

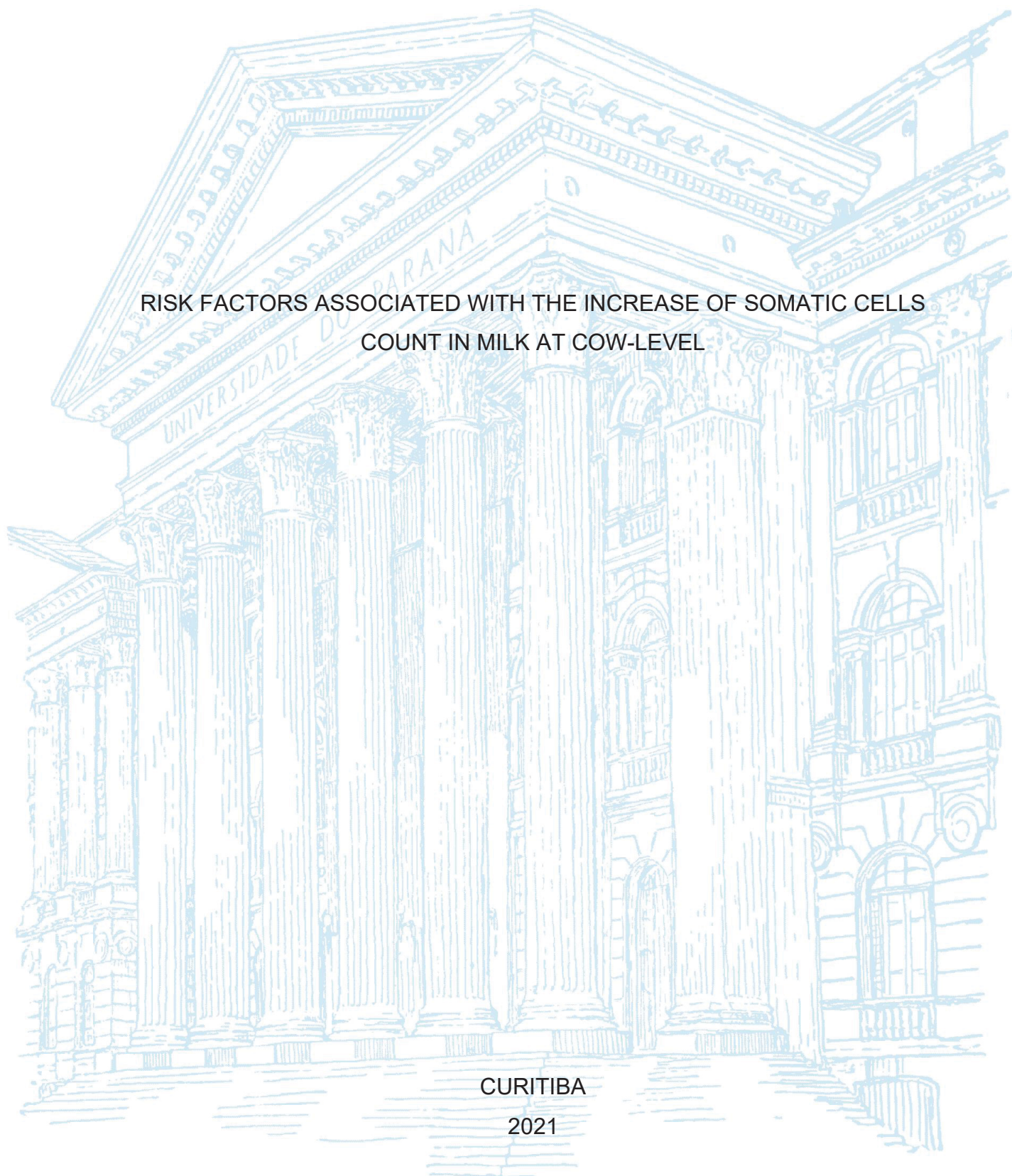
UNIVERSIDADE FEDERAL DO PARANÁ

RAFAELA SCHUNIG

RISK FACTORS ASSOCIATED WITH THE INCREASE OF SOMATIC CELLS  
COUNT IN MILK AT COW-LEVEL

CURITIBA

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RISK FACTORS ASSOCIATED WITH THE INCREASE OF SOMATIC CELLS  
COUNT AT COW-LEVEL

Dissertação apresentada ao Programa de Pós-Graduação em Zootecnia, Setor de Ciências Agrárias, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Mestre em Zootecnia.

Orientador(a): Dra. Maity Zopollatto

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“Our greatest weakness lies in giving up. The most certain way to succeed is  
always to try just one more time”.

(THOMAS EDISON)

## RESUMO

A mastite é uma doença que está presente em todas as propriedades leiteiras, e que está associada a diversas perdas na cadeia leiteira que se iniciam nas propriedades e seguem até as indústrias. Esse trabalho teve como objetivo entender fatores de risco relacionados ao animal e ao manejo, que estão associados ao aumento da contagem de células somáticas (CCS) em amostras individuais de vacas e conseqüentemente ao maior risco de desenvolvimento de mastite subclínica. Para isso, foi utilizado o banco de dados da Associação Paranaense de Criadores de Bovinos da Raça Holandesa (APCBRH) com dados individuais de CCS, bem como produção de leite diária, número de partos, dias em lactação e frequência de ordenha, para assim avaliar como essas variáveis estão relacionadas com o aumento da contagem de células somáticas. Foram utilizados dois modelos lineares generalizados, um objetivando estudar o efeito das variáveis sobre a CCS individual das vacas, e outro com o intuito de estudar a probabilidade de uma vaca apresentar ou não mastite subclínica. As vacas foram caracterizadas como sadias quando apresentaram  $CCS \leq 200.000$  céls/mL. Os resultados encontrados mostram que o aumento do número de lactações, está associado com o aumento da CCS individual e maiores probabilidades da vaca apresentar mastite subclínica. Uma vaca com quatro partos ou mais apresenta o dobro de células somáticas em relação a uma vaca primípara, quando o DEL é menor do que 105 dias ( $103.320 \pm 2.451$  vs  $51.820 \pm 1.186$  céls/mL), e 3,757 (95% IC 3,616-3,902) mais chances de apresentar mastite subclínica. Padrão semelhante foi observado com a diminuição da produção de leite, porém não foi observada diferença estatística na interação com frequência de ordenha quando a produção de leite foi menor que 30 litros/dia. De maneira geral, o aumento dos dias em lactação também foi prejudicial em relação à CCS, porém ao analisar a interação entre dias em lactação e frequência de ordenha, foi observado que até 205 dias em lactação o aumento da frequência de ordenha não altera os valores de CCS. Por fim, foram observados menores valores de CCS com o aumento da frequência de ordenha de 2x para 3x ao dia. Vacas secundíparas apresentaram CCS levemente menores quando ordenhadas 3x ao dia em comparação a vacas ordenhadas 2x ( $85.330 \pm 1.961$  vs  $87.290 \pm 1.980$  céls/mL), bem como 22% menos chances de apresentar mastite subclínica. O estudo nos permitiu concluir que todas as variáveis estudadas (dias em lactação, frequência de ordenha, número de lactações e produção de leite) afetam a CCS individual das vacas e a chance de apresentarem mastite subclínica. De uma forma geral, o aumento do número de lactações e dias em lactação elevam a CCS, enquanto o aumento da frequência de ordenha e da produção de leite reduzem a CCS.

Palavras-chave: Dias em lactação. Frequência de ordenha. Mastite subclínica. Número de lactações. Produção de leite. Qualidade do leite.

## ABSTRACT

Mastitis is a multifactorial disease that is present in all dairy farms and is associated with many losses in dairy chain that initiate at the farms and follow to the industries. This study had the objective of understanding risk factors related to cow-level and management that are associated with the increase of milk somatic cells count (SCC) in individual cow samples, and consequently higher risk of having subclinical mastitis. For this, data base from Holstein Breeders Association of Paraná (APCBRH) with individual SCC, daily milk yield, parity, days in milk and milking frequency, was used to evaluate how these factors are related with the increase of somatic cells count in milk. Two generalized linear models were used, one with the objective of studying how these variables are related to the increase of cow's individual SCC and another to study the probability of a cow having or not subclinical mastitis. Cows were classified as healthy if  $SCC \leq 200.000$  cells/mL. The results indicate that increasing parity number is related with higher SCC in milk and higher odds of a cow presenting subclinical mastitis. A cow with four or more calvings have the of double somatic cells count compared to a primiparous cow, when DIM is lower than 105 days ( $103,320 \pm 2,451$  vs  $51,820 \pm 1,186$  cells/mL), and 3.757 (95% CI 3.616-3.902) higher odds of having subclinical mastitis. The same pattern was observed as milk yield decreases, however it was not observed statistical difference in the interaction of milking frequency and milk production when milk yield was lower than 30 liters/day. In general, increasing days in milk was also negative for SCC, but when analyzing the interaction between days in milk and milking frequency, it was observed that until 205 days, the increase in milk frequency did not affect SCC. Lastly, it was observed lower SCC when increasing milking frequency from 2x to 3x per day. Secundiparous cows presented slightly lower SCC when milked 3x per day compared with cows milked 2x ( $85,330 \pm 1,961$  vs  $87,290 \pm 1,980$ ), as well as 22% lower odds of having subclinical mastitis. The study allowed us to conclude that all variables studied (days in milk, milk yield, milking frequency and parity) affect individual cow's SCC and the odds of having subclinical mastitis. In general, increasing parity and DIM elevates SCC, while increasing milking frequency and milk production decreases SCC.

Keywords: Days in milk. Milk quality. Milk yield. Milking frequency. Parity. Subclinical mastitis.

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## ABBREVIATION LIST

AMS	- Automatic milking system
APCBRH	- Holstein Breeders Association of Paraná
BTSCC	- Bulk tank somatic cells count
CM	- Clinical mastitis
CMT	- California mastitis test
CNS	- coagulase-negative staphylococci
DHI	- Dairy herd improvement programs
DIM	- Days in milk
IMI	- intramammary infection
LnSCC	- Log-transformed somatic cells count
MF	- Milking frequency
ML	- Milk losses
MY	- Milk yield
OCC	- Online cell counter
P	- Parity
SCC	- Somatic cells count
SCM	- Subclinical mastitis
TBC	- Total bacterial count
THK	- Teat end hyperkeratosis
WMT	- Wisconsin mastitis test

## SUMMARY

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## 1 INTRODUCTION

Mastitis is an inflammatory response of the mammary gland, mainly from bacterial infection (WATTS, 1988). Mastitis, in both its clinical (CM) and subclinical (SCM) state, is characterized by an increase in milk somatic cells count (SCC) (BOBBO et al., 2017). Somatic cells count has been widely used as a tool to monitor mammary gland health status, since it is a sensitive biomarker of SCM (PAAPE et al., 2002). Although there is a strong interdependence between intramammary infection (IMI) and SCC (BARKEMA et al., 1997), high SCC can only suggest an inflammatory process, not necessarily the presence of a pathogen (ZECCONI et al., 2020). Identifying SCM can be difficult, since there are no clinical symptoms, such as visible changes in the milk or udder. Subclinical mastitis can be characterized by SCC > 200,000 cells/mL (DOHOO; LESLIE, 1991).

It is well established that mastitis is responsible for many adverse economic effects for the dairy industry. A higher milk SCC is associated with lower contents of casein (HARMON, 1994; MAZAL et al., 2007; BOBBO et al., 2017) and lactose (WIELGOSZ-GROTH et al, 2003; BARŁOWSKA et al, 2009; BOBBO et al., 2017) and lower milk yield (BARŁOWSKA et al, 2009; HAND et al., 2012; GONÇALVES et al., 2018). Shelf life of products is also affected by higher SCC (BARBANO et al., 2006).

Likewise, mastitis imposes considerable economic losses at herd-level. Intramammary infections not only reduces milk yield, but also increase costs with culling, drugs, veterinary services and discarded milk (AGHAMOHAMMADI et al., 2018). Culling not only depends on individual factors (age, lactation stage, performance), but also on herd factors such as availability of replacement and milk and meat market (BEAUDEAU et al., 2000).

To define milk prices and fulfill legislation obligations, bulk tank analysis is done by the dairy industry. In Brazil, there is an obligation for at least a monthly analysis including milk composition (fat, protein, lactose, total solids, and non-fat total solids), SCC and total bacterial count (BRASIL, 2018). Bulk tank somatic cell count (BTSCC) is effective to monitor IMI at the herd level, but measurement of SCC at the cow-level is necessary to estimate the prevalence and incidence of subclinical mastitis (RUEGG; PANTOJA, 2013). Dairy herd improvement (DHI) programs are commonly used for udder health monitoring using SCC measurements (AGHAMOHAMMADI et al., 2018)

Increasing concern of lowering mastitis and consequently somatic cells count index is noted all over the world. Understanding risk factors related to increases in SCC is one of the key points to control mastitis and improve dairy welfare. The aim of this study was to evaluate the relationship between SCC and some cow-level and management factors such as milk yield (MY), days in milk (DIM), parity (P) and milking frequency (MF), in Holstein breed.

## 1.1 JUSTIFICATIVE

It is well established that mastitis and milk SCC is a big concern for dairy farms and industry. Understanding how somatic cells count risk factors are associated with management and cow characteristics is essential for decision-making at dairy farms.

## 1.2 OBJECTIVE

### 1.2.1 General objective

Analyze DHI program results of SCC at cow-level from Holstein Breeders Association of Paraná (APCBRH).

### 1.2.2 Specific objectives

- Evaluate the effect of milking frequency over individual somatic cells count;
- Evaluate the effect of parity over individual somatic cells count;
- Evaluate the effect of days in milk over individual somatic cells count;
- Evaluate the effect of milk yield over individual somatic cells count;
- Evaluate the odds of a Holstein cow having subclinical mastitis from risk factors mentioned above.

## 2 LITERATURE REVIEW

### 2.1 MASTITIS

Mastitis is the inflammation of the mammary gland caused by pathogenic microorganisms, potentially bacteria and less frequently molds, yeasts, or algae (BRANDT et al., 2010). More than 130 microorganisms isolated from the bovine mammary gland can be related to the development of IMI, mostly bacteria (WATTS, 1988).

The classification of mastitis is done by the presence or not of symptoms. Clinical mastitis is characterized by the presence of visible alteration in milk (such as flakes, clots and possibly blood), pain in the udder, swelling and redness (BRANDT et al., 2010). Systemic signs may follow, such as anorexia, lethargy and elevated rectal temperature (HARMON, 1994). Pinzón-Sánchez and Ruegg (2011) defined a scoring system for clinical cases according to the severity of the symptoms: mild (abnormal milk), moderate (abnormal milk followed by swelling or redness of the mammary gland) and severe (systemic signs of illness). Oliveira et al. (2013), after studying 800 cases of CM, showed that 50% of clinical cases presented only abnormal milk, while 35% presented abnormal milk and swelling of the affected quarter and 15% presented systemic symptoms.

Subclinical mastitis does not show any visible symptoms, however in both ways that the mastitis is manifested, there is an increase of somatic cell count in milk (BOBBO et al., 2017). Diagnosis of SCM can be made by measurement of somatic cells count by flow cytometry, electrical conductivity, California mastitis test (CMT, WHITESIDE, 1939), online cell counter (OCC, DALEN et al., 2019) and Wisconsin mastitis test (WMT, THOMPSON; POSTLE, 1964).

The mastitis-causing pathogens are separated according to the main transmission and reservoir. For environmental pathogens, as the name suggests, the main contamination source is the cow environment, primarily manure, but also includes bedding materials, soil and organic matter. The infection normally occurs between milkings (HOGAN; SMITH, 2003). Several studies have shown that environmental pathogens are the most prevalent causing clinical mastitis in dairy cows (LAGO et al., 2011; PINZÓN-SÁNCHEZ; RUEGG, 2011; SCHUKKEN et al., 2011).

Most of environmental pathogens are gram-negative bacteria, and they are the most often isolated from acute clinical cases of mastitis. Some are classified as coliforms, as *Escherichia* spp., *Klebsiella* spp. and *Enterobacter* spp. and others are gram-negative bacteria too, but incorrectly classified as coliforms, as *Serratia* spp., *Pseudomonas* spp. and *Proteus* spp. (HOGAN; SMITH, 2003; HOGAN; SMITH, 2012). *Streptococcus* spp. is a very important group of environmental pathogens as well (except *Streptococcus agalactiae*) and covers streptococci (*S. uberis* and *S. dysgalactiae*), enterococci (*Enterococcus faecium* and *E. faecalis*) and lactococci (*Lactococcus lactis* and *L. garvieae*) (KLAAS; ZADOKS, 2017).

The contagious pathogens are those adapted to survive within the mammary gland (BRADLEY, 2002). Those pathogens are spread from cow to cow, or between quarters of the same animal during milking (e.g., by contaminated milking equipment and milkers' hands) (CONTRERAS et al., 2011). Many mastitis pathogens have the biofilm-producing ability, which means they can adhere to different surfaces of the dairy environment, essential for dissemination (LEE et al., 2014). The most important are *Staphylococcus aureus*, *Streptococcus agalactiae* and *Mycoplasma* spp.

*Staphylococcus* gender species are divided into coagulase-positive staphylococci and coagulase-negative staphylococci (CNS), according to their ability to coagulate plasma. (PYÖRÄLÄ; TAPONEN, 2009). The CNS are opportunistic pathogens that are part of the normal flora of the teat skin (ENGER et al., 2015). According to Pyörälä and Taponen (2009), CNS are emerging mastitis pathogens, since they have become the predominant pathogen isolated from subclinical bovine mastitis in many countries. *Staphylococcus simulans* (43.6%), followed by *S. chromogenes* (23.3%), are the most common CNS species, and infections are normally subclinical or clinical with mild signs. On a research conducted by Persson et al. (2011), the most common bacteria isolated from subclinical mastitis were *S. aureus* (31%), CNS (27%), *S. dysgalactiae* (15%), *S. uberis* (14%), *E. coli* (4.8%) and *Streptococcus. spp.* (3.1%).

The importance of *S. agalactiae* tends to be minimized since it is normally found on farms in a non-clinical stage, causing severe-clinical mastitis less often than some other mastitis pathogens (MURPHY, 1956). In many countries with developed dairy industry, farms have become free of *S. agalactiae* (SAMPIMON et al., 2009). The implementation of mastitis control decreased the prevalence of contagious pathogens

and increased the importance of environmental pathogens (BRADLEY; GREEN, 2004).

## 2.2 THE ROLE OF SOMATIC CELLS

The somatic cell count (SCC) is an indicator of milk quality and health of the mammary gland. Somatic cells are normally present in milk and include macrophages, neutrophils, lymphocytes, and a smaller percentage of epithelial cells. In healthy lactating mammary gland total SCC is often < 100,000/mL of milk (SORDILLO et al., 1997; HAMANN, 2005), however the most practical value to reduce diagnostic error is < 200.000 cells/mL (DOHOO; LESLIE, 1991; DJABRI et al., 2002; PANTOJA et al., 2009).

Those cells are mainly macrophages (66-88%), while neutrophils are in a smaller proportion (1-11%), but they increase up to 90% in a quarter with IMI (PYÖRÄLÄ, 2003). There is a strong interdependence between intramammary infections and somatic cells count (BARKEMA et al., 1997). When intramammary infections occur, SCC can increase very rapidly, however high numbers can only suggest an inflammatory process, but not the presence of a pathogen (ZECCONI et al., 2020). Some other reasons for fluctuations in SCC include systemic disease, udder trauma, lactation stage, parity and seasonal variation (NØRSTEBØ et al., 2019). Furthermore, intramammary secretions (colostrum or milk) harbor diverse bacterial communities, regardless of the inflammatory status of the udder (DERAKHSHANI et al., 2018).

Bulk tank somatic cells count (BTSCC) values are used to define national and international standards of milk quality. Those limits are 400,000 cells/mL in the European Union (EU), Australia, New Zealand, and Canada (USDA, 2019) and 500,000 cells/mL in Brazil (BRASIL, 2018). The frequency that BTSCC analysis is done vary between dairy industries, and many of them use it to define milk prices, while others only to fulfill legislation obligations. In Brazil, bulk tank analysis of milk composition, SCC and total bacterial count (TBC) must be done at least once a month (BRASIL, 2018).

The BTSCC is used to monitor IMI in herd level, but measurement of SCC at the cow-level is necessary to estimate the prevalence and incidence of subclinical mastitis (RUEGG; PANTOJA, 2013). Although SCC are usually measured in

composite cow milk, the most accurate relationship between IMI and SCC exists at quarter level (SCHUKKEN et al., 2003). Clinical mastitis should not contribute to the increase of BTSCC, once Brazilian legislation require that abnormal milk must be discarded (BRASIL, 2018). In order to avoid adding abnormal milk to the bulk tank, foremilk must be observed before attaching the milking cluster.

### 2.3 THE IMMUNE SYSTEM

Mastitis develops in three phases, (1) invasion of an organism (with or without the establishment of infection), (2) infection (establishment of infection) and (3) inflammation (MURPHY, 1947). The development of IMI is determined by the efficiency of the bovine defense mechanism and the level of exposure to pathogens (HAMANN, 1991).

The efficiency of the mammary gland immune system reflects the ability of cows to resist the establishment of a new IMI. Mammary gland immunity uses a multifaceted network of physical, cellular and soluble factors. The immune system is responsible for preventing bacterial invasion of the mammary gland, to eliminate existing infections and to restore normal tissue function (SORDILLO, 2018).

The microorganism entry trigger leukocyte migratory response and bactericidal activity of the somatic cells at the site of infection. If this first response is effective, leukocyte recruitment stops and somatic cells return to healthful levels. If bacteria survives, the migration continues, resulting in the damage of mammary parenchyma tissue (SORDILLO et al., 1997).

The bovine defense mechanisms are separated in two different categories; (1) innate immunity (nonspecific responsiveness) mediated by the physical barrier of the teat end, macrophages, neutrophil, natural killer cells and certain soluble factors (lactoferrin, complement); and (2) specific immunity mediated by macrophages, antibody molecules and lymphoid population (SORDILLO et al., 1997).

The predominant defense during the early stages of the mammary gland infection is the innate immunity, which is present and is activated very fast at the site of infection by many stimuli (SORDILLO et al., 1997). This response is not specific and not long lasting (SORDILLO, 2018). On the other hand, the specific immunity can recognize pathogens by repeated exposure, that facilitates selective elimination

(SORDILLO et al., 1997). Both innate and specific immunity work together to optimize immune responses.

Immune and nonimmune cells present on the mammary gland are capable of sense pathogens thru pattern recognition receptors, as Toll-like receptors, which are transmembrane proteins expressed in leukocyte populations, endothelial cells, epithelial cells and fibroblasts (SORDILLO, 2018).

The inflammatory process is a normal response of the host immune system, and its purpose is to eliminate the source that is injuring the tissue, restore the immune homeostasis and return the tissue to its normal function (AITKEN et al., 2011). Many changes occur in the udder; (1) leaking of ions, proteins and enzymes from the blood into the milk, due to increased permeability; (2) invasion of phagocytosing cells into the milk compartment.; and (3) a decrease of the synthetic capacity of the gland, resulting in decreased concentrations of certain milk constituents (PYÖRÄLÄ, 2003).

## 2.4 PARITY

There is a higher incidence of clinical mastitis on initial months of lactation, and primiparous cows are less likely to develop CM when compared to animals on the third lactation and above (BREEN et al., 2009). The highest proportion of cows with SCC > 200,000 cells/mL is in the first lactation month, and it is more prominent for primiparous cows (FROSSLING et al., 2017). Cows that recorded an SCC >199,000 cells/mL and a milk protein percentage <3.2 at  $\leq 30$  DIM are at greater risk of developing CM after the first month of lactation, either because they are in the early stages of an active infection or because a subclinical infection may become clinical again (BREEN et al., 2009).

Older cows more often have elevated SCC when compared to younger cows and a lower proportion of older animals recover during the dry period (FROSSLING et al., 2017). Many studies have shown that increasing parity is associated with a lower chance of curing *S. aureus* mastitis (ZIV; STORPER, 1985; PYÖRÄLÄ; PYÖRÄLÄ, 1998; TAPONEN et al., 2003). Pyörälä and Pyörälä (1998) report 57% cure of heifers with clinical *S. aureus* mastitis and 27% cure of older animals when treated with penicillin G. According to Barkema et al. (2006), the bigger size of the mammary gland from older animals may contribute to reduced chances of mastitis cure.

Teat end hyperkeratosis (THK) is the thickening of the skin around the teat orifice, as a result of the cells hyperplasia. Many studies showed a significant association between SCC and THK, but in some studies, this link was not found. Pantoja et al. (2020), on a systematic review, showed that severe THK is a risk factor for both contagious and environmental CM and SCM, while mild THK is not, suggesting that the development of less severe cases of THK is a physiological response of the animal organism to milkings. Advancing parity number increases the incidence of THK scores 3 and 4, and higher SCC in milk (ASADPOUR et al., 2015).

## 2.5 MILK PRODUCTION

Clinical and subclinical mastitis have different effects on milk production, as well as the mastitis-causing organisms. Heikkilä et al. (2018) found that the extent of reduction on milk yield depends on the causal agent, lactation stage and type of disease (CM or SCM). Milk losses (ML) are larger when mastitis occurs in the beginning of lactation. Losses vary between 5.7% (1.8 kg/d) and 10.6% (3.4 kg/d) in early lactation, while diagnosis made after peak vary between 3.2% (1.0 kg/d) and 4.4% (1.4 kg/d).

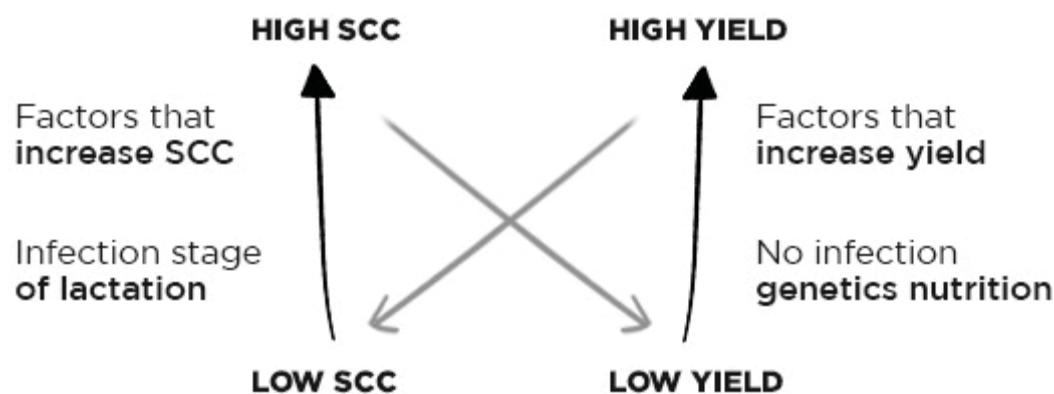
*Staphylococcus aureus* is one of the most important pathogens, and its prevalence worldwide ranges from 5 to 18%, while its prevalence in the herd is even higher, 30-85% (CONTRERAS; RODRÍGUEZ, 2011). *Staphylococcus aureus* infections are mainly subclinical (72%), however milk yield losses are very similar regardless of mastitis type. If the disease occurs before 120 DIM, losses of 7.1% (2.3 kg/d) on a 305-d lactation are observed. If it occurs after lactation peak, there is a decrease of 4.3% (1.4 kg/d) (HEIKKILÄ et al., 2018).

Gonçalves et al. (2018) evaluated milk losses due to SCM according to parity and lactation stage. Log-transformed somatic cells count (LnSCC) started affecting milk yield around 2.52 (approximately 12,400 cells/mL), much lower than what is normally defined as a healthy mammary gland. Primiparous cows have lower ML when compared to older cows. On first-lactation cows ML per unit increase of LnSCC varied between 0.68 kg/d in the beginning of the lactation, to 0.55 kg/d in mid-lactation, and 0.97 kg/d at the end of the lactation. For second-lactation cows, losses were 1.47 kg/d, 1.09 kg/d and 2.45 kg/d, respectively. For third lactation cows, milk losses per unit increase of LnSCC were 2.22 kg/d, 1.13 kg/d and 2.65 kg/d, respectively.

The relationship between 24-hour milk losses due to mastitis at cow level in Holstein cows was studied by Hand et al. (2012). The results showed that milk losses are greater as lactation average SCC increases. Primiparous cows with average 200.000 cells/mL in the first milk production quartile had losses of 0.35 kg, while cows with 500.000 cells/mL and 800.000 cells/mL had daily losses of 0.80 and 1.04 kg, respectively. These losses were also greater as the milk production quartile increases. Secundiparous cows with 200.000 cells/mL had increasing losses as milk production increases, and these losses varied from 0.61 kg to 1.07 kg. Parity was also related to higher losses, at average of 200.000 cells/mL, where primiparous cows had losses that varied between 0.35 to 0.80 kg, while for secundiparous losses were from 0.61 to 1.07, and for multiparous cows varied from 0.63 to 1.09 kg.

As MY increases, there is a dilution effect of milk somatic cells. According to Green et al. (2006), the effect of dilution is greater in lower SCC categories and there is a circularity between milk volume, SCC and intramammary status, which are illustrated in Figure 1. In the absence of an IMI, MY increases and SCC may be diluted.

FIGURE 1 - CIRCULAR ASSOCIATION BETWEEN SOMATIC CELLS COUNT AND MILK YIELD



SOURCE: Adapted from GREEN et al.(2006).

## 2.6 MILKING FREQUENCY AND DAYS IN MILK

The milking frequency is a management choice of the farm, although most cows are milked two times daily (2X). In high-producing herds, it is common for cows to be milked more frequently (STELWAGEN et al., 2013), once that increasing MF also increases milk production. According to Flint and Knight (1997), this increase in milk production is the result of combined effect of GH (growth hormone) that stimulates IGF-

1 production, acting as a survival factor for the mammary gland and prolactin, inhibiting local production of IGFBP-5, which inhibits IGF-1 and consequently lead to cell death.

According to Poole (1982), three times daily milking during can increase in 8,5% lactation yield for heifers (4498 vs 4881kg) and 13% for cows (5694 vs 6485 kg). Smith et al. (2002) also reported that herds milked 3x per day had an increase of 15-16% compared to those milked twice, during 3 years of study (8,853-9,013 kg vs 10,086-10,386 kg). They also noticed a difference on milk composition, where three times daily milking herds had lower fat (3.64 vs 3.57%), protein (3.22 vs 3.19%) and bulk tank somatic cells count (352,000-368,000 vs 331,000-341,000 cells/mL). Österman et al. (2005) also observed a decrease in milk SCC when comparing cows milked 2x per day and 3x per day in 12 months calving interval (74,131 vs 43,652 cells/mL).

Many studies have shown that as the lactation progress to the end, there is an increase in milk SCC. Berglund et al. (2002) studied two milking systems, conventional parlour with 2x milking per day and an automatic milking system (AMS). They divided the lactation in three periods, period I (5–16 week), period II, (week 17–23) and period III (week 24–29). In the AMS, no significant difference was observed in SCC from cow's quarter strip during period I and II, however difference was significant in period III (46,000 vs 49,000 vs 61,000 cells/mL). In the conventional parlour the difference was significant in all periods (56,000 vs 65,000 vs 126,000 cells/mL). When comparing both conventional parlour and AMS, differences were observed in period II and III.

Sebastino et al. (2020) evaluated three categories of days in milk: 6 to 100, 101 to 200, and 201 to 365 days. They observed that SCC increased as DIM progressed, an increase of 16% in the second third of lactation (75,013 vs 87,128 cells/mL) and 18% (102,613 cells/mL) in the last stage. Few clinical cases of mastitis are observed in late lactation, so the gradual increase of SCC across the lactation course is related to declining epithelial integrity and not to disease (SORENSEN et al., 2008).

### 3 MATERIAL AND METHODS

#### 3.1 DATA EDITION

Test-day records were obtained from lactating cows registered in the DHI program from APCBRH. The original data set included information of individual cow SCC, milk composition – including fat, protein, total solids, and lactose -, breed, daily milk yield, days in milk, number of lactations and daily milking times. The original data set is from lactating cows from January 2016 to December 2019 and comprised 1,048,575 test-day records from 69,035 cows and 812 herds. Editing was necessary to ensure reliability and consistency of the statistical analysis.

The first editing criteria was the breed, where only Holstein cows were studied in this research. A similar methodology, as described by Dürr et al. (2008) and Gonçalves et al. (2018), was used to edit milk composition, in which test-day records were required to have fat content between 2.5 and 7.5%, protein content between 2.5 and 5.5%, lactose content between 3.5 and 5.5%, TS content between 9.0 and 16%, SCC between 0 and 1,000,000 cells/mL, and milk yield between 2 and 70 kg. The objective of this study was not to evaluate milk composition, however it is a necessary editing criteria to avoid sampling mistakes. Test-day recordings <5 d and  $\geq 450$  d in milk were excluded from analysis. Parity was also restricted, and cows >8 lactations were not included. Each herd and cow needed at least 30 and 3 test-day records, respectively. Daily milking frequency was not limited, and cows milked two and three times a day were studied.

After editing, the final data set was submitted to statistical analysis comprised 694,704 test-day records from 49,089 Holstein cows and 476 herds. The number of test-day records excluded and retained at each step of the editing and the reasons for the exclusions are shown in Table 1.

TABLE 1 - EDITING CRITERIA AND NUMBER OF TEST-DAY RECORDS, COWS AND HERDS RETAINED AND EXCLUDED AT EACH STEP.

Excluded test-day records based on the following criteria	Initial number of test-day records at each edit step	Initial number of cows at each edit step	Initial number of herds at each edit step	Excluded test-day records at each edit step	Excluded cows at each edit step	Excluded herds at each edit step
-	1,048,575	69,035	812	-	-	-
Breed	1,009,792	65,976	796	38,783	3,059	16
Milk composition	881,752	64,787	789	128,04	1,189	7
Somatic cells count	814,199	63,555	785	67,553	1,232	4
Milk Yield	813,271	63,458	784	928	97	1
Days in Milk	763,472	62,316	777	49,799	1,142	7
Parity > 8	762,426	62,239	775	1,046	77	2
<30 analyses/ herd	759,577	61,152	514	2,849	1,087	261
<3 analyses/cow	747,52	52,954	498	12,057	8,198	16
Holstein final data	694,704	49,089	476	-	-	-

SOURCE: The author (2021).

### 3.2 STATISTICAL ANALYSIS

Initially, descriptive measures were calculated for the variables present in the database for Holstein breed. These measures were minimum and maximum values, quartiles and average. Such measures were calculated for the variables: parity, days in lactation, milk production, fat content, protein, lactose and total solids in milk, in addition to the somatic cell count.

To evaluate how somatic cells count is influenced by the intrinsic characteristics of the cow and management, it was necessary to separate the response variables in groups. Percentiles were used to categorize data on milk yield and days in milk, forming categories in each case and for both breeds. Days in milk were separated in four categories ( $DIM \leq 105$  d,  $105 < DIM \leq 205$  d,  $205 < DIM \leq 305$  d and  $DIM > 305$  d) and milk yield in four categories ( $MY < 20$  L,  $20 \leq MY < 30$  L,  $30 \leq MY < 40$  L and  $MY \geq 40$  L). Cows with four or more lactations were grouped together.

After exclusions and verifications were made regarding the biological validity of the data, generalized linear models were developed aiming to study the effects of number of milking, number of lactations, days in lactation and milk production on the individual SCC of cows. In these models, the variables of milking frequency, number

of lactations, days in milk, milk yield and their first order interactions were considered as fixed effects, whereas the year in which the milk analysis was performed was considered as a random effect, in addition to the random effect of repeated measures of the animal's effect within the herd.

Several variance-covariance matrices were tested in the modeling and the one that resulted in the best fit was the one maintained for the final model based on the Akaike and Bayesian information criteria (WOLFINGEER, 1993). In addition, the lognormal distribution was used to adjust the model due to the fact that SCC is a measure that presents a deviation to the left (mean>median). The standard errors and the mean were later re-transformed to the original scale of the variable. When the interaction effect resulted in significance, the unfolding of the interaction was performed through comparisons paired by the t test. Histograms were evaluated to verify the normality of the residues for these models.

Somatic cells count was categorized considering animals with subclinical mastitis or not, by using the limit value of 200,000 cells/mL (above represents animals with subclinical mastitis) (DOHOO; LESLIE, 1991). Again, generalized linear models (in this case logistic regression) were adjusted to the data for both breeds to study the probability of a cow presenting or not subclinical mastitis. These models were identical to the models described above in relation to the fixed effects studied, however, the random effects considered were only the year and the herd to which the animals belonged, since a cow effect within the herd resulted in non-convergence of the models. In addition, the binomial distribution was used to fit the model to the data. The probabilities were calculated from the probability of occurring an event (in this case the cow always has subclinical mastitis) depending on the category to which it belongs compared to a reference category. Again, when interactions showed significance, the unfolding of the interaction was performed using the odds ratio of the event to happen in a category compared to the reference category.

All statistical analysis were performed using the statistical software SAS OnDemand (2012). Descriptive analysis were performed using PROC MEANS, while verification of normality, histograms and percentiles were performed using PROC UNIVARIATE. Generalized linear models to assess the difference in SCC values and also the logistic regression models were conducted using PROC GLIMMIX. The results were considered significant at the 0.05 (5%) probability level.

## 4 RESULTS

### 4.1 DESCRIPTIVE RESULTS

In total, 694,704 test-day records from 49,089 cows were included in the study. The mean composition of raw milk was 3.72% of fat, 3.26% of protein, 4.47% of lactose, and 12.59% of total solids. The SCC had an average of 145,000 cells/mL and a median of 70,000 cells/mL. The mean number of lactations was equivalent to 1.2, DIM was 183 days and milk production was 33.07 L/day (TABLE 2).

TABLE 2 - DESCRIPTIVE ANALYSIS OF HOLSTEIN COWS TEST-DAY RECORDS.

Variables	Minimum	Lower Quartile	Mean	Median	Upper Quartile	Maximum
Parity	1	1	1.17	2	3	8
Days in milk (d)	5	88	183	175	265	450
Milk yield (L/d)	2.00	25.51	33.07	32.50	40.01	70.00
Fat (g/100g)	2.50	3.20	3.72	3.63	4.13	7.50
Protein (g/100g)	2.50	3.01	3.26	3.23	3.47	5.50
Lactose (g/100g)	3.50	4.52	4.47	4.68	4.81	5.50
Total solids (g/100g)	9.50	11.91	12.59	12.50	13.17	16.00
Somatic cells count ( $\times 10^3$ cells/mL)	1	28	145	70	177	1,000

SOURCE: The author (2021).

### 4.2 COW-LEVEL AND MANAGEMENT ON SOMATIC CELLS COUNT

The results indicated significant interactions for SCC between milking frequency and parity ( $P < 0.0001$ ), milking frequency and days in milk ( $P < 0.0001$ ), milking frequency and milk yield ( $P < 0.0001$ ), parity and days in milk ( $P < 0.0001$ ), parity and milk yield ( $P < 0.0001$ ) and days in milk and milk yield ( $P < 0.0001$ ). Adjusted SCC estimates for each category are presented in Table 3.

TABLE 3 - ADJUSTED SCC ESTIMATES FOR MILKING FREQUENCY, PARITY, DAYS IN MILK AND MILK YIELD CATEGORIES.

Variables	Ajusted values ( $\times 10^3$ cells/mL)	Standard error ( $\times 10^3$ cells/mL)	P-value
<b>Milking frequency</b>			<0.0001
2x	98.43	2.213	
3x	93.61	2.120	
<b>Parity</b>			<0.0001
Primiparous	60.57	1.366	
Secundiparous	86.31	1.953	
3 multiparous	114.81	2.614	
$\geq 4$ multiparous	141.47	3.225	
<b>Days in milk</b>			<0.0001
DIM $\leq 105$ d	70.57	1.604	
105 d > DIM $\geq 205$ d	84.77	1.928	
205 d > DIM $\geq 305$ d	107.93	2.443	
DIM > 305 d	131.51	2.997	
<b>Milk yield</b>			<0.0001
MY < 20 L	160.73	3.754	
20 L $\geq$ MY > 30 L	107.75	2.438	
30 L $\geq$ MY > 40 L	79.73	1.796	
MY $\geq 40$ L	61.49	1.397	
Milking frequency $\times$ Parity			<0.0001
Milking frequency $\times$ Days in milk			<0.0001
Milking frequency $\times$ Milk yield			<0.0001
Parity $\times$ Days in milk			<0.0001
Parity $\times$ Milk yield			<0.0001
Days in milk $\times$ Milk yield			<0.0001

SOURCE: The author (2021).

Somatic cells count estimates for the interaction of milking frequency and parity are presented in Table 4.

TABLE 4 - DIFFERENCES OF PARITY AND MILKING FREQUENCY ON SCC ( $\times 10^3$  CELLS/ML)

Parity	Milking frequency	
	2x	3x
Primiparous	64.23 $\pm$ 1.455 a D	57.12 $\pm$ 1.305 b D
Secundiparous	87.29 $\pm$ 1.980 a C	85.33 $\pm$ 1.961 b C
3 multiparous	117.14 $\pm$ 2.680 a B	112.53 $\pm$ 2.623 b B
$\geq 4$ multiparous	142.92 $\pm$ 3.272 a A	140.04 $\pm$ 3.281 b A

SOURCE: The author (2021).

NOTE: Lower case letters indicate difference in rows and uppercase in columns

With the increase in the number of calvings, a gradual increase in SCC was observed for cows milked two and three times a day. The difference between primiparous and  $\geq 4$  multiparous that were milked two times daily was 78,690 cells/mL. The increase in milking frequency revealed to be positive, decreasing the SCC of the different categories of parity, and for primiparous cows this difference was of 7,110 cells/mL.

Somatic cells count estimates for the interaction of milking frequency and days in milk are presented in Table 5.

TABLE 5 - DIFFERENCES OF MILKING FREQUENCY AND DAYS IN MILK ON SCC (X10<sup>3</sup> CELLS/ML).

Days in milk	Milking frequency	
	2x	3x
DIM $\leq$ 105 d	70.93 $\pm$ 1.613 a D	70.21 $\pm$ 1.627 a D
105 d > DIM $\geq$ 205 d	88.47 $\pm$ 2.012 a C	81.23 $\pm$ 1.887 b C
205 d > DIM $\geq$ 305 d	111.84 $\pm$ 2.544 a B	104.17 $\pm$ 2.398 b B
DIM > 305 d	133.76 $\pm$ 3.090 a A	129.29 $\pm$ 2.998 b A

SOURCE: The author (2021).

NOTE: Lower case letters indicate difference in rows and uppercase in columns.

As days in milk increased, higher adjusted SCC values were observed for both milking frequencies studied. Increasing milking frequency led to lower SCC in most of DIM categories, however no significant difference was observed when DIM  $\leq$  105 d for 2 and 3 milkings per day.

Table 6 shows the somatic cells count estimates for the interaction of milking frequency and milk yield.

TABLE 6 - DIFFERENCES OF MILKING FREQUENCY AND MILK YIELD ON SCC (X10<sup>3</sup> CELLS/ML).

Milk Yield	Milking frequency	
	2x	3x
MY < 20 L	160.95 ± 3.717 a A	160.52 ± 4.053 a A
20 L ≥ MY > 30 L	107.88 ± 2.438 a B	107.62 ± 2.489 a B
30 L ≥ MY > 40 L	81.22 ± 1.838 a C	78.26 ± 1.780 b C
MY ≥ 40 L	66.57 ± 1.540 a D	56.81 ± 1.291 b D

SOURCE: The author (2021).

NOTE: Lower case letters indicate difference in rows and uppercase in columns.

As milk yield increased, a gradual decrease of individual SCC was observed for cows milked twice and three times per day. The SCC difference between cows milked three times per day, producing less than 20 L/d and producing more than 40 L/d was 103,710 cells/mL. Higher milking frequency decreased SCC for higher milk yield categories (> 30L/d), however no significant difference was observed for lower production categories.

Somatic cells count estimates for the interaction of parity and days in milk are presented in Table 7.

TABLE 7 - DIFFERENCES OF PARITY AND DAYS IN MILK ON SCC (X10<sup>3</sup> CELLS/ML).

Days in milk	Parity			
	Primiparous	Secundiparous	3 multiparous	≥ 4 multiparous
DIM ≤ 105 d	51.82 ± 1.186 d D	58.71 ± 1.363 c D	78.89 ± 1.869 b D	103.32 ± 2.451 a D
105 d > DIM ≥ 205 d	52.81 ± 1.210 d C	74.58 ± 1.729 c C	102.13 ± 2.414 b C	128.40 ± 3.043 a C
205 d > DIM ≥ 305 d	62.43 ± 1.428 d B	101.49 ± 2.339 c B	134.84 ± 3.165 b B	158.88 ± 3.752 a B
DIM > 305 d	78.78 ± 1.824 d A	124.89 ± 2.926 c A	159.92 ± 3.845 b A	190.08 ± 4.612 a A

SOURCE: The author (2021).

NOTE: Lower case letters indicate difference in rows and uppercase in columns.

Increasing parity had a negative effect on SCC for all DIM categories. It was observed that somatic cells count doubled between primiparous cows and cows with four or more calvings when DIM ≤ 105 d. Similar behavior was observed for the other DIM categories, however in higher proportions as DIM increased. A gradual increase in SCC for all parity categories was observed as DIM increased, with a smaller increase for primiparous cows.

Table 8 shows the somatic cells count estimates for the interaction of parity and milk yield.

TABLE 8 - DIFFERENCES OF PARITY AND MILK YIELD ON SCC (X10<sup>3</sup> CELLS/ML).

Milk Yield	Parity			
	Primiparous	Secundiparous	3 multiparous	≥ 4 multiparous
MY < 20 L	97.91 ± 2.354 d A	157.15 ± 3.866 c A	194.97 ± 5.004 b A	222.54 ± 5.718 a A
20 L ≥ MY > 30 L	62.25 ± 1.417 d B	97.23 ± 2.243 c B	134.30 ± 3.167 b B	165.85 ± 3.916 a B
30 L ≥ MY > 40 L	49.03 ± 1.110 d C	69.87 ± 1.596 c C	95.74 ± 2.222 b C	123.20 ± 2.874 a C
MY ≥ 40 L	45.04 ± 1.045 d D	51.98 ± 1.200 c D	69.32 ± 1.620 b D	88.10 ± 2.074 a D

SOURCE: The author (2021).

NOTE: Lower case letters indicate difference in rows and uppercase in columns.

Higher milk yield resulted in lower SCC for all parity categories studied. It was observed that the amount of SCC when milk yield was lower than 20 L/day was more than double when comparing with milk yields higher than 40 L/day. This pattern was observed for all parity categories. Increasing parity resulted in higher SCC for all milk yield categories. The difference between primiparous and ≥ 4 multiparous was lower as milk yield increased. The lower difference was 43,060 cells/mL when MY ≥ 40 L.

Somatic cells count estimates for the interaction of days in milk and milk yield are shown in Table 9.

TABLE 9 - DIFFERENCES OF DAYS IN MILK AND MILK YIELD ON SCC (X10<sup>3</sup> CELLS/ML).

Milk Yield	Days in milk			
	DIM ≤ 105 d	105 d > DIM ≥ 205 d	205 d > DIM ≥ 305 d	DIM > 305 d
MY < 20 L	118.14 ± 3.030 d A	141.08 ± 3.644 c A	179.29 ± 4.383 b A	223.39 ± 5.355 a A
20 L ≥ MY > 30 L	81.41 ± 1.901 d B	94.98 ± 2.212 c B	119.64 ± 2.749 b B	145.72 ± 3.367 a B
30 L ≥ MY > 40 L	59.08 ± 1.351 d C	71.17 ± 1.625 c C	89.08 ± 2.035 b C	107.86 ± 2.525 a C
MY ≥ 40 L	43.64 ± 0.992 d D	54.15 ± 1.237 c D	71.03 ± 1.662 b D	85.18 ± 2.171 a D

SOURCE: The author (2021).

NOTE: Lower case letters indicate difference in rows and uppercase in columns.

Higher somatic cells count values were found for all milk yield categories as DIM progressed. SCC almost doubled when comparing DIM ≤ 105 d and DIM > 305 d, for

milk yield lower than 20 L/day. Increasing milk production resulted in lower SCC in milk for all DIM categories.

#### 4.3 ODDS OF A HOLSTEIN COW HAVING SUBCLINICAL MASTITIS

Frequencies of test-day records and percentage of cows classified as healthy ( $\leq 200,000$  cells/mL) or unhealthy ( $> 200,000$  cells/mL) for all categories studied are presented in Table 10.

TABLE 10 - FREQUENCY OF TEST-DAY RECORDS AND PERCENTAGE OF HEALTH AND UNHEALTHY COWS.

Variables	$\leq 200 \times 10^3$ cells/mL	Healthy cows (%)	$> 200 \times 10^3$ cells/mL	Unhealthy cows (%)
<b>Milking frequency</b>				
2x	308,502	44.41	103,015	14.83
3x	232,141	33.42	51,046	7.35
<b>Parity</b>				
Primiparous	236,567	34.05	40,064	5.77
Secundiparous	154,634	22.26	41,321	5.95
3 multiparous	83,054	11.96	33,895	4.88
$\geq 4$ multiparous	66,388	9.56	38,781	5.58
<b>Days in milk</b>				
DIM $\leq 105$ d	174,513	25.12	33,568	4.83
105 d $>$ DIM $\geq 205$ d	160,148	23.05	38,326	5.52
205 d $>$ DIM $\geq 305$ d	134,18	19.31	44,939	6.47
DIM $> 305$ d	71,802	10.34	37,228	5.36
<b>Milk yield</b>				
MY $< 20$ L	33,351	4.8	28,094	4.12
20 L $\geq$ MY $> 30$ L	144,926	20.86	52,607	7.57
30 L $\geq$ MY $> 40$ L	197,542	28.44	44,753	6.44
MY $\geq 40$ L	164,824	23.73	28,607	4.12

SOURCE: The author (2021).

The results indicated significant interactions for odds of having subclinical mastitis between milking frequency and parity ( $P=0.0026$ ), milking frequency and days in milk ( $P=0.0185$ ), milking frequency and milk yield ( $P<0.0001$ ), parity and days in milk ( $P<0.0001$ ), parity and milk yield ( $P<0.0001$ ) and days in milk and milk yield ( $P<0.0001$ ) (TABLE 11).

TABLE 11 - ODDS OF A HOLSTEIN COW HAVING SUBCLINICAL MASTITIS

Variables		Odds ratio	95% CI	P-value
<b>Milking frequency</b>				<0.0001
2x	3x	1.238	1.194-1.284	
<b>Parity</b>				<0.0001
Primiparous	Secundiparous	0.559	0.549-0.569	
3 multiparous	Primiparous	2.936	2.877-2.997	
≥ 4 multiparous	Primiparous	4.261	4.172-4.351	
3 multiparous	Secundiparous	1.640	1.607-1.674	
≥ 4 multiparous	Secundiparous	2.380	2.331-2.430	
3 multiparous	≥ 4 multiparous	0.689	0.674-0.705	
<b>Days in milk</b>				<0.0001
DIM ≤ 105 d	105 d > DIM ≥ 205 d	0.785	0.767-0.802	
DIM ≤ 105 d	205 d > DIM ≥ 305 d	0.619	0.606-0.632	
DIM > 305 d	DIM ≤ 105 d	2.134	2.083-2.187	
105 d > DIM ≥ 205 d	205 d > DIM ≥ 305 d	0.789	0.773-0.805	
DIM > 305 d	105 d > DIM ≥ 205 d	1.674	1.635-1.715	
DIM > 305 d	205 d > DIM ≥ 305 d	1.321	1.292-1.351	
<b>Milk Yield</b>				<0.0001
20 L ≥ MY > 30 L	MY < 20 L	0.506	0.491-0.521	
30 L ≥ MY > 40 L	MY < 20 L	0.335	0.326-0.345	
MY < 20 L	MY ≥ 40 L	3.939	3.815-4.068	
20 L ≥ MY > 30 L	30 L ≥ MY > 40 L	1.508	1.481-1.536	
20 L ≥ MY > 30 L	MY ≥ 40 L	1.992	1.946-2.039	
30 L ≥ MY > 40 L	MY ≥ 40 L	1.321	1.293-1.350	
Milking frequency × Parity				0.0026
Milking frequency × Days in milk				0.0185
Milking frequency × Milk yield				<0.0001
Parity × Days in milk				<0.0001
Parity × Milk yield				<0.0001
Days in milk × Milk yield				<0.0001

SOURCE: The author (2021).

Table 12 shows the odds of a Holstein cow having subclinical mastitis for the interaction of milking frequency and parity.

TABLE 12 - ODDS OF A HOLSTEIN COW HAVING SUBCLINICAL MASTITIS FOR THE INTERACTION OF PARITY AND MILKING FREQUENCY.

<b>Variables</b>	Odds ratio	95% CI
<b>Parity</b>		
2x Primiparous x 3x Primiparous	1.296	1.241-1.352
2x Secundiparous x 3x Secundiparous	1.216	1.165-1.268
2x 3 multiparous x 3x 3 multiparous	1.227	1.172-1.283
2x $\geq 4$ multiparous x 3x $\geq 4$ multiparous	1.217	1.163-1.274
<b>Milking frequency (2x)</b>		
Primiparous x Secundiparous	0.577	0.564-0.590
3 multiparous x Primiparous	2.857	2.786-2.930
$\geq 4$ multiparous x Primiparous	4.129	4.026-4.236
3 multiparous x Secundiparous	1.648	1.609-1.688
$\geq 4$ multiparous x Secundiparous	2.382	2.325-2.440
3 multiparous x $\geq 4$ multiparous	0.692	0.674-0.710
<b>Milking frequency (3x)</b>		
Primiparous x Secundiparous	0.541	0.526-0.557
3 multiparous x Primiparous	3.017	2.922-3.116
$\geq 4$ multiparous x Primiparous	4.396	4.254-4.542
3 multiparous x Secundiparous	1.633	1.581-1.686
$\geq 4$ multiparous x Secundiparous	2.379	2.302-2.458
3 multiparous x $\geq 4$ multiparous	0.686	0.663-0.711

SOURCE: The author (2021).

Higher odds of having SCM were observed for all parity categories when milking frequency was lower (2x), resulting in more than 20% higher odds than cows milked three times per day. Increasing parity was also related to higher odds of having SCM for both milking frequencies studied. Primiparous cows have 46% lower odds of having SCM than secundiparous cows when milked three times per day.

The odds of a Holstein cow having subclinical mastitis for the interaction of milking frequency and days in milk are presented in Table 13.

TABLE 13 - ODDS OF A HOLSTEIN COW HAVING SUBCLINICAL MASTITIS FOR THE INTERACTION OF MILKING FREQUENCY AND DAYS IN MILK.

<b>Variables</b>	<b>Odds ratio</b>	<b>95% CI</b>
<b>Days in milk</b>		
2x DIM ≤ 105 d x 3x DIM ≤ 105 d	1.276	1.221-1.334
2x 105 d > DIM ≥ 205 d x 3x 105 d > DIM ≥ 205 d	1.205	1.153-1.259
2x 205 d > DIM ≥ 305 d x 3x 205 d > DIM ≥ 305 d	1.252	1.199-1.307
2x DIM > 305 d x 3x DIM > 305 d	1.221	1.167-1.278
<b>Milking frequency (2x)</b>		
DIM ≤ 105 d x 105 d > DIM ≥ 205 d	0.807	0.788-0.827
DIM ≤ 105 d x 205 d > DIM ≥ 305 d	0.625	0.610-0.640
DIM > 305 d x DIM ≤ 105 d	2.088	2.026-2.151
105 d > DIM ≥ 205 d x 205 d > DIM ≥ 305 d	0.774	0.756-0.793
DIM > 305 d x 105 d > DIM ≥ 205 d	1.685	1.637-1.736
DIM > 305 d x 205 d > DIM ≥ 305 d	1.305	1.268-1.342
<b>Milking frequency (3x)</b>		
DIM ≤ 105 d x 105 d > DIM ≥ 205 d	0.762	0.737-0.789
DIM ≤ 105 d x 205 d > DIM ≥ 305 d	0.613	0.594-0.634
DIM > 305 d x DIM ≤ 105 d	2.181	2.105-2.260
105 d > DIM ≥ 205 d x 205d > DIM ≥ 305 d	0.804	0.779-0.830
DIM > 305 d x 105 d > DIM ≥ 205 d	1.663	1.606-1.722
DIM > 305 d x 205 d > DIM ≥ 305 d	1.338	1.296-1.381

SOURCE: The author (2021).

Cows milked three times daily had 20% or more lower odds of having subclinical mastitis compared to those milked two times per day for all DIM categories. Increasing DIM was related to higher odds of having SCM for both milking frequencies studied. When DIM > 305 d, odds of having SCM are 30% higher than 205 d > DIM ≥ 305 d for cows milked three times per day.

Table 14 shows the odds of a Holstein cow having subclinical mastitis for the interaction of milking frequency and milk yield.

TABLE 14 - ODDS OF A HOLSTEIN COW HAVING SUBCLINICAL MASTITIS FOR THE INTERACTION OF MILKING FREQUENCY AND MILK YIELD.

<b>Variables</b>	<b>Odds ratio</b>	<b>95% CI</b>
<b>Milk Yield</b>		
2x MY < 20 L x 3x MY < 20 L	1.074	1.013-1.138
2x 20 L ≥ MY > 30 L x 3x 20 L ≥ MY > 30 L	1.234	1.183-1.287
2x 30 L ≥ MY > 40 L x 3x 30 L ≥ MY > 40 L	1.308	1.256-1.361
2x MY ≥ 40 L x 3x MY ≥ 40 L	1.357	1.298-1.420
<b>Milking frequency (2x)</b>		
20 L ≥ MY > 30 L x MY < 20 L	0.542	0.528-0.557
30 L ≥ MY > 40 L x MY < 20 L	0.370	0.360-0.381
MY < 20 L x MY ≥ 40 L	3.503	3.372-3.640
20 L ≥ MY > 30 L x 30 L ≥ MY > 40 L	1.465	1.436-1.494
20 L ≥ MY > 30 L x MY ≥ 40 L	1.899	1.840-1.961
30 L ≥ MY > 40 L x MY ≥ 40 L	1.297	1.257-1.337
<b>Milking frequency (3x)</b>		
20 L ≥ MY > 30 L x MY < 20 L	0.472	0.449-0.496
30 L ≥ MY > 40 L x MY < 20 L	0.304	0.290-0.319
MY < 20 L x MY ≥ 40 L	4.430	4.219-4.651
20 L ≥ MY > 30 L x 30 L ≥ MY > 40 L	1.553	1.507-1.600
20 L ≥ MY > 30 L x MY ≥ 40 L	2.090	2.025-2.157
30 L ≥ MY > 40 L x MY ≥ 40 L	1.346	1.311-1.382

SOURCE: The author (2021).

Cows milked two times daily had higher odds of having a SCM compared to cows milked three times per day, for all milk yield categories, however it was observed a gradual increase in the odds as milk yield increases. For example, when MY < 20 L, cows milked with lower frequency have 7% higher odds than those milked three times per day, while when MY ≥ 40 L, the odds are 36% higher. As milk yield increases, lower odds of having subclinical mastitis were observed for both two and three-times milking frequency. The odds of a cow that is being milked two times daily and have a milk yield < 20 L/day is 3.503 (95% CI.372-3.640) higher than a cow in the same condition producing ≥ 40 L/day.

The odds of a Holstein cow having subclinical mastitis for the interaction of parity and days in milk are presented in Table 15.

TABLE 15 - ODDS OF A HOLSTEIN COW HAVING SUBCLINICAL MASTITIS FOR THE INTERACTION OF PARITY AND DAYS IN MILK.

continue

<b>Variables</b>	<b>Odds ratio</b>	<b>95% CI</b>
<b>Primiparous</b>		
DIM ≤ 105 d x 105 d > DIM ≥ 205 d	0.886	0.858-0.916
DIM ≤ 105 d x 205 d > DIM ≥ 305 d	0.700	0.678-0.723
DIM > 305 d x DIM ≤ 105 d	1.775	1.712-1.839
105 d > DIM ≥ 205 d x 205d > DIM ≥ 305 d	0.790	0.765-0.815
DIM > 305 d x 105 d > DIM ≥ 205 d	1.573	1.518-1.629
DIM > 305 d x 205 d > DIM ≥ 305 d	1.242	1.200-1.285
<b>Secundiparous</b>		
DIM ≤ 105 d x 105 d > DIM ≥ 205 d	0.748	0.722-0.776
DIM ≤ 105 d x 205 d > DIM ≥ 305 d	0.582	0.561-0.603
DIM > 305 d x DIM ≤ 105 d	2.256	2.167-2.349
105 d > DIM ≥ 205 d x 205d > DIM ≥ 305 d	0.777	0.752-0.804
DIM > 305 d x 105 d > DIM ≥ 205 d	1.688	1.625-1.755
DIM > 305 d x 205 d > DIM ≥ 305 d	1.312	1.267-1.360
<b>3 Multiparous</b>		
DIM ≤ 105 d x 105 d > DIM ≥ 205 d	0.748	0.718-0.779
DIM ≤ 105 d x 205 d > DIM ≥ 305 d	0.584	0.561-0.608
DIM > 305 d x DIM ≤ 105 d	2.318	2.212-2.429
105 d > DIM ≥ 205 d x 205d > DIM ≥ 305 d	0.781	0.751-0.812
DIM > 305 d x 105 d > DIM ≥ 205 d	1.733	1.657-1.812
DIM > 305 d x 205 d > DIM ≥ 305 d	1.354	1.298-1.412
<b>≥ 4 Multiparous</b>		
DIM ≤ 105 d x 105 d > DIM ≥ 205 d	0.764	0.735-0.795
DIM ≤ 105 d x 205 d > DIM ≥ 305 d	0.618	0.594-0.643
DIM > 305 d x DIM ≤ 105 d	2.235	2.133-2.341
105 d > DIM ≥ 205 d x 205d > DIM ≥ 305 d	0.809	0.778-0.841
DIM > 305 d x 105 d > DIM ≥ 205 d	1.708	1.632-1.787
DIM > 305 d x 205 d > DIM ≥ 305 d	1.381	1.322-1.442

TABLE 15 - ODDS OF A HOLSTEIN COW HAVING SUBCLINICAL MASTITIS FOR THE INTERACTION OF PARITY AND DAYS IN MILK.

conclusion

<b>Variables</b>	Odds ratio	95% CI
<b>DIM ≤ 105 d</b>		
Primiparous x Secundiparous	0.648	0.625-0.672
3 Multiparous x Primiparous	2.516	2.419-2.617
≥4 Multiparous x Primiparous	3.757	3.616-3.902
3 Multiparous x Secundiparous	1.631	1.567-1.697
≥4 Multiparous x Secundiparous	2.435	2.342-2.532
3 Multiparous x ≥4 Multiparous	0.670	0.643-0.698
<b>105 d &gt; DIM ≥ 205 d</b>		
Primiparous x Secundiparous	0.547	0.529-0.566
3 Multiparous x Primiparous	2.982	2.875-3.093
≥4 Multiparous x Primiparous	4.357	4.202-4.518
3 Multiparous x Secundiparous	1.632	1.573-1.694
≥4 Multiparous x Secundiparous	2.385	2.299-2.474
3 Multiparous x ≥4 Multiparous	0.684	0.658-0.712
<b>205 d &gt; DIM ≥ 305 d</b>		
Primiparous x Secundiparous	0.539	0.522-0.555
3 Multiparous x Primiparous	3.015	2.913-3.120
≥4 Multiparous x Primiparous	4.256	4.110-4.407
3 Multiparous x Secundiparous	1.624	1.569-1.681
≥4 Multiparous x Secundiparous	2.292	2.213-2.374
3 Multiparous x ≥4 Multiparous	0.708	0.682-0.736
<b>DIM &gt; 305 d</b>		
Primiparous x Secundiparous	0.510	0.492-0.528
3 Multiparous x Primiparous	3.286	3.156-3.422
≥4 Multiparous x Primiparous	4.731	4.537-4.934
3 Multiparous x Secundiparous	1.675	1.608-1.745
≥4 Multiparous x Secundiparous	2.412	2.312-2.516
3 Multiparous x ≥4 Multiparous	0.695	0.663-0.727

SOURCE: The author (2021).

As days in milk increased, higher odds of having subclinical mastitis were found for all parity categories. Increasing parity also increased the odds of having subclinical mastitis for all DIM categories.

Table 16 shows the odds of a Holstein cow having subclinical mastitis for the interaction of parity and milk yield.

TABLE 16 - ODDS OF A HOLSTEIN COW HAVING SUBCLINICAL MASTITIS FOR THE INTERACTION OF PARITY AND MILK YIELD.

continue

Variables	Odds ratio	95% CI
<b>Primiparous</b>		
20 L $\geq$ MY > 30 L x MY < 20 L	0.534	0.513-0.555
30 L $\geq$ MY > 40 L x MY < 20 L	0.378	0.363-0.394
MY < 20 L x MY $\geq$ 40 L	3.146	2.987-3.314
20 L $\geq$ MY > 30 L x 30 L $\geq$ MY > 40 L	1.412	1.372-1.452
20 L $\geq$ MY > 30 L x MY $\geq$ 40 L	1.679	1.610-1.751
30 L $\geq$ MY > 40 L x MY $\geq$ 40 L	1.190	1.143-1.238
<b>Secundiparous</b>		
20 L $\geq$ MY > 30 L x MY < 20 L	0.444	0.425-0.463
30 L $\geq$ MY > 40 L x MY < 20 L	0.287	0.275-0.300
MY < 20 L x MY $\geq$ 40 L	4.804	4.573-5.048
20 L $\geq$ MY > 30 L x 30 L $\geq$ MY > 40 L	1.548	1.501-1.597
20 L $\geq$ MY > 30 L x MY $\geq$ 40 L	2.132	2.052-2.216
30 L $\geq$ MY > 40 L x MY $\geq$ 40 L	1.378	1.330-1.428
<b>3 Multiparous</b>		
20 L $\geq$ MY > 30 L x MY < 20 L	0.498	0.473-0.525
30 L $\geq$ MY > 40 L x MY < 20 L	0.318	0.302-0.335
MY < 20 L x MY $\geq$ 40 L	4.302	4.064-4.554
20 L $\geq$ MY > 30 L x 30 L $\geq$ MY > 40 L	1.569	1.512-1.627
20 L $\geq$ MY > 30 L x MY $\geq$ 40 L	2.144	2.055-2.237
30 L $\geq$ MY > 40 L x MY $\geq$ 40 L	1.367	1.315-1.420
<b><math>\geq</math> 4 multiparous</b>		
20 L $\geq$ MY > 30 L x MY < 20 L	0.554	0.525-0.584
30 L $\geq$ MY > 40 L x MY < 20 L	0.367	0.348-0.387
MY < 20 L x MY $\geq$ 40 L	3.704	3.496-3.924
20 L $\geq$ MY > 30 L x 30 L $\geq$ MY > 40 L	1.509	1.455-1.565
20 L $\geq$ MY > 30 L x MY $\geq$ 40 L	2.052	1.968-2.139
30 L $\geq$ MY > 40 L x MY $\geq$ 40 L	1.360	1.309-1.413

TABLE 16 - ODDS OF A HOLSTEIN COW HAVING SUBCLINICAL MASTITIS FOR THE INTERACTION OF PARITY AND MILK YIELD.

conclusion

<b>Variables</b>	Odds ratio	95% CI
<b>MY &lt; 20 L</b>		
Primiparous x Secundiparous	0.448	0.428-0.469
3 Multiparous x Primiparous	3.373	3.197-3.560
≥4 Multiparous x Primiparous	4.429	4.194-4.678
3 Multiparous x Secundiparous	1.511	1.430-1.597
≥4 Multiparous x Secundiparous	1.984	1.875-2.099
3 Multiparous x ≥4 Multiparous	0.762	0.716-0.811
<b>20 L ≥ MY &gt; 30 L</b>		
Primiparous x Secundiparous	0.539	0.523-0.555
3 Multiparous x Primiparous	3.150	3.046-3.257
≥4 Multiparous x Primiparous	4.598	4.447-4.753
3 Multiparous x Secundiparous	1.696	1.638-1.757
≥4 Multiparous x Secundiparous	2.476	2.392-2.563
3 Multiparous x ≥4 Multiparous	0.685	0.660-0.712
<b>30 L ≥ MY &gt; 40 L</b>		
Primiparous x Secundiparous	0.591	0.574-0.608
3 Multiparous x Primiparous	2.835	2.747-2.925
≥4 Multiparous x Primiparous	4.301	4.167-4.438
3 Multiparous x Secundiparous	1.674	1.621-1.729
≥4 Multiparous x Secundiparous	2.540	2.459-2.623
3 Multiparous x ≥4 Multiparous	0.659	0.637-0.682
<b>MY ≥ 40 L</b>		
Primiparous x Secundiparous	0.684	0.656-0.713
3 Multiparous x Primiparous	2.467	2.363-2.576
≥4 Multiparous x Primiparous	3.763	3.603-3.930
3 Multiparous x Secundiparous	1.687	1.625-1.752
≥4 Multiparous x Secundiparous	2.573	2.479-2.672
3 Multiparous x ≥4 Multiparous	0.656	0.631-0.681

SOURCE: The author (2021).

Lower odds of having subclinical mastitis were observed as milk yield increased. For example, secundiparous cows producing less than 20 L/day have 4.804 more chances of having SCM compared to secundiparous cows producing more than 40 L/day. Higher odds of having SCM were found as the number of calvings increased for all milk yield categories.

The odds of a Holstein cow having subclinical mastitis for the interaction of days in milk and milk yield are shown in Table 17.

TABLE 17 - ODDS OF A HOLSTEIN COW HAVING SUBCLINICAL MASTITIS FOR THE INTERACTION OF DAYS IN MILK AND MILK YIELD.

continue

<b>Variables</b>	<b>Odds ratio</b>	<b>95% CI</b>
<b>DIM ≤ 105 d</b>		
20 L ≥ MY > 30 L x MY < 20 L	0.570	0.540-0.603
30 L ≥ MY > 40 L x MY < 20 L	0.398	0.378-0.420
MY < 20 L x MY ≥ 40 L	3.321	3.144-3.509
20 L ≥ MY > 30 L x 30 L ≥ MY > 40 L	1.432	1.383-1.484
20 L ≥ MY > 30 L x MY ≥ 40 L	1.895	1.825-1.967
30 L ≥ MY > 40 L x MY ≥ 40 L	1.323	1.281-1.365
<b>105 d &gt; DIM ≥ 205 d</b>		
20 L ≥ MY > 30 L x MY < 20 L	0.569	0.539-0.600
30 L ≥ MY > 40 L x MY < 20 L	0.396	0.375-0.417
MY < 20 L x MY ≥ 40 L	3.521	3.328-3.724
20 L ≥ MY > 30 L x 30 L ≥ MY > 40 L	1.437	1.392-1.485
20 L ≥ MY > 30 L x MY ≥ 40 L	2.002	1.930-2.077
30 L ≥ MY > 40 L x MY ≥ 40 L	1.393	1.350-1.437
<b>205 d &gt; DIM ≥ 305 d</b>		
20 L ≥ MY > 30 L x MY < 20 L	0.492	0.472-0.513
30 L ≥ MY > 40 L x MY < 20 L	0.312	0.300-0.326
MY < 20 L x MY ≥ 40 L	4.205	3.999-4.421
20 L ≥ MY > 30 L x 30 L ≥ MY > 40 L	1.575	1.530-1.621
20 L ≥ MY > 30 L x MY ≥ 40 L	2.069	1.988-2.152
30 L ≥ MY > 40 L x MY ≥ 40 L	1.313	1.264-1.364
<b>DIM &gt; 305 d</b>		
20 L ≥ MY > 30 L x MY < 20 L	0.410	0.394-0.426
30 L ≥ MY > 40 L x MY < 20 L	0.257	0.246-0.268
MY < 20 L x MY ≥ 40 L	4.898	4.591-5.226
20 L ≥ MY > 30 L x 30 L ≥ MY > 40 L	1.595	1.538-1.653
20 L ≥ MY > 30 L x MY ≥ 40 L	2.007	1.890-2.131
30 L ≥ MY > 40 L x MY ≥ 40 L	1.259	1.184-1.338

TABLE 17 - ODDS OF A HOLSTEIN COW HAVING SUBCLINICAL MASTITIS FOR THE INTERACTION OF DAYS IN MILK AND MILK YIELD.

conclusion

Variables	Odds ratio	95% CI
<b>MY &lt; 20 L</b>		
DIM ≤ 105 d x 105 d > DIM ≥ 205 d	0.771	0.723-0.823
DIM ≤ 105 d x 205 d > DIM ≥ 305 d	0.529	0.500-0.560
DIM > 305 d x DIM ≤ 105 d	2.850	2.695-3.014
105 d > DIM ≥ 205 d x 205d > DIM ≥ 305 d	0.686	0.649-0.726
DIM > 305 d x 105 d > DIM ≥ 205 d	2.198	2.080-2.324
DIM > 305 d x 205 d > DIM ≥ 305 d	1.509	1.443-1.577
<b>20 L ≥ MY &gt; 30 L</b>		
DIM ≤ 105 d x 105 d > DIM ≥ 205 d	0.774	0.745-0.803
DIM ≤ 105 d x 205 d > DIM ≥ 305 d	0.614	0.593-0.635
DIM > 305 d x DIM ≤ 105 d	2.047	1.975-2.122
105 d > DIM ≥ 205 d x 205d > DIM ≥ 305 d	0.793	0.768-0.819
DIM > 305 d x 105 d > DIM ≥ 205 d	1.584	1.531-1.639
DIM > 305 d x 205 d > DIM ≥ 305 d	1.256	1.220-1.294
<b>30 L ≥ MY &gt; 40 L</b>		
DIM ≤ 105 d x 105 d > DIM ≥ 205 d	0.776	0.754-0.800
DIM ≤ 105 d x 205 d > DIM ≥ 305 d	0.675	0.655-0.695
DIM > 305 d x DIM ≤ 105 d	1.839	1.773-1.907
105 d > DIM ≥ 205 d x 205d > DIM ≥ 305 d	0.869	0.845-0.894
DIM > 305 d x 105 d > DIM ≥ 205 d	1.428	1.379-1.479
DIM > 305 d x 205 d > DIM ≥ 305 d	1.241	1.198-1.285
<b>MY ≥ 40 L</b>		
DIM ≤ 105 d x 105 d > DIM ≥ 205 d	0.818	0.793-0.843
DIM ≤ 105 d x 205 d > DIM ≥ 305 d	0.670	0.645-0.696
DIM > 305 d x DIM ≤ 105 d	1.933	1.822-2.049
105 d > DIM ≥ 205 d x 205d > DIM ≥ 305 d	0.820	0.789-0.852
DIM > 305 d x 105 d > DIM ≥ 205 d	1.580	1.490-1.676
DIM > 305 d x 205 d > DIM ≥ 305 d	1.295	1.217-1.378

SOURCE: The author (2021).

Lower odds of having SCM were observed as milk yield increased for all DIM categories. For example, when 205 d > DIM ≥ 305 d, a cow with a production lower than 20 L/day have 4.205 (95% CI 3.999-4.421) more chances of having SCM than cows producing more than 40 L/day. As days in milk increases, the odds of a cow

having a SCM are higher for all milk yield categories. When  $MY \geq 40$  L, the odds of a cow having SCM when  $DIM > 305$  d is 93.3% higher than when  $DIM \leq 105$  d.

## 5 DISCUSSION

In general, the increase in milking frequency, from two to three times per day, has shown to decrease somatic cells count in milk in most cases (TABLES 4, 5 and 6) and lowered odds of a cow having subclinical mastitis (TABLES 12, 13 and 14). These results agree with other studies that compared different milking systems, showing that an increase in MF reduces SCC (BERGLUND et al., 2002; NØRGAARD et al., 2005; ÖSTERMAN et al., 2005). When milking frequency is increased, udder health may be improved by decreasing intramammary pressure by more frequently milk removal and lower time between milkings, which decreases the probability of pathogens penetrating the teat canal (GRINDAL, 1988), which is the first barrier against bacteria and IMI.

Milk routine is extremely important in order to reduce cow's exposure to mastitis pathogens. There is evidence that the hygiene conditions of dairy cows affect SCC (SCHREINER; RUEGG, 2003; SANT'ANNA; DA COSTA, 2011). The environment pathogens are isolated from the skin, teat ends and teat canals (CONTRERAS et al., 2011), and the number of mastitis pathogens on the teat end at milking is highly correlated to the incidence of IMI (PANKEY, 1989).

Three times milking is an interesting option for high producing herds, once it increases milk production and improves lactation persistency. The persistency in lactation curve is more apparent in primiparous than multiparous cows (ARBEL et al., 2001). According to Cole and Null (2009), high lactation persistency is associated with a slow rate of decline after lactation peak. This persistency is observed until the last 10 weeks of the recurring pregnancy (SORENSEN et al., 2008). Normally, the decrease in milk yield after lactation peak is due to the progressive loss of secretory cells mainly by programmed cell death, which lactation persistency is strongly influenced (STEFANON et al., 2002).

According to Flint and Knight (1997), this increase in milk production is the result of combined effect of GH (growth hormone), that stimulates IGF-1 production, acting as a survival factor for the mammary gland, and prolactin, inhibiting local production of IGFBP-5, which inhibits IGF-1, and consequently leads to cell death. The number of secretory cells in the mammary gland is determined by the balance between cell proliferation and apoptosis (KNIGHT, 2000). According to Nørgaard et al. (2005), increased milking frequency may increase cell proliferation and decrease apoptosis, thus giving rise to relatively more mammary epithelial cells.

It was observed that increasing milking frequency reduces individual SCC for all parities categories (TABLE 4), for DIM > 105 d (TABLE 5), and for milk yield higher than 30 L/day (TABLE 6). Milking frequency did not affect individual SCC when milk yield was lower ( $MY < 20 L$  and  $20 L \geq MY > 30 L$ ), although the odds of a cow having SCM was higher when cows are milked twice daily (TABLE 14). Milking frequency also had no effect when  $DIM \leq 105 d$ . Many studies have noticed and discussed that milk yield may have a dilution effect in SCC. According to Green et al (2006), there is a reduction in SCC with higher yields and dilution effect is more important in low SCC categories ( $<100,000$  cells/mL). They noticed that when  $200,000 < SCC < 400,000$  cells/mL, dilution accounted for 15% of estimated milk loss while 85% was the true milk loss resulted from mammary gland inflammation.

A combination of factors may be related to the fact that milking frequency did not have any effect in low production categories and in the beginning of lactation (TABLES 5 and 6). Three times daily milking increases milk production and milk persistency, which means that cows will be producing more milk for a longer period of time.. As we observed, it is expected lower SCC in the beginning of lactation, which will increase as lactation progresses, and consequently the dilution effect will be more prominent in the beginning of lactation, mostly for 2x milking cows that will have higher rate of decline in production after peak.

Higher milk yield had a positive impact in individual SCC for all parities (TABLE 8) and DIM (TABLE 9) categories studied and was related to lower odds of a Holstein cow having SCM (TABLES 16 and 17). Many studies have reported that one of the adverse economic effects of high SCC is the lower milk production (BARŁOWSKA et al, 2009; HAND et al., 2012; GONÇALVES et al., 2018; CHEN et al., 2021). Furthermore, it is important to remember that a healthy mammary gland will be producing more milk, with higher dilution effect and lower somatic cells count, which helps to explain why higher milk productions are associated with lower SCC. Keneddy et al (1982), observed that the lowest SCC value was found very close to lactation peak that occurs in early lactation.

Many studies have shown that increasing DIM is related to higher SCC in milk (BERGLUND et al., 2002; SORENSEN et al., 2008; SEBASTINO et al., 2020). Epithelial cells are part of the somatic cells present in milk (SORDILLO et al., 1997), and result of normal breakdown and repair, as the mammary gland prepares for the new lactation cycle, or as a mastitis consequence (SCHULTZ, 1977). The gradual

increase in SCC across lactation is not necessarily a sign of disease, but the declining and scurf of the epithelial integrity, although the odds of a cow having a mastitis are higher in late lactation (TABLE 15).

The increase in SCC toward the end of lactation is also associated with greater pathogen exposal and increasing udder manipulation, which may facilitate pathogens entrance and multiplication (RUEG; PANTOJA., 2013). Traditionally it is indicated a mean lactation period of 305 days or 10 months, and a dry period of 60 days. It was possible to observe by the results that increasing DIM over this period is harmful for milk quality by increasing milk somatic cells (TABLES 5, 7 and 9). Cows with four or more calvings almost doubled individual SCC when comparing early lactation (DIM  $\leq$  105 d) with lactations over 305 days (TABLE 7). In the beginning of lactation is expected lower SCC in milk and higher milk production, which will culminate with the highest dilution effect proposed by Green et al (2006).

In this study, increasing parity was a risk factor associated with the increase of somatic cells count at cow-level and higher odds of a Holstein cow having SCM for all milking frequency (TABLE 4), milk yield (TABLE 8) and DIM (TABLE 7) categories studied. Our results agree with many other studies that reported higher SCC as parity increases (NÓBREGA AND LANGONI, 2011; ASADPOUR et al., 2015; GONÇALVEZ et al., 2018; PANTOJA et al. 2020). Somatic cells count is normally high right after calving, which is attribute to excessive shedding of epithelial cells in a small in a small volume of milk (KENNEDY et al., .1982), but it decreases fast to normal levels until 6 days after calving (BARKEMA et al., 1999).The teat end is the primary barrier against mastitis pathogens, and as the number of parities increase, the functioning of the teat sphincter may be harmed as a physiological response to milking (PANTOJA et al., 2020). This changes on the teat tissue are a risk factor for clinical and subclinical mastitis for both environmental and contagious pathogens, which lead to increase in milk SCC. Asadpour et al. (2015) also showed that advancing parity number increases the incidence of THK and consequently higher SCC in milk.

Animals may have a decline in immune resistance as age progresses, which either can explain part of the increase of milk SCC (GONÇALVEZ et al., 2018). Older animals have a longer productive life, which means that their level of exposure to pathogens are a lot bigger than younger animals. Again, the increasing udder manipulation may facilitates pathogens entrance and multiplication (RUEG; PANTOJA., 2013). Some studies have reported that older animals have lower chances

of curing *S. aureus* mastitis, which is an important contagious pathogen (ZIV; STORPER, 1985; PYÖRÄLÄ; PYÖRÄLÄ, 1998; TAPONEN et al., 2003) and higher chances of the occurrence of clinical mastitis (WALLER et al., 2009; VERBEKE et al., 2014). According to Martins et al. (2017), primiparous cows have 49% and 54% lower odds to develop new IMI and acquiring a CM, respectively, than multiparous cows. Oliver and Mitchell (1983), reported that only 2.6% of infected quarters with major pathogens at calving were from cow completing first or second dry periods, while 23.8% were completing third or greater dry periods. There may also be a risk that chronic infection survives through lactations, as well as dry periods, and results in an accumulated risk for older cows (GREEN et al., 2007).

A big data of test-day records were used in this research, which allowed us to achieve great statistical results however, there were some limitations. There is no information of how those milk samples were collected, although we used editing criteria to avoid sampling mistakes. No microbial culture of milk was done to identify if cows were with the presence of a pathogen to conclude if they really were with mastitis or not. The data base did not content some information that would be helpful for a better understanding of the results, such as herd size, region, milking management or installations. Although we did not have this information, this does not invalidate the results found.

## 6 CONCLUSION

Mastitis is a multifactorial disease, and it is closely related to management adopted by dairy farms. In this study, we identified many risk factors associated with the increase in SCC at cow-level and higher odds of having SCM, such as lower milk yield, increasing parity, lower milking frequency and higher days in milk. In this study, milking frequency does only starts to affect individual SCC when DIM > 105 d and MY > 30 L. The importance of understanding how these factors are associated with high somatic cells is important when looking for explanations and solutions for high incidence of mastitis in the herd, high SCC at cow-level and consequently at bulk tank. This information is very useful when making decisions. Improving milk quality, and particularly SCC, is considered a priority and it is very important for farm profitability and success, once higher SCC and mastitis incidence is related to higher culling rates, antimicrobials use, discarded milk and lower milk production.

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