatic cell count (SCC) of milk from the affected quarter; they are generally spread from cow to cow at or around the time of milking. In contrast, environmental pathogens are best described as ‘opportunistic invaders’ of the mammary gland, not adapted to survival within the host; usually, they invade, multiply, engender a host immune response and are rapidly eliminated (BRADLEY, 2002).

Mastitis pathogens have also been classified as either major or minor, according to somatic cell count response. In general, the major contagious pathogens are *Streptococcus agalactiae*, *Staphylococcus aureus* and *Mycoplasma* sp. (KEEFE, 2012); as well as major environmental pathogens, such as *Streptococcus uberis*, *Streptococcus dysgalactiae*, and *Escherichia coli*. These pathogens elicit a greater somatic cell response than the minor ones, such as *Corynebacterium* species and coagulase-negative staphylococci. Infections involving

the major pathogens are more likely to result in an SCC level in cows of over 200,000 cells/mL when compared to the minor ones (BRADLEY; GREEN, 2005).

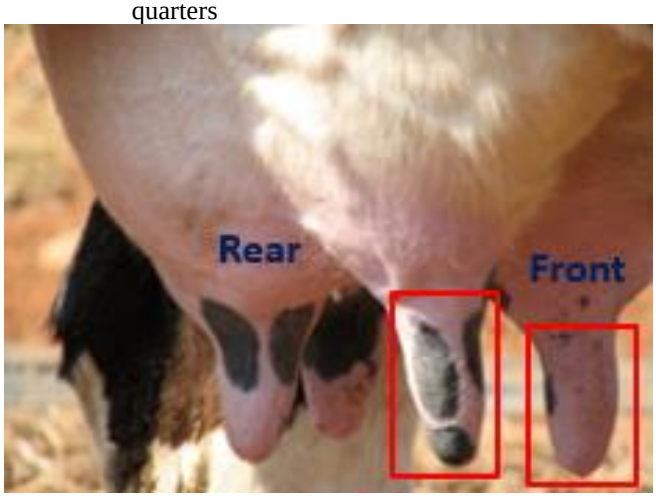
Mastitis can be classified into two pathogen forms: subclinical and clinical mastitis. Subclinical mastitis, which is the most prevalent form, displays no visible signs in the cow or the milk. However, the SCC is increased, milk composition is altered, milk yield is decreased, and pathogens isolated from milk. In the case of subclinical mastitis, detection is more difficult, and laboratory or cow-side tests are needed. Subclinical mastitis causes problems since it can go unnoticed and generate milk with lower quality than what is allowed to enter the bulk tank. Also, the affected cows remain contagious. The effects of milk from affected udder quarters with subclinical mastitis on cow composite milk have not been fully assessed (FÖRSBACK et al., 2009). On the other hand, the clinical form of mastitis can be characterized by hot, painful and swollen udder quarters, fever and loss of appetite in the cow. In clinical mastitis, the milk may contain flakes, clots, and blood. These visible symptoms of clinical mastitis facilitate detection, and the milk can be separated and not delivered to the dairy establishment.

The economic impact of subclinical mastitis is due to increased somatic cell count (SCC), quality deterioration, and reduction of milk yield (HALASA et al., 2007; FORSBACK et al., 2010a). The losses associated with milk yield may occur due to damage caused by the bacteria to milk secretory epithelia of the mammary gland. Additionally, this damage can result in permanent loss of capacity to synthesize milk (AULDIST et al., 1995). Furthermore, milk buyers demand milk with high quality for optimal production of dairy products (FORSBACK et al., 2010a), which does not occur when the milk comes from cows with subclinical mastitis.

Major pathogens are the most frequently isolated as the cause of subclinical intramammary infection (IMI). However, few studies have determined the effect of major pathogens causing subclinical IMI on the milk yield and composition at quarter level (LEITNER et al., 2006; BEZMAN et al., 2015) and none of them has used the evaluation of these variables by complete and individual milking data. The contralateral mammary glands correspond to the front and rear pairs of mammary glands (Figure 4). Considering that healthy contralateral mammary quarters produce similar amounts of milk, and have independent milk production, using the comparison between the pair of infected quarters versus their healthy contralateral within the cow, it would be possible to evaluate the effect of subclinical mastitis caused by specific pathogens on SCC, milk yield and composition. Thus, 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) that could decrease the accuracy of milk loss estimation (GONCALVES et al., 2016).

Figure 4 - Front and rear pairs of contralateral mammary quarters



The use of SCCs to estimate milk yield reduction, associated with subclinical mastitis, has been widely used (SCHUKKEN et al., 2003). However, this evaluation may be limited due to variables (e.g. breed, parity, days in milk) that influence the SCC, and, if not considered, may underestimate the evaluation of effects of subclinical mastitis on milk yield and composition (DÜRR et al., 2008). Another problem is the SCC threshold definition in which milk yield begins to be affected as a result of changes in SCC, especially when it is necessary to interpret the relationship between the alteration of milk yield at very low levels of milk somatic cells (GREEN; SCHUKKEN; GREEN, 2006). In addition, there are few studies based on estimates of milk yield losses and changes in milk composition from Brazilian herds through test day records. Therefore, the estimation of milk losses caused by subclinical mastitis is fundamental to the dairy industry from the point of view of prevention strategy planning for dairy herds (DÜRR et al., 2008).

Previous studies that evaluated the economic impact of mastitis have presented divergent estimates, mainly due to the diversity of utilized methodologies (PETROVSKI; TRAJCEV; BUNESKI, 2006; HUIJPS; HOGEVEEN 2007; HAGNESTAM-NIELSEN et al., 2009; HALASA et al., 2009; TESFAYE; REGASSA; KELAY, 2010; VAN ASSELDONK et al., 2010). Most of the studies evaluated the losses caused by clinical mastitis since this type of

mastitis is easily diagnosed (visible milk changes) and, consequently, the costs are greater observed by the farmer (e.g. milk discard, treatment costs and labor) (CHA et al., 2011). Most of the previous studies evaluated the difference on milk yield and composition, between healthy and infected mammary glands, based on SCC from different cows (BARKEMA et al., 1997; WILSON et al., 1997b; FORSBACK et al., 2010b; FORSBACK et al., 2010a) or even between identical twin cows (PEARSON et al., 2013). However, studies comparing contralateral mammary quarters of the same cow could be an alternative approach, since they are anatomically isolated and have similar milk yield when they are healthy (GONCALVES et al., 2016). Furthermore, milk yield losses caused by subclinical mastitis have already been calculated based on bulk milk tank (BMT) SCC. However, the most realistic estimates for assessing the impact of subclinical IMI are based on SCCs at the cow level (HUIJPS; LAM, 2008). Overall, subclinical mastitis causes losses in milk quantity and quality; therefore, this results in losses to farmers. For that reason, they have been implementing management strategies to reduce the incidence of subclinical mastitis-causing pathogens (HUIJPS; LAM, 2008).

Mastitis cost estimations vary between countries as well as between regions in the same country (HALASA et al., 2007; HUIJPS; LAM, 2008). Because of these differences, specific farm calculations are necessary, taking market and management differences among farms into account (HUIJPS; LAM, 2008). In addition, there are no studies related to the expenses generated by pathogens causing subclinical mastitis in Brazilian dairy herds. The knowledge of the losses caused by specific pathogens causing subclinical mastitis would aid the decision-making process of the farmer regarding management and prevention strategies, or perhaps decision on treatment/culling directed to the type of pathogen that is causing the disease.

1.3 HYPOTHESIS

Subclinical mastitis caused by major pathogens negatively affects milk yield and composition, and consequently the economic return under dairy farmers. The methodology of complete and individual milking per mammary quarter allows estimating production losses caused by intramammary infections caused by major pathogens.

1.4 GENERAL OBJECTIVES

The general objectives of the present thesis were to evaluate: (i) the effects of subclinical mastitis caused by major pathogens on SCC, milk leukocyte differentials (MLD) and milk yield; (ii) milk yield losses caused by subclinical mastitis, at cow and at quarter level; and (iii) the economic impact of subclinical mastitis caused by major pathogens.

1.5 SPECIFIC OBJECTIVES

The specific objectives were:

a) to evaluate the use of milk leukocyte differential (MLD) to identify milk quarters that are culture-positive;
b) to characterize the milk leukocyte responses to specific pathogen groups (minor, contagious, environmental and miscellaneous) causing subclinical mastitis;
c) to evaluate the effect of pathogen groups (minor, contagious, environmental and miscellaneous), causing subclinical mastitis, on SCC, milk yield and composition (crude protein and fat content), by comparison of contralateral mammary quarters within cows;
d) to determine the effect of pathogen groups (minor, contagious, environmental and miscellaneous) and specific pathogens, causing subclinical mastitis at the mammary quarter level, on milk prices and economic return (quarter milk yield × milk price), using the estimation based on a milk payment program;
e) to evaluate the effect of major pathogens (*Staph. aureus*, *Strep. agalactiae*, *Strep. uberis*, *Strep. dysgalactiae* and *Streptococci*-like bacteria), causing chronic subclinical mastitis, on SCC, milk yield and economic return using comparison of multiple versus single quarter milk samples;
f) to evaluate the relationship of milk yield and SCC from Brazilian dairy herds, using test day records to verify whether the association varies for different parities and stages of lactation, and whether this relationship should be interpreted at very low levels of cells in milk.

Chapter 2

Using milk leukocyte differentials for diagnosis of subclinical bovine mastitis.

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Submitted November, 2016.

2 USING MILK LEUKOCYTE DIFFERENTIALS FOR DIAGNOSIS OF SUBCLINICAL BOVINE MASTITIS

2.1 ABSTRACT

This research study aimed to evaluate the use of the milk leukocyte differential (MLD) to: (a) identify quarter milks that are culture-positive; and (b) characterize the milk leukocyte responses to specific groups of pathogens causing subclinical mastitis. The MLD measures the absolute number and relative percentage of inflammatory cells in milk samples. Using the MLD in two dairy herds (170 and 172 lactating cows, respectively), we studied all lactating cows with a most recent monthly Dairy Herd Improvement Association somatic cell count (SCC) > 200×10^3cells/mL. Quarter milk samples of all selected cows (n = 78) were analysed by MLD and aseptically collected milk samples were subjected to microbiological culture (MC). Positive MC were obtained from 102/156 (65.4%) of MLD-positive milk samples, and 28/135 (20.7%) of MLD-negative milk samples were MC-positive. When MC was considered the gold standard for mastitis diagnosis, the calculated diagnostic *Se* of the MLD was 65.4% ($IC_{95\%}$ = 57.4 to 72.8%) and the *Sp* was 79.3% ($IC_{95\%}$ = 71.4% to 85.7%). Quarter milks positive on MC had higher absolute numbers of neutrophils, lymphocytes and macrophages, with higher neutrophils% and lymphocytes% but lower macrophages%. The $Log_{10}\ ^{N}/_{L}$ ratios were the most useful ratio to differentiate specific subclinical mastitis quarters from healthy quarters. Use of the MLD on cows with monthly composite SCC > 200×10^3cells/mL for screening at quarter level identified quarters more likely to be culture-positive. In conclusion, the MLD can provide an analysis of mammary quarter status more detailed than provided by SCC alone; however, the MLD response to subclinical mastitis was not found useful to specifically identify the causative pathogen.

Keywords: Subclinical mastitis. Mammary quarters. Diagnoses. Leukocyte differentials. Bacteria.

2.2 INTRODUCTION

Subclinical mastitis (SM) is a common and economically significant disease of dairy cows, causing increased somatic cell counts (SCC) and decreased quality and yield of milk (WILSON; GONZALEZ; DAS, 1997a; PITKALA et al., 2004; HALASA et al., 2009). Approximately 70 to 80% of mastitis losses are due to subclinical mastitis (RENEAU; PACKARD, 1991). Subclinical mastitis infections are not evident and can persist in the mammary tissue throughout lactation (PILLA et al., 2013).

Subclinical mastitis is most commonly diagnosed by microbial culture-based (MC) methods or SCC, which are both traditional and well-established tests for detection of subclinical mastitis (OLIVER et al., 2004; HAND; GODKIN; KELTON, 2012). Although SCC is a robust quantitative measurement, it does not differentiate cell types. Microbiological culture is based on collection of quarter milks aseptically for inoculation on culture medium and further testing for microorganism identification. The requirement for aseptic collection of milk samples for MC can be a disadvantage as the process is susceptible to contamination. Furthermore, traditional methods using MC can be labor-intensive and it may take up to 2-7 days to reach a diagnosis (BARREIRO et al., 2010).

The milk leukocyte differential (MLD) has been investigated for potential in diagnosis of mastitis (DULIN; PAAPE; WEINLAND, 1982; KELLY et al., 2000; PILLAI et al., 2001; DOSOGNE et al., 2003; SCHWARZ et al., 2011a,b; PILLA et al., 2012; PILLA et al., 2013). The MLD can detect changes in proportions of cell types in milk independently of the SCC, which could provide information about inflammatory processes in quarters otherwise considered healthy (PILLA et al., 2012). This information could be useful when control programs for milk pathogens are being applied (PILLA et al., 2013). The changes in cell ratio have been used for the identification of inflammatory processes in cows with low SCC, with the potential to differentiate milk from healthy quarters from those with early or late inflammation (PILLA et al., 2012). The MLD patterns of 6 out of 41 quarter milk samples with SCC values from ≥9,000 to ≤46,000 cells/mL were described by SCHWARZ et al. (2011b) and their results revealed early inflammatory reactions based on the predominance of polymorphonuclear neutrophils (PMNL) (56–75%).

The MLD has been tested as an option to identify cows affected by any inflammatory process of the mammary gland, with the best results being reported by using logarithmic

PMNL:lymphocyte ratio as the variable (PILLA et al., 2012). However, there is still little knowledge about the MLD and its application under field conditions. Therefore, the aims of this study were to evaluate the use of MLD to (a) identify quarter milks that are culture-positive; and (b) characterize the milk leukocyte responses to specific groups of pathogens causing subclinical mastitis.

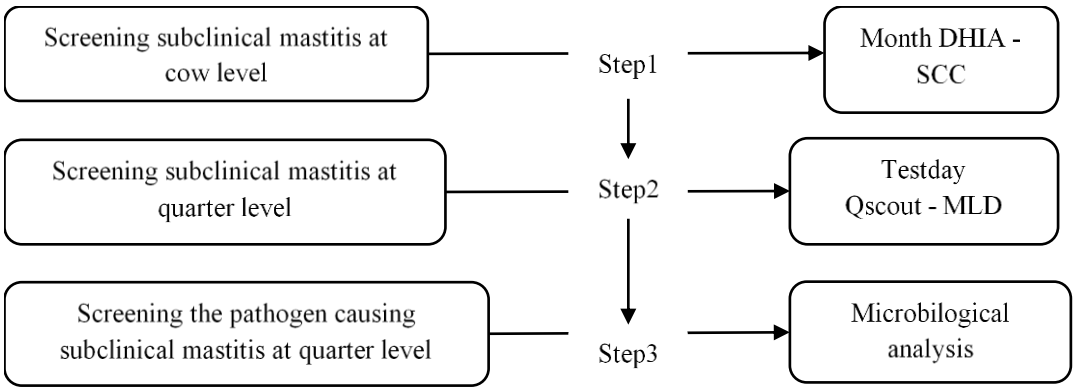
2.3 MATERIALS AND METHODS

This research was approved by the North Carolina (NC) State University Institutional Animal Care and Use Committee (Raleigh).

2.3.1 **Animals and herds**

Seventy-eight dairy cows were selected from 2 NC dairy herds (A and B) for detailed analysis of udder health status based on MLD and MC of aseptically collected quarter foremilk samples. Cows were selected on the basis of the most recent monthly Dairy Herd Improvement Association (DHIA) test results, with all lactating cows in each herd with a composite SCC > 200×10^3cells/mL, and with no history of clinical mastitis within the preceding month, considered eligible for the study (Step 1, figure 5). Selected cows included Holstein (n = 52), Jersey (n =19) and cross bred cows (n = 7) in various lactations (1 to 7) and stages of lactation.

Figure 5 - Flowchart explaining the use of QScout® Farm Lab integrated to the herd routine



Cows on farms A (n = 170; geometric mean bulk tank SCC = 112.3×10^3 cells/mL) and B (n = 172; geometric mean bulk tank SCC = 71.3×10^3 cells/mL) were housed in free-stall and pack barn facilities, respectively, and were milked twice a day in parlors. Both herds had consistent application of mastitis control programs based on the recommendations of the National Mastitis Council (NMC; http://www.nmconline.org). In both herds, cows were fed a total mixed ration composed of corn silage, grain concentrate, and minerals, with access to hay. Water was available *ad libitum*. Both farms were conventional milk producers with average milk yields of 9,015 (farm A) and 11,788 (farm B) Kg/yr per cow, respectively.

2.3.2 **Milk sampling**

Milk samples were collected from all functional quarters of the 78 eligible cows on the 2 farms. A total of 10 of the 312 possible quarters were non-functional. This left 302 quarter foremilk samples which were collected for MC according to NMC guidelines (OLIVER et al., 2004). Before milking, teat ends were scrubbed with 70% ethanol and the first three squirts of milk were discarded. Ten milliliters of milk per mammary quarter were collected aseptically. After the foremilk sampling for MC, milk from each quarter was collected into a quarter-based sampling chamber (Advanced Animal Diagnostics Company, AAD, Inc., Durham, NC) for MLD analysis. Quarter foremilk samples for MC were refrigerated (4-7 °C) until further analysis.

2.3.3 **Microbiologic analysis**

All microorganisms were isolated and categorized using procedures consistent with those recommended by the NMC (OLIVER et al., 2004). Milk samples were plated within 24 hours of collection. Milk samples were mixed and 0.1 mL of milk was inoculated onto trypticase soy agar plates with 5% sheep blood (Becton, Dickinson and Co., Sparks, MD). Inverted plates were incubated aerobically at 36 °C for 48 h and results were observed every 24 h regarding colonial characteristics (shape, size, number, and color), haemolytic ability (presence and type), and possible contamination. Isolates were Gram stained and catalase reaction determined. Creamy, grayish-white, or golden-yellow pigmented colonies, mannitol fermenting, coagulase and catalase positive Gram positive cocci that exhibited complete, incomplete, or both complete and incomplete hemolysis were identified as *Staphylococcus aureus* as described by ANDERSON and LYMAN (2006). All catalase positive *Staphylococcus* non-*aureus* or coagulase negative staphylococci (CNS) were speciated using the API Staph. identification system (bioMérieux SA, Marcy-L'Etoile, France). Gram positive, catalase negative cocci were identified as streptococci, enterococci, or aerococci and were speciated using the CAMP test and growth on bile-esculin and inulin agars. If necessary, API-20 Strep strips (bioMérieux SA, Marcy-L'Etoile, France) were used to speciate. Gram negative rods were identified using API-20E strips (bioMérieux SA, Marcy-L'Etoile, France). *Yeast* and *Nocardia* sp. identifications were based on morphology and Gram stain and *Prototheca* spp. by appearance after staining with lactophenol cotton blue. White-gray or yellowish color colonies with a slightly raised, dry and/or flaky, and nonhemolytic appearance (small and circular colonies approximately 1 mm in diameter) comprised of Gram-positive bacteria on Gram Stain that appeared at about 48 h of incubation were identified as *Corynebacterium* spp. as described by GONÇALVES et al. (2014). Milk samples with more than two morphologically different colonies were considered contaminated.

In 11 mammary quarters, two pathogens were isolated. This included five with environmental streptococci and CNS, two with environmental streptococci and *Staphylococcus aureus*, one with environmental *Streptococcus* and *Corynebacterium* spp., one with *Corynebacterium* spp. and CNS, one with *Enterococcus* spp. and CNS, and one with *Staph. chromogenes* and *Staph. hyicus*. Those cases were designated as follows: (1) samples with a major and a minor pathogen were designated as being caused by the major pathogen; (2)

samples with both *Staphylococcus aureus* and streptococci were designated as being infected by *Staphylococcus aureus*; and (3) samples with both *Corynebacterium* spp. and CNS were designated as being due to CNS.

2.3.4 **Somatic cell count**

Monthly milk SCC were recorded from DHIA analysis, using composite milk samples with preservative (United DHIA, Radford, VA).

2.3.5 **Milk leukocyte differential**

Milk leukocyte differentials were determined on fresh milk collected within 15 days after the most recent DHIA test day. The instrument (QScout MLD® test, Advanced Animal Diagnostics, Inc., Durham, NC) uses fluorescent microscopy technology to count and differentiate immune cells in milk. Milk from each quarter was collected into independent chambers of a collection and transfer device (CALDERWOOD et al., 2014, US patent #D720,468) that allows for milk to move into a test slide without the need for a pipette. Capillary action draws milk into the slide and a dried fluorescent stain reagent mixes into solution as the sample flows through the slide (WARDLAW; ROBERT, 1999, US patent #5,948,686). Stain uptake occurs quickly and the slide may be read automatically by the reader, which includes a fluorescent microscope (QScout Farm Lab, Advanced Animal Diagnostics, Inc., Durham, NC). Collected images are processed with patented software that identifies and distinguishes immune cells into three classes: neutrophil, lymphocyte, and macrophage, utilizing fluorescence emission of the cell, as described by WARDLAW; LEVINE; RODRIGUEZ, (2002) US patent #6,350,613, supplemented by analysis morphological characteristics. Specific indices are available for early lactation, mid-lactation/hospital, and dryoff. A user selects the index that corresponds with the sample type being processed. Samples may be processed in either a rapid mode (<4 min/cow) or a research mode (approximately 15 min/cow). The research mode collects a much larger and standardized number of images for

each quarter. The reader (QScout Farm Lab, Advanced Animal Diagnostics, Inc., Durham, NC) has programmable threshold levels within each index that may be selected by the user. By changing thresholds, a user can weight results towards higher sensitivity or higher specificity. Threshold settings for early lactation index range from 1-18, for mid-lactation range from 1-12, and for dryoff index range from 1-12. In addition to providing absolute values for each cell type (neutrophil, lymphocyte and macrophage), the total leukocyte count and percentage and total of each cell type were reported and also used in an index to produce a categorical quarter diagnosis of healthy versus infected. Phagocyte counts were calculated as the sum of macrophages and neutrophils. Because of the wide variations found within the cell populations, we evaluated the ratio among phagocytic cell groups expressed as a logarithm of base 10 with the aim of identifying a marker that indicated whether the quarters were more likely to be healthy or infected. The results were expressed as Log_{10} [$^{Neutrophils}/_{Lymphocytes}$] ($Log_{10}{}^{N}/_{L}$) and Log_{10} [$^{Phagocytes}/_{Lymphocytes}$] ($Log_{10}{}^{P}/_{L}$), as described previously by PILLA et al. (2012).

In the current study, samples were processed in research mode to increase accuracy of calculated differentials. The mid-lactation index was selected with the manufacturer-recommended threshold set at 7. In order to assess performance at various thresholds, settings were evaluated for the index range of 1-12 to allow sensitivity and specificity versus e MC to be evaluated at each threshold setting.

2.3.6 **Subclinical mastitis definition**

Mammary quarters were considered to have an intramammary infection (IMI) when quarter milk samples showed isolation of significant bacterial colony numbers as described by ARRUDA et al. (2013), with slight modification. Since we plated 0.1 mL of milk, we considered presence of IMI as detection of any pathogen at any level, similar to what was described by DOHOO et al. (2011).

Quarters selected from cows with SCC > 200×10^3cells/mL were categorized at quarter level according the following criteria as previously described (DVG, 2002; BANSAL et al., 2005): (a) healthy: culture-negative and total leukocyte count (TLC) ≤ 100×10^3 cells/mL; (b)

latent subclinical mastitis (latent-SM): culture-positive and TLC ≤ 100×10^3 cells/mL; (c) nonspecific subclinical mastitis (nonspecific-SM): culture-negative and TLC > 100×10^3 cells/mL; and (d) specific subclinical mastitis (specific-SM): culture-positive and TLC > 100×10^3 cells/mL.

2.3.7 **Experimental design and statistical analysis**

Data are presented as means ± SE. Associations between the MLD and MC status of the udder quarters were 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. We included data from all foremilk samples without contamination and with complete results for MLD and MC. The statistical model included the fixed effects of herd, cow, position of the udder quarter, milk yield, lactation number, parity number, breed and IMI. Statistical significance was defined at *P*-value < 0.05. The following statistical model was used:

$$Y_{ijklmno} = \mu + H_i + COW_j + Q_k + MY_l + DIM_m + P_n + B_O + IMI_p + e_{ijklmno}$$

where: $Y_{ijklmnop}$ was the dependent variable; μ was the overall mean, H_i and COW_j were the random effects of herd *i* (*i* = 1 to 2) and cow j (j = 1 to 78), Q_k was the fixed effect of quarter position *k* (*k* = 1 to 4); MY_l was the fixed effect of milk yield Kg/yr l [l = 1 to 3; MY_1 = high milk yield (≥ 10,675 Kg/yr is equal to >35 Kg/d in 305 days in milk), MY_2 = medium milk yield (6,100 to 10,674 Kg/yr is equal to 20 to 34.9 Kg/d in 305 d) and MY_3 = low milk yield (< 6,100 Kg/yr is equal to < 20 Kg/d in 305 d); DIM_m was the fixed effect of days in milk *m* (*m* = 1 to 3; DIM_1 = 4 to 100, DIM_2 = 101 to 200 and DIM_3 = 201 to 431); P_n was the number of parity *n* (*n* = 1 to 3;); B_o is the fixed effect of breed *o* (*o* = 1 to 3; B_1 = Holstein, B_2 = Jersey and B_3 = cross bred cows); IMI_P was the intramammary infection at quarter level focusing in the proposed objectives *p* [*p* = 1 to 2; IMI_1 = mastitis definition (healthy, latent infection, non-specific subclinical mastitis and specific subclinical mastitis); IMI_2 = specific groups of

pathogens causing subclinical mastitis (healthy, minor, environmental, contagious and miscellaneous)]; and $e_{ijklmno}$ was the random error term.

The analysis was performed on Log_{10} transformation for SCC, absolute values for each cell type, the total leukocyte count and percentage and total of each cell type to provide normal distribution of the data. Data was anti-Log_{10} transformed for presentation of the results and discussion. The MLD sensitivity (*Se*) and specificity (*Sp*) were determined comparing the MC as a standard methodology with the categorical quarter diagnosis from MLD technology (healthy versus infected) using on-line statistical software (MedCalc for Windows, version 16.8, MedCalc Statistical Software, 2016).

2.4 RESULTS

2.4.1 **Microbiologic analysis**

A total of 302 quarter milk samples was aseptically collected from eligible quarters. There were 8 contaminated samples, leaving 294 quarter samples with usable culture results. Frequency of mastitis pathogen identification by MC of 294 quarter samples is given in Table 1. Overall, 130 quarters (44.2%) were classified as culture-positive and 164 (55.8%) were negative on culture. Minor pathogens (n = 50) accounted for 17.0 % of total samples, being composed of CNS (n = 38) and *Corynebacterium* spp. (n = 12). Coagulase negative staphylococci (CNS) were the most commonly isolated mastitis-causing pathogen. Among the CNS group, *Staph. chromogenes* was the most frequent, being found in 24/38 CNS isolates (8.2% of all samples). A variety of other CNS species was found in the remainder (Table 1).

Table 1 - Frequency of mastitis pathogen identification by microbiological culture of mammary quarter foremilk samples (n = 294) from two herds in North Carolina

Microorganisms	Mammary quarter foremilk samples				
	No. Isolates by Farm			Absolute frequency (%)	Relative frequency (%)
	A	B	Total		
No.	202	92	294	100.00%	-
Negative culture	110	54	164	55.78%	-
Positive culture	92	38	130	44.22%	100.00%
Minor pathogens:	32	18	50	17.01%	38.46%
Coagulase negative Staphylococci	23	15	38	12.93%	29.23%
S. chromogenes	12	12	24	8.16%	18.46%
Other coagulase negative staph[1]	11	3	14	4.77%	10.77%
Corynebacterium spp.	9	3	12	4.08%	9.23%
Major pathogens:	51	17	68	25.2%	52.3%
Contagious pathogens—all *Staphylococcus aureus*	25	12	37	12.59%	28.46%
Environmental pathogens	26	5	31	10.54%	23.85%
Streptococcus spp.[2]	19	2	21	7.14%	16.15%
Enterococcus spp.[3]	5	1	6	2.04%	4.62%
Enterobacter cloacae	0	1	1	0.34%	0.77%
Escherichia coli	1	0	1	0.34%	0.77%
Serratia marcescens	0	1	1	0.34%	0.77%
Non-fermenter species	1	0	1	0.34%	0.77%
Miscellaneous pathogens:	9	3	12	4.08%	9.23%
Nocardia spp.	5	0	5	1.70%	3.85%
Yeast	3	2	5	1.70%	3.85%
Prototheca spp.	1	1	2	0.68%	1.54%

[1]Other coagulase-negative staphylococci for Farms A and B, respectively, included *Staph. capitis* (0 and 1), *Staph. hominis* (1 and 0), *Staph. hyicus* (2 and 1), *Staph. lugdnensis* (2 and 0), *Staph. sciuri* (1 and 0), *Staph. xylosus* (1 and 0), and other staphylococci (4 and 1), [2]Streptococci isolated from Farms A and B, respectively, included *Strep. bovis* (2 and 0), *Strep. dysgalactiae* (4 and 0), *Strep. uberis* (12 and 2), and *Aerococcus viridans* (1 and 0). [3]Enterococci isolated on farms A and B, respectively, included *Ent. avium* (1 and 0), *Ent. durans* (0 and 1), *Ent. faecium* (2 and 0), and other enterococci (2 and 0).

Major pathogens were identified in 68 quarters (23.1% of all samples) (Table 1). Out of them, 37 quarters had isolation of contagious pathogens, all. *S. aureus* (12.6%). There were 31 quarters with environmental pathogens (10.5%), primarily streptococci. A total of 12 quarters (4.1%) were identified as positive for other miscellaneous pathogens (*Nocardia* spp., yeast and *Prototheca* spp.).

2.4.2 **Somatic cell count and milk leukocyte differential**

2.4.2.1 Comparison of MLD results for quarters with variable mastitis definitions (healthy, latent-SM, non-specific-SM and specific-SM).

There were 102 mammary quarters classified as healthy (35%), 32 as latent-SM (11%), 59 as nonspecific-SM (20.3%) and 98 as specific-SM (33.7%) (Table 2). Mammary quarters with specific-SM (772.5×10^3cells/mL), nonspecific-SM (527.1×10^3cells/mL) and latent-SM (40.6×10^3cells/mL) had higher TLC than healthy quarters (25.1×10^3cells/mL). The neutrophils% were greater in specific-SM cases (65.7%) than nonspecific-SM cases (55.2%), latent-SM cases (55.0%) and healthy quarters (49.4%). Therefore, healthy quarters had the lowest mean value of absolute number of neutrophils (12.3×10^3cells/mL). Although mammary quarters with latent-SM, nonspecific-SM and specific-SM had higher TLC than healthy quarters, the macrophages% were lower in quarters with specific-SM (12.3%), nonspecific-SM (17.3%) and latent-SM (23.0%), when compared to healthy quarters (28.9%). The lymphocytes% and phagocytes% were similar among tested groups, but mammary quarters with specific-SM, nonspecific-SM and latent-SM had higher mean value of absolute number of lymphocytes and phagocytes than healthy quarters (Table 2).

Table 2 - Mean values for individual cell populations and combinations of cell populations from quarter milk samples considering the mastitis definition (n = 291)

Cell population[a]	Healthy	Latent	Non-specific Subclinical mastitis	Specific Subclinical mastitis
All quarters tested, No. (%)				
	102 (35)	32 (11)	59 (20.3)	98 (33.7)
Equipment results, No. (%)				
Negative (135)	98 (96.1)	26 (81.3)	9 (15.3)	2 (2)
Positive (156)	4 (3.9)	6 (18.7)	50 (84.7)	96 (98)
SCC[1]	570.61[a] ±148	705.72[a] ±302.24	561.72[a] ±103.66	701.87[a] ±164.81
TLC[2]	4.40[d] ±0.05 (25.12)	4.61[c] ±0.08 (40.63)	5.72[b] ±0.06 (527.11)	5.89[a] ±0.05 (772.50)
Neutrophils%	4.69[c] ±0.02 (49.37)	4.74[b] ±0.02 (54.99)	4.74[b] ±0.02 (55.16)	4.82[a] ±0.02 (65.72)
Neutrophils	4.09[d] ±0.05 (12.34)	4.35[c] ±0.09 (22.49)	5.46[b] ±0.07 (291.61)	5.71[a] ±0.06 (510.15)
Macrophages%	4.46[a] ±0.04 (28.93)	4.36[b] ±0.05 (23.03)	4.24[c] ±0.04 (17.29)	4.09[d] ±0.04 (12.29)
Macrophages	3.87[b] ±0.05 (7.36)	3.95[b] ±0.08 (8.91)	4.95[a] ±0.06 (90.07)	4.98[a] ±0.05 (96.58)
Lymphocytes%	4.25[a] ±0.03 (17.71)	4.25[a] ±0.04 (17.79)	4.24[a] ±0.03 (17.30)	4.25[a] ±0.03 (17.89)
Lymphocytes	3.66[d] ±0.06 (4.53)	3.86[c] ±0.09 (7.23)	4.96[b] ±0.07 (91.01)	5.14[a] ±0.06 (138.39)
Phagocytes%	4.92[a] ±0.01 (82.87)	4.92[a] ±0.02 (82.64)	4.90[a] ±0.02 (79.41)	4.91[a] ±0.01 (82.07)
Phagocytes[3]	4.32[d] ±0.05 (20.73)	4.53[c] ±0.08 (33.54)	5.62[b] ±0.06 (420.15)	5.80[a] ±0.05 (631.98)
Log_{10} $^{N}/_{L}$[4]	0.44[b,c] ±0.04	0.49[a,b] ±0.05	0.50[a,b] ±0.04	0.57[a] ±0.04
Log_{10} $^{P}/_{L}$[5]	0.67[a] ±0.04	0.67[a] ±0.05	0.66[a] ±0.05	0.66[a] ±0.04

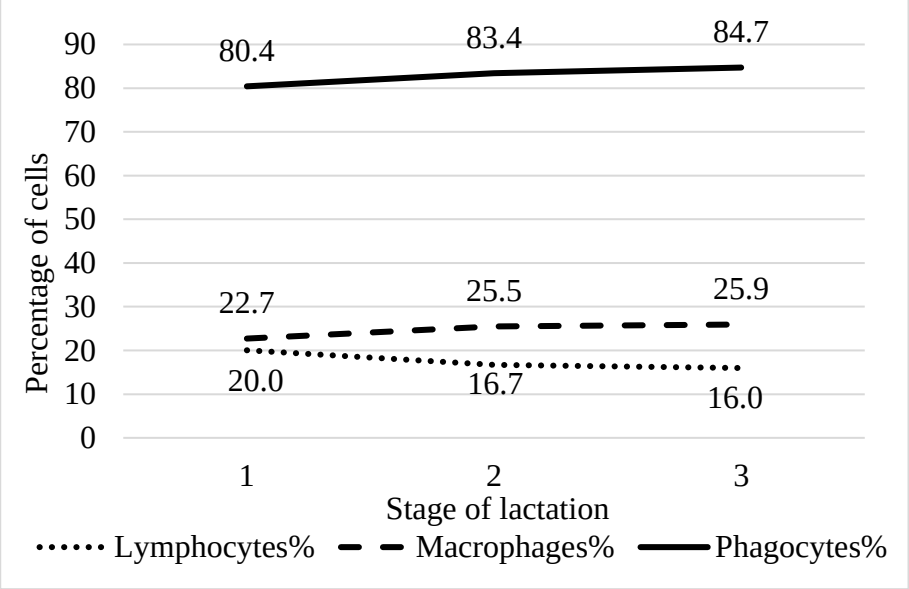
[a]Cells were presented as absolute number and in ratio. [a]Data are presented as means on Log10 transformation ± SE. [a]Data are presented as means on antiLog_{10} transformation between parentheses. [a]Different letters within row were significantly different ($P < 0.05$). [a]Three quarter milk samples were reported as disabled by the MLD automated technology. [1]Geometric mean of somatic cell count at cow level from most recent DHIA test day prior to quarter sample collection. [2]Total Leukocyte Count and other measures on quarter basis. [3]Phagocyte count were based on the sum of macrophages and neutrophils. [4]Log_{10} N/L = Log_{10} [$^{Neutrophils}/_{Lymphocytes}$]; [5]$Log_{10}$ P/L = Log_{10} [$^{Phagocytes}/_{Lymphocytes}$].

We evaluated the ratio among phagocytic cell groups expressed as a logarithm of base 10 aiming to identify cows more likely to be milk culture-positive according our definition of mastitis. We found that the cell ratio Log_{10} $^{N}/_{L}$ was higher in quarters with specific-SM (0.57), nonspecific-SM (0.50) and latent-SM (0.49) than healthy quarters (0.44). Using the cell ratio Log_{10} $^{N}/_{L}$ would provide some differentiation of the quarters classified according to our mastitis definition. On the other hand, there was no difference of the cell ratio Log_{10} $^{P}/_{L}$ between quarters

with specific-SM (0.66), nonspecific-SM (0.66), latent-SM (0.67) and healthy quarters (0.67) (Table 2).

No influence of quarter position, milk yield, parity and breed could be found on milk MLD results. However, we observed an effect of stage of lactation (DIM_1 = 4 to 100, DIM_2 = 101 to 200 and DIM_3 = 201 to 431) on milk MLD results. The greater the DIM, the greater the macrophages% ($P < 0.04$) and the phagocytes% ($P < 0.01$), but lower the lymphocytes% ($P < 0.01$) (Figure 6).

Figure 6 - Effect of stage of lactation (DIM1 = 4 to 100, DIM2 = 101 to 200 and DIM3 = 201 to 431) on milk macrophages%, lymphocytes% and phagocytes%



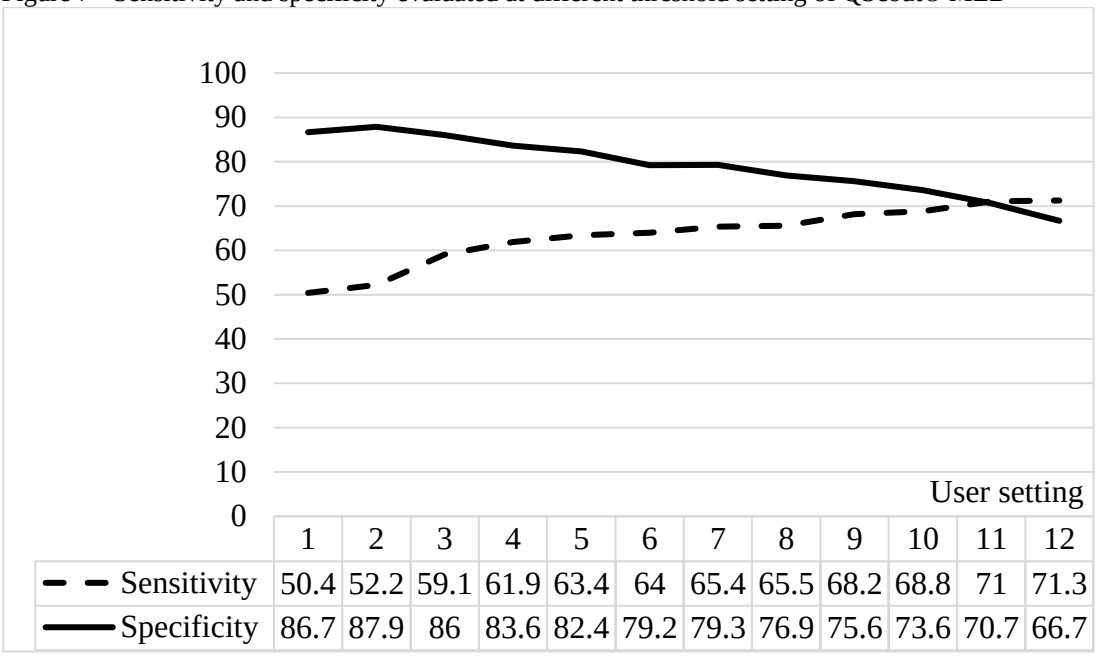
2.4.3 **Performance of categorical analysis on instrument readout (negative or healthy vs. positive or infected)**

A total of 294 quarter samples were submitted to the automated technology based upon readout results. Three quarter milk samples were reported as disabled by the MLD automated technology, leaving results available for a total of 291 quarters (Table 2). Out of 102 mammary quarters designated as healthy, 98 quarters (96.1%) were categorized as negative and 4 (3.9%) as positive by the automated technology based upon readout results. A total of 32 quarters were classified as latent-SM, with 26 quarters (81.3%) classified negative and 6 as a positive. There

were 59 quarters designated as having nonspecific SM, and the automated technology categorized 9 quarters as negative (15.3%) and 50 (84.7%) as positive. Ninety-eight mammary quarters with specific-SM cases were categorized as 2 negative (2%) and as 96 (98%) as positive.

As shown in Table 2, the MLD categorized 156 mammary quarters as positive (53.6%) and 135 as a negative (46.4%). Out of all quarters categorized as negative (n = 135) by the MLD, 79.3% had negative cultures (n = 107) and 20.7% had positive cultures (n = 28). On the other hand, out of all quarters considered positive (n = 156) by the automated technology, 65.4% had positive cultures (n = 102) and 34.6% had negative cultures (n = 54). When MC was considered the gold standard for mastitis diagnosis, the calculated diagnostic *Se* of the MLD was 65.4% ($IC_{95\%}$ = 57.4 to 72.8%) and the *Sp* was 79.3% ($IC_{95\%}$ = 71.4% to 85.7%). Using MC results as the "gold standard," *Se* and *Sp* of the categorical instrument readout results (healthy or infected) based upon cut-offs ranging from 1-12 are shown in Figure 7. Sensitivity progressively increased from a minimum of 50.4% at a user setting of 1 to a maximum of 71.3% at a setting of 12 (Figure 7). Specificity progressively decreased from a maximum of 86.7% at user setting 1 to 66.7% at setting 12 (Figure 7).

Figure 7 - Sensitivity and specificity evaluated at different threshold setting of QScout® MLD



	1	2	3	4	5	6	7	8	9	10	11	12
Sensitivity	50.4	52.2	59.1	61.9	63.4	64	65.4	65.5	68.2	68.8	71	71.3
Specificity	86.7	87.9	86	83.6	82.4	79.2	79.3	76.9	75.6	73.6	70.7	66.7

2.4.3.1 Comparison of MLD results for quarters following categorization by mastitis pathogen groups (minor, environmental, contagious and miscellaneous).

A total of 161 healthy quarters (culture-negative) were selected and compared to 130 infected quarters (culture-positive) according to the pathogen category. The MC and MLD results from mammary quarters infected with minor pathogens (n = 50; 17%), environmental pathogens (n = 31; 10.5%), contagious pathogens (n = 37; 12.6%), and miscellaneous pathogens (n = 12; 4.1%) were compared to healthy quarters (Table 3).

Mammary quarters subclinically infected by miscellaneous (992.4×10^3 cells/mL) and contagious (973.6×10^3 cells/mL) pathogens had a similar TLC, but both group of pathogens had higher TLC than healthy quarters (76.3×10^3 cells/mL) and quarters infected by environmental (332.2×10^3 cells/mL) and minor pathogens (134.4×10^3 cells/mL). Mammary quarters subclinically positive with miscellaneous, contagious, environmental and minor pathogens had higher mean values of absolute number of neutrophils and neutrophils% than healthy quarters. The absolute number of macrophages was higher in all infected quarters as compared to healthy quarters, however, the % macrophages was higher in healthy quarters than quarters infected by any pathogen. This represented a proportional decrease of macrophages% but increase of neutrophils% when a quarter became infected. The lymphocytes% and phagocytes% were similar among tested groups, but mammary quarters infected by any pathogen had higher mean numbers of lymphocytes and phagocytes as compared to healthy quarters (Table 3). The cell ratio $Log_{10}\ {}^{N}/_{L}$ was significantly higher in quarters infected by miscellaneous (0.62), contagious (0.57), environmental (0.53) and minor pathogens (0.52) than in healthy contralateral quarters (0.47). On the other hand, there was no difference in the cell ratio $Log_{10}\ {}^{P}/_{L}$ between healthy quarters (0.67) and quarters infected with miscellaneous (0.69), contagious (0.67), environmental (0.66) and minor pathogens (0.65) (Table 3).

Table 3 - Mean values for individual cell populations and combinations of cell populations from quarter milk samples considering the category of pathogens subclinical mastitis-causing (n = 291)

Cell population[a]	Healthy	Minor	Environmental	Contagious	Miscellaneous
All quarters tested, No. (%)					
	161 (55.3)	50 (17.2)	31 (10.7)	37 (12.7)	12 (4.1)
Equipment results, No. (%)					
Negative	107 (65.2)	17 (34)	6 (19.4)	4 (10.8)	1 (8.3)
Positive	54 (34.8)	33 (66)	25 (80.6)	33 (89.2)	11 (91.7)
SCC[1]	577.47^{a} ±99.49	587.16^{a} ±192.11	1151.93^{a} ±487.43	630.59^{a} ±80.43	684.47^{a} ±232.21
TLC[2]	4.88^{e} ±0.08 (76.37)	5.13^{d} ±0.12 (134.40)	5.52^{c} ±0.14 (332.20)	5.99^{a,b} ±0.13 (973.64)	6.00^{a} ±0.21 (992.43)
Neutrophils %	4.71^{e}±0.01 (51.57)	4.77^{c,d} ±0.02 (58.99)	4.79^{b,c} ±0.02 (61.08)	4.82^{a,b} ±0.02 (66.31)	4.86^{a} ±0.03 (71.99)
Neutrophils	4.59^{e} ±0.09 (39.24)	4.91^{d} ±0.13 (80.82)	5.32^{c} ±0.16 (207.87)	5.81^{a,b} ±0.14 (648.04)	5.85^{a} ±0.23 (706.32)
Macrophages %	4.38^{a} ±0.04 (23.91)	4.20^{b} ±0.05 (15.83)	4.19^{b} ±0.06 (15.35)	4.09^{b} ±0.05 (12.34)	4.10^{b} ±0.08 (12.70)
Macrophages	4.27^{d,e} ±0.10 (18.43)	4.33^{d} ±0.12 (21.25)	4.70^{c} ±0.14 (50.47)	5.10^{a,b} ±0.13 (126.27)	5.15^{a} ±0.19 (142.40)
Lymphocytes %	4.24^{a} ±0.03 (17.55)	4.26^{a} ±0.04 (18.06)	4.26^{a} ±0.04 (18.11)	4.25^{a} ±0.04 (17.73)	4.23^{a} ±0.06 (17.15)
Lymphocytes	4.14^{e} ±0.07 (13.86)	4.39^{d} ±0.12 (24.46)	4.78^{c} ±0.14 (59.91)	5.23^{a,b} ±0.12 (171.44)	5.23^{a} ±0.22 (171.20)
Phagocytes %	4.91^{a} ±0.01 (81.60)	4.91^{a} ±0.02 (81.10)	4.92^{a} ±0.02 (82.64)	4.92^{a} ±0.02 (82.55)	4.93^{a} ±0.02 (84.61)
Phagocytes[3]	4.79^{e} ±0.09 (62.33)	5.04^{d} ±0.13 (108.84)	5.44^{c} ±0.15 (275.74)	5.91^{a,b} ±0.14 (807.98)	5.93^{a} ±0.22 (849.77)
Log_{10} $^{N}/_{L}$[4]	0.47^{b} ±0.04	0.52^{a,b} ±0.05	0.53^{a,b} ±0.05	0.57^{a} ±0.05	0.62^{a} ±0.07
Log_{10} $^{P}/_{L}$[5]	0.67^{a} ±0.04	0.65^{a} ±0.05	0.66^{a} ±0.05	0.67^{a} ±0.05	0.69^{a} ±0.07

Cells were presented as absolute number and in ratio. Data are presented as means on Log_{10} transformation ± SE. Data are presented as means on antiLog_{10} transformation between parentheses. Different letters within row were significantly different ($P < 0.05$). [1]Geometric mean of somatic cell count at cow level from most recent DHIA test day prior to quarter sample collection. [2]Total Leukocyte Count and other measures on quarter basis. [3]Phagocyte count were based on the sum of macrophages and neutrophils. [4]Log_{10} N/L = Log_{10} [$^{Neutrophils}/_{Lymphocytes}$]; [5]$Log_{10}$ P/L = Log_{10} [$^{Phagocytes}/_{Lymphocytes}$].

2.5 DISCUSSION

It has been proposed that the MLD can identify changes in relative cell populations before the increase in TLC occurs in the course of inflammatory process (PILLA et al., 2012; PILLA et al., 2013). Based upon this, we asked if the use of MLD would be able to (a) identify quarter milks more likely to be culture-positive; and (b) characterize the milk leukocyte

responses to specific groups of pathogens causing subclinical mastitis. We found that 65.4% of quarters producing MLD-positive test results were positive for MC, while 20.7% of quarters testing MLD-negative were culture-positive. The $Log_{10}\ {}^{N}/_{L}$ ratios were shown to be the most useful ratio to differentiate specific subclinical mastitis cases from healthy quarters. In addition to giving a total cell count, the MLD can be used for more detailed evaluation of udder health status.

2.5.1 **Microbiologic Analysis**

Both farms used for this study were representative of smaller farms with mastitis problems warranting investigation, in that a considerable number of various pathogens were detected including *Staphylococcus aureus*. The validity of using elevated composite cow SCC (>200×10^3cells/mL in most recent test) as a criterion for selection was affirmed by the finding that an average of 44.2% of quarter samples tested produced a positive microbiological result (45.6% for farm A and 41.3% for farm B). Both farms had approximately the same profile of pathogens. We have found *Staphylococcus aureus* as a frequent problem in some dairies in our region. The CNS were frequently isolated, similar to other studies (e.g., MAKOVEC and RUEGG (2003); TOMAZI et al. (2015)). Considering all isolates, there were 38.5% minor pathogens, with CNS predominating, 28.5% *Staphylococcus aureus*, 23.8% environmental pathogens with streptococci predominating, and 9.2% infrequent pathogens such as *Nocardia* spp., yeasts and *Prototheca* spp. The profile of pathogens found in positive cultures makes the herds used appropriate for an investigation of mastitis diagnostics considering multiple etiologies.

2.5.2 **Somatic cell count and milk leukocyte differential**

Comparison of MLD results for quarters with variable mastitis definition (healthy, latent-SM, non-specific-SM and specific-SM). The significantly higher TLC for specific-SM (772.5×10^3 cells/mL) versus healthy quarters (25.1×10^3 cells/mL) samples was not surprising,

as it was part of the selection criteria. The magnitude of the difference, as well as the significantly higher total neutrophils, total macrophages, total lymphocytes, and total phagocytes was consistent with expectations, similar to other studies (PILLAI et al., 2001; DOSOGNE et al., 2003; SCHWARZ et al., 2011a,b; PILLA et al., 2012; PILLA et al., 2013). SCHWARZ et al. (2011b) showed that PMNL in milk samples with SCC values < 6.25×10^3 cells/mL were rare (mean proportion = 15%). PILLAI et al. (2001) evaluated the MLD from mammary quarters with high SCC (>250×10^3 cells/mL) in comparison to the quarters with low SCC (<250×10^3 cells/mL), and they observed that the TLC and PMNL were consistently higher in quarters with high SCC. Additionally, it was reported that quarters with high SCC, TLC and PMNL were more often positive on MC (62 to 87%) compared with those with low SCC, TLC and PMNL (37 to 51%).

Similar to our study, PILLAI et al. (2001) observed that 33 to 49% (mean = 40%) of the inflammatory cells from infected quarters were PMNL; while PMNL constituted only 17 to 25% (mean = 20%) of the inflammatory cells counted from uninfected quarters. SCHWARZ et al. (2011b) observed that PMNL were the dominant cell population in milk samples of diseased quarters, with proportions of PMNL ≥ 65%.

Results of our study are most comparable to those of PILLA et al. (2012), who compared differential cell counts from 96 normal quarters with 92 abnormal quarters categorized as latent mastitis, unspecific mastitis and subclinical mastitis. Similar to our findings, PILLA et al. (2012) found that lymphocytes, neutrophils, and $Log_{10}\ {}^{N}/_{L}$ were significantly higher in abnormal quarters. Macrophages were not significantly affected in the study of PILLA et al. (2012).

Our numerical results for neutrophils% were very similar to those reported by PILLA et al. (2012). Although we detected differences in macrophages% between quarters with specific-SM versus those with nonspecific-SM, latent-SM and healthy quarters, the absolute values we obtained for macrophages were very similar to those of PILLA et al. (2012). In general, in the present study there was a proportional decrease of macrophages% with increases of neutrophils% when the quarter became infected. This result was similar to those described by SCHWARZ et al. (2011a), who reported a significant negative correlation between macrophage% and SCC.

In our study, in which we classified the mammary quarters in a slightly different manner, $Log_{10}\ {}^{N}/_{L}$ mean values from healthy quarters (0.44) were significantly lower the latent-SM (0.49), nonspecific-SM (0.50) and specific-SM (0.57) groups (Table 2). These mean values of

$Log_{10}\ ^{N}/_{L}$ were lower than what PILLA et al. (2012) reported. PILLA et al. (2012) categorized quarters in four groups (healthy quarters, latent mastitis-LM, nonspecific mastitis-UM and subclinical mastitis-SM) according to the SCC and MC results. They found that the $Log_{10}{}^{PMNL}/_{Lymphocytes}$ mean values in healthy quarters (0.11) were significantly lower than those in groups with latent mastitis (0.57), nonspecific mastitis (0.73), and subclinical mastitis (0.94). Similar to our study, $Log_{10}\ ^{N}/_{L}$ was significantly different in quarters with specific-SM (0.57) versus healthy quarters (0.44), but not for $Log_{10}\ ^{P}/_{L}$, indicating the merit of investigating quarters with $Log_{10}\ ^{N}/_{L} > 0.44$ because they may be more often infected. This value is similar to what PILLA et al. (2013) reported as a cutoff value of 0.49. This categorization of the quarters in different types of mastitis is important since it may minimize the effect of positive and false negatives, as an example the nonspecific-SM cases (even in absence of bacteria has high SCC).

According to PILLA et al. (2013), no influence of sampling day, parity, lactation stage, or quarter position could be found on either milk or blood MLD results. We did not observe a similar finding based on our results, because the greater the DIM the greater the macrophages% and the phagocytes%, but lower the lymphocytes% which is in agreement with previous results (DOSOGNE et al., 2003).

2.5.3 **Performance of categorical analysis on readout (negative or healthy vs. positive or infected).**

The MLD readout results corresponded reasonably well with the quarter culture results, with 79.3% of negative MLD results being negative on culture, while 65.4% of MLD-positive quarters were culture-positive. Our reported *Se* of 65.4% and *Sp* of 79.3% were similar to those reported in prior studies. PILLA et al. (2013) reported *Se* of 73.3% and *Sp* of 73.6%. Adjustment of user settings from 1 to 12 would allow user optimization of settings. Sensitivities progressively increased from 50.4% at setting 1 to 71.3% at setting 12, while specificities decreased from 86.7% at setting 1 to 66.7% at setting 12 (Figure 7).

2.5.4 **Comparison of MLD results for quarters following categorization by mastitis pathogen groups (minor, environmental, contagious and miscellaneous).**

The difference in TLC was striking when quarters infected by any pathogen were compared to healthy quarters, as was the difference in neutrophils%, macrophages% and Log_{10} $^{N}/_{L}$. SCHWARZ et al. (2011a) described results similar to our study, reporting significant differences of cellular components in milk between quarters infected with pathogens as compared to healthy quarters. Although we have observed differences of MLD between quarters infected by any pathogen versus healthy quarters, we found that the MLD in response to SM cannot be used to specifically identify the causative pathogen.

One purpose of our study was to consider the actual field application of this technology. Milk culture or other forms of microbiological analysis can be costly to the producer. An obvious use of the MLD would be to focus on cows with monthly SCC above some cut-off point (here, > 200×10^{3}cells/mL) with screening the infection at quarter level by providing a more rapid diagnosis performed by automated technology based upon 'on-farm differential cells' readout results.

Two recent studies (HOCKETT; PAYNE; RODRIGUEZ, 2014a;b) have evaluated the automated technology readout results for selective dry cow therapy after diagnosis of infection by MLD compared to blanket dry cow treatment with cephapirin benzathine and cloxacillin. These studies from HOCKETT; PAYNE and RODRIGUEZ (2014a;b) indicated that the use of MLD to guide selective treatment of infected cows reduced the use of cephapirin benzathine (47%) and cloxacillin (58%), and resulted in similar rate of infection, SCC and milk compared to blanket antibiotic therapy.

2.6 CONCLUSION

The MLD response to subclinical mastitis can provide more detailed diagnostic evaluation of than provided by SCC alone. We found that 65.4% of quarters producing MLD-positive test results were positive for MC, with 20.7% of quarters testing MLD-negative found as culture-positive. Similar to other previous studies, quarters positive on culture had higher

absolute numbers of neutrophils, lymphocytes and macrophages, with higher neutrophils% and lymphocytes% but lower macrophages%. The $Log_{10}{}^{N}/_{L}$ ratios were shown to be the most useful ratio to differentiate specific subclinical mastitis cases from healthy quarters. An obvious use of the MLD would be to help focus on the cows with monthly SCC above some limit (here > 200×10^{3}cells/mL) for screening the infection at quarter level by providing a more rapid diagnosis performed by automated technology based upon 'on-farm differential cells' readout results. Although, the MLD could identify quarter more likely to be culture-positive, it was not possible to identify the response caused by a specific agent.

Chapter 3

Bovine subclinical mastitis reduces milk yield and alters composition at contralateral mammary quarter level within cow

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3 BOVINE SUBCLINICAL MASTITIS REDUCES MILK YIELD AND ALTERS COMPOSITION AT CONTRALATERAL MAMMARY QUARTER LEVEL WITHIN COW

3.1 ABSTRACT

Subclinical mastitis (SM) caused by specific groups of pathogens results in distinctive degrees of changes of milk yield and composition in affected mammary quarters. Comparing healthy and infected contralateral mammary quarters can 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). Therefore, 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 versus 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. Healthy quarters (124 pairs) had lower geometric mean SCC (153.60×10^3 cells/mL SEM 63.35) than infected contralateral quarters (SCC of 337.53×10^3 cells/mL SEM 169.70). 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 2.9 Kg/quarter.milking according to the pathogen causing SM. Economic losses were higher in SM caused by *Enterococcus* spp. (US$ 0.43/quarter.milking), *Streptococcus dysgalactiae* (US$ 0.74/quarter.milking) and *Escherichia coli* (US$ 0.98/quarter.milking). Additionally, there was a trend of *Staphylococcus aureus* and *Citrobacter* spp. induce economic losses of US$ 0.26 and 0.29/quarter.milking, respectively.

Keywords: Milk quality. Subclinical mastitis. Contagious. Environmental. Milk price.

3.2 INTRODUCTION

Mastitis is one of the most common diseases of dairy cattle, present in both clinical and subclinical form. Subclinical mastitis (SM) is an asymptomatic form of intramammary inflammation that affects 20 to 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; GAY, 1993; SMITH; 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; LAURENT; MOUSSAOUI, 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., 2010b).

Milk quality payment programs (MQPP) are strategies of dairy companies to motivate farmers to produce high quality milk (BOTARO; GAMEIRO; SANTOS, 2013) and previous

studies suggested its effectiveness in influencing milk quality (NIGHTINGALE et al., 2008). Considering that, we believed that the MQPP would 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; HELLER; LEITNER, 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; REGASSA; KELAY, 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; GODKIN; KELTON, 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 ($<100\times10^3$cells/mL versus $>100\times10^3$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 versus their contralateral quarters infected by *Corynebacterium* spp., a minor pathogen, of the same cow (LEVAN; EBERHART; KESLER, 1985; GONCALVES et al., 2016). However, to 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) (GONCALVES 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; LAURENT; MOUSSAOUI, 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).

3.3 MATERIAL AND METHODS

3.3.1 **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 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.

3.3.2 **Milk sampling and quarter milking**

First, composite milk samples 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 (Table 4). 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 > 200×10^3 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 were selected and sampled a second time within 15 days and had quarter foremilk samples collected aseptically for MC as previously described (Step 2).

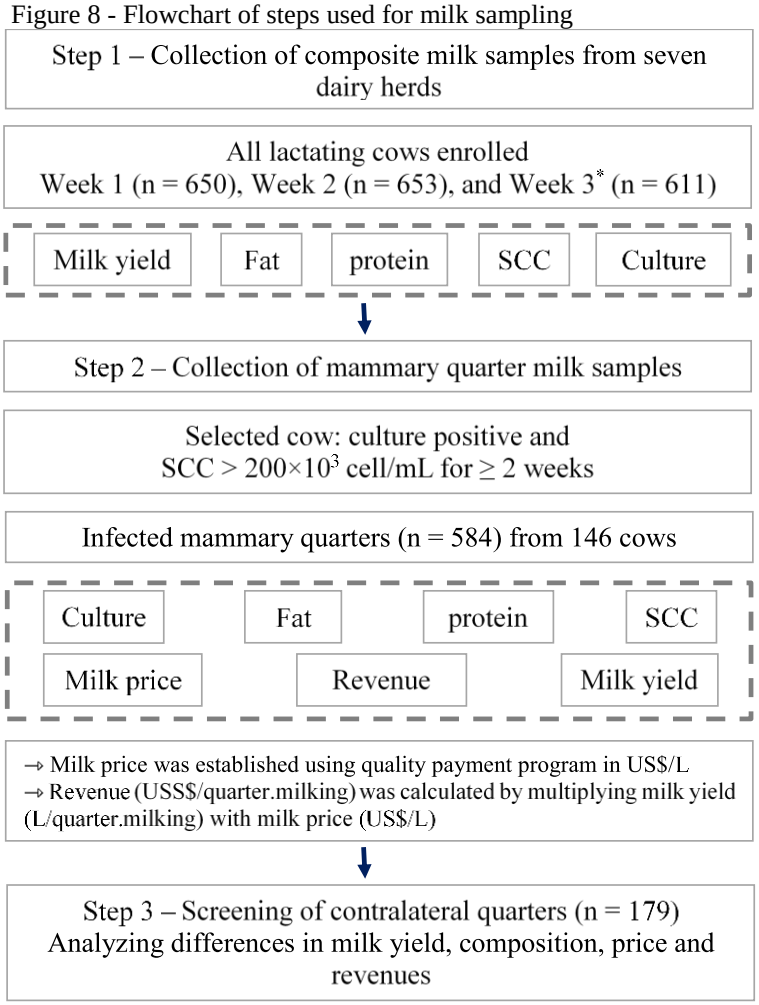
Table 4 - Data from all lactating cows (Step 1a) collected weekly on dairy farms (n = 7): number of lactation, days in milk, components of milk and somatic cell count at the cow level

Variables[b]	Farm A	Farm B	Farm C	Farm D	Farm E	Farm F	Farm G
Week 1							
n	118	23	58	93	159	124	75
DIM	223 ± 15.30	188± 37.39	227± 18.13	184± 17.74	145± 9.83	211± 14.40	236± 12.38
NL	2.21 ± 0.11	1.65 ± 0.17	2.69 ± 0.18	2.51 ± 0.18	2.61 ± 0.11	1.88 ± 0.10	2.51 ± 0.19
MY[1]	14.10 ± 0.51	15.51 ± 0.96	23.42 ± 1.27	22.17 ± 0.79	30.74 ± 0.84	25.78 ± 0.82	14.89 ± 0.60
Protein %	3.79 ± 0.06	3.28 ± 0.09	3.28 ± 0.05	3.46 ± 0.04	3.12 ± 0.03	3.53 ± 0.04	3.38 ± 0.04
Fat %	4.60 ± 0.12	3.30 ± 0.18	4.54 ± 0.13	4.63 ± 0.11	3.81 ± 0.10	4.38 ± 0.11	4.25 ± 0.10
SCC[2]	583.37 ± 187.5	101.4 ± 92.9	425.5 ± 184.9	223.2 ± 115.9	133.0 ± 69.9	224.3 ± 72.0	323.7 ± 175.8
Week 2							
n	121	21	56	95	165	122	74
DIM	232± 16.36	174± 36.83	227± 18.96	188± 17.48	144± 9.60	215± 14.49	239± 12.91
NL	2.21 ± 0.11	1.71 ± 0.18	2.69 ± 0.19	2.48 ± 0.17	2.63 ± 0.11	1.81 ± 0.09	2.49 ± 0.16
MY[1]	16.02 ± 0.60	15.43 ± 0.83	22.64 ± 1.20	23.55 ± 0.81	27.49 ± 0.80	25.84 ± 0.93	12.84 ± 0.06
Protein %	3.54 ± 0.03	3.27 ± 0.08	3.46 ± 0.05	3.40 ± 0.05	3.06 ± 0.03	3.51 ± 0.05	3.32 ± 0.04
Fat %	4.53 ± 0.09	3.35 ± 0.15	4.58 ± 0.12	3.99 ± 0.12	3.70 ± 0.10	4.49 ± 0.11	4.16 ± 0.12
SCC[2]	474.9 ± 145.3	97.3 ± 45.2	516.9 ± 228.0	173.1 ± 74.3	148.4 ± 77.2	235.1 ± 89.8	353.7 ± 98.2
Week 3							
n	110	23	52	94	143	120	69
DIM	231± 17.58	188± 37.76	230± 20.14	175± 16.95	148± 9.56	214± 14.38	234± 12.13
NL	2.15 ± 0.12	1.65 ± 0.17	2.64 ± 0.20	2.45 ± 0.16	2.57 ± 0.11	1.83 ± 0.09	2.47 ± 0.14
MY[1]	12.61 ± 0.63	15.82 ± 1.02	24.02 ± 1.44	23.31 ± 0.68	27.05 ± 0.77	24.82 ± 0.71	13.84 ± 0.56
Protein %	3.84 ± 0.06	3.41 ± 0.07	3.47 ± 0.05	3.50 ± 0.05	2.97 ± 0.03	3.48 ± 0.04	3.42 ± 0.04
Fat%	4.29 ± 0.09	4.31 ± 0.16	4.46 ± 0.11	4.00 ± 0.11	3.35 ± 0.10	3.78 ± 0.09	4.15 ± 0.10
SCC[2]	519.5 ± 182.4	360.5 ± 286.0	325.2 ± 232.6	152.6 ± 51.6	99.4 ± 64.7	201.7 ± 121.3	292.2 ± 108.2
Number and percentage of SCC < 200×10³cells/mL							
n	79	47	53	163	297	176	74
%	22.7	69.1	31.9	57.8	63.6	48.1	33.9
SCC	102.2 ± 5.3	65.6 ± 7.6	97.8 ± 7.0	80.9 ± 4.0	53.3 ± 3.0	70.8 ± 3.8	87.6 ± 5.8

[a]Composite milk samples. [b]Variables were represented in average and standard error (±). [1]L/cow.day. [2]Geometric mean somatic cell count (×10³cells/mL).

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 liters. 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). Milk samples were kept refrigerated (4-7 °C) until they were transported to the laboratory for MC analysis (Figure 8).

Figure 8 - Flowchart of steps used for milk sampling



*In step 1, bacteriological culturing of composite milk was performed only in the 3rd week.

3.3.3 **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 hours and observed every 24 hours 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 Table 5 and Table 6 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).

Table 5 - Summary of steps used for identification of bacteria in the genus *Staphylococcus* spp.

Biochemical tests[a]	*Staphylococcus* spp.		
	S. aureus	CPS non-*aureus*[1]	CNS[2]
Morphology (cocci)	grape-like clusters	clusters	grape-like clusters
Gram staining	+	+	+
KOH	-	-	-
Catalase	+	+	+
Coagulase	+	+	-
Acetoin	+	-	-

[a]Adapted from (OLIVER et al., 2004). [1]Non-*aureus* coagulase positive staphylococci*;* the most subclinical cases were caused by *Staph. hyicus* and *Staph. intermedius*. [2]coagulase negative staphylococci.

Table 6 - Summary of steps used for identification of bacteria in the genus *Streptococcus* spp. and *Enterococcus* spp.

Biochemical tests[a]	*Streptococcus* spp.				*Enterococcus* spp.
	S. agalactiae	*S. dysgalactiae*	*S. uberis*	Other *Streptococci*	
Morphology (cocci)	tendency to form chains	Single or short chains	short chains	pairs or chains	singles, pairs (diplococci) or short chains
Gram staining	+	+	+	+	+
KOH	-	-	-	-	-
Catalase	-	-	-	-	-
CAMP	+	-	+/-	-	-
Esculin	-	+/-	+	+	+
Bile esculin	-	-	-	+	+
Pyr test	-	-	+	-	+

[a]Adapted from (MURRAY et al., 2003; OLIVER et al., 2004)

3.3.4 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 (1,000 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-hour 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.

3.3.5 Experimental design and statistical analysis

Data are presented as means ± SEM. 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 versus 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 versus infected contralateral quarter and between right healthy quarters versus left healthy contralateral quarters within cow. For all statistical analyses, significance was declared at $P \leq 0.05$ and trends at $P \leq 0.10$. The following statistical model was used:

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

where Y_{ijklmn} was the dependent variable; μ is the overall mean; H_i was the herd (i = 1 to 7) that was considered as random effect; $Q_j(C_k)$ was the fixed effect of contralateral quarter (j = 1 to 2, front and rear quarters splitting in halves) nested within cow k; D_l was the days in milk (l = 62 to 483) as covariate in the model; P_m was the parity (m = 1 to 6) as covariate in the model; M_n was the presence or absence of subclinical mastitis (n = 1 to 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 versus 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.

3.3.6 **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:

$$\mathrm{MP}_{\mathrm{corrected},t} = \mathrm{MP}_{\mathrm{nominal},t} \times \left(\frac{\mathrm{INPC\ January}_{2015}}{\mathrm{INPC}t}\right)$$

Where, $MP_{corrected,\ t}$ 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; $MP_{nominal,\ t}$ 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 ($MP_{corrected,t}$) was set at US$ 0.306/L (R$ 0.935/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 Table 7. 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:

$$\mathrm{R}_i = \mathrm{MP}_{\mathrm{f}i} \times \mathrm{MY}_i$$

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 R*i* were calculated in Brazilian currency (Real; R$) and were converted to US$ dollar (1 US$ ≡ 3.05 R$).

Table 7 - The concentrations of milk fat and protein distributed in bonus tracks, neutrality and penalty

Protein[1]			Fat		
From	to	R$/L[2]	from	to	R$/L
2.00	2.09	-0.1017	2.00	2.09	-0.0520
2.10	2.19	-0.0904	2.10	2.19	-0.0468
2.20	2.29	-0.0791	2.20	2.29	-0.0416
2.30	2.39	-0.0678	2.30	2.39	-0.0364
2.40	2.49	-0.0565	2.40	2.49	-0.0312
2.50	2.59	-0.0452	2.50	2.59	-0.0260
2.60	2.69	-0.0339	2.60	2.69	-0.0208
2.70	2.79	-0.0226	2.70	2.79	-0.0156
2.80	2.89	-0.0113	2.80	2.89	-0.0104
2.90	3.09	0.0000	2.90	2.99	-0.0052
3.10	3.19	0.0113	3.00	3.29	0.0000
3.20	3.29	0.0226	3.30	3.39	0.0052
3.30	3.39	0.0452	3.40	3.49	0.0104
3.40	3.49	0.0565	3.50	3.59	0.0182
3.50	3.59	0.0678	3.60	3.69	0.0234
3.60	3.69	0.0735	3.70	3.79	0.0286
3.70	3.79	0.0791	3.80	3.89	0.0338
3.80	3.89	0.0848	3.90	3.99	0.0364
3.90	3.99	0.0904	4.00	4.09	0.0390
4.00	4.09	0.0961	4.10	4.19	0.0416
4.10	4.19	0.1017	4.20	4.29	0.0442
4.20	4.29	0.1017	4.30	4.39	0.0468
4.30	4.39	0.1017	4.40	4.49	0.0473
4.40	4.49	0.1017	4.50	4.59	0.0478
4.50	4.59	0.1017	4.60	4.69	0.0483
4.60	4.69	0.1017	4.70	4.79	0.0488
4.70	4.79	0.1017	4.80	4.89	0.0494
4.80	4.99	0.1017	4.90	4.99	0.0499
≥ 5.00		0.1017	≥ 5.00		0.0504

[1]Mammary quarters with concentrations of milk fat and protein < 2g/100g were not included in the statistical analysis; [2]Brazilian real (R$); (1 US$ ≡ 3.05 R$).

3.4 RESULTS

3.4.1 **Cow level results**

A total of 1,915 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. The MC results of composite milk samples collected during the 3rd week (Step 1) are summarized in Table 8. Of the 611 composite milk samples, 397 (65%) 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.

3.4.2 **Mammary quarter level analysis**

3.4.2.1 Bacteriological culturing results.

A total of 146 lactating cows were considered as having a subclinical IMI and were selected for further analysis. Of all 584 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 (9.25%). Miscellaneous pathogens were isolated in 13 quarters milk samples (2.23%) (Table 8). The most frequently isolated bacteria at the quarter level were *Corynebacterium* spp. (7.9%), followed by CNS (5.8%), *Staphylococcus aureus* (5.31%), *Streptococcus uberis* (4.62%), *Streptococcus agalactiae* (3.94%), other environmental streptococci (2.4%), Gram-negative isolates (2.4%),

Enterococcus spp. (1.37%) and *Streptococcus dysgalactiae* (0.68%). Mixed culture (2 pathogens) represented 0.51% of all quarter milk samples submitted to MC (Table 8). Table 9 summarizes descriptive data from the 146 dairy cows that were selected for mammary quarter analysis according to the IMI status (step 2).

Table 8 - 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[1]	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[2]	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

[1]Coagulase negative staphylococci.
[2] Non-*aureus* coagulase positive stapholococci.

Table 9 - 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[1]	Environment[2]	Contagious[3]	Miscellaneous [4]
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 ± 1.1
Milk yield[5]	24.28 ± 10.2	23.14 ± 9	17.55 ± 9.9	19.77 ± 7.6
Protein%	3.37 ± 0.48	3.26 ± 0.42	3.5 ± 0.52	3.69 ± 0.64
Fat%	3.62 ± 1.1	3.69 ± 0.94	4.03 ± 0.98	4.82 ± 1.11
SCC[6]	862.9 ± 265.6	730.4 ± 286.8	1,058.2 ± 289.5	819.2 ± 395.9

[a]Variables were represented in average and standard error mean (±). [1]*Corynebacterium* spp. and coagulase negative staphylococci; [2]Enterobacteriaceae and environmental *Streptococcus;* [3]*Staphylococcus aureus* and *Streptococcus agalactiae*; [4]*Enterococcus* spp., *Nocardia* spp., non-*aureus* coagulase positive stapholococci, *Trueperella pyogenes* and yeast; [5]L/day; [6]Geometric mean somatic cell count ($\times 10^3$cells/mL).

3.4.2.2 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 versus infected contralateral quarters were selected and distributed according to the pathogen category (Table 10). 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 = 0.15) was observed between healthy (208.8×10^3cells/mL) and contralateral quarters infected by minor pathogens (505.7×10^3cells/mL) (Table 10).

Healthy quarters had lower geometric mean SCC (207.2×10^3 cells/mL) than contralateral quarters infected by environmental pathogens ($1{,}278.7\times10^3$ cells/mL). Thus, healthy quarters had higher milk yield (3.64 Kg/quarter.milking) when compared with contralateral quarter infected by environmental pathogens (3.08 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 10).

Healthy quarters had lower geometric mean SCC (250.9×10^3 cells/mL) than contralateral quarters infected by contagious pathogens ($1{,}623.4\times10^3$ cells/mL). Therefore, healthy quarters had higher milk yield (3.51 Kg/quarter.milking) than contralateral quarters

infected by contagious pathogens (2.78 Kg/quarter.milking). Concentration of milk protein and fat was lower in healthy quarters than contralateral counterparts that were infected by contagious pathogens (Table 10).

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 10).

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 ($P = 0.10$) to reduce the milk economic return (US$0.30/quarter.milking) when compared with healthy contralateral quarters (Table 10).

Table 10 - Effect of mastitis on milk yield, composition and economic return using difference between contralateral mammary quarters (n = 179 pairs) distributed by groups of pathogens causing subclinical mastitis

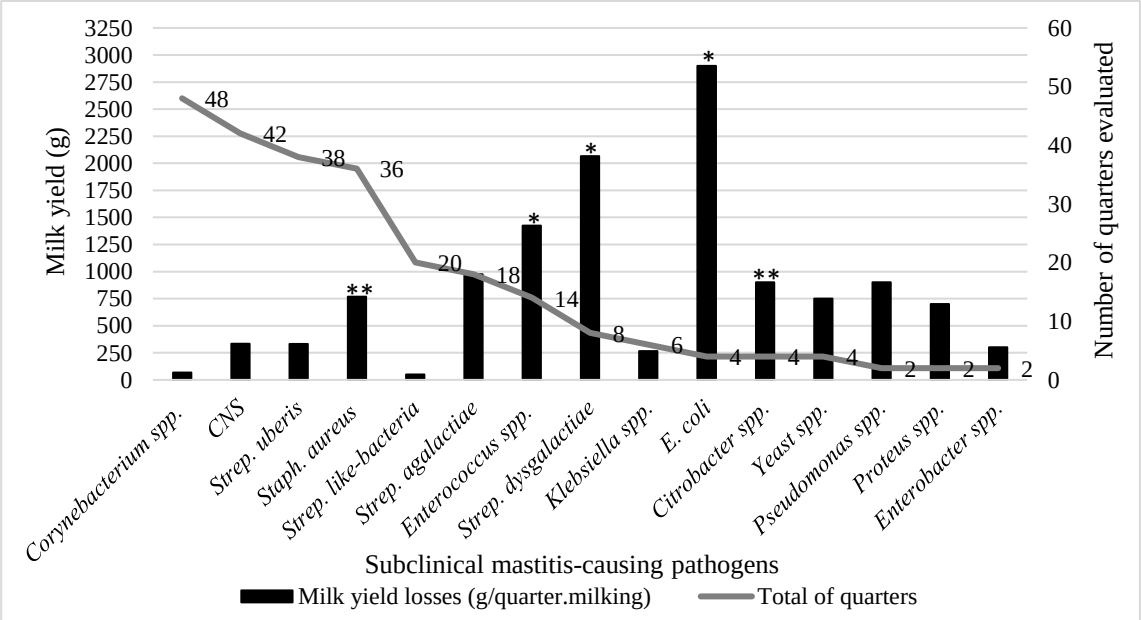
Variables[a]	Pairwise contralateral comparison					Residual Error	P-value
	Healthy[1]	Minor[2]	Environment[3]	Contagious[4]	Miscellaneous [5]		
N°. Pairs	55	45	43	27	9	-	-
Milk yield[6] H[a]	3.95[A,*]	3.54[A]	3.64[A]	3.51[A]	3.92[A]		
Milk yield[6] I[b]	3.79 [A]	3.31[A]	3.08[B]	2.78[B]	2.85[A]		
Δ[†] Milk yield[6] losses	0.19[a]	0.23[ab]	0.61[b]	0.70[b]	1.04[b]	19.839	0.0376
SCC[7] H	87.08[A]	208.80[A]	207.24[B]	250.86[B]	171.28[B]		
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]	2789.10	0.0306
Concentration of milk components (g/100g)							
Protein H	3.38[A]	3.34[A]	3.41[A]	3.47[B]	3.21[A]		
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]	0.0328	0.0149
Fat H	3.56[A]	3.34[A]	3.49[A]	3.47[B]	3.21[A]		
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]	0.3175	0.0161
Economic approach							
Economic return[8] H	1.2179[A]	1.1256[A]	1.1659[A]	1.1291[A]	1.2117[A]		
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]	0.2010	0.0091

*Variables were represented in average and †Δ represents the adjust values of healthy quarter minus infected; aH = represents the healthy quarters; bI = represents the infected quarters, except for group Healthy1, whose comparison was made between healthy contralateral quarters;1Right healthy quarters were subtracted from left healthy contralateral quarter; 2Corynebacterium spp. and CNS; 3Enterobacteriaceae and environmental Streptococci; 4Staphylococcus aureus and Streptococcus agalactiae; 5Enterococcus spp., Nocardia spp., non-aureus coagulase positive staphylococci, Trueperella pyogenes and yeast; 6Kg/quarter.milking; 7Geometric mean somatic cell count (×103cells/mL); 8Economic return (quarter milk yield × milk price) = US$/quarter.milking. Values per variable within a columns with different captal letters represents the difference between healthy quarter and their contralateral ($P < 0.05$). Values per variable within a row with different lowercase letters differ significantly at $P<0.05$.

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

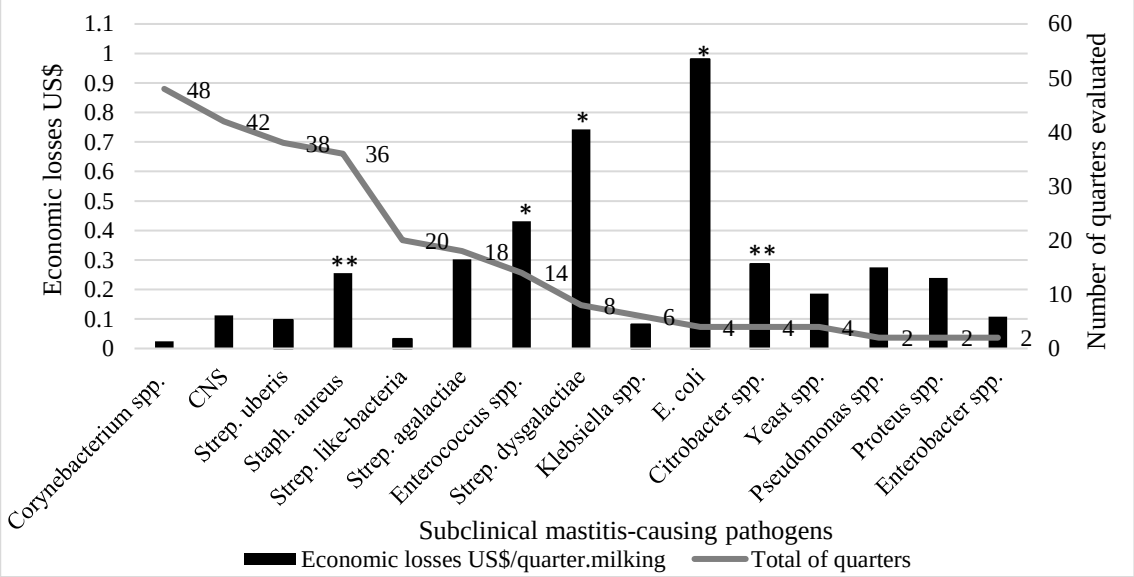
Among the isolated pathogens causing subclinical mastitis (n = 15), the milk losses ranged from 0.07 Kg/quarter.milking when mammary quarters were infected by *Corynebacterium* spp. to 2.9 Kg/quarter.milking when IMI was caused by *Escherichia coli* (Figure 9). Economic losses ranged from US$ 0.02 to 0.98/quarter.milking being higher in SM cases caused by *Enterococcus* spp. (US$ 0.43/quarter.milking), *Streptococcus dysgalactiae* (US$ 0.74/quarter.milking) and *Escherichia coli* (US$ 0.98/quarter.milking). There was a trend of *Staphylococcus aureus* and *Citrobacter* spp. induce economic losses of US$ 0.26 and 0.29/quarter.milking, respectively (Figure 10).

Figure 9 - 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).

Figure 10 - 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 ($P < 0.10$).

3.5 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 versus 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 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 (13.16%) and *Staphylococcus aureus* (11.1%) than what was found in the current study; but lower

isolation of *Corynebacterium* spp. (6.69%) and *Streptococcus uberis* (1.4%) causing SM. Interestingly, there was a lower isolation of *Streptococcus agalactiae* (3.94%) 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; RUEGG, 2003; TAPONEN; 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 between quarters have been previously reported by MERLE; SCHRODER and HAMANN (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 < 100×10^3 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. This is in agreement with recent results from other studies that evaluated natural IMI (TOMAZI et al., 2015; GONÇALVES et al., 2016), 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; POUTREL, 1982; LEVAN; EBERHART;

KESLER, 1985). Some studies reported a significant negative effect of mastitis caused by CNS on milk yield (GROHN et al., 2004; LEITNER et al., 2006). 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. 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 (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; PUHAN; SCHALLIBAUM, 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; 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. 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. Moreover, 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. 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 versus 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$ 712.80 from their profit per month when they had contagious cases [(-0.22 × $\%_{\text{contagious IMI quarters}}$) × two milking/day] and US$ 637.20 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$ 8,553.6 (contagious IMI) and US$ 7,646.4 (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; RUEGG, 2003), but relatively large compared with other studies at the quarter level (BEZMAN et al., 2015; TOMAZI et al.,

2015; GONÇALVES et al., 2016). 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*.

3.6 CONCLUSION

Specific groups of pathogens causing subclinical mastitis results in distinctly degrees of changes of quality deterioration and milk yield reduction. 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. 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 (US$ 0.18 and 0.22/quarter.milking, respectively) when compared to their healthy contralateral quarters.

Chapter 4

Chronic subclinical mastitis reduces milk yield and economic return: An evaluation using successive milk sampling over time.

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4 CHRONIC SUBCLINICAL MASTITIS REDUCES MILK YIELD AND ECONOMIC RETURN: AN EVALUATION USING SUCCESSIVE MILK SAMPLING OVER TIME.

4.1 ABSTRACT

The aim of this study was to evaluate the effects of non-chronic and chronic subclinical mastitis caused by major pathogens (*Staph. aureus*, *Strep. agalactiae*, *Strep. uberis*, *Strep. dysgalactiae* and *Streptococci*-like bacteria) on somatic cell count (SCC), milk yield and economic returns using milk sampling over time. A total of 146 out of 650 lactating cows were selected from seven dairy herds for having high composite milk SCC (> 200,000 cells/mL) in combination with the isolation of a major mastitis pathogen. From these selected cows, 1,436 quarter milk samples were collected during three successive sampling occasions with intervals of 15-20 days. From these quarter milk samples SCC, protein% and fat% were determined, as well as bacteriological culturing. Additionally, quarter milk yield was measured by milking the mammary quarters individually using three successive milk sampling over time. Bacterial isolates identified as being major pathogens by microbiological culture, but that exhibited divergence regarding the MALDI-TOF MS results, were submitted to partial sequencing of the 16S rRNA gene. Using the measured quarter milk yields and the concentration of fat and protein, the economic returns per quarter were calculated based upon the Brazilian base milk price. Quarters were classified as having chronic subclinical mastitis when the causative pathogen was detected by positive (P) culture, during three consecutive milk samplings, with 15 -20 day intervals ($P_1P_2P_3$). In turn, quarters were considered as having non-chronic subclinical mastitis when they exhibited at least one negative (N) culture result among the three-successive milk sampling over time ($P_1P_2N_3$, $P_1N_2P_3$, $P_1N_2N_3$). Quarters exhibiting non-chronic subclinical mastitis in the first sampling, but that was considered healthy after two consecutive culture-negative samplings (category $P_1N_2N_3$), produced 0.18-0.68 kg/quarter.milking more milk when they went from culture-positive to culture-negative; 0.06-0.89 kg/quarter.milking more milk when they went from culture-positive caused by major pathogens to culture-negative; 0.24-0.87 kg/quarter.milking more milk when they went from culture-positive caused by environmental streptococci to culture-negative; and 1.57-1.69 kg/quarter.milking more milk when they went from culture-positive caused by *Staph. aureus* to culture-negative. Overall,

milk losses and economic returns varied according to the type of mastitis-causing pathogen. Mammary quarters that were recovered from subclinical mastitis caused by *Staph. aureus* and environmental streptococci exhibited an increase in economic returns of approximately 0.47 and 0.69 US$/quarter.milking, respectively.

Keywords: Mastitis. Subclinical. Chronic. Non-chronic. *Staph. aureus.* Environmental streptococci. Milk loss. Economic return.

4.2 INTRODUCTION

Mastitis is the most prevalent production disease in dairy herds worldwide and is associated with several negative production effects (HALASA et al., 2007). Milk yield and composition can be affected by a more or less severe short-term depression and, in case of no cure, by a long-acting effect, sometimes overlapping effect to the next lactation (SEEGERS; FOURICHON; BEAUDEAU, 2003). The severity of the inflammation can be classified into subclinical, clinical and chronic forms, and its degree is dependent on the nature of the causative pathogen and on the age, breed, immunological health and lactation state of the animal (BRADLEY, 2002). Subclinical mastitis cannot be detected visually but still has major cost implications. The effects caused by subclinical mastitis can be further intensified when the pathogen resists the immune defense and adapts to the mammary tissue which become the infection as chronic. Chronic subclinical mastitis is an another form of the disease that results in persistent inflammation of the mammary gland (VIGUIER et al., 2009). Therefore, the losses associated with milk production may be more intense due to the damage caused by invading bacteria to milk secretory epithelia of the mammary gland (VIGUIER et al., 2009), which also could result in permanent loss of capacity to synthesize milk when the quarter are infected by chronic cases (AULDIST et al., 1995).

Numerous studies have documented that cows with subclinical infections, indicated by an increased somatic cell count (SCC), which are associated with reduced milk production per cow (LOSINGER, 2005). Despite that SCC has routinely been used to diagnose intramammary infection (IMI), other methodologies like microbiology culture, MALDI-TOF MS and partial sequencing of genes can be used to identify the specific mastitis-causing pathogen (BARREIRO et al., 2010). It is already known that changes in milk yield associated with

mastitis strongly depend on the mastitis causing pathogen (COULON et al., 2002). In previous studies, BEZMAN et al. (2015) described that the infection caused by *Strep. dysgalactiae* decreased the quarter milk yield by approximately 20%. BOTARO et al. (2015) reported that the replacement of the secretory tissue by a fibrotic one may gradually occur as a consequence of the *Staph. aureus* IMI, leading to the chronic form of the disease. COULON et al. (2002) described that *Escherichia coli* mastitis induces the greatest and most durable milk yield reductions, when compared with other types of mastitis. However, the effects of pathogens causing subclinical mastitis on milk yield and composition have been reported less frequently (LEITNER et al., 2000; COULON et al., 2002; BERGLUND et al., 2007; FORSBACK et al., 2009; BEZMAN et al., 2015).

Different approaches were used to estimate production losses due to subclinical mastitis (DÜRR et al., 2008; HAND; GODKIN; KELTON, 2012). The most commonly used in research is based on analysis of milk SCC for comparison among herds and at the cow level, but less frequently reported at the quarter level. However, for quantification of the effects caused by mastitis, fixed effects such as parity and lactation stage needs to be included in the evaluation to minimize the sources of variation (HALASA et al., 2009). Most previous studies evaluated the difference in milk yield and composition between healthy and infected quarters but based on SCC (BARKEMA et al., 1997; WILSON et al., 1997b; FORSBACK et al., 2009). Moreover, sampling at the quarter level seems to be beneficial, since it avoid the dilution effect from composite milk sampling upon a single quarter with high SCC (GREEN; SCHUKKEN; GREEN, 2006). In this context, we believe that successive milk quarter sampling occasions over time with the intension to evaluate milk loss caused by major pathogens would be the best approach to determine the effect caused by chronic subclinical mastitis cases. To date, there are few studies that specifically evaluated the effects of chronic subclinical mastitis per type of pathogen at the mammary quarter level (SWINKELS et al., 2005a; STEENEVELD; SWINKELS; HOGEVEEN, 2007). The studies are almost scarce if considered our proposed comparison using successive milk quarter sampling occasions over time. The precise information about the milk production losses caused by subclinical mastitis, specially by major pathogens, at quarter level will help the dairy industry establish control measures to avoid losses both in milk yield and quality of dairy products (BOTARO et al., 2015). For that reason, the aim of this study was to evaluate the effects of non-chronic and chronic subclinical mastitis caused by major pathogens (*Staph. aureus*, *Strep. agalactiae*, *Strep. uberis*, *Strep. dysgalactiae* and *Streptococci*-like bacteria) on SCC, milk yield and economic returns using comparison of successive milk sampling over time.

4.3 MATERIAL AND METHODS

4.3.1 **Ethics approval**

Before the commencement of the study, approval was obtained from the Ethical Committee for the Use of Animals of the School of Veterinary Medicine and Animal Sciences at the University of São Paulo, Brazil (protocol number 3020/2013).

4.3.2 **Dairy herds and cow selection**

In order to be included in this study, herds were required to have cow identification and data recording systems in place. Lactating Holstein cows (n = 650), with an average parity of 2.3 (SEM 0.03) and 192 (SEM 3.3) days in milk, from seven dairy herds (located in the Midwest area of São Paulo State, Brazil), and with no history of clinical mastitis within the preceding month, were used (for details: GONÇALVES et al., 2017 and see chapter 3). Selected cows had an average milk yield of 22.3 ± 0.2 L/cow.day during the nine-month sampling period (Feb-Oct 2014). All lactating cows were housed in free-stall barn facilities and were milked twice a day in herringbone milking parlors, using similar milking routines among farms. In all herds, cows were fed a total mixed ration (TMR) composed of corn silage, grain concentrate, and minerals.

Composite milk samples were collected from all lactating cows for SCC analyses and bacteriological culturing (BC) for the identification of pathogens causing IMI (Step 1). Milk yield (L/cow.day) data, information with respect to parity and days in milk was recorded at the cow level. All foremilk samples submitted for BC analyses were collected using an aseptic technique, following National Mastitis Council guidelines (OLIVER et al., 2004). Dairy cows with SCC > 200×10^3 cells/mL and positive isolation of pathogens were considered to be subclinically infected (n = 146) and were selected for evaluation at the mammary quarter level within 15 days (Step 2) Table 11.

4.3.3 Milk sampling and quarter milking

Quarter milk samples (*n* = 1,436; Step 2) from cows considered to be subclinically infected were collected during three successive sampling occasions, with an interval of 15 to 20 days. The milk yield was measured at the quarter level by milking mammary quarters individually using a bucket milking system (GONÇALVES et al., 2017, see chapter 3). Briefly, the equipment included a pulsator and a cluster of four liners connected to individual silicone tubes, equipped with valves for vacuum release. Each liner was fitted with a milk meter (MM6 DeLaval, Campinas, Brazil) to estimate milk yield by quarter. 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 center stand held a bucket with a capacity for 50 liters. The system allowed the milk to flow separately from each mammary quarter to a milk meter and then into the bucket. After milking, quarter milk samples (40 mL) from the milk meter were collected into plastic tubes containing Bronopol (2-bromo-2-nitropropane-1,3-diol) as a preservative (0.05 g/100 mL milk), according to International Dairy Federation guidelines (IDF-FIL, 1995). Samples were stored in a refrigerator (4-7°C) until they were transported to the laboratory for milk composition and SCC analyses.

4.3.4 Microbiological and milk composition analyses

Microbiological cultures of the milk samples were performed in accordance with the National Mastitis Council guidelines (OLIVER et al., 2004), with the addition of the acetoin biochemical test. Briefly, a total of 10.0 µL of milk was inoculated on blood agar plaques containing 5% defibrinated bovine blood. The plates were inverted and incubated in an aerobic environment at 37°C for 48 hours and observed every 24 hours for colony characterization (format, size, number, and color) and hemolysis. Gram staining and KOH and catalase testing were carried out in order to determine morphology, differentiation and bacterial grouping. The biochemical tests used for bacterial species identification were performed as previously reported by GONÇALVES et al. (2017). Isolates were cryopreserved at -80ºC in sterile plastic tubes containing 1.0 mL of brain heart infusion broth (BBL-Becton Dickinson and Co., Cockeysville, MD, USA) and 0.5 mL of 20% glycerine solution. Following preservation, all

bacterial isolates that were classified as major pathogens were thawed, recultured, and submitted to species-specific identification using the MALDI-TOF MS method. Isolates identified as major pathogens by microbiological culture, but that exhibited divergent identification by MALDI-TOF MS, were submitted to partial sequencing of the 16S rRNA gene.

Milk components (concentration of protein and fat) were determined by infrared absorption using a milk analyzer (Bentley 2000®, Bentley Instruments Inc., Chaska, MN, USA), and SCC, by the use of flow cytometry equipment (Somacount300®, Bentley Instruments Inc., Chaska, MN, USA).

4.3.5 **Bacterial species identification by MALDI-TOF MS**

A loop of the bacterial colony was added to 300 µL of autoclaved Milli-Q® water (Millipore Corporation, Bedford, MA, USA) and 900 µL of HPLC grade ethanol, followed by homogenization for 1 minute. In order to completely remove the supernatant, centrifugation was carried out (all centrifugations were performed at 13,000 x g during 2 min) in a 5417R model Epperndof® centrifuge (Eppendorf do Brasil, São Paulo, Brazil). Bacterial pellets were dried at room temperature during 5 to 10 minutes.

Formic acid solution 70% was added (10-50 µL) to the bacterial pellets, in proportion with sediment size, in order for complete dissolution. Next, acetonitrile 100% was added to each sample in equal volumes to the formic acid 70% solution, with an interval of 10 min between each reagent, thus producing a bacterial extract at a 1:1 ratio of formic acid 70% and acetonitrile 100%. One final centrifugation step was carried out in order to separate bacterial cell debris of the supernatant, which contained the extracted ribosomal proteins (BARREIRO et al., 2012).

A total of 1.0 µL of each bacterial extract was spotted onto the steel plate spots (MSP 384 polished-steel target; Bruker Daltonik, Bremen, Germany) and left to dry at room temperature. The dry spots were layered with 1.0 µL of matrix solution, composed of α-cyano-4-hydroxycinnamic acid, diluted in acetonitrile 50% and trifluoroacetic acid 2.5%. Following matrix addition, the spots were dried at room temperature during approximately 7 minutes in order to perform mass spectrometry analyses.

4.3.6 **16S rRNA gene sequence analyses**

An aliquot of 1.0 mL of cryopreserved bacterial samples was centrifuged at 10,000 x g during 10 min. Next, 100 µL of lysozyme buffer solution and 4.0 µL of lysozyme (10 mg/mL, Merck, Whitehouse Station, NJ, USA) was added to the obtained bacterial pellet, and the suspensions were incubated at room temperature for 15 min. Subsequently, DNA extraction was performed using an extraction kit with 20.0 µL of proteinase K (Illustra® blood genomic Prep Mini Spin, GE Healthcare, Little Chalfont, Buckinghamshire, UK). All PCR reactions were adjusted to a 25.0 µL total volume, of which 2.0 µL aliquots were added to the 23.0 µL of PCR mixture [8.5 µL of distilled water, 12.5 µL of Go Taq Colorless Master Mix® (Promega, Madison, WI, USA), 1.0 µL of forward primer and 1.0 µL of reverse primer].

The degenerate primer pair Bac-16SF-5'AGAGTTTGATCATGGCTCAG3' and Bac-16SR-5'CGGTTACCTTGTTACGACTT' were initially used to amplify pathogens causing subclinical mastitis. Cycling conditions were 94°C for 5 min, 35 cycles of 94°C for 20 s, 50°C for 20 s, 75°C for 45 s and 72°C for 5 min. The PCR was performed in a Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, USA).

The resulting PCR products were analyzed by electrophoresis in 1.5% (wt/vol) agarose gels prepared with TBE (Tris/borate/EDTA). An aliquot of 3.0 µL of molecular marker, containing a 100 base pair DNA Ladder® (Applied Biosystems, Foster City, CA, USA), was added to the first well of each gel. Negative control (Mix+primers) and positive control (*Staph. aureus,* ATCC 29.213) were placed into the remaining wells of each gel.

After 30 minutes of electrophoresis, of which 5 were at 80 volts and 25 at 110 volts, DNA amplification blots were visualized by ultraviolet translumination. The bands of interest were excised and purified using a gel band purification kit (Wizard® PCR Preps DNA Purification System, Promega, Madison, WI), according to the manufacturer's instructions.

The purified PCR products were sequenced unidirectionally using the reverse (R) primer. The sequencing reaction was carried out adding 1.0 µL of the R primer [5 pmoL], 1.5 µL of 5X Buffer, 2.0 µL of purified DNA [20ng/µL]; 4.5 µL of nuclease-free water, and 1.0 µL of BigDye® (Terminator v3.1 Cycle Sequencing kit - Applied Biosystems, Foster City, CA, USA), and cycling conditions were identical to that applied in the polymerase chain reaction described previously.

The precipitation procedure was carried out with two ethanol washes (Sigma, St. Louis, MO, USA). In the first washing step, 21.0 µL of absolute ethanol was used, and the solution

was maintained at room temperature for 5 min. Next, the solution was centrifuged at 3,000 x g for 30 min at 15°C. In the second washing step, 35.0 µL of ethanol (75%) were used and centrifuged at 1,650 x g for 15 minutes at 15°C. The ethanol was discarded by inversion of the plates, which were submitted to heating at 95°C for 8 minutes, and 10.0 µL of Hi-Di formamide (Life TechnologiesTM, Foster City, CA, USA) was added. The sequencing reaction products were analyzed by automatic sequencer capillaries (ABI 3500 Genetic Analyzer® - Applied Biosystems, Foster City, CA, USA).

All sequences obtained from 16S rRNA gene sequences were confirmed using the GenBank online data Reference Library. Isolates were regarded as identified at the species level when the similarity to a reference sequence was ≥ 99%.

4.3.7 **Mastitis characterization and experimental design**

Mammary quarters were characterized as having subclinical mastitis when the milk samples exhibited SCC > 100×10^3 cells/mL. Furthermore, they were considered to be infected by minor pathogens (*Corynebacterium* spp. and Coagulase-negative staphylococci, CNS) when > 10 colonies (1,000 CFU/mL) were isolated; for environmental agents (environmental or Gram-negative streptococci) when > 3 colonies (300 CFU/mL) were isolated; for contagious pathogenic agents (*Staph. aureus* or *Strep. agalactiae*) when ≥ 1 colony (100 CFU/mL) were isolated; or for other mastitis-causing pathogens, as described by DOHOO et al. (2011). The mammary quarters were classified as having chronic subclinical mastitis when the same causative pathogen was detected by positive (P) culture in the milk samples from the quarters, during three consecutive samplings, with 15-20 day intervals ($P_1P_2P_3$) as described by Berglund et al (2007), but using the mastitis definition based on SCC > 200×10^3 cells/mL. In turn, the mammary quarters were considered as having non-chronic subclinical mastitis when they exhibited at least one negative (N) culture result among the three-successive milk sampling over time ($P_1P_2N_3$, $P_1N_2P_3$, $P_1N_2N_3$). Quarters were considered healthy when the SCC ≤ 100×10^3 cells/mL, with the absence of bacterial growth after 48 hours of milk incubation.

The study was carried out using three assays (Tables 11 to 13):

During the first assay (1), four types of approaches were applied. Regarding approach 1, the positive (P) and negative (N) culture results were considered. Therefore, the quarters that exhibited chronic subclinical mastitis during milk sampling ($P_1P_2P_3$, n = 114) were compared

with the quarters classified as non-chronic subclinical mastitis ($P_1N_2N_3$, n = 135; $P_1N_2P_3$, n = 39; and $P_1P_2N_3$, n = 66). With regard to approach 2, culture results showing isolation of major pathogens (all isolated pathogens, except CNS and *Corynebacterium* spp.) were considered. Thus, quarters exhibiting chronic subclinical mastitis caused by major pathogens during the three samplings ($P_1P_2P_3$, n = 54) were compared with the quarters with non-chronic subclinical mastitis also caused by major pathogens ($P_1N_2N_3$, n = 66; $P_1N_2P_3$, n = 15; and $P_1P_2N_3$, n = 15). With respect to approach 3, quarters with chronic subclinical mastitis caused by environmental streptococci ($P_1P_2P_3$, n = 24) were compared with the quarters exhibiting non-chronic subclinical mastitis, also caused by environmental streptococci ($P_1N_2N_3$, n = 12; $P_1N_2P_3$, n = 6; and $P_1P_2N_3$, n = 9). Regarding approach 4, mammary quarters with chronic subclinical mastitis caused by *Staph. aureus* ($P_1P_2P_3$, n = 27) were compared with quarters exhibiting non-chronic subclinical mastitis, also caused by *Staph. aureus* ($P_1N_2N_3$, n = 3 and $P_1N_2P_3$, n = 3).

In the second assay, mammary quarters exhibiting chronic subclinical mastitis caused by *Staph. aureus* ($P_1P_2P_3$, n = 27) and environmental streptococci ($P_1P_2P_3$, n = 24) were compared with quarters that were deemed healthy due to culture-negative during the three milk samplings ($N_1N_2N_3$, n = 264).

Regarding the third assay, two approaches were carried out. In the first approach, mammary quarters exhibiting non-chronic subclinical mastitis cause by *Strep. agalactiae* ($P_1N_2N_3$, n = 18), environmental streptococci ($P_1N_2N_3$, n = 12), *Strep. like-bacteria* ($P_1N_2N_3$, n = 12), *Enterococcus* spp. ($P_1N_2N_3$, n = 12), Gram-negative bacteria ($P_1N_2N_3$, n = 6), *Staph. aureus* ($P_1N_2N_3$, n = 3) and yeast ($P_1N_2N_3$, n = 3) were compared with quarters that were considered healthy, due to culture-negative results during the three milks samplings ($N_1N_2N_3$, n = 264). With respect to the second approach, quarters with non-chronic subclinical mastitis caused by environmental streptococci ($P_1P_2N_3$, n = 9) and *Strep. agalactiae* ($P_1P_2N_3$, n = 3), were compared with quarters that were considered healthy, since they were culture-negative during the three milk samplings ($N_1N_2N_3$, n = 264).

Table 11 - Total of steps performed in the current study and the performed variables

Evaluated variables	*Sampling occasion*					
	Step 1. sampling at cow level A total of 650 cows			Step 2. sampling at quarter level A total of 1,436 enrolled quarters		
	-3	-2	-1	0	1	2
The day of sampling	1 d	7 d	14 d	15 d	30 d	45 d
Criteria of selection	Cow at least with two sampling occasion with SCC > 200,000 cells/mL and positive-culture at the last sampling of the step 1			Quarters culture-positive *quarters with SCC < 100,000 cells/mL and culture-negative were considered healthy		
SCC	✓	✓	✓	✓	✓	✓
Protein%	✓	✓	✓	✓	✓	✓
Fat%	✓	✓	✓	✓	✓	✓
Culture			✓	✓	✓	✓
Measurement of milk yield	✓	✓	✓	✓	✓	✓

Table 12 - Classification of mammary quarters according to microbiological culture results

Sampling occasions at the step 2			Intramammary infection quarter status
15 d	30 d	45 d	
N[a]	N	N	Healthy
N	P[b]	P	*not included*
N	P	N	*not included*
N	N	P	*not included*
P	P	P	Chronic subclinical cases
P	P	N	Non-chronic subclinical cases
P	N	P	Non-chronic subclinical cases
P	P	N	Non-chronic subclinical cases

[a]Culture negative (N); [b]Culture-positive (P)

Table 13 - Assays and approaches used in statistical models for evaluating effect of mastitis

Assay	Approach		Categories comparison	Possibilities of comparison among categories
1	1	positive or negative culture	Chronic subclinical cases vs. non-chronic	PPP × (PPN[a], P[b]NP, PPN)
1	2	group of pathogen level	Chronic subclinical cases vs. non-chronic	PPP × (PPN, PNP, PPN)
1	3	pathogen specie level	Chronic subclinical cases vs. non-chronic	PPP × (PPN, PNP, PPN)
1	4	pathogen specie level	Chronic subclinical cases vs. non-chronic	PPP × (PPN, PNP, PPN)
2	1	pathogen specie level	Chronic subclinical cases vs. healthy quarters	PPP × NNN
3	1	pathogen specie level	Non-chronic subclinical cases vs. healthy quarters	NNN × (PPN, PNP, PPN)
3	2	pathogen specie level	Non-chronic subclinical cases vs. healthy quarters	NNN × (PPN, PNP, PPN)

[a]Culture negative (N); [b]Culture-positive (P)

4.3.8 **Economic return**

At the quarter level, the milk price per liter was simulated using the milk quality payment programs for milk protein and fat, from a Brazilian commercial dairy processing company, as described previously (GONÇALVES et al., 2017, see chapter 3). The Brazilian base milk price was set at US$ 0.306/L, based on price data over the last 20 years. After these preliminary calculations, simulation of milk quality payment at the quarter level was performed using the concentrations of milk fat and protein at the quarter level. The final milk price (US$), considering the milk quality payment at quarter level, was calculated as the sum of the Brazilian base milk price and each adjustment due to quality milk prices. Additionally, the economic return per milking at the quarter level (US$/milking.quarter) was calculated multiplying the final milk price by the milk yield (Kg/milking.quarter).

4.3.9 **Statistical analyses**

The effect of chronic subclinical mastitis on SCC, milk production and economic returns was assessed using the comparison of successive milk sampling over time. Regarding the proposed assays, the following statistical model was applied:

$$Y_{ijklmnop} = \mu + R_{i(random)} + C_{j(random)} + Q_k \times C_l(R_i) + D_m + P_n + Cat_o + S_p + Cat_o \times S_p + e_{ijklmnop}$$

in which $Y_{ijklmno}$ was considered as the dependent variable; μ represented the general average; R_i represented the herd (i = 1 to 7) and C_j, the cow (j = 1 to 650), in which both were considered as random effect; $Q_k \times C_l(R_i)$ represented the fixed effect regarding quarter-cow within herd; D_m was considered as the effect of the days in milk (m = 62 to 483) and P_n, the number of calvings (n = 1 to 6), both inserted as covariables in the model; Cat_o represented the categories regarding presence or absence of subclinical mastitis during the three milk samplings (o = 1 to 8; N = culture-negative; P = culture-positive, type of the pathogen according to the proposed assay; 1. $N_1N_2N_3$; 2. $N_1N_2P_3$; 3. $N_1P_2P_3$; 4. $N_1P_2N_3$; 5. $P_1P_2P_3$; 6. $P_1P_2N_3$; 7. $P_1N_2N_3$; and 8. $P_1N_2P_3$); S_p represented the effect of time, mammary quarter sampling (p = 1 to 3); $Cat_o \times S_p$ was the interaction between infection status and sampling period, and $e_{ijklmno}$ represented the random deviation.

Variance heterogeneity was removed from all SCC data by way of transformation to SCC linear score (LS) values, using the formula described as follows (SCHUKKEN et al., 2003):

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

Afterward, the SCC was shown as a geometric mean. Statistical models were assessed using the SAS MIXED procedure (version 9.3, SAS Institute, Cary, NC, USA). With regard to all of the statistical analyses, $P \leq 0.05$ for significance and $P \leq 0.10$ for tendency were applied.

4.4 RESULTS

4.4.1 Effect of subclinical mastitis on milk production and composition by comparison of multiple samplings of mammary quarters

The groups of pathogens that most frequently caused IMI, isolated from the three-successive quarter milking sampling occasions over time were: minor pathogens (CNS and *Corynebacterium* spp., 14%), environmental pathogens (*Strep. uberis*, *Strep. dysgalactiae*, *Strep.* like-bacteria and Gram-negative bacteria; 7.7%), contagious bacteria (*Staph aureus* and *Strep. agalactiae*; 7.3%), and infrequent bacteria (CPS, *Enterococcus* spp., *Trueperella pyogenes* and yeast; 1.3%) (Table 14). In the three successive quarter sampling occasions, a reduction in IMI could be observed over time as the percentage of culture-negative from the quarters increased: 64.2% (sampling 1) to 70.6% (sampling 2) and 74.3% (sampling 3).

4.4.2 Identification by MALDI-TOF MS and sequencing of the 16S rRNA gene

A total of 212 isolates of major mastitis-causing pathogens (*Staph. aureus*, $n = 79$; *Strep. uberis*, $n = 69$; *Strep. agalactiae*, $n = 29$; *Strep.* like-bacteria, $n = 16$; *Enterococcus* spp., $n = 13$; and *Strep. dysgalactiae*, $n = 6$) that caused subclinical mastitis ($n = 212$, distributed over three samplings) underwent identification at the bacterial species level by MALDI-TOF mass spectrometry (Table 15). A total of 92.4% of the *Staph. aureus* isolates ($n = 79$, identified by microbiological culture, were confirmed at the species level by MALDI-TOF MS ($n = 73$, score > 2). Only six *Staph. aureus* isolates, identified by microbiological culture, displayed diverging identification of bacterial species by MALDI-TOF MS. Among these isolates, three were identified in the first sampling (1 *Staph. epidermidis*, 1 *Staph. haemolyticus* and 1 *Staph.*

chromogenes), two were isolated in the second sampling (2 *Staph. chromogenes*) and one during sampling 3 (1 *Staph. chromogenes*) (Table 15).

A total of 91.3% of the *Strep. uberis* isolates (*n* = 69), identified by microbiological culture, were confirmed at the species level by MALDI-TOF MS (*n* = 63, score > 2). A remaining six isolates, identified as *Strep. uberis* by microbiological culture, exhibited different genus and bacterial species identification by MALDI-TOF MS, of which 2 were identified as *Aerococcus viridans* and 1 was *Lactococcus garvieae*, from the first sampling; 2 were *Aerococcus viridans*, from the second sampling; and 1 was an *Aerococcus viridans*, from the third sampling (Table 15).

Strep. agalactiae isolates (*n* = 29), identified by microbiological culture, exhibited 100% similarity at the species identification level by MALDI-TOF MS (*n* = 29, score > 2). A total of six *Strep. dysgalactiae* isolates, identified by microbiological culture, were identified as 5 *Strep. dysgalactiae* and 1 *Aerococcus viridans* by MALDI-TOF MS (Table 15).

The identification of the *Enterococcus* spp. isolates (*n* = 13) by microbiological culture showed 92.3% similarity to the MALDI-TOF MS identification results (*n* = 12, score > 2). In the first sampling, the MALDI-TOF MS enabled the identification of 5 isolates of *Enterococcus faecalis*, 2 of *Enterococcus gallinarum*, 1 of *Enterococcus faecium* and 1 of *Streptococcus lutetiensis*. During sampling 2, identification by mass spectrometry, at the species level, was possible for 1 *Enterococcus faecalis* and 1 *Enterococcus faecium*. In sampling 3, two isolates of *Enterococcus* spp. were identified by MALDI-TOF MS as *Enterococcus faecium* and *Enterococcus gallinarum* (Table 15). The *Strep. lutetiensis* isolate, identified by MALDI-TOF MS (score = 2), may have been wrongly identified as *Enterococcus* spp. by the culture, since the pathogen was not inserted into the laboratory routine microbiological identification clad.

A total of 16 isolates were identified by microbiological culture as *Strep*. like-bacteria. From this total (*n* = 16), using MALDI-TOF MS, it was possible to determine the following pathogens at the species level: 5 *Aerococcus viridans* (score > 2), 5 *Aerococcus viridans* (score > 1.9), 3 *Lactococcus lactis* (score > 2), 2 *Lactococcus garvieae* (score > 2) and 1 *Enterococcus hirae* (score > 2).

A total of 22 isolates had divergent identification results between microbiological culture and MALDI-TOF MS, and were submitted to 16S rRNA gene sequencing. The 16S rRNA gene sequencing results are described in Table 16. In general, the sequencing of the 16S rRNA gene confirmed 95.6% of the identification achieved by mass spectrometry. Only one isolate, identified by microbiological culture as *Strep*. like-bacteria, displayed divergence between MALDI-TOF MS and sequencing. Using mass spectrometry, it was determined as

Aerococcus viridans (score = 2.09), and by sequencing, as *Streptococcus uberis* (ID% ≥ 99%). In Figure 11, the spectra (m/z), containing the mass peaks, are shown, regarding a few of the bacterial isolates that cause subclinical mastitis. Some bacterial species exhibit high similarity and may be differentiated by mass peak observation.

Table 14 - Microbiological culture results of mammary quarter milk samples (Step 2: sampling 1, n = 584; sampling 2, n = 470; and sampling 3, n = 382) from 7 dairy herds

Microorganism	Nº. isolates from step2 (quarter sampling stage)					
	Sampling 1	(%)	Sampling 2	(%)	Sampling 3	(%)
Nº.	584	100	470	100	382	100
Culture-negative	375	64.2	332	70.6	284	74.3
Culture-positive	209	35.8	138	29.4	98	25.7
Secondary agents	80	13.7	66	14.0	54	14.1
CNS[1]	34	5.8	30	6.4	31	8.1
Corynebacterium spp.	46	7.9	36	7.7	23	6.0
Environmental agents	59	10.1	37	7.9	20	5.2
Streptococcus uberis	34	5.8	21	4.5	14	3.7
Streptococcus dysgalactiae	4	0.7	2	0.4	0	-
Strep. like-bacteria	7	1.2	4	0.9	5	1.3
Gram-negative bacteria	14	2.4	10	2.1	1	0.3
Contagious agents	54	9.2	32	6.8	22	5.8
Staphylococcus aureus	31	5.3	27	5.7	21	5.5
Streptococcus agalactiae	23	3.9	5	1.1	1	0.3
Uncommon agents	16	2.7	3	0.6	2	0.5
CPS[2]	1	0.2	0	-	0	-
Enterococcus spp.	9	1.5	2	0.4	2	0.5
Trueperella pyogenes	2	0.3	0	-	0	-
Yeast	2	0.3	1	0.2	0	-
Contamination	2	0.3	0	-	0	-

[1] Coagulase-negative staphylococci; [2]Non-aureus coagulase-positive staphylococci.

Table 15 - Identification of the species that cause subclinical mastitis by MALDI-TOF MS of the quarter milk samples from three sampling periods (herds, n = 7)

Microbiological culture		MALDI-TOF MS Tube extraction				
No.	Suggested identification	No.	Suggested identification	Score	SD[1]	SCC[2]
Sampling 1						
34	*Streptococcus uberis*	31	*Streptococcus uberis*	2.22	0.10	871
		2	*Aerococcus viridans*	2.00	0.04	14
		1	*Lactococcus garvieae*	2.15	-	596
31	*Staphylococcus aureus*	28	*Staphylococcus aureus*	2.21	0.13	731
		1	*Staphylococcus epidermidis*	2.33	-	563
		1	*Staphylococcus haemolyticus*	2.07	-	1632
		1	*Staphylococcus chromogenes*	1.96	-	249
23	*Streptococcus agalactiae*	23	*Streptococcus agalactiae*	2.18	0.10	737
4	*Streptococcus dysgalactiae*	3	*Streptococcus dysgalactiae*	2.39	0.18	815
		1	*Streptococcus dysgalactiae*	1.88	-	253
9	*Enterococcus* spp.	5	*Enterococcus faecalis*	2.00	0.11	872
		2	*Enterococcus gallinarum*	2.21	0.02	311
		1	*Enterococcus faecium*	2.45	-	5814
		1	*Streptococcus lutetiensis*	2.00	-	180
7	*Strep. like-bacteria*	3	*Lactococcus lactis*	2.25	0.03	3311
		1	*Lactococcus garvieae*	2.07	-	6555
		1	*Aerococcus viridans*	2.08	-	38
		2	*Aerococcus viridans*	1.98	0.02	48
Sampling 2						
27	*Staphylococcus aureus*	25	*Staphylococcus aureus*	2.28	0.06	350
		2	*Staphylococcus chromogenes*	1.83	0.02	117
21	*Streptococcus uberis*	19	*Streptococcus uberis*	2.21	0.1	825
		2	*Aerococcus viridans*	2.05	0.02	29
5	*Streptococcus agalactiae*	5	*Streptococcus agalactiae*	2.22	0.11	945
4	*Strep. like-bacteria*	1	*Enterococcus hirae*	2.40	-	505
		3	*Aerococcus viridans*	1.96	0.01	111
2	*Streptococcus dysgalactiae*	1	*Streptococcus dysgalactiae*	2.33	-	127
		1	*Aerococcus viridans*	2.06	-	589
2	*Enterococcus* spp.	1	*Enterococcus faecalis*	2.22	-	1367
		1	*Enterococcus faecium*	2.17	-	7977
Sampling 3						
21	*Staphylococcus aureus*	20	*Staphylococcus aureus*	2.29	0.12	453
		1	*Staphylococcus chromogenes*	2.11	-	1403
14	*Streptococcus uberis*	13	*Streptococcus uberis*	2.30	0.09	686
		1	*Aerococcus viridans*	2.09	-	54
5	*Strep. like-bacteria*	4	*Aerococcus viridans*	2.04	0.04	129
		1	*Lactococcus garvieae*	2.06	-	108
2	*Enterococcus* spp.	1	*Enterococcus gallinarum*	2.17	-	2945
		1	*Enterococcus faecium*	2.24	-	3564
1	*Streptococcus agalactiae*	1	*Streptococcus agalactiae*	2.37	-	2682

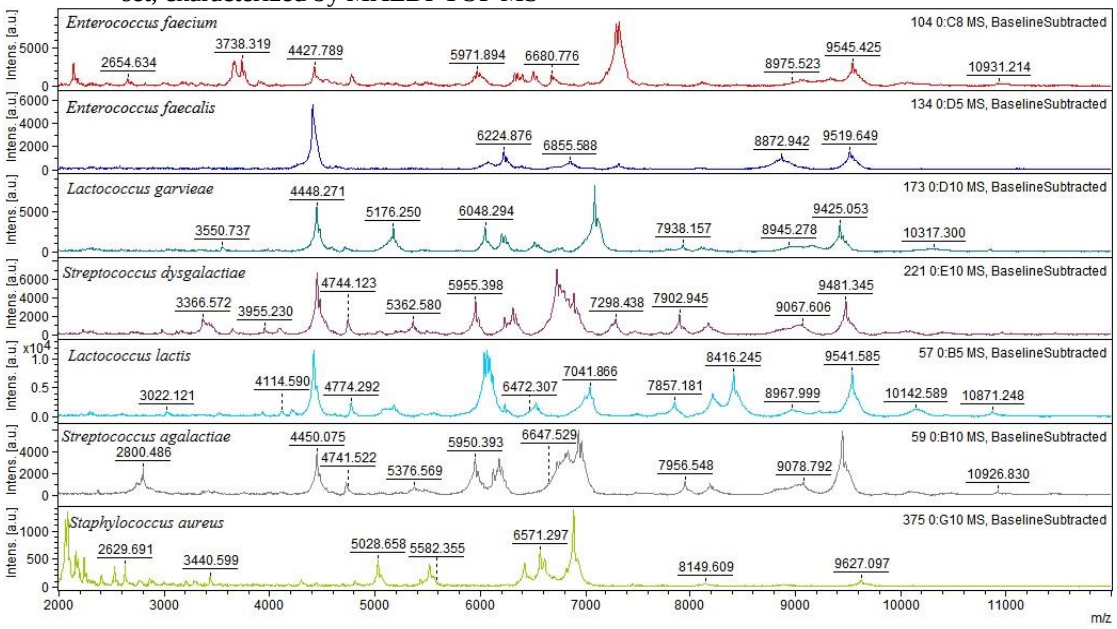
[1]Standard deviation; [2]Geometric mean of somatic cell count SCC ($\times 10^3$células/mL).

Table 16 - Divergent results between microbiological cultures and MALDI-TOF MS, confirmed by 16S rRNA gene sequencing

Microbiological culture		MALDI-TOF MS Tube extraction			16S rRNA sequencing		
No.	Suggested identification	No.	Suggested identification	Score	No.	Suggested identification	ID%[1]
14	Other *Streptococcus*	3	*Aerococcus viridans*	2.04	3	*Aerococcus viridans*	≥ 99%
		1	*Aerococcus viridans*	2.09	1	*Streptococcus uberis*	≥ 99%
		5	*Aerococcus viridans*	1.97	5	*Aerococcus viridans*	≥ 99%
		1	*Enterococcus hirae*	2.40	1	*Enterococcus hirae*	≥ 99%
		2	*Lactococcus garvieae*	2.06	2	*Lactococcus garvieae*	100%
		2	*Lactococcus lactis*	2.25	2	*Lactococcus lactis*	≥ 99%
6	*Streptococcus uberis*	5	*Aerococcus viridans*	2.03	5	*Aerococcus viridans*	≥ 99%
		1	*Lactococcus garvieae*	2.15	1	*Lactococcus garvieae*	≥ 99%
1	*Streptococcus dysgalactiae*	1	*Aerococcus viridans*	2.06	1	*Aerococcus viridans*	≥ 99%
1	*Enterococcus* spp.	1	*Streptococcus lutetiensis*	2.00	1	*Streptococcus lutetiensis*	≥ 99%

[1]Similarity percentage of the sequences found on GenBank ≥ 99%

Figure 11 - Identification of species of subclinical mastitis-causing bacteria based on ribosomal protein set, characterized by MALDI-TOF MS



4.4.3 **Assay 1: effect of chronic subclinical mastitis on milk yield, somatic cell count, and economic return versus non-chronic subclinical mastitis.**

Approach 1. When quarters remained infected with chronic subclinical mastitis, no difference of milk yield among quarter sampling occasions were observed (P_1 = 2.59 kg/quarter.milking; P_2 = 2.66 kg/quarter.milking; and P_3 = 2.26 kg/quarter.milking; P-values > 0.05). On the other hand, considering the quarter categories with non-chronic subclinical mastitis ($P_1N_2N_3$; $P_1N_2P_3$ and $P_1P_2N_3$), culture-positive quarters in the first milk sampling (P_1 = 2.82 kg/quarter.milking), had lower milk yield than culture-negative quarters in the last two milk samplings (N_2 = 3.5 kg/quarter.milking and N_3 = 3.0 kg/quarter.milking; P-values < 0.05). In other words, quarters that were infected at the first sampling had no pathogen isolation in the next two samplings had increased milk yield, varying from 0.18 to 0.68 kg/quarter.milking. The SCC accompanied the culture results, in which if the culture was positive (detection of the colony causing subclinical mastitis), the SCC was greater than 100,000 cells/mL. The economic return did not differ among quarters with chronic subclinical mastitis when comparing successive milk sampling occasions over time (P_1 = 0.86 US$/quarter.milking; P_2 = 0.87 US$/quarter.milking, and P_3 = 0.74 US$/quarter.milking). However, quarters classified as non-chronic subclinical mastitis, from a category ($P_1N_2N_3$) that presented a culture-positive result

in the first milk sampling, had lower economic return (P_1 = 0.92 US$/quarter.milking) when compared to the quarters that were culture-negative in the second milk sampling (N_2 = 1.15 US$/quarter.milking) (Table 17).

Approach 2. As observed in approach 1, quarters of category $P_1N_2N_3$ with non-chronic subclinical mastitis caused by major pathogens, had lower milk yield in the first milk sampling (P_1 = 2.74 kg/quarter.milking) than culture-negative quarters in the second sampling (N_2 = 3.63 kg/quarter.milking; P-value > 0.05). The SCC was greater than 100,000 cells/mL when major pathogens were detected in BC. The economic return was also higher for culture-negative quarters in the second sampling (N_2 = 1.17 US$/quarter.milking) than for culture-positive quarters infected by major pathogens in the first sampling (P_1 = 0.87 US$/quarter.milking) when considered quarters classified as non-chronic subclinical mastitis belonged to category $P_1N_2N_3$ (Table 18).

Approach 3. Considering the quarters classified as being of the $P_1N_2N_3$ category, in which initially it exhibited infection (P_1 = 2,451,880 cells/mL) caused by environmental streptococci, and afterward had no pathogen isolation, it was observe both a decrease of SCC (N_2 = 150,470 cells/mL and N_3 =30,720 cells/mL), accompanied with an increase of milk yield (varying from 0.24 to 0.87 kg/quarter.milking). If the quarters being of the $P_1P_2N_3$ category had culture-negative results in the last one of the three successive milk samplings occasions, an increase of approximately 0.86 kg/quarter.milking was observed. The economic returns differed over time among quarters that had been culture-positive in the first sampling (P_1 = 1.08 US$/quarter.milking), culture-positive in the second sampling (P_2 = 0.39 US$/quarter.milking) and culture-negative in the third sampling (N_3 = 1.4 US$/quarter.milking). Quarters that had a positive culture results, followed by negative and positive results in the last two milk samplings ($P_1N_2P_3$), exhibited lower economic returns in the third sampling (P_3 = 0.39 US$/quarter.milking), when compared to the previous one (N_2 = 1.08 US$/quarter.milking). Interestingly, this result suggests that the economic return of the infection status caused by environmental streptococci might cause an approximate loss of -0.69 US$/quarter.milking (Table 19).

Approach 4. The detection of *Staph. aureus* in the first milk quarter sampling (P_1 = 2.31 kg/quarter.milking), followed by two consecutive culture-negative milk sampling results, suggested that these quarters possibly recovered from subclinical mastitis, since it was observed an increase of milk yield (N_2 = 3.9 kg/quarter.milking; N_3 = 4.0 kg/quarter.milking). In summary, these results show that quarters infected by *Staph. aureus* might have produced less milk (1.57 to 1.68 kg/quarter.milking) when compared to the milk yield from the same

recovered quarter. Quarters of category $P_1N_2N_3$ which the last two milk samplings occasions had culture-negative results, had lower SCC than the SCC observed in the first milk sampling with culture-positive by *Staph. aureus*. The economic returns were higher when the infected quarter in the first sampling became culture-negative, as observed in the $P_1N_2N_3$ category (P_1 = 0.79 US$/quarter.milking versus N_2 = 1.20 US$/quarter.milking and N_3 = 1.26 US$/quarter.milking). No effect of chronic ($P_1P_2P_3$) and non-chronic subclinical mastitis, caused by *Staph. aureus*, of category $P_1N_2P_3$, was observed on SCC, milk yield, and economic return, when results were evaluated among samplings (Table 20).

Table 17 - Effect of chronic subclinical mastitis (PPP, n = 114) on milk yield, somatic cell count, and economic return versus non-chronic subclinical mastitis (PNN, n = 135; PNP, n = 39; and PPN, n = 66)

Variables	Marginal means			SEM	Interaction between categories		
					P-values		
	Sampling 1 (15d)	Sampling 2 (30d)	Sampling 3 (45d)		PPP vs. PNN	PPP vs. PNP	PPP vs. PPN
Milk yield (Kg/quarter.milking)							
PPP[1]	2.59[a]	2.66[a]	2.26[a]	0.8375	0.07	0.37	0.10
PNN[2]	2.82[b]	3.50[a]	3.00[a]	0.7892			
PNP[3]	2.86[a]	2.87[a]	2.91[a]	10.495			
PPN[4]	2.94[a]	3.05[a]	3.43[a]	10.147			
SCC (×10^3 cells/mL)							
PPP	1,221.40[a]	1,061.01[a]	1,133.38[a]	189.80	0.002	0.37	0.14
PNN	1,235.47[a]	526.86[c]	559.29[b]	154.93			
PNP	1,130.66[a]	408.18[b]	872.6[a]	314.03			
PPN	1,133.34[a]	1,564.99[a]	728.79[a]	294.42			
Economic return (US$/quarter.milking)							
PPP	0.86[a]	0.87[a]	0.74[a]	0.7675	0.06	0.36	0.09
PNN	0.92[b]	1.15[a]	0.98[a,b]	0.7174			
PNP	0.93[a]	0.95[a]	0.95[a]	0.9827			
PPN	0.96[a]	0.97[a]	1.15[a]	0.9470			

SEM: Standard error. [1]PPP: chronic subclinical mastitis cases, culture positive (P) during the three moments of milk sampling. [2,3,4]PNN, PNP, PPN: non-chronic subclinical mastitis cases, culture negative (N) at least in one moment of milk sampling. Values per variable within a row represent the same quarter during the three moments of milk sampling, and values with different lowercase letters differ significantly at $P < 0.05$.

Table 18 - Effect of chronic subclinical mastitis caused by major pathogens (PPP, n=54) on milk yield, somatic cell count, and economic return versus non-chronic subclinical mastitis caused by major pathogens (PNN, n=66; PNP, n=15; and PPN, n=15)

Variables	Marginal means			SEM	Interaction between categories *P-values*		
	Sampling 1 (15d)	Sampling 2 (30d)	Sampling 3 (45d)		PPP vs. PNN	PPP vs. PNP	PPP vs. PPN
Milk yield (Kg/quarter.milking)							
PPP[1]	2.47[a]	2.66[a]	2.22[a]	0.4090	0.25	0.50	0.96
PNN[2]	2.74[b]	3.63[a]	2.80[b]	0.3468			
PNP[3]	1.94[a]	1.92[a]	2.06[a]	0.5081			
PPN[4]	2.23[a]	2.80[a]	2.50[a]	0.7698			
SCC (×10^3 cells/mL)							
PPP	1,289.10[a]	1,485.75[a]	1,963.23[a]	734.13	0.14	0.17	0.40
PNN	1,731.68[a]	385.96[b]	463.84[b]	622.84			
PNP	473.42[a]	763.31[a]	756.32[a]	917.82			
PPN	412.16[a]	856.72[a]	941.02[a]	1,393.90			
Economic return (US$/quarter.milking)							
PPP	0.83[a]	0.88[a]	0.73[a]	0.1294	0.29	0.46	0.90
PNN	0.87[a]	1.17[b]	0.89[a]	0.1098			
PNP	0.64[a]	0.64[a]	0.69[a]	0.1583			
PPN	0.77[a]	0.94[a]	0.85[a]	0.2379			

SEM: Standard error. [1]PPP: chronic subclinical mastitis cases, culture positive (P) during the three moments of milk sampling. [2,3,4] PNN, PNP, PPN: non-chronic subclinical mastitis cases, culture negative (N) at least in one moment of milk sampling. Values per variable within a row represent the same quarter during the three moments of milk sampling, and values with different lowercase letters differ significantly at $P < 0.05$.

Table 19 - Effect of chronic subclinical mastitis caused by environmental streptococci (PPP, n=24) on milk yield, somatic cell count, and economic return versus non-chronic subclinical mastitis caused by environmental streptococci (PNN, n=12; PNP, n=6; and PPN, n=9)

Variables	Marginal means			SEM	Interaction between categories *P-values*		
	Sampling 1 (15d)	Sampling 2 (30d)	Sampling 3 (45d)		PPP vs. PNN	PPP vs. PNP	PPP vs. PPN
Milk yield (Kg/quarter.milking)							
PPP[1]	3.31[a]	3.51[a]	2.52[a]	0.6571	0.21	0.19	0.61
PNN[2]	3.71[a]	4.58[b]	3.95[b]	0.5887			
PNP[3]	4.05[a]	4.64[a]	4.15[a]	0.7442			
PPN[4]	2.72[a]	2.71[a]	3.57[b]	0.9600			
SCC (×10^3 cells/mL)							
PPP	2,894.73[a]	2,797.37[a]	4,549.28[a]	432.27	<.0001	<.0001	0.32
PNN	2,451.88[a]	150.47[b]	30.72[b]	377.10			
PNP	210.29[a]	64.44[b]	431.21[a]	523.79			
PPN	1,183.68[a]	2,098.13[a]	2,476.37[a]	714.34			
Economic return (US$/quarter.milking)							
PPP	0.98[a]	1.17[a]	0.84[a]	0.6083	0.38	0.27	0.69
PNN	1.17[a]	0.84[a]	1.08[a]	0.5475			
PNP	0.84[a]	1.08[a]	0.39[c]	0.6999			
PPN	1.08[a]	0.39[b]	1.4[c]	0.9143			

SEM: Standard error.[1]PPP: chronic subclinical mastitis cases, culture positive (P) during the three moments of milk sampling. [2,3,4] PNN, PNP, PPN: non-chronic subclinical mastitis cases, culture negative (N) at least in one moment of milk sampling. Values per variable within a row represent the same quarter during the three moments of milk sampling, and values with different lowercase letters differ significantly at $P < 0.05$.

Table 20 - Effect of chronic subclinical mastitis caused by Staph. aureus (PPP, n=27) on milk yield, somatic cell count, and economic return versus non-chronic subclinical mastitis caused by Staph. aureus (PNN, n=3; and PNP, n=3)

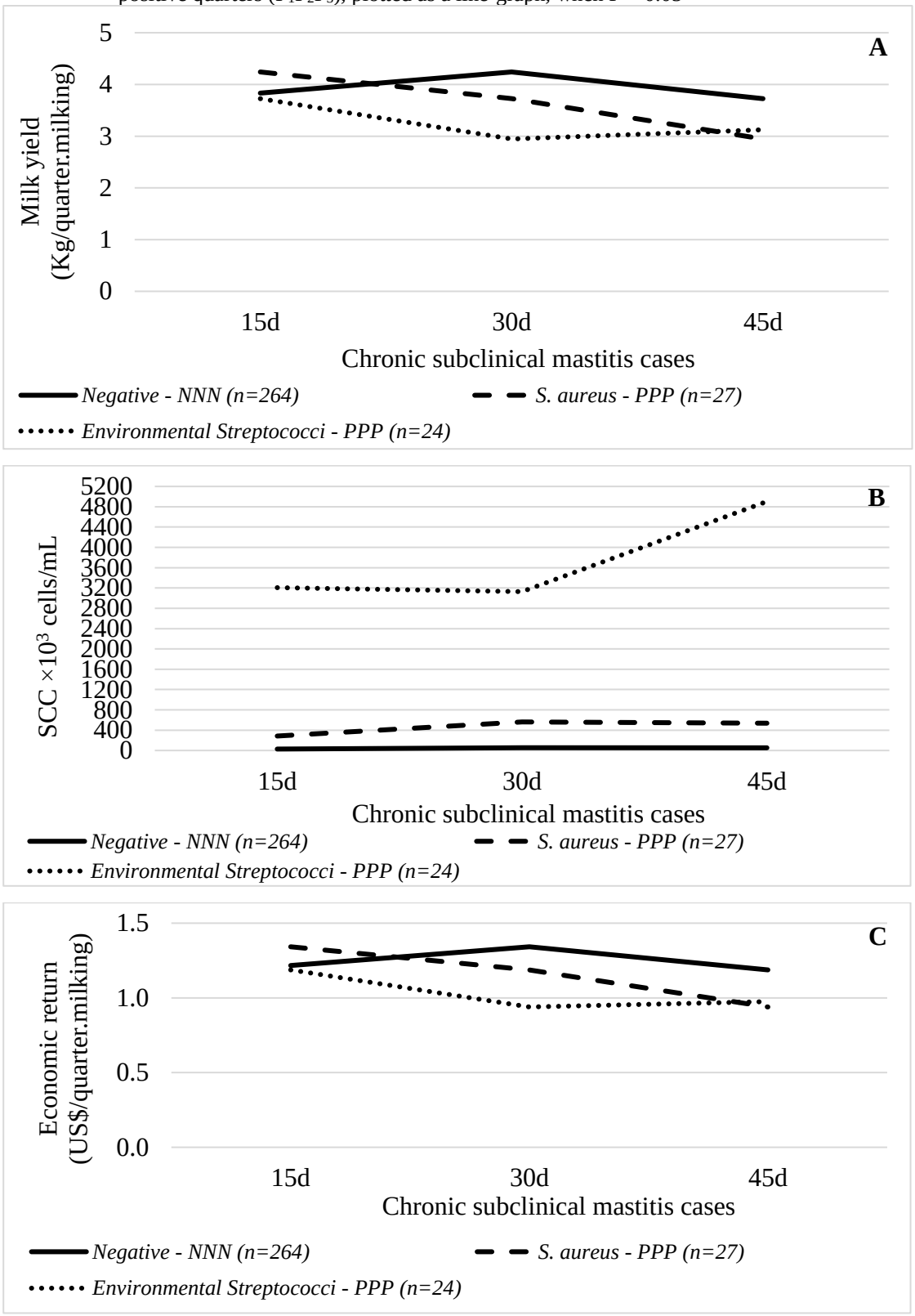
Variables	Marginal means			SEM	Interaction between categories *P-values*	
	Sampling 1 (15d)	Sampling 2 (30d)	Sampling 3 (45d)		PPP vs. PNN	PPP vs. PNP
Milk yield (Kg/quarter.milking)						
PPP[1]	2.50[a]	2.73[a]	2.54[a]	2.5899		
PNN[2]	2.31[a]	3.88[b]	3.99[b]	3.3928	0.42	0.50
PNP[3]	1.82[a]	1.71[a]	2.18[a]	1.9043		
SCC (×10³ cells/mL)						
PPP	369.72[a]	607.8[a]	562.75[a]	4.6126		
PNN	665.92[a]	227.16[b]	227.71[b]	4.0172	0.82	0.89
PNP	415.53[a]	417.93[a]	446.86[a]	5.1897		
Economic return (US$/quarter.milking)						
PPP	0.82[a]	0.87[a]	0.80[a]	2.5262		
PNN	0.79[a]	1.20[b]	1.26[b]	3.3012	0.42	0.50
PNP	0.60[a]	0.54[a]	0.70[a]	1.8650		

SEM: Standard error.[1]PPP: chronic subclinical mastitis cases, culture positive (P) during the three moments of milk sampling. [2,3] PNN, PNP, PPN: non-chronic subclinical mastitis cases, culture negative (N) at least in one moment of milk sampling. Values per variable within a row represent the same quarter during the three moments of milk sampling, and values with different lowercase letters differ significantly at $P < 0.05$.

4.4.4 **Assay 2: effect of chronic subclinical mastitis caused by specific pathogens on milk yield, somatic cell count, and economic returns versus healthy quarters.**

Culture-negative mammary quarters during the three samplings ($N_1N_2N_3$ = 264) exhibited higher milk yield (3.93 ±0.42kg/quarter.milking) than chronic subclinical mastitis quarters infected by *Staph. aureus* ($P_1P_2P_3$ = 27; 2.98 ±0.57kg/quarter.milking and environmental streptococci ($P_1P_2P_3$ = 27; 2.89 ±0.73kg/quarter.milking) (Figure 12-A). The SCC were lower for healthy quarters ($N_1N_2N_3$ = 44.8×10³ ±84.2 cells/mL) than for chronic subclinical mastitis quarters infected by *Staph. aureus* ($P_1P_2P_3$ = 461.7×10³ ±167.7 cells/mL) and environmental streptococci ($P_1P_2P_3$ = 3,745.8×10³ ±244.3 cells/mL) (Figure 12-B). The economic return was also higher for healthy quarters ($N_1N_2N_3$ = 1.25 ±0.12 US$/quarter.milking) when compared with chronic subclinical mastitis quarters infected by *Staph. aureus* ($P_1P_2P_3$ = 0.94 ±0.17 US$/quarter.milking) and environmental streptococci ($P_1P_2P_3$ = 0.95 ±0.22 US$/quarter.milking) (Figure 12-C).

Figure 12 - Effect of chronic subclinical mastitis caused by specific pathogens on (A) milk yield (Kg/quarter.milking), (B) linear score SCC and (C) economic return (US$/quarter.milking) versus healthy quarters using the comparison from assay 2: dynamic of the same quarter during three milk samplings distributed in categories of negative quarters ($N_1N_2N_3$) versus positive quarters ($P_1P_2P_3$), plotted as a line-graph, when $P < 0.05$



4.4.5 Assay 3: effect of non-chronic subclinical mastitis caused by specific pathogens versus healthy quarters on milk yield, somatic cell count, and economic return.

Approach 1 and 2. Culture-negative mammary quarters during the three samplings ($N_1N_2N_3$ = 264) had similar milk yield and economic return than non-chronic subclinical mastitis ($P_1N_2N_3$ = 135, and $P_1P_2N_3$ = 27), regardless of the type of pathogen. The SCC was lower in healthy quarters of category $N_1N_2N_3$ than non-chronic subclinical mastitis quarters $P_1N_2N_3$ (Figure 13) and $P_1P_2N_3$ (Figure 14).

Figure 13 - Effect of non-chronic subclinical mastitis caused by specific pathogens on somatic cell count ($\times10^3$cells/mL) versus healthy quarters using the comparison from assay 3: dynamic of the same quarter during three milk samplings distributed in categories o of negative quarters ($N_1N_2N_3$) versus positive-negative-negative quarters ($P_1N_2N_3$), plotted as a line-graph, when $P < 0.05$

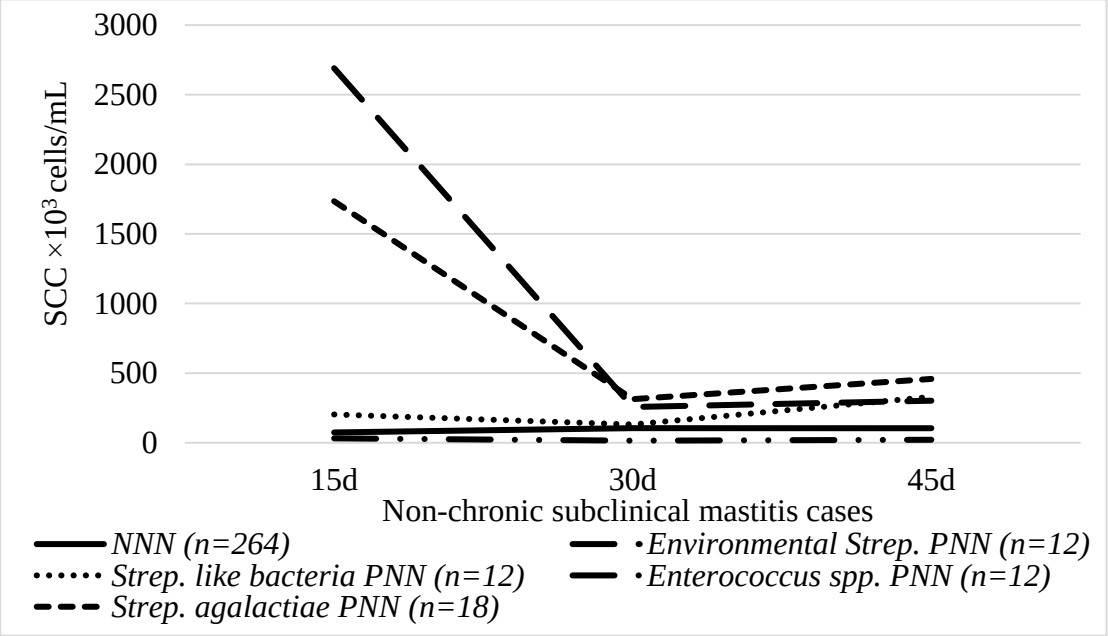
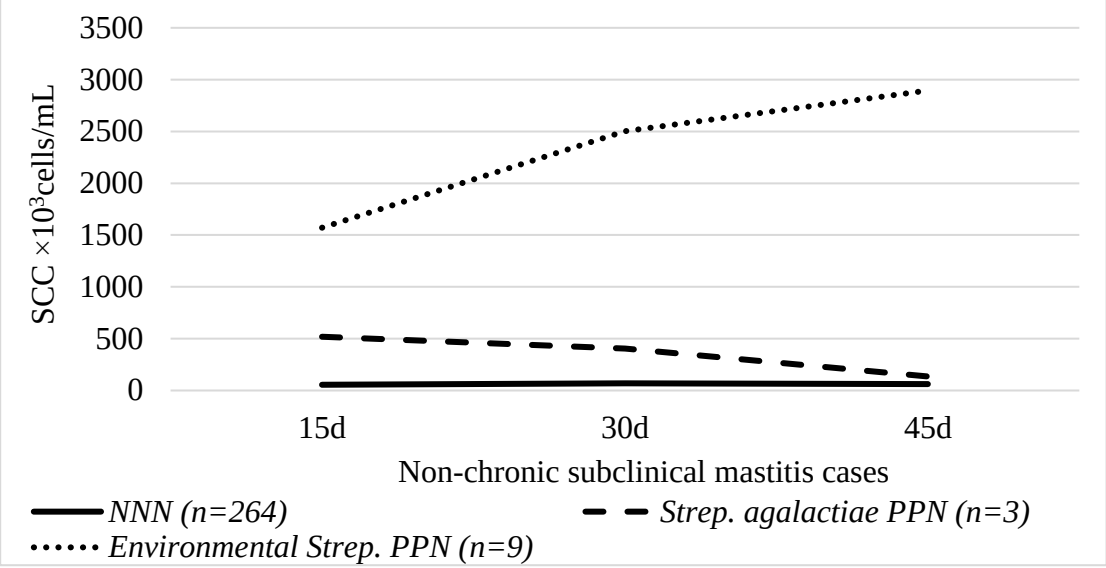


Figure 14 - Effect of non-chronic subclinical mastitis caused by specific pathogens on somatic cell count ($\times 10^3$cells/mL) versus healthy quarters using the comparison from assay 3: dynamic of the same quarter during three milk samplings distributed in categories of negative quarters ($N_1N_2N_3$) versus positive-positive-negative quarters ($P_1P_2N_3$), plotted as a line-graph, when $P < 0.05$



4.5 DISCUSSION

In the present study, we questioned whether there were differences of milk yield, SCC and consequently in the economic returns when chronic and non-chronic subclinical mastitis quarters were compared, using successive milk sampling occasion over time. In general, mammary quarters diagnosed with chronic subclinical mastitis exhibited lower milk yield, higher SCC, and, had consequently lower economic returns, when compared to quarters diagnosed with non-chronic subclinical mastitis or healthy ones. The changes observed regarding milk yield and economic returns depended on the type of mastitis (chronic versus non-chronic) and the type of pathogen causing subclinical mastitis.

4.5.1.1 Methodologies for evaluating milk loss: different points of view.

To date, there have been few studies that evaluated the milk losses associated with mastitis at quarter level (LEVAN; EBERHART; KESLER, 1985; COULON et al., 2002;

LEITNER et al., 2006; DÜRR et al., 2008; HALASA et al., 2009; PIEPERS et al., 2009; FORSBACK et al., 2010a; PEARSON et al., 2013; BEZMAN et al., 2015; BOTARO et al., 2015). The majority of the studies evaluated milk losses caused by clinical mastitis (HAGNESTAM; EMANUELSON; BERGLUND, 2007; SCHUKKEN et al., 2009), because of the ease in determining how much the cow ceased to contribute to the herd. Thus, few studies have estimated the milk losses according the type of pathogen causing subclinical mastitis (HALASA et al., 2009; TESFAYE; REGASSA; KELAY, 2010). Some studies carried out evaluations based on results of CMT or SCC test-day for the evaluation of milk losses (DE GRAAF; DWINGER, 1996; BERGLUND et al., 2007; FORSBACK et al., 2010a). However, the ideal would be to evaluate these losses by type of pathogen, preferably followed by species confirmation using advanced technologies. The use of rapid platforms for diagnosis has been gaining interest since misidentifications have been reported by microbiological culture of bacterial groups, which are known to have high genotypic similarity among them (WERNER et al., 2014). In this context, mass spectrometry and gene sequencing were used in the present study to confirm the bacterial species causing subclinical mastitis, avoiding underestimation at the time in which milk loss per pathogen was calculated. MALDI-TOF MS enabled > 90% identification at the species level (score > 2) of pathogens isolated from chronic subclinical mastitis cases, which concurs with the results published by our research group (BARREIRO et al., 2010). Furthermore, by way of MALDI-TOF MS, it was possible to identify, at the species level, all the isolates (7.5%; n = 16/212) determined by culture as *Strep.* like-bacteria (*Aerococcus* spp. and *Lactococcus* spp.). Our results are in accordance with those described by WERNER et al. (2014), who observed high genotypic similarity among the species *Strep. uberis* and *Lactococcus* spp., which could be the cause of the misidentification by microbiological culture.

Another important factor for evaluating milk losses is the frequency of sampling used. Most of the studies evaluated milk losses at the herd or cow level using test day records, either by assessing daily milk loss (kg/cow/day) or milk loss by lactation (kg/cow/305d) (AULDIST et al., 1995; DÜRR et al., 2008; HAND; GODKIN; KELTON, 2012). However, few studies have evaluated the losses of milk yield at the mammary quarter level (LEITNER et al., 2006; BERGLUND et al., 2007; FORSBACK et al., 2009; BEZMAN et al., 2015).To our knowledge, there are few studies to date that specifically evaluated the effects of chronic subclinical mastitis per type of pathogen at the mammary quarter level (WHITE et al., 1937; SWINKELS et al., 2005a; STEENEVELD; SWINKELS; HOGEVEEN, 2007). The studies are almost inexistent if considered our proposed comparison using successive milk quarter level could increase the

reliability of the estimation since mammary quarters are independent anatomical milk producing units. Also, the assessment of losses of milk production at the quarter level is more accurate than those performed at the cow level, due to the dilution effect that three healthy quarters could exert on a single infected quarter (GREEN; SCHUKKEN; GREEN, 2006). Reliable estimates of milk yield reduction and milk composition changes are needed to carry out reliable economic calculations that may assist producers in decision making, and the adoption of some strategic management (HALASA et al., 2009).

Another relevant factor is the calculation method used to evaluate milk losses (HUIJPS; LAM, 2008). Several models of evaluation have been described so far, which makes it difficult to compare results between studies (SEEGERS; FOURICHON; BEAUDEAU, 2003; LOSINGER, 2005). The applied methodologies include the comparison of healthy cows versus infected or non-infected cows (BEZMAN et al., 2015; BOTARO et al., 2015). However, most studies do not consider the parity and days in milk as adjustment factors, as recommended by DE ROOS; HARBERS and DE JONG (2004). Some innovative studies have used different ways of comparing mammary quarters. For example, pairs of healthy and infected contralateral quarters were compared recently to evaluate the effect of subclinical mastitis caused by contagious and environmental pathogens on milk yield (GONÇALVES et al., 2017). Another interesting study evaluated milk losses using quarters from twin cows (PEARSON et al., 2013).

The uniqueness of the current study. Given the scenario of the need to control a series of factors to obtain an accurate estimate of milk losses caused by chronic subclinical mastitis, we believe that our study may be considered a pioneer in this field of research. For the present study, measurements of total milk yield per mammary quarter/milking were performed, which made the study extremely laborious.

The detection of IMI status by microbiological culture enabled us to compare quarters that exhibited positive versus negative cultures according to the sampling visit. Therefore, based on the results of the IMI status according to the sampling visit, categories were created to facilitate data comparison from cases of chronic subclinical mastitis ($P_1P_2P_3$), non-chronic subclinical mastitis ($P_1P_2N_3$, $P_1N_2N_3$, $P_1N_2P_3$) and healthy mammary quarters ($N_1N_2N_3$). Also, given the complexity of the study, different approaches were engendered depending on how the results of pathogen identification (e.g. positive or negative culture, per pathogen group or pathogen characterization at the species level) were determined.

4.5.1.2 Effect of chronic subclinical mastitis on SCC, milk yield, and economic return.

The primary losses associated with subclinical mastitis are milk losses and changes in composition (HALASA et al., 2007; HALASA et al., 2009). Therefore, in the present study, we evaluated the quality of milk according to the SCC, and protein and fat contents per US$/L. It is already well established that these alterations depend on the causative pathogen (COULON et al., 2002; LE ROUX; LAURENT; MOUSSAOUI, 2003; LEITNER et al., 2006). Thus, the price/L of milk was calculated taking into account the type of pathogen causing subclinical mastitis. Based on this, the economic returns were calculated by multiplying the quarter's production by the price of the liter of milk. According to LEITNER et al. (2006), the severity of the disease depends on the pathogen's site of action within the mammary tissue. For example, some strains of *Staph. aureus*, have virulence factors, which help them to adapt in the alveolar mammary tissue, leading to chronic mastitis. Therefore, in the present study it was evaluated the effect of chronic subclinical mastitis in same quarter using successive milk samplings occasions over time. Chronic mammary quarters $P_1P_2P_3$, regardless of the approach used [positive (P) and negative (N) culture; group of pathogens (major or minor) detected; or by type of pathogen determined], had no difference of milk yield, SCC, and economic returns, among multiple samplings over a period of time. These results are in accordance with previous scientific studies, that reported when a quarter is chronically infected, it is difficult to return to its previous milk-producing capacity possibly because of milking-producing tissue damage (VIGUIER et al., 2009). To our knowledge, no previous studies evaluated the effects of chronic subclinical mastitis according to the type of pathogen at the mammary quarter level over time.

The mammary quarters of categories $P_1P_2N_3$, $P_1N_2P_3$, and $P_1N_2N_3$, which displayed IMI in the first sampling, but which had one or two culture-negative results in the other samplings, were designated as quarters with non-chronic mastitis and were compared among each other. In these categories, the detection of a single sampling as culture-negative did not guarantee that the quarter had recovered from subclinical mastitis. Therefore, two of the three categories of quarters, subclinically infected with non-chronic mastitis ($P_1P_2N_3$, $P_1N_2P_3$), showed no difference in milk yield and economic return, when compared over time. However, quarters infected with non-chronic subclinical mastitis had higher SCC than culture-negative quarters. We observed, regardless of the type of approach [positive (P) and negative (N) culture; group of pathogens (major or minor) detected; or type of pathogen identified], that when the result of a culture was positive, the SCC increased above the cutoff value for healthy quarters (> 100,000

cells/mL), as described by SCHWARZ et al. (2011a). Even when the quarter was culture-positive in the first sampling (P_1) and culture-negative in the second sampling (N_2), the SCC remained above the cutoff. We believe that these SCC results may be explained by the presence of false negatives in the microbiological culture.

Previous results have shown that at least two consecutive samplings exhibiting low SCC (< 200,000 cells/mL) or culture-negative are needed to diagnose cows as healthy (SCHUKKEN et al., 2003). A similar approach could be used based on SCC at the quarter level, but using a lower cutoff point for SCC (< 100,000 cells/mL) to classify as a healthy (SCHWARZ et al., 2011a). Therefore, lower milk yield and economic return were observed when compared quarters with non-chronic subclinical mastitis from the first sampling to the quarters that presented negative culture in two other samplings in the course of time (category $P_1N_2N_3$). The SCC of mammary quarters with chronic subclinical mastitis in the first sampling was higher than the observed SCC in the two posterior samplings that displayed culture-negative. However, the quarters that showed culture-negative samplings, shortly after infection, had SCC above the cutoff. We believe that even if a microbiological cure had occurred in these culture-negative quarters, there may be a residual effect of the previously IMI case on the immune system, which would maintain a high SCC (SCHUKKEN et al., 2003).

The group of quarters initially classified as exhibiting non-chronic subclinical mastitis, but that was considered healthy after two consecutive culture-negative samplings (category $P_1N_2N_3$), produced 0.24-0.87 kg/quarter.milking more milk when they went from culture-positive caused by environmental streptococci to culture-negative; and 1.57-1.69 kg/quarter.milking more milk when they went from culture-positive caused by *Staph. aureus* to culture-negative. Results of milk yield losses from our study were higher than those described by TESFAYE; REGASSA and KELAY (2010), of 0.40-0.78 kg/quarter.milking. However, TESFAYE; REGASSA and KELAY (2010) compared non-chronic infected subclinically quarters by *Staph. aureus* with negative quarter using a single milk sampling, differing from the present study which evaluated quarters during different samplings. Inferior values were described by BOTARO et al. (2015), which reported a loss of 0.12 kg/quarter.milking of milk when they compared milk yield of quarters infected by *Staph. aureus* versus their contralateral healthy quarters within cow. Milk losses caused by species of *Streptococcus* spp. was of 0.63 kg/quarter.milking, when comparing healthy quarters versus infected ones within cow (BEZMAN et al., 2015), with was similar with the results of the present study. Also, milk loss associated with infection caused by *Escherichia coli*, at the quarter level, was 1.2 kg/quarter.milking (BEZMAN et al., 2015), and because of the low frequency of cows with

IMI caused by enterobacteria in the present study, these animals were excluded from statistical analysis. In general, the evaluated milk losses using the approach of culture results (culture-positive) was lower than the evaluated milk losses using the approach of major pathogens, environmental streptococci or *Staph. aureus*.

4.5.1.3 Effect of chronic subclinical mastitis on SCC, milk yield, and economic return in comparison with healthy quarters.

Altogether, our results were based on the comparison of the average milk production observed in healthy mammary quarters from three milk samplings (category $N_1N_2N_3$) with the mean obtained from mammary quarters having chronic infection (category $P_1P_2P_3$) caused by environmental streptococci and *Staph. aureus*. Milk losses estimated in Assay 2 vary from 0.95-2.25 kg/quarter.milking, depending on the type of pathogen causing chronic subclinical mastitis. BERGLUND et al. (2007) compared milk production from mammary quarters with chronic subclinical mastitis (three samplings, SCC > 200,000 cells/mL) with healthy quarters, but did not find significant differences. FORSBACK et al. (2009) observed lower milk loss than in the present study (0.32 kg/quarter.milking) when they compared quarters with subclinical mastitis (a single sampling, SCC > 100,000 cells/mL) versus healthy quarters.

4.5.1.4 Effect of non-chronic subclinical mastitis on SCC, milk yield, and economic return in comparison with healthy quarters.

Quarters with chronic subclinical mastitis, belonging to categories $P_1P_2N_3$ and $P_1N_2N_3$, had similar milk yield and economic return when compared to the healthy quarters category ($N_1N_2N_3$). On the other hand, the SCC was higher in the categories that had one or two culture-positive quarters ($P_1P_2N_3$ or $P_1N_2N_3$) than in the healthy quarter category ($N_1N_2N_3$). These results were similar to those described by FORSBACK et al. (2009) and BERGLUND et al. (2007) when they compared the SCC of healthy quarters versus infected ones (> 100,000 and > 200,000 cells/mL; respectively).

Overall, the comparison using multiple versus single quarter milk samples allowed to estimate the milk losses caused by chronic and non-chronic subclinical cases using different approaches. As our study was a cross-sectional investigation for a supportive cause and effect inference of the major pathogens chronic or non-chronic subclinical mastitis on quarter milk yield, SCC and return economic, our model did not take into account information of mastitis cases from cows in previous lactation. Therefore, some selected quarters may have suffered a damage caused by previous occurrence which may be considered one limitation of the present study.

4.6 CONCLUSION

Milk losses and economic returns vary according to the type of mastitis-causing pathogen -0.24 to -0.87 kg/quarter.milking, and -1.57 to -1.69 kg/quarter.milking, when infected by environmental streptococci and *Staph. aureus*, respectively. Mammary quarters that were cured from subclinical mastitis caused by *Staph. aureus* and environmental streptococci exhibited an increase in economic return of approximately 0.47 and 0.69 US$/quarter.milking, respectively.

Chapter 5

Milk losses associated with somatic cell counts per parity and stage of lactation: a cow-level analysis.

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5 MILK LOSSES ASSOCIATED WITH SOMATIC CELL COUNTS PER PARITY AND STAGE OF LACTATION: A COW-LEVEL ANALYSIS.

5.1 ABSTRACT

The reduction in milk production caused by subclinical mastitis in dairy cows was evaluated through the regression of test day milk yield on log-transformed somatic cell counts ($Log_{10}SCC$). Test day records (n = 1,200,002) were obtained from the milk recording agency for the Brazilian state of Paraná, and included 781 herds with data from 92,560 Holstein cows lactating from January 2010 to December 2015. A segmented regression was fitted to estimate the cutoff point in the $Log_{10}SCC$ scale where milk yield starts to be affected by mastitis. The statistical model used to explain daily milk yield included the effects of herd–cow of test (random), days in milk, parity and $Log_{10}SCC$, and analyses were performed by parity and stage of lactation. The cutoff point where milk yield starts to be affected by changes in $Log_{10}SCC$ was estimated from data to be around 0.90 (~7,963 cells/mL) for Holsteins cows from Brazilian herds. Milk losses per unit increase in $Log_{10}SCC$ had estimates around -1.77 kg in the beginning of the first lactation (5 to 20 days), -1.37 kg in the mid-lactation (126 to 140 days), and -2.28 kg at the end of the lactation (291 to 305 days), and started approximately -3.28 kg (5 to 20 days), decreased to -2.36 kg in the mid-lactation (126 to 140 days), and reached – 4.20 kg (291 to 305 days) in adult Holsteins. Daily milk losses caused by changes in $Log_{10}SCC$ were dependent on parity and stage of lactation, and these factors should be considered when estimating losses associated with subclinical mastitis.

Keywords: Subclinical. Mastitis. Holstein. Test day. Milk loss.

5.2 INTRODUCTION

Brazil has displayed a continuous growth of bovine milk production. During the last 20 years, production has more than doubled (103.1%), from 15.1 billion in 1991 to 30.7 billion liters of milk in 2010. Currently, Brazil has been considered the sixth largest milk producer in

the world, with an average 35 billion liters, behind only the European Union, the United States, India, China and Russia (IBGE, 2015). National milk production is expected to increase at an annual rate of 1.9%, which will correspond to a production of 41.3 billion liters of raw milk at the end of 2023 (BRASIL, 2013). Given the current scenario of milk production in Brazil, one possible way to achieve the target of producing this much milk will be an increase in cow productivity, associated with reducing production costs. Despite, a total of 21.75 million Brazilian dairy cows been milked in 2015, 29.8% of dairy farms in Brazil have a herd of up to nine lactating cows, 59.7% with 10 to 99 lactating cows, and 10.5% with more than 100 lactating cows which suggest a discrepancy of cows' productivity among herds and consequently the lower average productivity in Brazil (1,609 liters/cow/year) (IBGE, 2015). Therefore, animal health improvement is one of the main goals in achieving an efficient production system (HALASA et al., 2007; HALASA et al., 2009).

Economic losses are a consequence of control costs and decrease production, the most significant loss resulting from diminished milk production (SEEGERS; FOURICHON; BEAUDEAU, 2003; HUIJPS; LAM, 2008). In this context, subclinical mastitis is measured in terms of milk somatic cell count (SCC), mainly because no clinical signs of the disease are evident. However, as SCC is increased, milk composition is altered and milk yield decreases (FORSBACK et al., 2009). The weight of evidence indicates that cows with high SCC produce a lower milk volume than cows with low SCC (GREEN; SCHUKKEN; GREEN, 2006). To quantify the impact of subclinical mastitis on dairy herds, it is therefore crucial to quantify the relationship between milk SCC and yield (HAND; GODKIN; KELTON, 2012). Thus, the cost of subclinical mastitis cases will largely depend on the extent of the associated yield loss (HAGNESTAM-NIELSEN et al., 2009).

Numerous studies have been published attempting to quantify the relationship between SCC concentration and milk production (HORTET; SEEGERS, 1998; DÜRR et al., 2008; HAND; GODKIN; KELTON, 2012). Some studies were focused on estimating test-day, 24-h milk losses or milk yield losses of complete lactations. Several approaches have been proposed to estimate milk production loss at a herd level, for instance, the comparison between milk yield of healthy and infected cows, or healthy and infected cows before and after infection in the same animal (HALASA et al., 2009). However, some factors need to be controlled since milk loss can be overestimated due to population differences, the used methods to detect mastitis or the used statistical models. Moreover, clear effects of parity as well as stage of lactation on the magnitude of milk losses have been reported (HORTET; SEEGERS, 1998).

Somatic cell count < 100,000 cells/mL is often considered to be normal, reflecting a healthy mammary gland, whereas a SCC > 200,000 cells/mL is suggestive of bacterial infection. These information were affirmed by BRADLEY and GREEN (2005) since 75% of cows had SCC > 200,000 cells/mL and around 75% of cows without an intramammary infection had a SCC ≤ 200,000 cells/mL. However, authors have assumed different SCC levels as the threshold where milk yield starts to be affected by changes in cell counts (GREEN; SCHUKKEN; GREEN, 2006). DÜRR et al. (2008) reported that milk yield already started to be affected when there were more than 7,400 cells/mL. Therefore, a lack of research exists where the relationship between milk yield loss and SCC at the cow level is quantified (HAND; GODKIN; KELTON, 2012), and it is still not clear how the relationship between milk yield and SCC should be interpreted at very low levels of cells in milk. According to DÜRR et al. (2008), having a good approximation of milk losses is key information to the dairy industry for estimating costs of disease and planning preventive strategies for dairy herds. The primary hypothesis of this study was that there is a cause-and-effect relationship between SCC with milk losses which depends on both the cows' parity and the stage of lactation. We also hypothesized that milk yield is not affected by increasing SCC level up to some (unknown) level of SCC. However, after reaching a certain cutoff (threshold) point, milk yield would be affected as SCC increases. Hence, the aim of this study was to examine the magnitude of the effect of SCC on milk yield from Holstein cows in Brazilian dairy herds using test day records to verify whether the association varies for different parities and stages of lactation. Another question to address was the threshold where the association between SCC and milk yield starts. In other words, at which level of SCC does milk yield begin to be affected.

5.3 MATERIAL AND METHODS

5.3.1 **Data**

Test day records were obtained from Associação Paranaense de Criadores de Bovinos da Raça Holandesa (APCBRH), the milk recording agency for the Brazilian state of Paraná, and included data from Holstein cows lactating from January 2010 to December 2015. Descriptive statistics of records used herein are presented in Table 21 and Figures 15 and 16.

Editing was performed to ensure both reliability and consistency for the statistical analysis. In order to be included in the dataset, test day records were required to meet the following criteria: fat content between 2.5 and 6.5%, protein content between 2.5 and 5.5%, lactose content between 3.5 and 6%, total solids content between 8.5 and 14.5%, SCC between 0 and 3,000,000 cells/mL and milk yield between 2 and 70 kg, as described by DÜRR et al. (2008). Only records from parities 1 to 6 and from 5 to 305 days in milk were included. Test-day recordings after 305 days in milk were excluded from analysis to avoid problems with unequal lengths of lactations and misclassification of parity due to failure to register subsequent calving. Twenty stages of lactation groups were defined (stage 1 = days 5 to 20, stage 2 = days 21 to 35, …, stage 20 = days 291 to 305). As per the retrospective, longitudinal design featured in the dataset available and to fit for the purpose of our study, observations from cows that had more than one record available per parity–stage combination were randomly subsampled in order to avoid the asymmetric distribution of measurements of individuals. The sum of all previously mentioned edits caused the elimination of approximately two thirds of the records. In order to allow the inclusion of a random herd–testday effect in the statistical model, we imposed a minimum of 100 records per herd and the constraint that the herd-testday needed > 4 records to be kept. Descriptive statistics of both kept and eliminated data sets showed that records used for analysis are representative of the Brazilian dairy cow population in the period studied (CUNHA et al., 2008).

5.3.2 **Statistical analysis**

In order to assess the first aim of this study, statistical analyses were performed separately by parity and by stage of lactation. The majority of herds in Brazil is monthly tested and hence within one 15-day stage of lactation there will generally be only one herd-test visit. Therefore, apart from any surprise spot-test retests of herds, there will only be one record per cow per stage, hence obviating any issues of repeated records per cow. In order to avoid problems of the occasional spot herd retest when more than one test of the same cow was recorded within the same parity–stage interval, only the first one was kept for analysis.

Figure 15 - Somatic cell count mean per parity number and stage of lactation (n = 1,200,002 test day records)

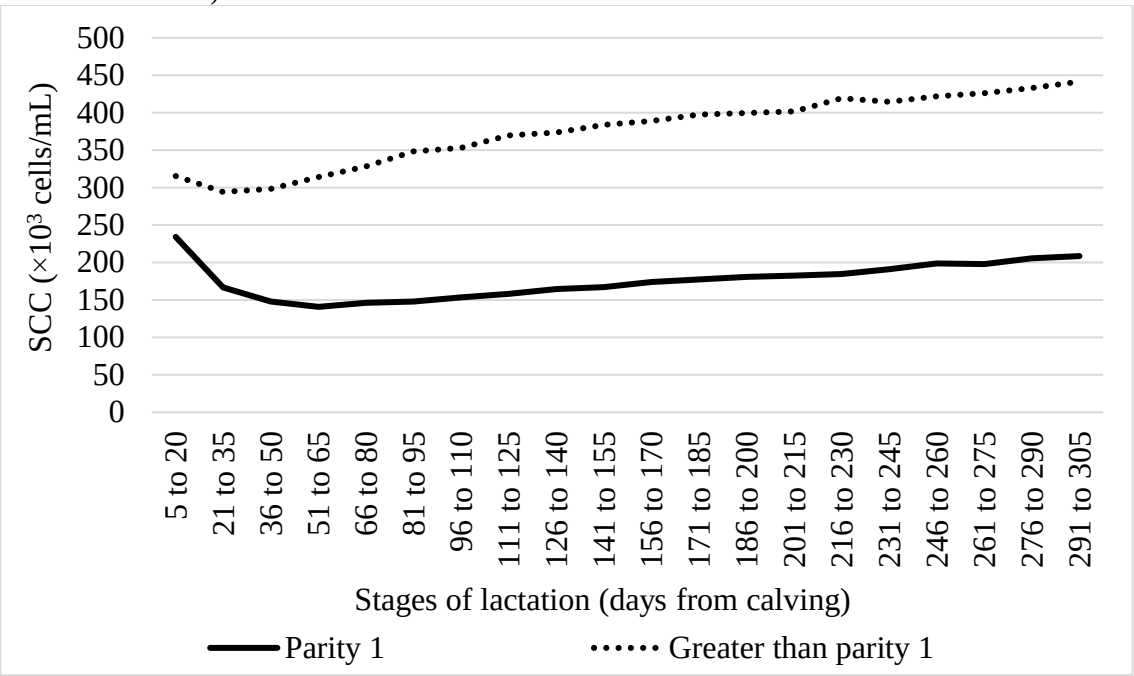


Figure 16 - Milk yield mean per parity number and stage of lactation (n = 1,200,002 test day records)

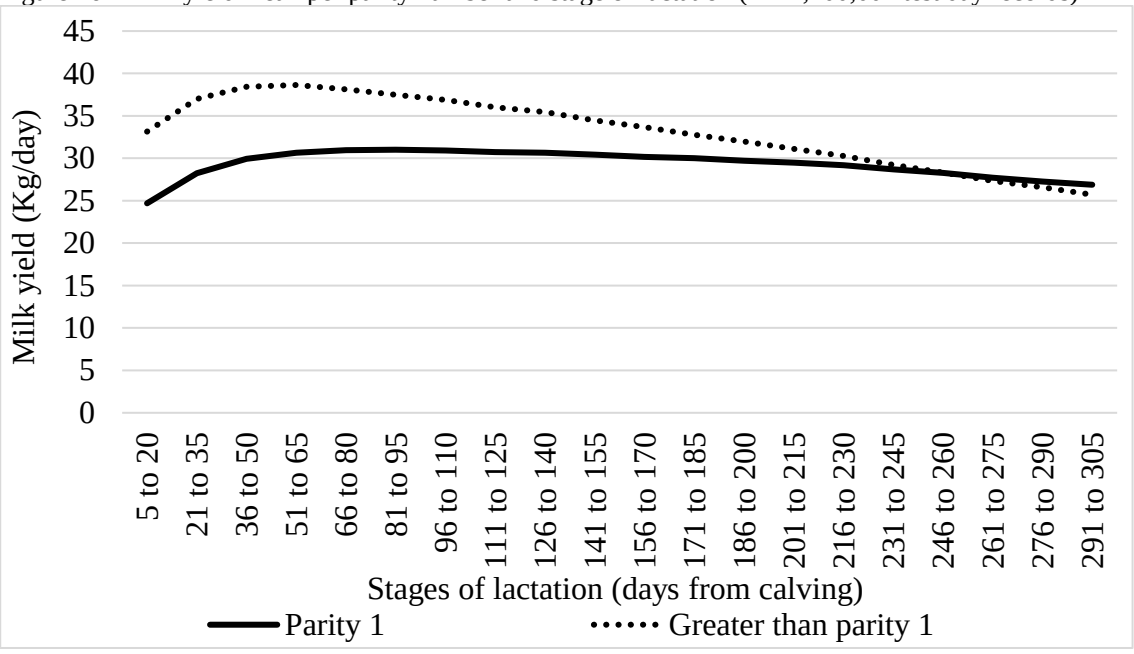


Table 21 - Counts and descriptive statistics of test day records included in the study of milk losses associated with somatic cell counts in Brazilian dairy herds (2010 to 2015)

	Breed
	Holstein
Total number of test day records before editing	3,578,621
Total number of test day records after editing	1,200,002
Number of cows included	92,560
Number of herds included	781

Parity number	*N*	TDMY[1]		SCC[2]		$Log_{10}SCC$		Fat%		Protein%		Lactose%		Total solids%	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	464,799	29.28	8.26	176.01	350.97	1.81	0.59	3.55	0.62	3.13	0.30	4.71	0.20	12.36	0.83
2	331,303	33.18	10.34	243.65	431.51	1.94	0.63	3.55	0.65	3.16	0.32	4.60	0.22	12.27	0.87
3	206,831	34.12	10.87	322.76	505.14	2.09	0.64	3.54	0.64	3.13	0.32	4.54	0.23	12.16	0.87
4	115,039	34.04	10.84	383.35	552.30	2.18	0.63	3.53	0.64	3.11	0.32	4.51	0.24	12.08	0.87
5	56,362	33.27	10.72	437.74	597.73	2.25	0.64	3.52	0.64	3.09	0.32	4.48	0.24	12.02	0.87
6	25,668	32.24	10.49	476.42	622.19	2.30	0.64	3.53	0.63	3.08	0.31	4.46	0.25	12.00	0.86

[1]TDMY, test day milk yield per cow (in kg). [2]SCC, test day somatic cell count per cow ($\times 10^3$ cells/mL). [3]$Log_{10}SCC$, natural logarithm of test day somatic cell count per cow (e.g. $Log_{10}SCC = 2$ is equivalent to 100,000 cells/mL).

In order to assess the SCC threshold at which a milk yield drop, the approach adopted here was to estimate the threshold from the data by fitting a segmented regression. Since healthy cows do maintain low cell counts in milk (CAPUCO et al., 2003) it is hypothesized here that milk yield is not affected by increasing SCC level up to some (unknown) level of SCC. After reaching this cutoff point, milk yield would be affected as SCC increases. This required the estimation of three parameters: the intercept (a), the cutoff (threshold) point where milk losses start (c) and the regression coefficient of milk yield on SCC (b), for values of SCC above the cutoff. The basic statistical model used to analyze data was:

[1] For observations where the Log_{10}SCC was greater than the cutoff.

$$X_{ij} = \mu + (1 \mid HTD)_i + \beta(Log_{10}SCC)_{ij} + \beta_1(DIM)_{ij} + \beta_2(P)_{ij} + \varepsilon_{ij}$$

[2] Observations in which the Log_{10}SCC was less than or equal to the cutoff, the model was:

$$X_{ij} = \mu + (1 \mid HTD)_i + \beta(c)_{ij} + \beta_1(DIM)_{ij} + \beta_2(P)_{ij} + \varepsilon_{ij}$$

where X_{ij} is the milk yield at test day of the *j*th cow in the *i*th herd–testday, μ is the mean milk yield of the population, $(1|HTD)_i$ is the random effect of herd-testday (data from 2010 to 2015), Log_{10}SCC is the logarithm of the SCC at test day (e.g. Log_{10}SCC = 2 is equivalent to 100,000 cells/mL), *c* is the cutoff as described above, DIM_{ij} is days in milk, P_{ij} is the parity number of lactating cow, β, β_1 and β_2 are the respective regression coefficients and the error term $\varepsilon \sim N(0, \sigma^2 I)$.

The statistical analyses were carried out using the NLMIXED procedure of SAS® program, which accommodates non-linear mixed models and allows fitting segmented regressions (version 9.3; SAS Institute Inc., Cary, NC, USA).

5.3.3 **Post-analysis calculations**

In order to describe the trend of the linear regression coefficients of daily milk yield on Log_{10}SCC over the course of each lactation, the regression coefficient estimates for the 20 stages defined here were, in turn, used as data points in a weighted regression on DIM, weighted by the inverse of their sampling variance. This allows an estimate of a milk loss regression coefficient appropriate to any given DIM and not just to the 20 stages of lactation. The weighted regressions also provide a means of testing differences between linear regression coefficients of daily milk yield on Log_{10}SCC obtained for parity-stage combinations. The GLM procedure of SAS was used to carry out the weighted regressions (version 9.3; SAS Institute Inc., Cary, NC, USA). Similarly, $\chi 2$ tests were carried out to test the hypothesis of equality between linear regression coefficients of different parity-stage combinations.

The expected daily milk losses distributed in different groups of SCC according the parity-stage of lactation combination were calculated. Briefly, the greater the Log_{10}SCC, the greater the milk losses both in kg and percentage. For example, parity 1 had the average of all milk losses estimates of -1.48 kg ±0.57 when the SCC = 100,000 cells/mL (n = 299,191), -1.96 kg ±0.63 with SCC = 200,000 cells/mL (n = 73,603), -2.26 kg ±0.66 with SCC = 300,000 cells/mL (n = 28,968), -2.45 kg ±0.69 with SCC = 400,000 cells/mL (n = 15,410), -2.62 kg ±0.71 with SCC = 500,000 cells/mL (n = 9,499), -3.02 kg ±0.76 with SCC = 900,000 cells/mL (n = 21,169), and -3.10 kg ±0.77 with SCC = 1,000,000 cells/mL (n = 16,959).

The actual milk losses for individual cows may be estimated by applying the equation:

$$ML = (\mathrm{Log_{10}SCC} - \text{ cutoff } c) \times \mathrm{EML}$$

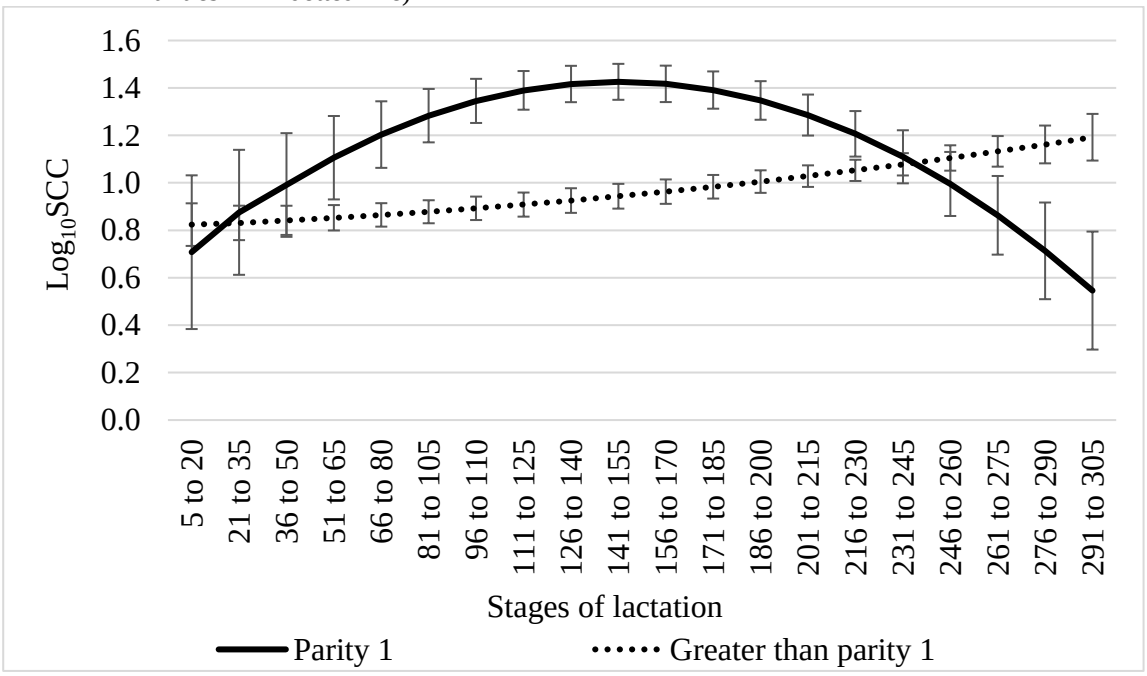
where ML is the milk loss (kg) for given cow, Log_{10}SCC is the actual SCC on the logarithm scale in that cow's milk, cutoff c is the point in the log scale where losses start and EML is the expected milk loss per unit increased in Log_{10}SCC according to parity-stage lactation combination.

5.4 RESULTS

5.4.1 **Cutoff point**

The estimate of the cutoff point (*c*) where milk losses associated with $Log_{10}SCC$ start for different parity-stage of lactation combinations in the Holstein cows in Brazil were reported in Figure 17. $Log_{10}SCC$ ranged from 0.55 ±0.25 to 1.43 ±0.08 for parity 1 and from 0.82 ±0.09 to 1.19 ±0.10 for parities ≥ 2, and the average of all estimates was 0.90 ±0.32. Figures presented here suggest that no milk losses due to SCC (subclinical mastitis) occurs up to approximately $Log_{10}SCC$ = 0.90 ±0.32 (~7,963 cells/mL).

Figure 17 - Estimates and standard error of the cutoff point (c) where milk losses associated with $Log_{10}SCC$ start for the Holstein cows in Brazilian herds from 2010 to 2015 (Parity 1 = solid line and Parities > 1 = dotted line)



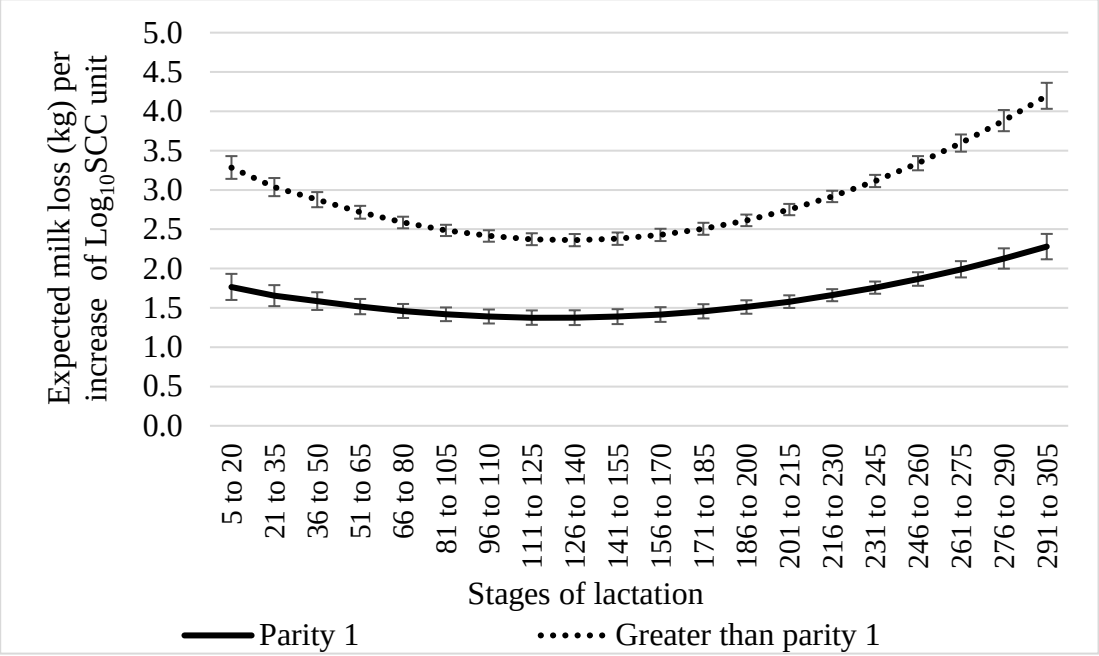
5.4.2 **Regression on days in milk, on parity and on Log_{10}SCC**

The estimates of linear regression coefficient of daily milk yield were positive and had similar values across parities and stages of lactation. The mean value and standard deviation for the estimates were 0.1086 kg/d ±0.0162 for parity 1, and 0.1928 kg/d ±0.0189 for parities > 1. These results are in accordance with the description that the greater the parity number of the cow, the higher the cow's daily milk yield (DUR et al., 2008; HAND; GODKIN; KELTON, 2012).

The aim of this study was to examine the magnitude of the effect of SCC on milk yield from Brazilian dairy herds using test-day records to verify whether the association varies for different parities and stages of lactation. For this reason, we determined the estimates of linear regression coefficients of daily milk yield on Log_{10}SCC for each parity-stage of lactation combination, the standard deviation, and the weighted regression between these estimates. Weighted regressions using linear regression coefficients of each lactation as data points were all statistically significant, indicating that milk losses due to Log_{10}SCC vary across the lactation. Linear regression coefficients presented a quadratic trend in all parity-stage combinations. The $\chi 2$ tests showed that first parity differs significantly from estimates for later parities, but no significant difference was encountered between estimates for the same stage of lactation in 2^{nd}, 3^{rd}, 4^{th}, 5^{th} and 6^{th} parities. Overall, milk losses associated with changes in Log_{10}SCC are not the same in different stages of the lactation and are lower in first parity than in later parities.

The linear regression coefficient of daily milk yield on Log_{10}SCC had estimates around -1.77 kg in the beginning of the first lactation (5 to 20 days), -1.37 kg in the mid-lactation (126 to 140 days), and -2.28 kg at the end of the lactation (291 to 305 days). These results indicate that in first parity cows any increase of one unit in Log_{10}SCC over the cutoff point is expected to cause a reduction in daily milk yield from 1.37 to 2.28 kg. Differences were also observed for the regression coefficients in later parities. As a general trend for second and later parities, losses in daily milk yield per unit increase in Log_{10}SCC started approximately -3.28 kg (5 to 20 days), decreased to -2.36 kg in the mid-lactation (126 to 140 days), and reached - 4.20 kg (291 to 305 days) (Figure 18). According to the $\chi 2$ test, there was no difference when comparing the estimates from second and later parities.

Figure 18 - Estimates and standard error of the linear regression coefficients of expected milk loss per unit increase in Log_{10}SCC (result = 2 is equivalent to 100,000 cells/mL, b2) for the Holstein cows in southeast Brazil from 2010 to 2015 (Parity 1 = solid line and Parities > 1 = dotted line).



5.4.3 **Estimated losses**

The expected milk loss (EML) per unit increased in Log_{10}SCC according to parity-stage lactation combination was provided in Table 22. Figures 19 A–B represent the predicted milk losses over the course of a full lactation from primiparous (Figure 19-A) and multiparous (Figure 19-B) cows according to the final model estimates based on the covariates investigated herein. By means of illustration, losses expected from a freshly calved at the 50th day of its first lactation cow with a SCC 500,000 cells/mL could have its milk production increased to 1.11 kg [-2.71 kg - (-1.60 kg)] at a SCC 100,000 cells/mL (See formula 1 and 2 below).

$$ML = (\text{Log}_{10}\text{SCC} - \text{cutoff } c) \times \text{EML}$$

[1] Considering first lactation cow, at 50 DIM, with SCC = 100,000 cells/mL

$$(2.00 - 0.99) \times 1.59 = -1.60$$

[2] Considering first lactation cow, at 50 DIM, with SCC = 500,000 cells/mL

$$(2.70 - 0.99) \times 1.59 = -2.71$$

A third lactation cow, for instance, with a SCC 500,000 cells/mL (Log_{10}SCC ~ 2.7) at 100 DIM is expected to yield 4.36 kg lesser than that its milk production projected at a Log_{10}SCC ~ 0.89 (7,762 cells/mL) (See formula 3 below).

$$ML = (\text{Log}_{10}\text{SCC} - \text{ cutoff } c) \times \text{EML}$$

[3] Considering third lactation cow, at 100 DIM, with SCC = 500,000 cells/mL

$$(2.70 - 0.89) \times 2.41 = -4.36$$

Figure 19 - The expected daily milk losses: (A) and (B) milk losses% distributed in different groups of SCC according to parity 1 and group of parities > 1, respectively

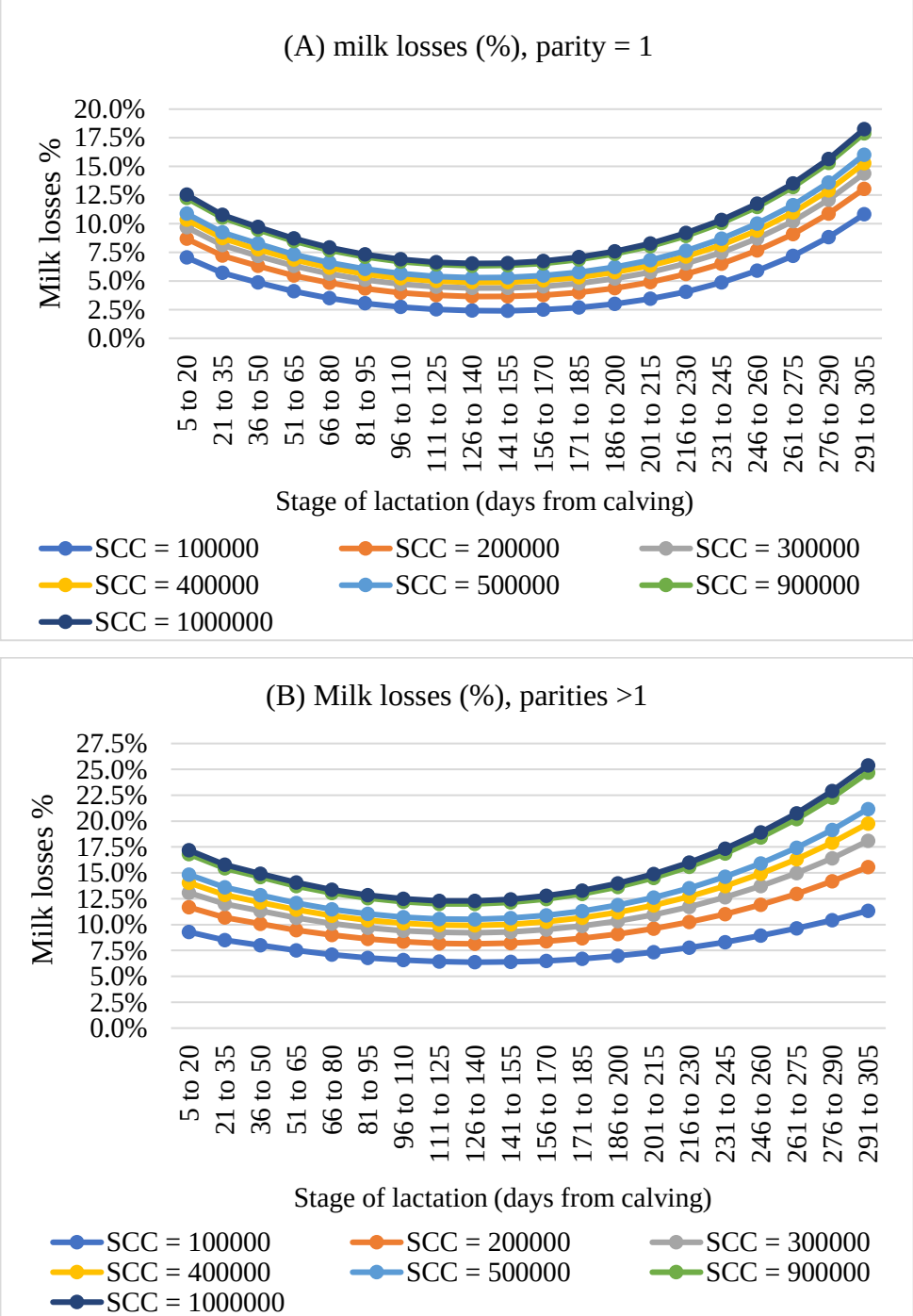


Table 22 - Results of the final model estimating milk losses of Holstein cows at parities 1 and greater than 1 at the stages of lactation (1, 2, 3…20), according to the SCC cutoff 100,000 cells/mL

Stage of lactation (days)	Parity 1 (n = 299,191)		Parity > 1 (n = 348,218)	
	Estimate (Kg)	Estimate (%)	Estimate (Kg)	Estimate (%)
5 to 20	-2.28	-7.1	-3.86	-9.3
21 to 35	-1.86	-5.7	-3.55	-8.5
36 to 50	-1.60	-4.9	-3.33	-8.0
51 to 65	-1.36	-4.1	-3.12	-7.5
66 to 80	-1.16	-3.5	-2.93	-7.1
81 to 95	-1.02	-3.1	-2.79	-6.8
96 to 110	-0.91	-2.7	-2.67	-6.6
111 to 125	-0.84	-2.5	-2.59	-6.4
126 to 140	-0.80	-2.4	-2.54	-6.4
141 to 155	-0.80	-2.4	-2.51	-6.4
156 to 170	-0.82	-2.5	-2.52	-6.5
171 to 185	-0.89	-2.7	-2.55	-6.7
186 to 200	-0.99	-3.0	-2.60	-7.0
201 to 215	-1.13	-3.4	-2.67	-7.3
216 to 230	-1.32	-4.1	-2.76	-7.8
231 to 245	-1.56	-4.9	-2.87	-8.3
246 to 260	-1.88	-5.9	-2.99	-8.9
261 to 275	-2.26	-7.2	-3.12	-9.6
276 to 290	-2.74	-8.8	-3.25	-10.4
291 to 305	-3.31	-10.8	-3.39	-11.3

$Log_{10}SCC = 2$ is equivalent to 100,000 cells/mL.

5.5 DISCUSSION

Results presented here depicted a typical proportion the Holstein breed population in Brazil. Increases in the SCC records of dairy cows are generally associated with an onset of mastitis (HAND; GODKIN; KELTON, 2012). Conversely, recent models (GRÆSBØLL et al., 2016) demonstrated that SCC from healthy cows are less correlated with milk production compared to the measure of SCC per milliliter - which indicates that for these cows, the production of somatic cells in the udder may be detached from the daily milk production. However, both factors investigated here consistently proved to be key components associated with the increase of somatic cell count in milk of Holstein cows that are not necessarily undergoing an intramammary infection or inflammation response. Findings here reinforces that the relationship between udder health status and test-day SCC (HAND; GODKIN; KELTON, 2012) has to feature both factors to accurate assess the milk losses associated with a given SCC

measured at test-day record. Covariates investigated were found to affect SCC throughout a 305-day lactation length from Holstein cows in Brazil. The input of both parity and stage of lactation to the model provided a refinement of the Log_{10}SCC cutoff at which milk losses expected among the population investigated herein seem to be different until the second third of lactation (from day 201 after calving) for both first-lactation and ≥2 lactation cows. Figures suggest that first-lactation Holstein cows at early stages of lactation (at day 5 - 20) may start experiencing milk losses at Log_{10}SCC 0.71 whereas multiparous cows may have their milk production begin to be affected at Log_{10}SCC 0.82 (at day 5 – 20). SCC thresholds at which losses are expected to occur increase as lactation progresses towards the 141st day (Log_{10} SCC 1.43) after calving from first parity cows and for multiparous cows SCC thresholds increase as lactation progresses (Log_{10} SCC 0.82 to 1.19). LAEVENS et al. (1997) reported that parity and stage of lactation, neither individually analyzed nor as an interaction between the two factors were influential to the SCC test-day recorded from cows that were cultured negative for mastitis-causing pathogens. Our estimates obtained upon the analysis of individual test-day records across parities over an entire lactation support the fact that milk losses do occur irrespectively of the mammary gland infection, also recently reported elsewhere (MILLER et al., 2004; DÜRR et al., 2008; ARCHER et al., 2013). Similarly to our study, a relationship between test-day somatic cell scores (SCS) and the stage of lactation from first and second lactation Holstein cows was also investigated (MILLER et al., 2004), but the highest test-days somatic cell score (SCS) was more often observed from the freshly calved heifers and late lactation cows. We also observed the same trend of highest SCC among the early stages lactation from first parity cows (Figure 1, day 5 – 20 after calving) and that from all parity numbers on later stages of lactation.

The variation among SCC at low levels associated with the potential of a dilution effect due to the relatively constant SCC throughout the lactation of healthy animals was not expected to strongly impact milk losses from early lactation heifers and multiparous cows until the days 66 – 80 of lactation (MILLER et al., 2004; GREEN; SCHUKKEN; GREEN, 2006). Our assumptions were based on the weakest association between SCC and daily milk yield found by HAGNESTAM-NIELSEN et al. (2009) from days 21 – 56 and 21 – 112 of lactation in primiparous and multiparous cows, respectively. However, our estimates indicate that with an increase of one unit point in Log_{10}SCC in the beginning of lactation milk losses can be expected to be more pronounced for multiparous cows (-3.28 kg, at day 5 – 20) than that for first parity cows right after calving (-1.77 kg, at day 5 – 20), although the first month of lactation, high somatic cell count, rainy season and history of clinical mastitis cases are factors associated with

mastitis for both primiparous and multiparous cows (OLIVEIRA et al., 2015). Similar trend across parity categories was observed by DÜRR et al. (2008), who found these losses to be -0.490 kg/day in Holstein heifers at day 1 – 15 after calving, and of -0.880 kg/day in adult cows at the same stage of lactation, per unit increase of log-transformed somatic cell counts. According to MILLER et al. (2004), increased losses are likely to happen among adult animals due to the heavy adoption of mastitis control measures towards heifers as opposed to those adopted for second lactation cows, which are expected to experience a decline on the immune resistance with age and be more prone to the consequences of high SCC over milk secretion. The milk losses from first parity animals on the onset of lactation that were identified herein exceeded estimates from ARCHER et al. (2013), who found that a 1-unit increase in the mean natural logarithm SCC over the first 5-30 days after calving of first lactation Irish dairy heifers was associated with a median decrease of 0.442 kg/day. Although those authors (ARCHER et al., 2013) accounted for the age of animals at calving in their model, they have not (or did not appear to) taken the breed of animals into consideration for their analysis. This could possibly suggest that estimates from higher-yielding breeds, such as Holstein animals, that comprised the dataset used in this study, might be associated with more pronounced losses as the Log_{10}SCC cutoff increases.

Estimated losses at a one unit increase of Log_{10}SCC of mid-lactation animals were lower than those observed from early-lactation heifers and cows (as discussed before). A one-unit increase in the variance of Log_{10}SCC around days 126 and 140 from calving of cows at first lactation was associated with an average milk decrease of -1.37 kg/day, and of -2.36 kg/day for animals at later lactations with a one unit increase of Log_{10}SCC. The decrease in milk production estimated in our study was greater than that reported by DÜRR et al. (2008) for Holstein cows at around the same stage of lactation. Losses observed at the 121st day of lactation by DÜRR et al. (2008) from heifers were -0.340 kg/day and of -0.810 kg/day in adult cows with the increase in the natural logarithm of test day SCC of one unit. Similarly to DÜRR et al. (2008), estimates of milk yield we present here originated from records of cows in which the exclusion criteria did not take into considerations cases of clinical or subclinical mastitis. Thus, our estimates of milk loss with an increase unit of Log_{10}SCC should be expected to be higher than that reported by DÜRR et al. (2008) given that the mean SCC score across all cows tested here were 340,000 cells/mL (Table 1) as opposed to that found by DÜRR et al. (2008) (~ 220,000 cells/mL). Mid-lactation dairy cows (at 101 – 200 DIM) from southern herds in Brazil are at increased risk to the occurrence of chronic cases of subclinical mastitis compared to animals at 100 DIM (CARDOZO et al., 2015), which could possibly explain the higher

increased estimates of milk losses found in our study and the Log_{10}SCC cutoff point associated with it.

The lowest cutoff Log_{10}SCC at which multiparous cows start experiencing milk losses was identified during the first third of lactation (5 – 20 days, Log_{10}SCC 0.82 cells/mL ± 0.09). Interestingly, primiparous cows had the first and last lowest cutoff Log_{10}SCC very similar, being observed at the first and last third of lactation (5 – 20 days, Log_{10}SCC 0.71 cells/mL ± 0.32 and 291 – 305 days, Log_{10}SCC 0.55 cells/mL ± 0.25). Elsewhere DÜRR et al. (2008), the lowest SCC cutoff points where milk losses were observed differed from ours (around 106 – 120 after calving), which for them coincided with the yield increase of cows towards the peak of lactation (around 90 – 100 DIM). This is biologically plausible as cows are in negative energy balance, and udder defenses may be impaired due to the metabolic stress (HAGNESTAM-NIELSEN et al., 2009).

Findings reported here substantiate the SCC level, parity and the stage of lactation at which milk losses are expected to be a matter of concern to the Brazilian dairy farmer. Previous studies addressed the issue by showing milk losses around 0.61 kg/day and 3.26kg/day from primiparous and multiparous cows, respectively, with the increase of individual test-day SCC at a cutoff from 14,270 cells/mL (COLDEBELLA et al., 2003). However, the use of those results may be of limited application given the diversity of herd management nationwide, as their findings were based on a single herd with exceptional on-farm practices in place. Figures presented corroborate with their estimates (COLDEBELLA et al., 2003), but rather may contribute on a broader extent to the Brazilian dairy industry, as we assessed a relevant proportion of the Holstein breed cows in Brazil. The average SCC below which cows are expected to out-produce their herdmates is 7,963 cells/mL. Over this threshold, however, our estimates of milk losses were higher and, on average, -1.88 kg/day and -3.53 kg/day, for heifers and multiparous Brazilian Holstein cows, respectively. Given the concerns around the SCC at cow-level and its economic implication - especially on levels at which milk losses are still overlooked by the dairy farmer – implementation of any mastitis control strategy at herd level has to be defined by understanding what acceptable level of associated losses with the bulk tank SCC is aimed by the dairy farmer (TROENDLE; TAUER; GRÖHN, 2017). This understanding, therefore, may be pivotal for the successful implementation of such programs and assist the dairy industry to accurately estimate losses associated with high SCC, especially at cow-level.

5.6 CONCLUSION

Daily milk losses caused by changes in Log_{10}SCC were dependent on parity and stage of lactation, and these factors should be considered when estimating losses associated with subclinical mastitis. Lactation milk loss (kg) increased significantly as lactation average SCC increased. Milk yield starts to be affected by changes in Log_{10}SCC only after a cutoff point, which is around 0.90 (approximately 7,963 cells/mL) for Holsteins cows from Brazilian herds. Second and later parities demonstrated greater milk loss than did first parity cows. A reduction in daily milk yield ranged from 1.37 to 2.28 kg is expected when first parity cows have an increase of one unit in Log_{10}SCC over the cutoff point, whereas second and later parities is expected ranging from 2.36 to 4.20 kg. The milk losses were lower during the lactation pick.

Chapter 6

Final considerations

6 FINAL CONSIDERATIONS

In chapter 1, we observed that during the last 20 years, milk production in Brazil has more than doubled (103.1%), from 15.1 billion in 1991 to 30.7 billion liters of milk in 2010. Currently, Brazil is the sixth largest milk producer in the world, with an average production of approximately 35 billion/year. However, Brazil still has low productivity and wide diversity among herds. In this context, one possible way to achieve the target of producing 40.3 billion liters of milk by 2023 will be to increase cow productivity, along with reducing milk production costs and practicing strict sanitary management measures with the aim of reducing mastitis and improving Brazilian milk quality.

In chapter 2, we described that subclinical mastitis is most commonly diagnosed by microbial culture-based (MC) methods or SCC, which are both traditional and well-established tests for detection of subclinical mastitis. However, milk culture or other forms of microbiological analysis can be costly to the producer. Therefore, the current study aimed to evaluate the use of the MLD to identify quarter milks that are most likely to be culture-positive. When MC was considered the gold standard for mastitis diagnosis, the calculated diagnostic *Se* of the MLD was 65.4% ($IC_{95\%}$ = 57.4 to 72.8%) and the *Sp* was 79.3% ($IC_{95\%}$ = 71.4% to 85.7%). An obvious use of the MLD would be to focus on cows with monthly SCC above some cut-off point (here, > 200×10^3cells/mL) with screening the infection at quarter level by providing a more rapid diagnosis performed by automated technology based upon 'on-farm differential cells' readout results. The MLD can provide an analysis of mammary quarter status more detailed than provided by SCC alone; however, the MLD response to subclinical mastitis was not found useful to specifically identify the causative pathogen.

In chapter 3, we emphasized that bacterial infections cause damage to milk secretory epithelia of the mammary gland and affect the yield of total milk and milk components. This damage can even result in a permanent loss of the capacity to synthesize milk by the mammary tissue. As a consequence, SM caused by specific groups of pathogens results in distinctive degrees of changes of milk yield and composition in affected mammary quarters. For that reason, the effect of different pathogens was studied by evaluating the contralateral (healthy and infected) mammary quarters of 146 lactating cows. We used this approach of contralateral quarters comparison since it 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). The milk losses ranged from 0.07 Kg/quarter.milking to 2.9

Kg/quarter.milking according to the pathogen causing SM. Economic losses were higher in SM caused by *Enterococcus* spp. (US$ 0.43/quarter.milking), *Streptococcus dysgalactiae* (US$ 0.74/quarter.milking) and *Escherichia coli* (US$ 0.98/quarter.milking). Additionally, there was a trend of *Staphy. aureus* and *Citrobacter* spp. to induce economic losses of US$ 0.26 and 0.29/quarter.milking, respectively. Overall, the economic return was lower in quarters with SM caused by environmental and contagious pathogens (US$ 0.18 and 0.22/quarter.milking, respectively) when compared to their healthy contralateral quarters.

In chapter 4, we discussed about different approaches were used to estimate production losses due to SM. The most commonly used research approach is based on analysis of milk SCC for comparison among herds and at the cow level, but less frequently reported at the quarter level. Since the effects caused by SM can be further intensified when the pathogen resists the immune defense and adapts to the mammary tissue, we proposed to accompany infected mammary quarters using successive milk sampling occasions over time. Thus, the aim of this study was to evaluate the effects of non-chronic and chronic subclinical mastitis caused by major pathogens (*Staph. aureus*, *Strep. agalactiae, Strep. uberis, Strep. dysgalactiae* and *Streptococci*-like bacteria) on SCC, milk yield and economic returns using milk sampling over time. As a final conclusion, milk losses and economic returns varied according to the type of mastitis-causing pathogen -0.24 to -0.87 kg/quarter.milking, and -1.57 to -1.69 kg/quarter.milking, when infected by environmental streptococci and *Staph. aureus*, respectively. Mammary quarters that were cured from SM caused by *Staph. aureus* and environmental streptococci exhibited an increase in economic return of approximately 0.47 and 0.69 US$/quarter.milking, respectively.

After determined the milk and losses by type of pathogen causing SM, we have evaluated the effect of SCC on milk yield from Holstein cows in Brazilian dairy herds using test day records to verify whether the association varies for different parities and stages of lactation. Additionally, we evaluated the threshold where the association between SCC and milk yield starts. In other words, at which level of SCC does milk yield begin to be affected? Therefore, in Chapter 5 we reported that milk yield starts to be affected by changes in Log_{10}SCC around 0.90 (~7,963 cells/mL) for Holstein cows from Brazilian herds. Daily milk losses caused by changes in Log_{10}SCC were dependent on parity and stage of lactation, and these factors should be considered when estimating losses associated with subclinical mastitis. Lactation milk loss (kg) increased significantly as lactation average SCC increased. A reduction in daily milk yield ranged from 1.37 to 2.28 kg is expected when first parity cows have an increase of

one unit in Log_{10}SCC over the cutoff point, whereas second and later parities is expected ranging from 2.36 to 4.20 kg. The milk losses were lower during the lactation peak.

Overall, the results of this thesis indicated that milk losses depend on the type of pathogen causing SM. Major pathogens have showed greater effects on milk quality than when it was observed using the approach of culture results of negative or positive. The methodology for evaluation of subclinical mastitis effect on milk yield interferes in the estimation of milk losses, and should include factors such as DIM and number of parity. First parity cows had lower milk losses when compared to the cows with parities ≥ 2. The greater the number of parity and the stage of lactation, the greater the milk losses associated with high SCC.

REFERENCES

ARCHER, S. C.; MC COY, F.; WAPENAAR, W.; GREEN, M. J. Association between somatic cell count early in the first lactation and the lifetime milk yield of cows in Irish dairy herds. **J Dairy Sci**, v. 96, n. 5, p. 2951-2959, 2013.

ARRUDA, A. G.; GODDEN, S.; RAPNICKI, P.; GORDEN, P.; TIMMS, L.; ALY, S. S.; LEHENBAUER, T. W.; CHAMPAGNE, J. Randomized noninferiority clinical trial evaluating 3 commercial dry cow mastitis preparations: I. Quarter-level outcomes. **J Dairy Sci**, v. 96, n. 7, p. 4419-4435, 2013.

AULDIST, M.; COATS, S.; ROGERS, G.; MCDOWELL, G. Changes in the composition of milk from healthy and mastitic dairy cows during the lactation cycle. **Aust J Exp Agric**, v. 35, n. 4, p. 427-436, 1995.

AULDIST, M. J.; HUBBLE, I. B. Effects of mastitis on raw milk and dairy products. **Aust J Dairy Tech**, v. 53, n. 1, p. 28-36, 1998.

BANSAL, B. K.; HAMANN, J.; GRABOWSKIT, N. T.; SINGH, K. B. Variation in the composition of selected milk fraction samples from healthy and mastitic quarters, and its significance for mastitis diagnosis. **J Dairy Res**, v. 72, n. 2, p. 144-152, 2005.

BARKEMA, H. W.; SCHUKKEN, Y. H.; LAM, T. J.; GALLIGAN, D. T.; BEIBOER, M. L.; BRAND, A. Estimation of interdependence among quarters of the bovine udder with subclinical mastitis and implications for analysis. **J Dairy Sci**, v. 80, n. 8, p. 1592-1599, 1997.

BARREIRO, J. R.; BRAGA, P. A.; FERREIRA, C. R.; KOSTRZEWA, M.; MAIER, T.; WEGEMANN, B.; BOETTCHER, V.; EBERLIN, M. N.; DOS SANTOS, M. V. Nonculture-based identification of bacteria in milk by protein fingerprinting. **Proteomics**, v. 12, n. 17, p. 2739-2745, 2012.

BARREIRO, J. R.; FERREIRA, C. R.; SANVIDO, G. B.; KOSTRZEWA, M.; MAIER, T.; WEGEMANN, B.; BOTTCHER, V.; EBERLIN, M. N.; DOS SANTOS, M. V. Short communication: Identification of subclinical cow mastitis pathogens in milk by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. **J Dairy Sci**, v. 93, n. 12, p. 5661-5667, 2010.

BENNETT, R. The 'Direct Costs'of Livestock Disease: The Development of a System of Models for the Analysis of 30 Endemic Livestock Diseases in Great Britain. **J Agric Econ**, v. 54, n. 1, p. 55-71, 2003.

BERGLUND, I.; PETTERSSON, G.; OSTENSSON, K.; SVENNERSTEN-SJAUNJA, K. Quarter milking for improved detection of increased SCC. **Reprod Domest Anim**, v. 42, n. 4, p. 427-432, 2007.

BEZMAN, D.; LEMBERSKIY-KUZIN, L.; KATZ, G.; MERIN, U.; LEITNER, G. Influence of intramammary infection of a single gland in dairy cows on the cow's milk quality. **J Dairy Res**, v., n., p. 1-8, 2015.

BLUM, S. E.; HELLER, E. D.; LEITNER, G. Long term effects of Escherichia coli mastitis. **Vet J**, v. 201, n. 1, p. 72-77, 2014.

BOTARO, B. G.; CORTINHAS, C. S.; DIBBERN, A. G.; E SILVA, L. F.; BENITES, N. R.; DOS SANTOS, M. V. *Staphylococcus aureus* intramammary infection affects milk yield and SCC of dairy cows. **Trop Anim Health Prod**, v. 47, n. 1, p. 61-66, 2015.

BOTARO, B. G.; GAMEIRO, A. H.; SANTOS, M. V. D. Quality based payment program and milk quality in dairy cooperatives of Southern Brazil: an econometric analysis. **Sci agric**, v. 70, p. 21-26, 2013.

BRADLEY, A. Bovine mastitis: an evolving disease. **Vet J**, v. 164, n. 2, p. 116-128, 2002.

BRASIL. MINISTÉRIO DA AGRICULTURA, PECUÁRIA E ABASTECIMENTO - MAPA. **Projeções do Agronegócio: Brasil 2012/2013 a 2022/2013.** Assessoria de Gestão Estratégica, Distrito Federal, Brasília: MAPA/ACS, 2013. 96 p.

CALDERWOOD, D.; DRACH, J.P.; PAUL, C.; RODRIGUEZ, R.; HOCKETT, M.; MARCUSON, R.; MIGGELS, S.G.; MACK, H.J.; YOUNG, D.; POLLARD, J.N. **Sample collection device assembly**. USA720,468, 30 dez. 2014.

CAPUCO, A. V.; ELLIS, S. E.; HALE, S. A.; LONG, E.; ERDMAN, R. A.; ZHAO, X.; PAAPE, M. J. Lactation persistency: insights from mammary cell proliferation studies. **J Anim Sci**, v. 81 Suppl 3, p. 18-31, 2003.

CARDOZO, L. L.; THALER NETO, A.; SOUZA, G. N.; PICININ, L. C. A.; FELIPUS, N. C.; RECHE, N. L. M.; SCHMIDT, F. A.; WERNCKE, D.; SIMON, E. E. Risk factors for the occurrence of new and chronic cases of subclinical mastitis in dairy herds in southern Brazil. **J Dairy Sci**, v. 98, n. 11, p. 7675-7685, 2015.

CHA, E.; BAR, D.; HERTL, J. A.; TAUER, L. W.; BENNETT, G.; GONZALEZ, R. N.; SCHUKKEN, Y. H.; WELCOME, F. L.; GROHN, Y. T. The cost and management of different types of clinical mastitis in dairy cows estimated by dynamic programming. **J Dairy Sci**, v. 94, n. 9, p. 4476-4487, 2011.

COLDEBELLA, A.; MACHADO, P. F.; DEMÉTRIO, C. G. B.; RIBEIRO JÚNIOR, P. J.; CORASSIN, C. H.; MEYER, P. M.; CASSOLI, L. D. Contagem de células somáticas e produção de leite em vacas holandesas de alta produção. **Pesq Agropec Bras**, v. 38, 1451-1457, 2003.

CONAB. COMPANHIA NACIONAL DE ABASTECIMENTO. **Conjuntura mensal: Leite e derivados.** Distrito Federal, Brasília: CONAB, abril/2016. 9 p.

COULON, J. B.; GASQUI, P.; BARNOUIN, J.; OLLIER, A.; PRADEL, P.; POMIÈS, D. Effect of mastitis and related-germ on milk yield and composition during naturally-occurring udder infections in dairy cows. **Anim Res**, v. 51, n. 05, p. 383-393, 2002.

CUNHA, R. P. L.; MOLINA, L. R.; CARVALHO, A. U.; FACURY FILHO, E. J.; FERREIRA, P. M.; GENTILINI, M. B. Mastite subclínica e relação da contagem de células

somáticas com número de lactações, produção e composição química do leite em vacas da raça Holandesa. **Arq Bras Med Vet Zootec**, v. 60, 19-24, 2008.

DE GRAAF, T.; DWINGER, R. H. Estimation of milk production losses due to sub-clinical mastitis in dairy cattle in Costa Rica. **Prev Vet Med**, v. 26, n. 3–4, p. 215-222, 1996.

DE ROOS, A. P.; HARBERS, A. G.; DE JONG, G. Random herd curves in a test-day model for milk, fat, and protein production of dairy cattle in The Netherlands. **J Dairy Sci**, v. 87, n. 8, p. 2693-2701, 2004.

DOHOO, I.; ANDERSEN, S.; DINGWELL, R.; HAND, K.; KELTON, D.; LESLIE, K.; SCHUKKEN, Y.; GODDEN, S. Diagnosing intramammary infections: comparison of multiple versus single quarter milk samples for the identification of intramammary infections in lactating dairy cows. **J Dairy Sci**, v. 94, n. 11, p. 5515-5522, 2011.

DOSOGNE, H.; VANGROENWEGHE, F.; MEHRZAD, J.; MASSART-LEEN, A. M.; BURVENICH, C. Differential leukocyte count method for bovine low somatic cell count milk. **J Dairy Sci**, v. 86, n. 3, p. 828-834, 2003.

DULIN, A. M.; PAAPE, M. J.; WEINLAND, B. T. Cytospin centrifuge in differential counts of milk somatic cells. **J Dairy Sci**, v. 65, n. 7, p. 1247-1251, 1982.

DÜRR, J. W.; CUE, R. I.; MONARDES, H. G.; MORO-MÉNDEZ, J.; WADE, K. M. Milk losses associated with somatic cell counts per breed, parity and stage of lactation in Canadian dairy cattle. **Livest Sci**, v. 117, n. 2–3, p. 225-232, 2008.

DVG (German Veterinary Society). 2002. Leitlinien zur Bekämpfung der Mastitis als Bestandsproblem. DVG, Giessen, Germany.

FLEISCHMANN, W. **The Book of the Dairy**. Blackie and Son: London, United Kingdom. 1986.

FORSBACK, L.; LINDMARK-MANSSON, H.; ANDREN, A.; AKERSTEDT, M.; ANDREE, L.; SVENNERSTEN-SJAUNJA, K. Day-to-day variation in milk yield and milk composition at the udder-quarter level. **J Dairy Sci**, v. 93, n. 8, p. 3569-3577, 2010b.

FORSBACK, L.; LINDMARK-MANSSON, H.; ANDREN, A.; AKERSTEDT, M.; SVENNERSTEN-SJAUNJA, K. Udder quarter milk composition at different levels of somatic cell count in cow composite milk. **Animal**, v. 3, n. 5, p. 710-717, 2009.

FORSBACK, L.; LINDMARK-MANSSON, H.; ANDREN, A.; SVENNERSTEN-SJAUNJA, K. Evaluation of quality changes in udder quarter milk from cows with low-to-moderate somatic cell counts. **Animal**, v. 4, n. 4, p. 617-626, 2010a.

FOX, L.K. AND GAY, J.M. **Contagious mastitis**. Veterinary clinics of north America, v.3, 1993, 475–488.

GONCALVES, J. L.; TOMAZI, T.; BARREIRO, J. R.; BEURON, D. C.; ARCARI, M. A.; LEE, S. H.; MARTINS, C. M.; ARAUJO JUNIOR, J. P.; DOS SANTOS, M. V. Effects of

bovine subclinical mastitis caused by *Corynebacterium* spp. on somatic cell count, milk yield and composition by comparing contralateral quarters. **Vet J**, v. 209, p. 87-92, 2016.

GRÆSBØLL, K.; KIRKEBY, C.; NIELSEN, S. S.; HALASA, T.; TOFT, N.; CHRISTIANSEN, L. E. Models to Estimate Lactation Curves of Milk Yield and Somatic Cell Count in Dairy Cows at the Herd Level for the Use in Simulations and Predictive Models. **Front Vet Sci**, v. 3, 115, 2016.

GREEN, L. E.; SCHUKKEN, Y. H.; GREEN, M. J. On distinguishing cause and consequence: do high somatic cell counts lead to lower milk yield or does high milk yield lead to lower somatic cell count? **Prev Vet Med**, v. 76, n. 1-2, p. 74-89, 2006.

GROHN, Y. T.; WILSON, D. J.; GONZALEZ, R. N.; HERTL, J. A.; SCHULTE, H.; BENNETT, G.; SCHUKKEN, Y. H. Effect of pathogen-specific clinical mastitis on milk yield in dairy cows. **J Dairy Sci**, v. 87, n. 10, p. 3358-3374, 2004.

HAGNESTAM, C.; EMANUELSON, U.; BERGLUND, B. Yield losses associated with clinical mastitis occurring in different weeks of lactation. **J Dairy Sci**, v. 90, n. 5, p. 2260-2270, 2007.

HAGNESTAM-NIELSEN, C.; EMANUELSON, U.; BERGLUND, B.; STRANDBERG, E. Relationship between somatic cell count and milk yield in different stages of lactation. **J Dairy Sci**, v. 92, n. 7, p. 3124-3133, 2009.

HALASA, T.; HUIJPS, K.; OSTERAS, O.; HOGEVEEN, H. Economic effects of bovine mastitis and mastitis management: a review. **Vet Q**, v. 29, n. 1, p. 18-31, 2007.

HALASA, T.; NIELEN, M.; DE ROOS, A. P.; VAN HOORNE, R.; DE JONG, G.; LAM, T. J.; VAN WERVEN, T.; HOGEVEEN, H. Production loss due to new subclinical mastitis in Dutch dairy cows estimated with a test-day model. **J Dairy Sci**, v. 92, n. 2, p. 599-606, 2009.

HAND, K. J.; GODKIN, A.; KELTON, D. F. Milk production and somatic cell counts: a cow-level analysis. **J Dairy Sci**, v. 95, n. 3, p. 1358-1362, 2012.

HOCKETT, M.; PAYNE, M. AND RODRIGUEZ, R. Evaluation of cow-level selective dry cow therapy based on diagnosis by milk leukocyte differential. In: NATIONAL MASTITIS COUNCIL REGIONAL MEETING. **Preceding**… Ghent, BE. Natl. Mastitis Counc., Inc., Madison, WI, USA, 2014a, p.207.

HOCKETT, M.; PAYNE, M. AND RODRIGUEZ, R. Milk leukocyte differential as a tool to guide quarter-level, selective dry cow therapy. In: NATIONAL MASTITIS COUNCIL REGIONAL MEETING. **Preceding**… Ghent, BE. Natl. Mastitis Counc., Inc., Madison, WI, USA, 2014b, p.208.

HOGEVEEN, H.; HUIJPS, K.; LAM, T. J. Economic aspects of mastitis: new developments. **N Z Vet J**, v. 59, n. 1, p. 16-23, 2011.

HORTET, P.; SEEGERS, H. Calculated milk production losses associated with elevated somatic cell counts in dairy cows: review and critical discussion. **Vet Res**, v. 29, n. 6, p. 497-510, 1998.

HUIJPS, K.; HOGEVEEN, H. Stochastic modeling to determine the economic effects of blanket, selective, and no dry cow therapy. **J Dairy Sci**, v. 90, n. 3, p. 1225-1234, 2007.

HUIJPS, K.; LAM, T. J.; HOGEVEEN, H. Costs of mastitis: facts and perception. **J Dairy Res**, v. 75, n. 1, p. 113-120, 2008.

IBGE. INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA. **Produção da pecuária municipal – 2015**. Rio de Janeiro, RJ: IBGE, v. 43, 2015. 49 p.

KELLY, A. L.; TIERNAN, D.; O'SULLIVAN, C.; JOYCE, P. Correlation between bovine milk somatic cell count and polymorphonuclear leukocyte level for samples of bulk milk and milk from individual cows. **J Dairy Sci**, v. 83, n. 2, p. 300-304, 2000.

KITCHEN, B. J. Review of the progress of dairy science: bovine mastitis: milk compositional changes and related diagnostic tests. **J Dairy Res**, v. 48, n. 1, p. 167-188, 1981.

KOSKINEN, M. T.; WELLENBERG, G. J.; SAMPIMON, O. C.; HOLOPAINEN, J.; ROTHKAMP, A.; SALMIKIVI, L.; VAN HAERINGEN, W. A.; LAM, T. J.; PYORALA, S. Field comparison of real-time polymerase chain reaction and bacterial culture for identification of bovine mastitis bacteria. **J Dairy Sci**, v. 93, n. 12, p. 5707-5715, 2010.

LAEVENS, H.; DELUYKER, H.; SCHUKKEN, Y. H.; DE MEULEMEESTER, L.; VANDERMEERSCH, R.; DE MUELENAERE, E.; DE KRUIF, A. Influence of parity and stage of lactation on the somatic cell count in bacteriologically negative dairy cows. **J Dairy Sci**, v. 80, n. 12, p. 3219-3226, 1997.

LE ROUX, Y.; LAURENT, F.; MOUSSAOUI, F. Polymorphonuclear proteolytic activity and milk composition change. **Vet Res**, v. 34, n. 5, p. 629-645, 2003.

LEITNER, G.; CHAFFER, M.; KRIFUCKS, O.; GLICKMAN, A.; EZRA, E.; SARAN, A. Milk leucocyte populations in heifers free of udder infection. **J Vet Med B Infect Dis Vet Public Health**, v. 47, n. 2, p. 133-138, 2000.

LEITNER, G.; KRIFUCKS, O.; MERIN, U.; LAVI, Y.; SILANIKOVE, N. Interactions between bacteria type, proteolysis of casein and physico-chemical properties of bovine milk. **Int Dairy J**, v. 16, n. 6, p. 648-654, 2006.

LEVAN, P. L.; EBERHART, R. J.; KESLER, E. M. Effects of Natural Intramammary *Corynebacterium bovis* Infection on Milk Yield and Composition1. **J Dairy Sci**, v. 68, n. 12, p. 3329-3336, 1985.

LOSINGER, W. C. Economic impacts of reduced milk production associated with an increase in bulk-tank somatic cell count on US dairies. **J Am Vet Med Assoc**, v. 226, n. 10, p. 1652-1658, 2005.

MAKOVEC, J. A.; RUEGG, P. L. Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001. **J Dairy Sci**, v. 86, n. 11, p. 3466-3472, 2003.

MedCalc Statistical Software version 16.8. 2016. Accessed Aug. 4, 2016. https://www.medcalc.org/calc/diagnostic_test.php

MERLE, R.; SCHRODER, A.; HAMANN, J. Cell function in the bovine mammary gland: a preliminary study on interdependence of healthy and infected udder quarters. **J Dairy Res**, v. 74, n. 2, p. 174-179, 2007.

MILLER, R. H.; NORMAN, H. D.; WIGGANS, G. R.; WRIGHT, J. R. Relationship of test-day somatic cell score with test-day and lactation milk yields. **J Dairy Sci**, v. 87, n. 7, p. 2299-2306, 2004.

MURRAY, P. R.; BARON, E. J.; JORGENSEN, J. J.; PFALLER, M. A.; YOLKEN, R. H. Manual of Clinical Microbiology. **ASM Press: Washington, DC**, v. 8th ed., 2003.

NIGHTINGALE, C.; DHUYVETTER, K.; MITCHELL, R.; SCHUKKEN, Y. Influence of variable milk quality premiums on observed milk quality. **J Dairy Sci**, v. 91, n. 3, p. 1236-1244, 2008.

OLIVEIRA, C. S. F.; HOGEVEEN, H.; BOTELHO, A. M.; MAIA, P. V.; COELHO, S. G.; HADDAD, J. P. A. Cow-specific risk factors for clinical mastitis in Brazilian dairy cattle. **Prev Vet Med**, v. 121, n. 3–4, p. 297-305, 2015.

OLIVER, S. P. O.; GONZÁLEZ, R. N.; HOGAN, J. S.; JAYARAO, B. M.; OWENS, W. E. Microbiological Procedures for the Diagnosis of bovine udder infection and determination of milk quality. In: **A Global Organization for Mastitis Control and Milk Quality.** Fourth Ed. National Mastitis Council Inc., Verona, WI, USA, pp. 1-40, 44-46, 2004.

PEARSON, L. J.; WILLIAMSON, J. H.; TURNER, S. A.; LACY-HULBERT, S. J.; HILLERTON, J. E. Peripartum infection with *Streptococcus uberis* but not coagulase-negative staphylococci reduced milk production in primiparous cows. **J Dairy Sci**, v. 96, n. 1, p. 158-164, 2013.

PETROVSKI, K. R.; TRAJCEV, M.; BUNESKI, G. A review of the factors affecting the costs of bovine mastitis. **J S Afr Vet Assoc**, v. 77, n. 2, p. 52-60, 2006.

PIEPERS, S.; DE VLIEGHER, S.; DE KRUIF, A.; OPSOMER, G.; BARKEMA, H. W. Impact of intramammary infections in dairy heifers on future udder health, milk production, and culling. **Vet Microbiol**, v. 134, n. 1-2, p. 113-120, 2009.

PIEPERS, S.; SCHUKKEN, Y. H.; PASSCHYN, P.; DE VLIEGHER, S. The effect of intramammary infection with coagulase-negative staphylococci in early lactating heifers on milk yield throughout first lactation revisited. **J Dairy Sci**, v. 96, n. 8, p. 5095-5105, 2013.

PILLA, R.; MALVISI, M.; SNEL, G. G.; SCHWARZ, D.; KONIG, S.; CZERNY, C. P.; PICCININI, R. Differential cell count as an alternative method to diagnose dairy cow mastitis. **J Dairy Sci**, v. 96, n. 3, p. 1653-1660, 2013.

PILLA, R.; SCHWARZ, D.; KONIG, S.; PICCININI, R. Microscopic differential cell counting to identify inflammatory reactions in dairy cow quarter milk samples. **J Dairy Sci**, v. 95, n. 8, p. 4410-4420, 2012.

PILLAI, S. R.; KUNZE, E.; SORDILLO, L. M.; JAYARAO, B. M. Application of differential inflammatory cell count as a tool to monitor udder health. **J Dairy Sci**, v. 84, n. 6, p. 1413-1420, 2001.

PITKALA, A.; HAVERI, M.; PYORALA, S.; MYLLYS, V.; HONKANEN-BUZALSKI, T. Bovine mastitis in Finland 2001--prevalence, distribution of bacteria, and antimicrobial resistance. **J Dairy Sci**, v. 87, n. 8, p. 2433-2441, 2004.

RAINARD, P.; POUTREL, B. Dynamics of nonclinical bovine intramammary infections with major and minor pathogens. **Am J Vet Res**, v. 43, n. 12, p. 2143-2146, 1982.

RENEAU, J.K.; PACKARD, V.L. Monitoring mastitis, milk quality and economic losses in dairy fields. **Dairy food environ sanit**, v.11, 1991, 4–11.

SANTOS, M. V.; MA, Y.; BARBANO, D. M. Effect of somatic cell count on proteolysis and lipolysis in pasteurized fluid milk during shelf-life storage. **J Dairy Sci**, v. 86, n. 8, p. 2491-2503, 2003.

SCHUKKEN, Y. H.; HERTL, J.; BAR, D.; BENNETT, G. J.; GONZALEZ, R. N.; RAUCH, B. J.; SANTISTEBAN, C.; SCHULTE, H. F.; TAUER, L.; WELCOME, F. L.; GROHN, Y. T. Effects of repeated gram-positive and gram-negative clinical mastitis episodes on milk yield loss in Holstein dairy cows. **J Dairy Sci**, v. 92, n. 7, p. 3091-3105, 2009.

SCHUKKEN, Y. H.; WILSON, D. J.; WELCOME, F.; GARRISON-TIKOFSKY, L.; GONZALEZ, R. N. Monitoring udder health and milk quality using somatic cell counts. **Vet Res**, v. 34, n. 5, p. 579-596, 2003.

SCHWARZ, D.; DIESTERBECK, U. S.; KONIG, S.; BRUGEMANN, K.; SCHLEZ, K.; ZSCHOCK, M.; WOLTER, W.; CZERNY, C. P. Microscopic differential cell counts in milk for the evaluation of inflammatory reactions in clinically healthy and subclinically infected bovine mammary glands. **J Dairy Res**, v. 78, n. 4, p. 448-455, 2011a.

SCHWARZ, D.; DIESTERBECK, U. S.; KONIG, S.; BRUGEMANN, K.; SCHLEZ, K.; ZSCHOCK, M.; WOLTER, W.; CZERNY, C. P. Flow cytometric differential cell counts in milk for the evaluation of inflammatory reactions in clinically healthy and subclinically infected bovine mammary glands. **J Dairy Sci**, v. 94, n. 10, p. 5033-5044, 2011b.

SEBRAE. SERVIÇO BRASILEIRO DE APOIO ÀS MICRO E PEQUENAS EMPRESAS. **Cenários para o leite e derivados na região Nordeste em 2020.** Pernambuco, Recife: SEBRAE, 2013. 154 p.

SEEGERS, H.; FOURICHON, C.; BEAUDEAU, F. Production effects related to mastitis and mastitis economics in dairy cattle herds. **Vet Res**, v. 34, n. 5, p. 475-491, 2003.

SMITH, K.L.; HOGAN, J.S. **Environmental mastitis**. Veterinary Clinics of North America, v.3, 1993, 489–498.

STEENEVELD, W.; SWINKELS, J.; HOGEVEEN, H. Stochastic modelling to assess economic effects of treatment of chronic subclinical mastitis caused by *Streptococcus uberis*. **J Dairy Res**, v. 74, n. 4, p. 459-467, 2007.

SWINKELS, J. M.; HOGEVEEN, H.; ZADOKS, R. N. A partial budget model to estimate economic benefits of lactational treatment of subclinical *Staphylococcus aureus* mastitis. **J Dairy Sci**, v. 88, n. 12, p. 4273-4287, 2005b.

SWINKELS, J. M.; ROOIJENDIJK, J. G.; ZADOKS, R. N.; HOGEVEEN, H. Use of partial budgeting to determine the economic benefits of antibiotic treatment of chronic subclinical mastitis caused by *Streptococcus uberis* or *Streptococcus dysgalactiae*. **J Dairy Res**, v. 72, n. 1, p. 75-85, 2005a.

TAPONEN, S.; PYÖRÄLÄ, S. Coagulase-negative staphylococci as cause of bovine mastitis—Not so different from *Staphylococcus aureus*? **Vet Microbiol**, v. 134, n. 1–2, p. 29-36, 2009.

TESFAYE, G. Y.; REGASSA, F. G.; KELAY, B. Milk yield and associated economic losses in quarters with subclinical mastitis due to *Staphylococcus aureus* in Ethiopian crossbred dairy cows. **Trop Anim Health Prod**, v. 42, n. 5, p. 925-931, 2010.

TOMAZI, T.; GONCALVES, J. L.; BARREIRO, J. R.; ARCARI, M. A.; DOS SANTOS, M. V. Bovine subclinical intramammary infection caused by coagulase-negative staphylococci increases somatic cell count but has no effect on milk yield or composition. **J Dairy Sci**, v. 98, n. 5, p. 3071-3078, 2015.

TROENDLE, J. A.; TAUER, L. W.; GRÖHN, Y. T. Optimally achieving milk bulk tank somatic cell count thresholds. **J Dairy Sci**, v. 100, n. 1, p. 731-738, 2017.

URECH, E.; PUHAN, Z.; SCHALLIBAUM, M. Changes in milk protein fraction as affected by subclinical mastitis. **J Dairy Sci**, v. 82, n. 11, p. 2402-2411, 1999.

VAN ASSELDONK, M. A.; RENES, R. J.; LAM, T. J.; HOGEVEEN, H. Awareness and perceived value of economic information in controlling somatic cell count. **Vet Rec**, v. 166, n. 9, p. 263-267, 2010.

VIGUIER, C.; ARORA, S.; GILMARTIN, N.; WELBECK, K.; O'KENNEDY, R. Mastitis detection: current trends and future perspectives. **Trends Biotechnol**, v. 27, n. 8, p. 486-493, 2009.

WARDLAW, S.C.; LEVINE, R.A.; RODRIGUEZ, R.R. **Determination of white blood cell differential and reticulocyte counts**. USA6,350,613, 26 fev. 2002.

WARDLAW, S.C.; ROBERT, A.L. **Method for performing blood cell counts.** USA5,948,686, 4 mar. 1999.

WERNER, B.; MORONI, P.; GIOIA, G.; LAVIN-ALCONERO, L.; YOUSAF, A.; CHARTER, M. E.; CARTER, B. M.; BENNETT, J.; NYDAM, D. V.; WELCOME, F.; SCHUKKEN, Y. H. Short communication: Genotypic and phenotypic identification of environmental streptococci and association of *Lactococcus lactis* ssp. *lactis* with intramammary infections among different dairy farms. **J Dairy Sci**, v. 97, n. 11, p. 6964-6969, 2014.

WEVER, P.; EMANUELSON, U. Effects of systematic influences and intramammary infection on differential and total somatic cell counts in quarter milk samples from dairy cows. **Acta Vet Scand**, v. 30, n. 4, p. 465-474, 1989.

WHITE, G. C.; COUTURE, G. W.; ANDERSON, E. O.; JOHNSON, R. E.; PLASTRIDGE, W. N.; WEIRETHER, F. J. Chronic Bovine Mastitis and Milk Yield. **J Dairy Sci**, v. 20, n. 4, p. 171-180, 1937.

WILSON, D. J.; DAS, H. H.; GONZALEZ, R. N.; SEARS, P. M. Association between management practices, dairy herd characteristics, and somatic cell count of bulk tank milk. **J Am Vet Med Assoc**, v. 210, n. 10, p. 1499-1502, 1997b.

WILSON, D. J.; GONZALEZ, R. N.; DAS, H. H. Bovine mastitis pathogens in New York and Pennsylvania: prevalence and effects on somatic cell count and milk production. **J Dairy Sci**, v. 80, n. 10, p. 2592-2598, 1997a.

ZOCCAL, R. Alguns números do leite. **Revista Balde Branco**, São Paulo, Ed. set., 2016. Available at: <http://www.baldebranco.com.br/alguns-numeros-do-leite/>. Accessed on: Jan 26, 2016.

APPENDIX

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Biofilm-producing ability and efficiency of sanitizing agents against *Prototheca zopfii* isolates from bovine subclinical mastitis

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ABSTRACT

The objectives of the present study were to evaluate (1) the capacity of the microalga *Prototheca zopfii* isolated from subclinical bovine mastitis cases to form biofilms; and (2) the resistance of these isolates to sanitizing agents. Ten isolates of *P. zopfii* from cows with subclinical mastitis (somatic cell count >200 × 10^3 cells/mL), distributed in 5 dairy farms, were evaluated for their capacity to form biofilms in polystyrene microplate assays and stainless steel coupons, at 25°C and 37°C ± 1°C. *Prototheca zopfii* were isolated from milk samples via microbiological culture and analyzed by 18S rRNA gene sequencing. Biofilm formation on the coupons was observed by scanning electron microscopy. The resistance to sanitizing agents was assessed using the biofilm-forming *P. zopfii* isolates in stainless steel coupon assays, which were subjected to 3 sanitizers: peracetic acid, sodium hypochlorite, and iodine solution. To evaluate resistance to the sanitizers, the minimum inhibitory concentration (MIC) technique was performed using decreasing concentrations of the sanitizing agents (20, 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, and 0.019 g/L). After inoculating the isolates, all concentrations were evaluated at 3 distinct incubation periods (24, 48, and 72 h) to assess the effect of incubation time on the MIC. Using the polystyrene microplate assays, 1 isolate showed weak biofilm production, 5 moderate, and 4 strong, when incubated at 25°C ± 1. For isolates incubated at 37°C ± 1, 6 showed weak biofilm production and 4 moderate. All *P. zopfii* isolates (n = 10) had the capacity to form biofilms on stainless steel coupons. The longer the incubation period of the *P. zopfii* isolates at different dilutions, the greater the concentrations of sanitizer needed to prevent growth of the microalgae under the tested conditions. We detected a significant effect of sanitizer and time of incubation (24, 48, and 72 h) on MIC values against *P. zopfii* isolates. The isolates were sensitive in vitro to peracetic acid (MIC_{90} ≥0.019 g/L), sodium hypochlorite (MIC_{90} ≥0.312 g/L), and iodine solution (MIC_{90} ≥0.625 g/L), after 24 h of incubation (where MIC_{90} = concentration needed to inhibit 90% of isolates). Of the tested sanitizers, peracetic acid had the greatest efficiency against *P. zopfii*. We conclude that *P. zopfii* isolates are capable of biofilm production, which may contribute to their persistence in a milking and dairy environment.

Key words: subclinical mastitis, biofilm-producing isolate, sanitizer, *Prototheca zopfii*

INTRODUCTION

The occurrence of mastitis caused by the microalga *Prototheca zopfii* has been described in several countries (Corbellini et al., 2001; Möller et al., 2007; Osumi et al., 2008; Marques et al., 2010b; Ricchi et al., 2010; Pieper et al., 2012). The frequency of bovine protothecal mastitis caused by *P. zopfii* has been increasing worldwide, which may represent a serious problem due to the inherent resistance to routine therapy of these microalgae (Cunha et al., 2010; Pieper et al., 2012; Ricchi et al., 2010). This resistance is associated with the capacity of the microalgae to infect and survive in macrophages and to invade mammary tissue, making them responsible for a persistent infection with intermittent shedding of *P. zopfii* in milk (Marques et al., 2006).

The treatment of mastitis caused by *Prototheca* spp. with antimicrobials produces only temporary improvement of clinical signs due to the low rate of cure in vivo, and because of this, the causative agent is not eliminated (Costa et al., 1996). Therefore, culling cows infected with *P. zopfii* is one of the recommended control measures to reduce the disease (Jáncsó et al., 2001).

The main risk factors associated with mastitis caused by *P. zopfii* in dairy herds are transmission between

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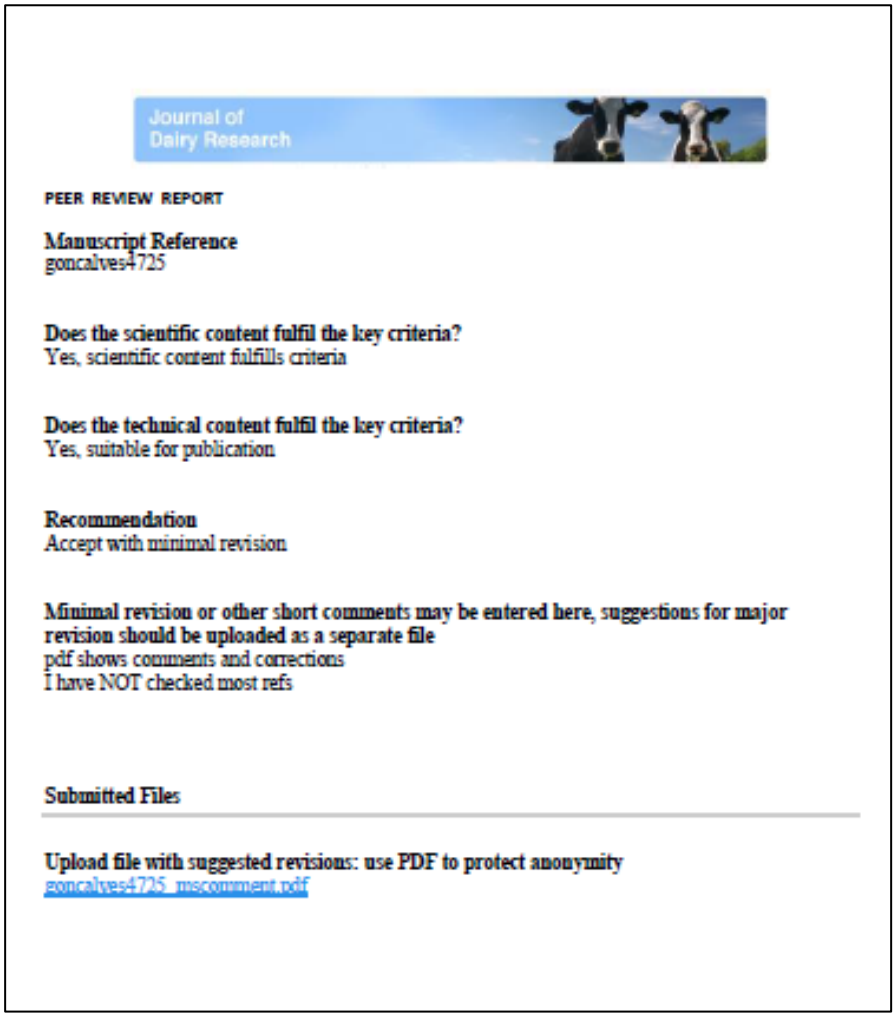
CHAPTER 2: SUBMISSION TO JOURNAL OF DAIRY RESEARCH.



Journal of
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PEER REVIEW REPORT

Manuscript Reference
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Does the scientific content fulfil the key criteria?
Yes, scientific content fulfills criteria

Does the technical content fulfil the key criteria?
Yes, suitable for publication

Recommendation
Accept with minimal revision

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CHAPTER 3: SUBMISSION TO ANIMAL JOURNAL.

Animal: An International Journal of Animal Bioscience

Bovine subclinical mastitis reduces milk yield and alters composition at contralateral mammary quarter level within cow

--Manuscript Draft--

Manuscript Number:	ANIMAL-16-40994R1
Full Title:	Bovine subclinical mastitis reduces milk yield and alters composition at contralateral mammary quarter level within cow
Short Title:	Subclinical mastitis at quarter level
Article Type:	Research Article
Section/Category:	4. Behaviour, welfare and health
Keywords:	milk quality; subclinical mastitis; contagious; environmental; milk price.
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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 > 200×103 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. Healthy quarters (124 pairs) had lower geometric mean SCC (153.60×103 cells/mL SEM 63.35) than infected contralateral quarters (SCC of 337.53×103 cells/mL SEM 169.70). 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