DISSERTATION

EVALUATION OF NOVEL STRATEGIES FOR IMPROVING HEALTH AND WELLBEING OF DAIRY CATTLE

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ABSTRACT

EVALUATION OF NOVEL STRATEGIES FOR IMPROVING HEALTH AND WELLBEING OF DAIRY CATTLE

The research projects covered in this dissertation were carried out in commercial conventional and organic certified dairy farms and were intended to provide basic information on strategies for early detection and management of diseases in dairy cows. Organic dairy systems are regulated by specific requirements that must be met all times and therefore have restricted treatment options. Detection of disorders during the subclinical phase is therefore critical for prompt action leading to quicker recovery in both conventional and organic dairy herds. In addition, evidence generated in studies carried out on organic dairy animals could be applied to conventional dairy farms in the USA, as restrictions in the use of antimicrobials are increasing in food animals. Early detection leading to prompt treatment decreases milk yield reduction, treatment cost, and labor. The application of precision dairy technology is increasing and becoming more popular and feasible. Producers who have adopted the technology seek validated results to more fully utilize the technology for management decisions since these technologies have not been utilized to their full potential.

This dissertation contains results from five research studies conducted in lactating dairy cows, with an emphasis on early detection and alternative treatment options of health disorders. The study presented in chapter I evaluates the use of differential leucocyte count obtained from a commercial on-farm tool to detect subclinical mastitis. The objective was to analyze the association between milk leukocyte proportions provided by a commercial automated milk

leukocyte differential (MLD) test and multiple cow and quarter-level variables. The study population consisted of 104 Holstein cows (32 primiparous and 72 multiparous) in one farm. Cows were categorized by days in milk as early (<50 DIM; n=29), middle (50–250 DIM; n=25), and late lactation (>250 DIM; n = 50). Milk from 416 quarters was collected and analyzed for lymphocytes (LYM), neutrophils (NEU), and macrophages (MAC) counts using an automated milk fluorescence microscopy system. Concurrently, a sterile composite milk sample was collected from each cow for pathogen identification through microbiological culture. Culture results were classified as no growth (NOG), gram-negative (GN), gram-positive (GP), or other (OTH). Milk leukocyte proportions varied depending on the level of total leukocyte counts (TLC; $P < 0.001$). Similarly, leukocyte ratios (NEU:LYM, NEU:MAC, and phagocyte:LYM) were different for multiple total leucocyte count (TLC) categories ($P < 0.05$). There was no association between parity number and MLD; however, cows in early lactation had the greatest proportions of NEU and LYM. Leukocyte ratios varied depending on parity number and stage of lactation. Cows in the medium milk-yield category had the smallest proportions of NEU and LYM, and there was significant variation in leukocyte ratios, depending on the level of milk yield. In healthy quarters, MLD were not associated with quarter position; however, the NEU:MAC ratio was greater in rear quarters than in front quarters. In quarters with TLC >100,000, NEU% was greater in rear quarters than in front quarters ($P = 0.03$). For quarters with pathogen growth, TLC was greatest for GN followed by OTH and GP ($P < 0.001$). Milk LD depended on the isolated pathogen group, although the magnitudes of the differences were small. Although the changes in the proportions of leukocytes in milk were associated with categories of TLC, levels of milk yield, and mastitis-causing pathogen groups, the deviations were small in magnitude.

The research presented in chapter II was an extension of the previous study, as we used electrical conductivity of milk to identify subclinical mastitis pathogens. The objective was to characterize the pattern of electrical conductivity (EC) in milk during intramammary infection, considering specific mastitis-causing pathogen groups involvement. Cows (n=200) identified by an in-line mastitis detection system with a positive deviation >15% in the manufacturer's proprietary algorithm for EC (HEC) were considered cases and enrolled in the study at the subsequent milking. One control (CON) cow, within normal ranges for EC, was matched to each case based on parity and stage of lactation. A sterile composite milk sample was collected from each cow for bacteriological culture. Milk yield (MY) and EC were recorded for each milking during ±7 d relative to enrolment. Milk cultures were categorized into Gram positive (GP), Gram negative (GN), other (OTH), and no growth (NOG). Data were submitted for repeated measures analysis with EC as the dependent variable and EC status at d -1, bacteriological culture category, parity number, stage of lactation, and days relative to sampling as independent variables. Average EC (± SE) was significantly greater in HEC than in CON cows (12.5 ± 0.51 mS/cm vs. 10.8 ± 0.49 mS/cm) on the day of identification (d -1). Milk yield on d -1 was greater in CON than in HEC (37.6 ± 5.12 kg vs. 33.5 ± 5.19 kg). Average EC on d -1 were similar for the different bacteriological culture categories: 11.4 ± 0.57 mS/cm, 11.7 ± 0.52 mS/cm, 12.3 ± 0.82 mS/cm, and 11.7 ± 0.51 mS/cm in GN, GP, OTH, and NOG, respectively. Parity number was only associated with d -1 EC in HEC group, with the greatest EC values in parity 3 (12.3 ± 0.25 mS/cm), followed by parity 2 (11.9 ± 0.21 mS/cm), parity ≥3 (11.6 ± 0.46 mS/cm), and primiparous animals (11.2 ± 0.2 mS/cm). A significant effect on EC for the interaction of day relative to identification by pathogen category was observed. The same interaction effect was observed on daily MY. We

concluded that characteristic temporal patterns in EC and MY in particular pathogen groups may provide indications for differentiation of groups of mastitis-causing pathogens.

Chapter III covers the evaluation of the use of milk components ratios for monitoring of health disorders during early lactation. The objective was to evaluate the potential of milk fat to protein (FPR), milk protein to lactose (PLR), and milk fat to lactose (FLR) ratios for detection of subclinical disease before evident clinical signs. Milk component data from 198 Holstein cows were recorded from beginning of lactation through 60 days in milk (DIM), using the AfiLab® milk analysis system at the University of Florida (UF) Dairy Unit. Milk components were recorded as an average of AM and PM milkings. Occurrence of health disorders (mastitis [MAS], metritis [MET], clinical hypocalcemia [HYC], digestive disorders [DIG], lameness [LAM], and ketosis [KET]) were assessed by UF veterinarians and farm personnel. For each of the three parameters, two indices were developed: (i) Cow index (CIx) = measurement on the day of diagnosis (d 0) minus –3 to –5 d average relative to d 0, divided by the –3 to –5 d average; and (ii) mates index (MIx) = [(affected cow d0 − avg −3 to −5 days) − (pen mates d0 − avg −3 to −5 days)]/pen mates d0. Cow alert value (CAV) and mates alert value (MAV) were set when the respective index value was less than –0.1 or more than +0.1. The correlation between FPR and FLR was intermediate for both sick and healthy cows (r = 0.47 and 0.51, respectively). The correlation between FLR and PLR were high (r = 0.66 and 0.69) for healthy and sick animals respectively. Interestingly, the correlation between PLR and FPR were -0.27 and -0.26 respectively. The odds (95% CI) of MAS multiplied by 1.2 (1.13–1.27), 1.32 (1.27–1.37), and 1.27(1.19-1.29) for each decimal unit increment in FPR, FLR and PLR, respectively. For each decimal unit increment in FPR, FLR and PLR, the odds of MET multiplied by 1.42 (1.34-1.51) , 1.32 (1.26–1.38), and 1.67 (1.09-1.24), respectively; the odds of KET multiplied by 1.52 (1.43–1.62), 1.33 (1.27-1.39), and 1.34 (1.24–

1.44); the odds of HYC multiplied by 0.53 (0.308–0.92), 1.34(1.15-1.57), and 1.36 (1.2–1.55); and the odds of DIG multiplied by 1.35 (1.27–1.44), 1.29(1.23-1.35), and 1.16 (1.09–1.24). The odds of LAM were only significant for changes in FLR and PLR [1.22 (1.6–1.29) and 1.22(1.15-1.28)]. Sensitivity (Se) of CAV based on FPR ranged from 58% (LAM) to 100% (HYC) with 62% specificity (Sp). The Se of CAV based on FLR ranged from 60% (LAM) to 100% (HYC) with 63% Sp. The Se of CAV based on PLR ranged from 45% (LAM) to 100% (HYC) with 72% Sp. Sensitivity (Se) of MAV based on FPR ranged from 57% (LAM) to 100% (HYC) with 63% specificity (Sp). The Se of MAV based on FLR ranged from 55% (LAM) to 100% (HYC) with 65% Sp. The Se of CAV based on PLR ranged from 45% (LAM) to 100% (HYC) with 73% Sp. The results suggested that changes in FPR, FLR and PLR may be used as indicators of disease; MAS, LAM, and KET were better detected using FLR, MET and HYC were better detected using PLR, and FPR was more effective for DIG detection. Overall, CAV was more effective than MAV on disease detection.

In chapter IV, we evaluated the use a leg mounted sensor to detect specific lameness disorders. Our objective was to characterize the dynamics of lying time, daily steps, bouts frequency, and milk yield in animals submitted for hoof trimming (HT) during early lactation. A total of 310 multiparous Holstein cows from a USDA organic certified dairy herd in Northern Colorado were enrolled and monitored for 7 months for lying time (min/d), steps (n/d), bouts (n/d), motion index (units/d), and milk yield (kg/d). Cows submitted for HT were differentiated as receiving corrective interventions (TRM) or as being diagnosed with a lameness disorder (DIS). Cows not submitted to HT were considered as healthy controls. A rolling 7-day average was created for each activity and production parameter. Relative to HT, a deviation from the rolling average was calculated daily. Data were examined by logistic regression and mixed model

analyses for associations between the behavior and performance parameters and lameness disorders. Cows in the study were evaluated for locomotion scores fortnightly. Cows with locomotion score 4 and 5 had greater lying time and greater lameness probabilities obtained from the system; whereas animals with locomotion score 4 were also associated with higher daily steps and motion indices ($P < 0.05$). Lying time and number of daily bouts were greater in DIS whereas daily milk yield and the number of steps were smaller compared to healthy and TRM cows. Greater lying time category, greater bouts frequency category, and early lactation were associated with greater odds of an animal being affected with DIS, whereas high steps category and transition period were associated with reduced odds of DIS. Similarly, greater daily steps category and first parity were associated with reduced odds of TRM whereas early lactation was associated with greater odds of TRM. Specific lameness conditions demonstrated different dynamics for activity behavior, which could be utilized to detect locomotion disorders. We conclude that the dynamics of lying time, bout frequency, daily steps, and milk yield around HT are disorder specific and require different recovery times after treatment to reach normal levels.

Finally, the study presented in chapter V evaluated treatment options for digital dermatitis in an organic dairy herd. A randomized clinical trial was conducted using 70 multiparous Holstein cows with an early digital dermatitis (DD) lesion at a USDA certified organic dairy farm in Northern Colorado. Cows were examined on day 0 (d0) for demonstration of pain in the hind feet and were enrolled in the study based on the presence of early DD lesions (scores M1 and M2). Cows with acute DD lesions were randomly assigned to one of three treatment groups on d0: i) topical application of copper sulfate and iodine (CUI; n = 24); ii) topical application of honey and iodine (HOI; n = 23); and iii) control subject to no treatment (CON; n = 23). Cows were evaluated on days 3 (d3), 12 (d12), 28 (d28), and 120 (d120) post treatment for pain response, lesion size

and received a locomotion and a lesion score. Cure was defined as the transition from active to non-active stages (M1/M2 to M0 or M4). The formulations tested in this study had variable effects on the treatment of DD lesions. The proportion of animals with early stage lesions (M1 and M2) was reduced from d0 in all treatment groups. Gross visual examination suggested that the cure rate was greater in CUI cows on all follow up days. The odds of experiencing pain on d3 after treatment application were numerically greater in CON, followed by HOI and CUI. However, the odds of pain increased in HOI and CUI during the follow up period. The change in lesion size was significantly ($P = 0.0016$) affected by the interaction of treatment by day, with persistence of larger lesions in HOI group and a greater reduction in lesion size for cows in the CUI group. Non-antibiotic treatment formulations were partially effective in the treatment of DD in organic dairy farms. Clinical assessment of animals and evaluation of lesions suggested that the CUI combination was superior to both HOI and CON group.

As these studies provide new knowledge on the use of precision dairy technologies, more opportunities for improving management strategies to enhance animal health, production and reproductive performance are available. Additionally, novel therapeutic strategies for use will prove beneficial in controlling the infection in organic herds as well as reducing the antibiotic use in conventional herds. Thus, the focus of this dissertation on early detection combined with novel treatment strategy brings new perspective in animal health and veterinary medicine research.

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INTRODUCTION

The dairy industry in the USA is undergoing a rapid structural change in herd size and production levels. In synchrony, health management systems in the dairy farms have also evolved. In recent years, the focus of dairy health management has been shifted to herd level strategies of disease management rather than treatment of individual animals (LeBlanc et al., 2006). Although the knowledge of physiology and herd management has improved over the time, the incidence of the most common health disorders has remained constant (LeBlanc et al., 2006). In this scenario, improving wellbeing of dairy cattle needs a multifactorial approach. Through robust preventative management and effective therapeutic practices, it may be possible to reduce the prevalence of disease in dairy cattle. In addition, early detection could reduce the treatment cost as well as reduce losses due to milk withdrawal and lowered performance. To make the situation more complex, the use of therapeutic resources, such as antimicrobials is going through serious questioning by the consumers (Barkema et al., 2015). Therefore, this dissertation focuses on two factors of animal wellbeing and health; 1) exploring strategies to use precision dairy technologies for early disease detection, and 2) evaluation of novel options for treatment of health disorders.

Although precision dairy technologies have increasingly been available to the dairy producers, their adoption is relatively slow (Russel and Bewley, 2013). These resources provide objective measurement of behavioral and physiological parameters and eliminate error due to subjectivity in the measurement. Dairy producers implement precision technologies to improve individual animal management, group or pen management, whole-farm management, and overall farm production efficiency (Wathes et al., 2008). In addition to improving health and production efficiency, technologies accurately monitor individual animals, groups of cattle, and farms, which

can improve the public perception on dairy animal welfare (Laca, 2009). Although these technologies are able to perform wide range of functions, there is the perception that they are not utilized to their potential by dairy producers. Therefore, this dissertation explores broad uses of precision dairy technologies, which could help dairy farmers to make more effective farm management decisions.

Mastitis and lameness are the conditions that affect the highest proportion of dairy cows, 24.8% and 16.8%, respectively (USDA, 2018). With the availability of modern technologies and tools, early detection of these conditions is possible based on monitoring of behavioral and physiological parameters. Electrical conductivity and milk leucocyte differentials are among the technologies that are being accepted among producers as effective detection devices for mastitis. Since timely information regarding the involvement of specific mastitis causing pathogens during mastitis has significant effects on mastitis control programs and improves treatment outcomes (Lago et al., 2011), we evaluated milk leucocyte differential and electrical conductivity to identify their relation with different factors associated with mastitis and their potential to identify the pathogen involvement in mastitis conditions of various severity. In chapter I of this dissertation, we explored the use of differential count of leucocytes in milk and their relative abundance in sick and healthy quarters. In chapter II of the dissertation, we evaluated electrical conductivity in milk and its possible use in pathogen identification in different categories of mastitis events ranging from latent mastitis to non-specific mastitis.

Another developing area in health monitoring of cows by inline milk analysis is the use of milk component ratios to identify health disorders. In chapter III, we evaluated use of combinations of milk components to identify various transition dairy cattle diseases. We developed indices based on milk components comparing the cow with herself and herd mates. These indices were based on

ratios of milk components because of their robustness and reduced fluctuation compared to individual components (Reist et al., 2002).

The USDA's National Animal Health Monitoring System (NAHMS) has estimated an overall lameness prevalence of 16.8% with the highest percentage (18%) in larger operations (USDA, 2018). Another precision technology that we evaluated in chapter IV, used activity behavior to identify changes in behavior that indicate lameness. Sensors measure locomotion behavior automatically and, in this case, a 3-axis accelerometer previously validated using direct visual observation was employed (Brochers et al, 2011). This technology was evaluated for early stage detection, and to measure time to recovery in cows that required corrective hoof trimming or treatment for lameness.

In a different area, commercial dairy producers are under constant pressure to reduce the use of antimicrobials used on their farms. In organic management systems, there are limited therapies that meets the requirements for use in sick cows (Richert et al, 2013). Digital dermatitis is one of the most common infectious cause of lameness with 31.1% of all operations reporting at least one case (USDA, 2018). In the concluding chapter, we evaluated 2 treatment options for digital dermatitis using constituents that are permitted for use on certified organic dairy farms. The research assessed the effect of treatment on short term and long-term resolution of this disorder. The results from this chapter provided scientific basis for the treatment in organic dairy farms as well as for reducing antimicrobial use in conventional dairy farms.

The novel strategies evaluated in this dissertation will help on improving the animal wellbeing at both herd and individual level. The conclusions from this dissertation will provide expanded horizon for the use of available precision dairy technologies in disease detection.

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CHAPTER 1 - RELATIONSHIPS AMONG QUARTER MILK LEUKOCYTE PROPORTIONS AND COW AND QUARTER-LEVEL VARIABLES UNDER DIFFERENT INTRAMAMMARY INFECTION STATUSES

Introduction

Mastitis remains one of the most prevalent and costly diseases in dairy systems (Damm et al., 2017). The use of somatic cell count (SCC) for the diagnosis of intramammary infection (IMI) is widely accepted and is considered a reliable procedure for the detection of subclinical mastitis (SCM). Although SCC is a robust methodology, it does not provide a differentiation of cell types (Damm et al., 2017). The use of a milk leukocyte differential (MLD) test, providing the proportions of specific leukocytes in milk, has been proposed as a complement to SCC to assess the presence and the severity of IMI (Dohoo et al., 1981; Leitner et al., 2003; Godden et al., 2017). Moreover, in recent studies, MLD has been suggested as a screening option for IMI in selective dry cow therapy and fresh cow assessment (Godden et al., 2017; Gonçalves et al., 2017). According to a recent study (Godden et al., 2017), costs for the MLD test are approximately $18,000 for the reader, $5.00 per cow for the cassette, in addition to the cost of labor for the sampling and the analysis procedures.

The proportions of leukocytes in milk have been shown to change depending on the degree of IMI, suggesting that different frequencies of white cell types may be an indication of the diverse stages of progression of infection (Sarikaya et al., 2006). In addition, it is plausible to speculate that these fluctuations in cell proportions in milk from inflamed mammary glands may exhibit characteristic patterns, depending on the presence of specific groups of pathogens.

Microbiological culture of milk is the accepted gold standard for determining IMI status in both, clinical or SCM. In addition, this information is of significant value when selecting for the appropriate treatment options. However, the cost and time requirements associated with milk

culture, together with the risk for contamination and mishandling of samples, limit this approach as a routine diagnostic procedure in commercial farms (Godden et al., 2017). Consequently, a rapid, cow-side test to determine IMI, providing an indication of the potential groups of pathogens involved, would be a beneficial tool in the control of mastitis.

As the application of the differential cell count in milk develops, more details on this parameter behavior under different physiological or pathological stages of the cow are required. The study hypothesis was that specific white cell proportions in milk will deviate depending upon multiple cows and quarter-level variables, including the presence of IMI and the infectious pathogens. Therefore, the study objective was to analyze the association between milk leukocyte proportions provided by a commercial automated MLD test and multiple cows and quarter-level variables, including SCC, parity number, stage of lactation, level of milk production, and presence of mastitis-causing pathogens.

Materials and methods

Study design, animals and management

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Colorado State University (protocol ID: 16-6775A). The research was conducted in a commercial dairy herd in West Texas (Plainview, TX). Cows were housed in a freestall barn and milked three times daily in a rotary milking parlor (Afimilk, Kibbutz Afikim, Israel). Cows were not vaccinated against any mastitis pathogen. A total of 104 clinically healthy Holstein cows were randomly selected, among which 32 animals were primiparous, and 72 were multiparous cows (26, 20, and 26 cows in 2nd, 3rd, and ≥3rd lactation).

Milk leukocyte differential test and bacteriological cultures

Milk samples obtained from 416 quarters were evaluated for MLD using Qscout (Advanced Animal Diagnostics, Durham, NC). Milk was collected following standard aseptic procedures (National Mastitis Council, 1999) that included teat-end disinfection with alcohol and disposal of the first streams of milk. The device reader has programmable threshold levels on a scale of 1 to 18 that may be selected by the user, and different thresholds would result in higher sensitivity or higher specificity (Godden et al., 2017; Gonçalves et al., 2017). The reported sensitivity and specificity for this technology at threshold level 7, using microbiological culture as a gold standard, were 65.4% and 79.3%, respectively (Gonçalves et al., 2017).

Milk from each quarter was applied into a plastic container connected to one of four quadrants of a single-use cassette. The cassette was immediately loaded into the automated reading device, using the research mode (threshold level 6) that required about 15 min per cassette and offered increased accuracy for the differentials. The device uses a fluorescence microscopy imaging system taking images to identify and count lymphocytes (LYM), neutrophils (NEU), and macrophages (MAC) (Godden et al., 2017; Gonçalves et al., 2017). Results are provided as total leukocyte counts (TLC) and as absolute values for each cell type (NEU, LYM, and MAC). In addition, the percentages of NEU, LYM, and MAC for each sample were directly obtained from the device for statistical analyses. Finally, absolute cell counts from MLD were used for determination of NEU:LYM, NEU:MAC, and phagocyte (PHAG):LYM (indicated by NEU + MAC) ratios.

Right before collection for MLD, a composite milk sample from the four quarters was collected from each study cow in a sterile tube using aseptic technique (National Mastitis Council, 1999). Briefly, the teats were cleaned and disinfected using 70% alcohol in a cotton gauze. The

first few streams of milk were discarded, and 10 mL of foremilk was aseptically collected in a sterile tube as a composite sample from each of the four quarters. Samples were immediately frozen before lab submission for bacteriological culture.

All milk samples were submitted to and received at The Dairy Authority, LLC (Greely, CO). Approximately, 0.01 mL of each milk sample was inoculated using a disposable inoculation loop (Hardy Diagnostics, Santa Maria, CA) onto blood agar plates containing 4% washed bovine blood (Quad Five, Ryegate, MT) and 0.1% esculin (Sigma–Aldrich, St. Louis, MO) and MacConkey agar plates (Oxoid, ThermoFisher Scientific, Waltham, MA) and incubated aerobically at 37 °C. Bacterial growth was identified after 24 and 48 h of incubation according to National Mastitis Council (1999). Briefly, *Staphylococcus aureus* and *Staphylococcus* spp. were identified by hemolytic pattern and tube coagulase test. *Streptococcus* spp. was identified by a catalase test (Hydrogen Peroxide) and gram stain (JorVet gram stain kit, Sigma–Aldrich). *Escherichia coli* and *Klebsiella* spp. were identified using morphologic characteristics of colonies on MacConkey agar, production of indole, motility, and utilization of citrate. Approximately, 0.1 mL of each milk sample was also inoculated onto a Modified Eaton's Mycoplasma Agar (The Dairy Authority Labs) with a polyester swab (Hardy Diagnostics). Mycoplasma agar plates were incubated in an 8–10% CO_2 incubator at 37 °C for 10 d. Mycoplasma agar plates were examined under a dissecting microscope at 3, 7, and 10 d.

Cow and quarter-level variable categorization

Eight categories were created for quarter TLC (≤100,000; 101,000–200,000; 201,000–400,000; 401,000–700,000; 701,000–1,000,000; 1,001,000–3,000,000; 3,001,000–10,000,000; and >10,000,000 cells/mL). Results from bacterial culture (cow-level composite milk sample) were classified as gram-negative (GN) pathogens (*Acinetobacter* spp., *E. coli*, *Pasteurella* spp.,

and *Pseudomonas* spp.); gram-positive (GP; CN *Staphylococcus*, *Corynebacterium* spp., S. *aureus*, *Streptococcus* spp., and *Trueperella pyogenes*); other (OTH; *Mycoplasma* spp. and *Prototheca* spp.); and culture negative (NOG). Other variables at the cow level included parity number (1; 2; 3; >3) and stage of lactation (early [<50 DIM], middle [50–250 DIM], and late [>250 DIM]). Individual milk yield provided by in-line milk meters (Afimilk, Kibbutz Afikim, Israel) at the time of sampling was available. The average (SD) milk yield per day for the study cows was 32.6 (10.9) kg, with a range from 6.7 to 52.1 kg. Milk yield at the day of sampling was categorized into quartiles (Q1 <20 kg; Q2–Q3 = 20.1–29.0 kg; and Q4 >29.0 kg). To evaluate the effect of quarter position, quarters were grouped into front and rear quarters and were subsequently categorized as healthy if TLC ≤100,000 and as affected if TLC >100,000.

Statistical Analyses

Cow and quarter-level data were entered into spreadsheets (Excel 2016, Microsoft, Redmond, WA) and analyzed using SAS version 9.3 (SAS Institute Inc., Cary, NC). Data were checked for normal distribution, and subsequently, MLD values were arcsine transformed, while TLC data were reciprocally transformed, where they did not show a normal distribution. After completion of the analyses, the results were back-transformed to be reported in the original scales.

Pathogen data originated from bacteriological cultures from composite samples (cow-level data). Therefore, in cows with positive cultures, only the quarter with the greatest TLC was considered for the analyses testing associations between MLD and mastitis-causing pathogen groups. For cows with negative cultures, the average for the four quarters was considered. Statistical models were tested using PROC MIXED in SAS and included pathogen category, parity number, stage of lactation, milk-yield category at the time of sampling, and quarter position.

In general, depending on the analysis, the models were defined as follows:

$Y_{ijklm} = \mu + Path_i + Par_j + St_k + Mlk_l + Qp_m + e_{ijklm}$

Where:

Y_{ijklm} = dependent variable (TLC, quarter-level MLD, or leukocyte ratios)

μ = overall population mean

$Path_i$ = effect of pathogen category (NOG, GN, GP, or OTH)

Par_j = effect of parity number (1, 2, 3, or >3)

St_k = effect of stage of lactation (early, mid, or late)

Mlk_l = effect of milk yield (low, medium, or high)

Qp_m = effect of quarter position (front or rear)

e_{ijklm} = error term

For all the analyses, statistical significance was defined at P value <0.05.

Results and Discussion

Overall, milk leukocyte proportions from 411 quarters were available. Samples from five quarters did not return MLD results from Qscout and were removed from the analysis. Composite milk samples from 104 cows were submitted for bacteriologic testing. Cultures indicated no growth (NOG) for 44 samples, and 5 samples registered multiple pathogens growth (>2 different bacteria) and were considered contaminated and removed from the subsequent analyses. Main categories of reported pathogens were Coagulase Negative S. aureus (n = 30), Streptococcus spp. (8), Corynebacterium spp. (5), Mycoplasma spp. (4), E. coli (2), S. aureus (2), T. pyogenes (2), Prototheca spp. (1), and Pasteurella spp. (1).

Mean (median) for quarter TLC was 1,553,000 (209,000) cells/mL. Milk leukocyte proportions varied depending on the category of TLC ($P < 0.001$; Figure 1). NEU% consistently increased as categories of TLC augmented ($P < 0.001$). This response was opposite for MAC%, which were larger for small TLC categories ($P < 0.001$). LYM% also varied by category of TLC ($P < 0.0001$) but in a smaller magnitude. Similarly, the three presented leukocyte ratios (NEU:LYM, NEU:MAC, and PHAG:LYM) were different depending on the TLC category ($P < 0.001$, $P < 0.001$, and $P = 0.01$, respectively; Figure 1). There was no association between parity number and MLD; however, LYM% were greatest in early lactation ($P < 0.001$). Contrarily, the greatest value for MAC% was determined in late lactation ($P = 0.009$; Figure 2). Only the NEU:MAC ratio was associated with parity, with the greatest value in parity 1 ($P = 0.01$). Conversely, the NEU:LYM ratio and the PHAG:LYM ratio were associated with time after calving and were smallest during early lactation ($P < 0.001$ and $P < 0.001$, respectively; Figure 3). Leukocyte proportions and ratios were both associated with milk-yield category ($P < 0.05$). NEU% was greater in the high milk-yield category compared with the medium-yield category ($P = 0.01$). LYM% was greater in the high milk-yield level than in both the medium- and the low-yield categories ($P < 0.005$). Finally, MAC% was greater in the low and medium categories ($P < 0.001$), and the NEU:MAC and the PHAG:LYM ratios were both greater in the low and medium milk-yield categories than in the high-yield category ($P = 0.002$ and $P = 0.02$, respectively; Figure 4).

No associations between quarter position and NEU, LYM, and MAC% were determined in healthy quarters (TLC $\leq$ 100,000 cells/mL). Similarly, no associations were found for the NEU:LYM and the PHAG:LYM ratios. Interestingly, the PHAG:LYM ratio was greater in rear quarters than in front quarters ($P = 0.01$; Table 1). In high TLC quarters, only NEU (%) was greater in the rear quarters than in front quarters ($P = 0.03$).

Values for TLC varied by pathogen group ($P < 0.001$) and were largest in GN, followed by OTH and GP pathogens (Table 2). However, TLC were not different for GP and GN groups. Milk leukocyte proportions also varied according to the pathogen group involved. NEU% were highest in the OTH and GP groups, with the lowest level in GN and NOG ($P < 0.001$). However, no significant difference was established for GP vs. GN groups. On the other hand, LYM% were reduced in all GN, GP, and OTH pathogens as compared with the NOG group ($P < 0.001$). Furthermore, LYM% was greater for the GN compared with the GP group ($P < 0.01$). Finally, MAC% was increased in the GN group of pathogens and decreased in the OTH and the GP groups ($P = 0.03$), as compared with the NOG group (Table 2).

The smallest NEU:LYM ratio (mean ± SE) was for the NOG group (3.68 ± 0.27), and values increased to 5.16 (±0.28) in the GP group, 6.08 (± 0.78) in the OTH group, and 7.16 (± 1.25) in the GN group ($P< 0.001$). No difference among pathogen groups was observed for the NEU:MAC ratio. The PHAG:LYM ratio varied by pathogen group with 5.79 (±0.59) in the NO group, 23.5 (±2.64) in the GN group, and 7.93 (±1.68) in the OTH, followed by 7.40 (±0.59) in the GP group ($P < 0.001$). The ratio was greater in the GN group, compared with the GP group ($P = 0.001$).

Discussion

We analyzed the association between milk leukocyte proportions provided by a commercial automated MLD test and multiple cow and quarter-level variables. Values for milk leukocyte proportions in normal milk reported in previous studies are widely variable (Koess and Hamann, 2008; Schwarz et al., 2011b). According to some researchers, MAC are the predominant cell type (Leitner et al., 2000; Riollet et al., 2001; Lindmark-Mansson et al., 2006), whereas others have indicated that LYM are the major population (Park et al., 1992; Schwarz et al. 2011a, 2011b).

Mielke and Koblenz (1981) reported that milk from cows with no evidence of clinical mastitis had 37–38% NEU, 13–20% LYM, and 17–20% MAC. In more recent reports, NEU proportions ranged between 6 and 50%, LYM proportions between 14 and 80%, and MAC proportions between 12 and 46% (Rivas et al., 2001; Merle et al., 2007; Koess and Hamann, 2008; Schwarz et al., 2011b). Interestingly, our observed distribution of white cells in milk from healthy quarters was similar to that recently described by Gonçalves et al. (2017). The main cell type for healthy quarters in our study was NEU (51.4%), followed by MAC (30.1%) and LYM (17.4%). In agreement, Gonçalves et al. (2017) reported NEU, MAC, and LYM percentages in milk of culture negative udder quarters of 49.4, 28.9, and 17.7%, respectively.

As reported previously (Leitner et al., 2000; Pillai et al., 2001; Dosogne et al., 2003; Pilla et al., 2013), stage of lactation was a significant factor affecting milk leukocyte proportions in our study. Dosogne et al. (2003) found that in early lactation, the percentage of LYM was greater and the percentages of mature MAC and NEU were lower than in the other stages of lactation. In another study (Pilla et al., 2013), percentages of mature MAC and NEU in early lactation were only about half of the values found in mid and late lactation. These findings have not been studied in detail, but a possible explanation may be that specific pathogens prevail in IMI at different stages of lactation, and each pathogen will recruit different proportions of white cell populations (Leitner et al., 2000). Moreover, parity number was another factor influencing the proportions of white cells in healthy milk.

In healthy quarters, the position of quarters did not affect the MLD, as well as milk white cells ratios; however, there were significant differences in the NEU:MAC ratios when quarters were categorized as front vs. rear. This difference in front and rear quarters may be related to greater volume of milk (about 60 vs. 40%) secreted from the rear quarter than from the front quarter

(Tancin et al., 2006). Nevertheless, this difference is not observed in affected quarters, which may be a result of inflammation during mastitis. Furthermore, in the affected quarters, NEU% were higher in rear quarters, which may also be attributed to disproportionate milk production.

With increasing TLC levels, we observed increasing NEU% and decreasing MAC%. This phenomenon illustrates that MLD may be dependent on the severity of the inflammation, as determined here across the various levels of TLC, and thus can be used to indicate a specific level of inflammation in the quarter. However, in the case of leukocyte ratios, the patterns illustrated that the ratios can only be used to differentiate extreme low and high categories. Nonetheless, it should be noted that the accuracy of cell differentiation may be affected when TLC are greater and cell overlapping results on conflicting image interpretation.

The association of MLD and leukocyte ratios for milk-yield categories, as observed in this study, suggests an effect for different milk-yield categories. The MLD distribution in high-yield category is different from medium- and low-yield categories. Thus, any mastitis detection algorithm based on MLD should consider milk yield in the model to best identify the alterations of MLD related to infections.

As reviewed by Schwarz et al. (2011b), LYM, MAC, and NEU play specific roles in inflammatory responses within the mammary gland. LYM regulate immune responses recognizing specific antigens through membrane receptors. MAC are active-phagocytic cells, ingesting bacteria, and cellular debris. In addition, the release of chemoattractants from MAC induces the recruitment of NEU that will act against bacteria at the beginning of an acute inflammatory process. In consequence, the distribution of leukocyte numbers is important for the success of intramammary defenses against invading pathogens (Leitner et al., 2003), making plausible the idea that cell proportions would change depending on the type of pathogen and the severity and

chronicity of infection. In fact, different cell patterns have been documented in the presence of different pathogens and during the course of infection. In acute mastitis, NEU are the predominant cell type, whereas in chronic infections, the MAC cell type is more prevalent (Leitner et al., 2003).

Our results evidenced significant differences in white cell proportions between specific pathogen groups, but the magnitude of the changes was small. In agreement with our findings, Damm et al. (2017) found increased proportions of NEU and reduced proportions of MAC in quarters with elevated SCC. However, LYM remained fairly constant, which was termed as antidromic trend of NEU and MAC. In a report by Schwarz et al. (2011b), the proportion of LYM decreased from >60% at SCC values <10,000 cells/mL to 18.7% when SCC was 1,394,000 cells/mL. Conversely, NEU increased from <30% within the SCC range of <10,000 cells/mL to 63.65% at SCC of 1,394,000 cells/mL.

Results from our study are similar to those observed by Gonçalves et al. (2017), who also reported higher MAC% in healthy quarters than in quarters infected by any pathogen. The proportional decrease of MAC% with increases in NEU% was evident in both studies. More in detail, Gonçalves et al. (2017) indicated that the NEU% were greater in specific-SCM (culture positive) cases (65.7%) than in nonspecific-SCM (culture negative) cases (55.2%), latent-SCM (cases with culture positive but low TLC; 55.0%), and healthy quarters (49.4%). The MAC% were lesser in quarters with specific-SCM (12.3%), nonspecific-SCM (17.3%), and latent-SCM TLC (23.0%), when compared with healthy quarters (28.9%). The LYM and PHAG% were similar among tested groups, but mammary quarters with specific-SCM, nonspecific-SCM, and latent-SCM had greater mean value of absolute number of LYM and PHAG than healthy quarters.

An interesting finding in our study is that the change in relative proportions of leukocytes partially depended on the mastitis pathogen group isolated from milk. In a study presented by Emanuelson et al. (1989), a different classification system considering minor and major pathogen groups resulted on differential SCC classifying 96 and 38% of infections due to minor and major pathogens correctly. However, in our study, when milk with GP and GN isolates were compared, LYM were the only population that indicated significant differences. This inability for differentiating GP from GN infections, probably due to a smaller sample size, limits the practical use of this analysis, as one the most relevant applications would be to direct the therapeutic approach for these groups of infections.

All the presented cell type ratios were moderately elevated in affected quarters when compared with those from healthy cows. In the three ratios, affected quarters showing NOG had slight increase while the greatest changes occurred in the GN group. Our results are in partial agreement with those from Gonçalves et al. (2017) where the cell ratio Log10 (NEU:LYM) 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 Log10 (PHAG:LYM) between healthy quarters (0.67) and quarters infected with miscellaneous (0.69), contagious (0.67), environmental (0.66), and minor pathogens (0.65).

Pathogens included in the GN group would normally produce acute inflammation (Wenz et al., 2001), thus resulting in high NEU percentages and very high NEU:LYM and PHAG:LYM ratios. However, in our study, low percentages of LYM can be attributed to this scenario and may be regarded as characteristic feature of this type of pathogen. On the other hand, the GP group had higher proportions of NEU and low MAC values, compared with the healthy quarters. Pathogens

in the OTH group normally result in chronic infections, and therefore, moderate higher levels of all the leukocyte are expected (Oviedo-Boyso et al., 2007). This peculiar behavior in GN, OTH, and GP pathogens may be a useful tool for orientating toward specific pathogen groups.

It is important to notice that we had a significant proportion of culture negative samples in our study. Shedding of too low numbers of pathogens or ceased growth may be reasons for negative bacteriological results. Although epithelial cells are also detected from fluorescence microscopy, their immunological function is not well established; therefore, we did not consider them for analysis. The number of cell fractions sampled also depends on milk sampled because cisternal milk shows a reduced NEU counts, compared with alveolar milk (Pilla et al., 2012). We used squirts of milk after discarding first few streams of the milk from the teats, and this could have altered our results.

Another limitation of the study was the use of pooled milk samples for microbiological culture. This is a cost-effective method that does not allow for quarter-level discrimination. To partially address this problem, we considered using the MLD values corresponding to greatest TLC quarter for culture positive animals and an average MLD of all four quarters for the culture negative animals. Although the procedure reduced the number of quarters used for the analyses, we are more confident about the values for each category.

Conclusion

Our results demonstrated significant differences in white cell proportions in different physiological and pathological stages of the lactating dairy cow, although the magnitudes of the differences were small. Altered MLD in multiple categories of TLC suggests its value in detecting mastitis of varying severity. Differentiable patterns in the changes in cell proportions and leukocyte ratios observed in the presence of specific pathogen groups may provide useful

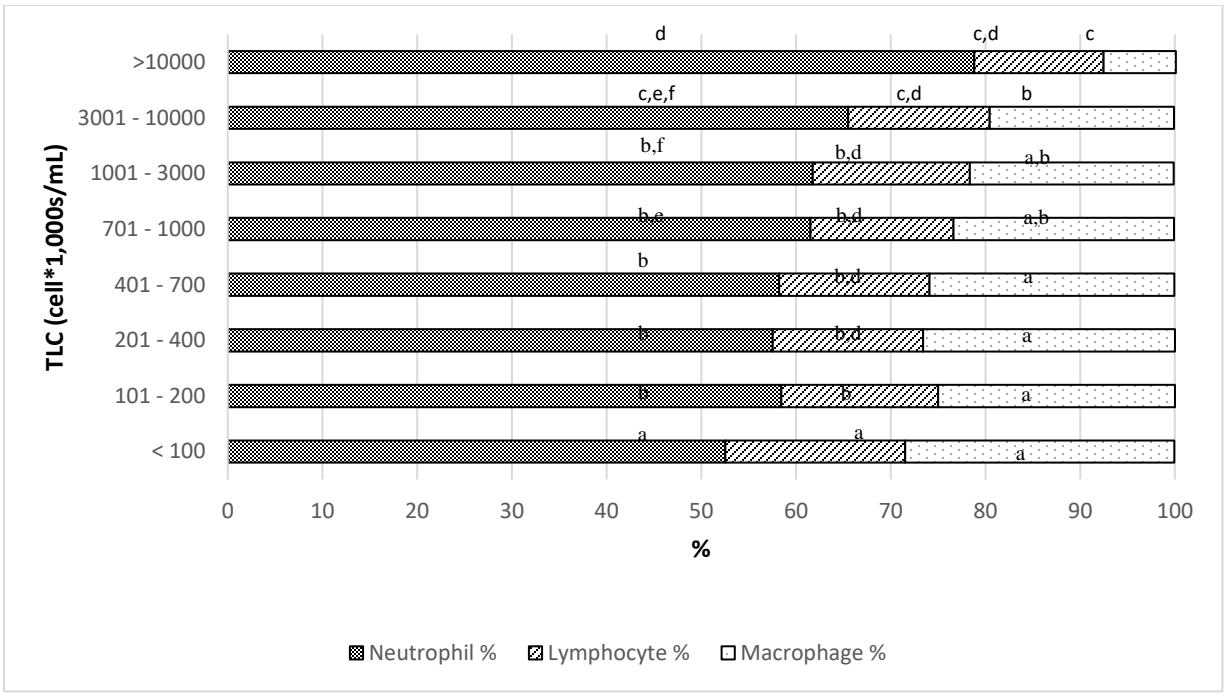
information for the identification of specific causal agent groups in mastitis cases. Additional research is necessary to determine the potential applications for this methodology.

Table 1.1. Milk leukocyte proportions (mean ± SE) and milk leukocyte ratios by quarter position (front vs. rear) in healthy (TLC ≤ 100,000) and affected quarters (n = 411 quarters)

Parameter	Front quarters	Rear quarters	P-value
TLC ≤ 100.000 (n= 151)			
Neutrophil (%)	51.4 ± 0.02	52.5 ± 0.02	0.50
Lymphocyte (%)	17.4 ± 0.02	19.1 ± 0.02	0.21
Macrophages (%)	30.1 ± 0.04	26.7 ± 0.03	0.11
Neutrophil:Lymphocyte ratio	3.47 ± 0.28	3.48 ± 0.27	0.99
Neutrophil:Macrophage ratio	1.84 ± 0.25	2.64 ± 0.24	0.01
Phagocyte:Lymphocyte ratio	5.78 ± 0.48	5.58 ± 0.46	0.75
TLC > 100.000 (n = 260)			
Neutrophil (%)	58.4 ± 0.017	62.4 ± 0.02	0.03
Lymphocyte (%)	14.6 ± 0.005	14.6 ± 0.01	0.99
Macrophages (%)	25.0 ± 0.02	21.5 ± 0.03	0.06
Neutrophil:Lymphocyte ratio	4.50 ± 0.17	4.62 ± 0.18	0.61
Neutrophil:Macrophage ratio	4.31 ± 0.35	4.50 ± 0.35	0.71
Phagocyte:Lymphocyte ratio	7.12 ± 0.33	6.59 ± 0.34	0.26

Table 1.2. Least square means (±SE) for total milk leukocyte counts, milk leukocyte proportions, and milk leukocyte ratios by group of mastitis-causing pathogen (n = 99 quarters)

Parameter	No growth	GN	GP	OTH	Group *P*-value	GP vs. GN *P*-value
TLC (cells*1000/ml)	263 ± 11.2	39,174 ± 1,245	1,290 ± 110	2,585 ± 481	<0.001	0.16
Neutrophil (%)	55.4 ± 0.05	52.7 ± 1.02	66.9 ± 0.05	69.0 ± 0.42	0.001	0.16
Lymphocyte (%)	16.5 ± 0.11	6.54 ± 2.18	13.4 ± 0.01	11.6 ± 0.08	<0.001	0.01
Macrophages (%)	26.9 ± 0.08	35.5 ± 1.85	18.3 ± 0.77	17.7 ± 0.62	0.03	0.12
Neutrophil:Lymphocyte ratio	3.68 ± 0.28	7.16 ± 1.25	5.16 ± 0.28	6.08 ± 0.80	<0.001	0.12
Neutrophil:Macrophage ratio	3.57 ± 0.66	6.86 ± 2.95	4.97 ± 0.66	7.68 ± 1.88	0.11	0.53
Phagocyte:Lymphocyte ratio	5.79 ± 0.59	23.6 ± 2.64	7.40 ± 0.59	7.93 ± 1.68	<0.001	<0.001



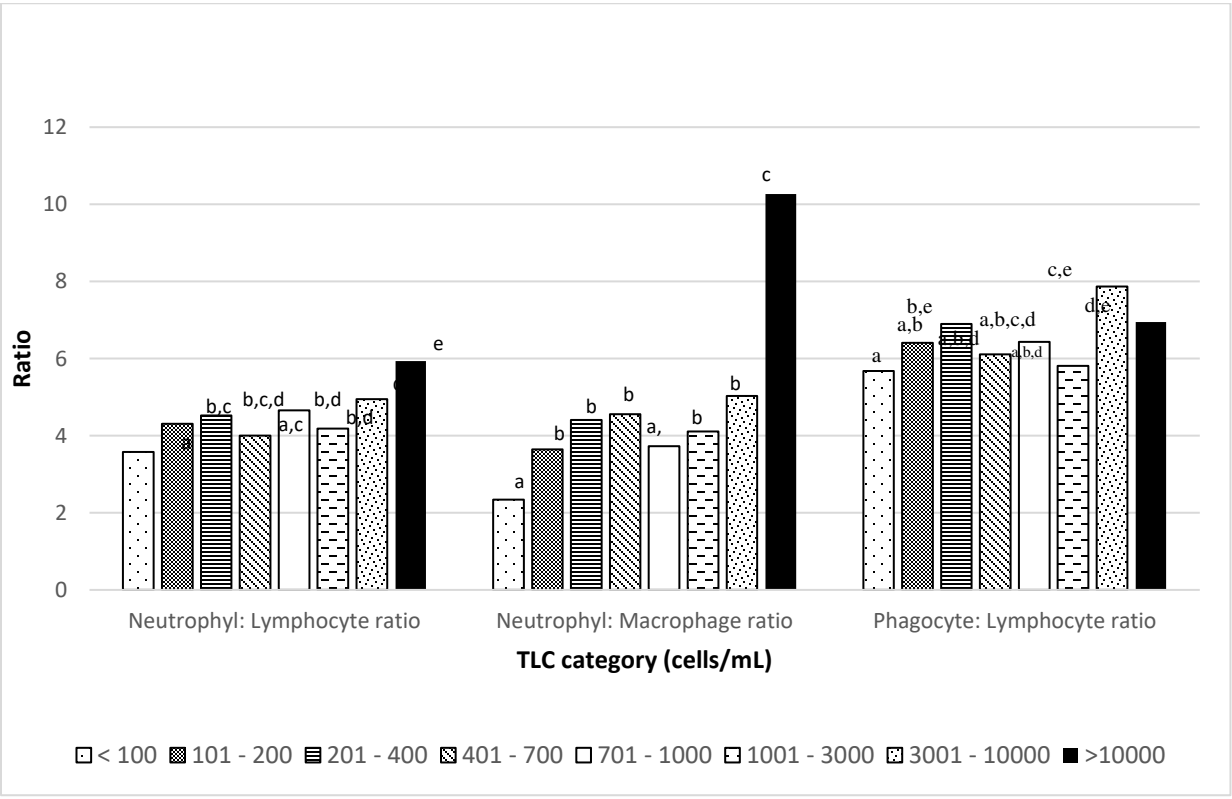
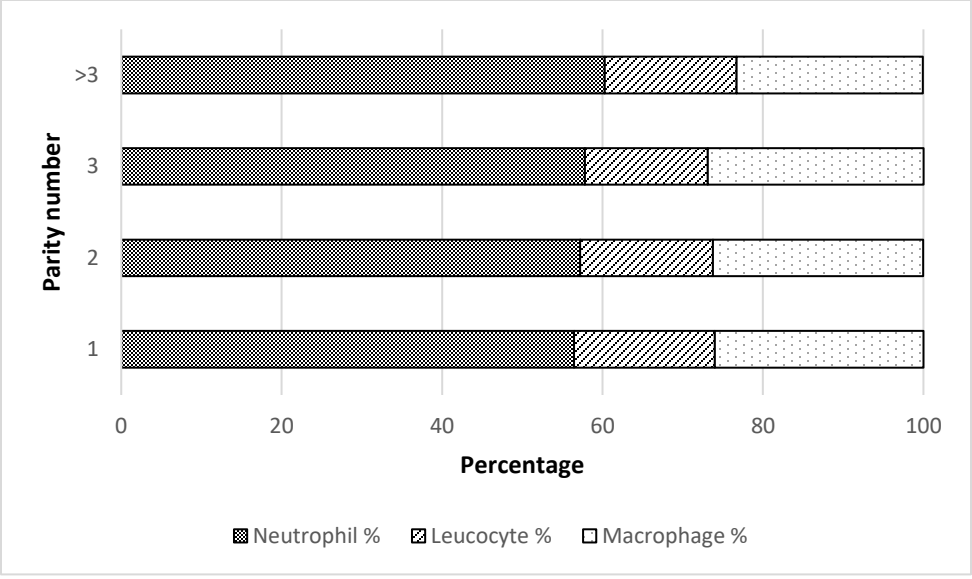


Figure 1.1. Milk leukocyte proportions (a) and milk leukocyte ratios (b) by category of total leukocyte count (TLC). Categories within same group (leukocyte type or leukocyte ratio) with different letters indicate statistically significant difference at $P < 0.05$



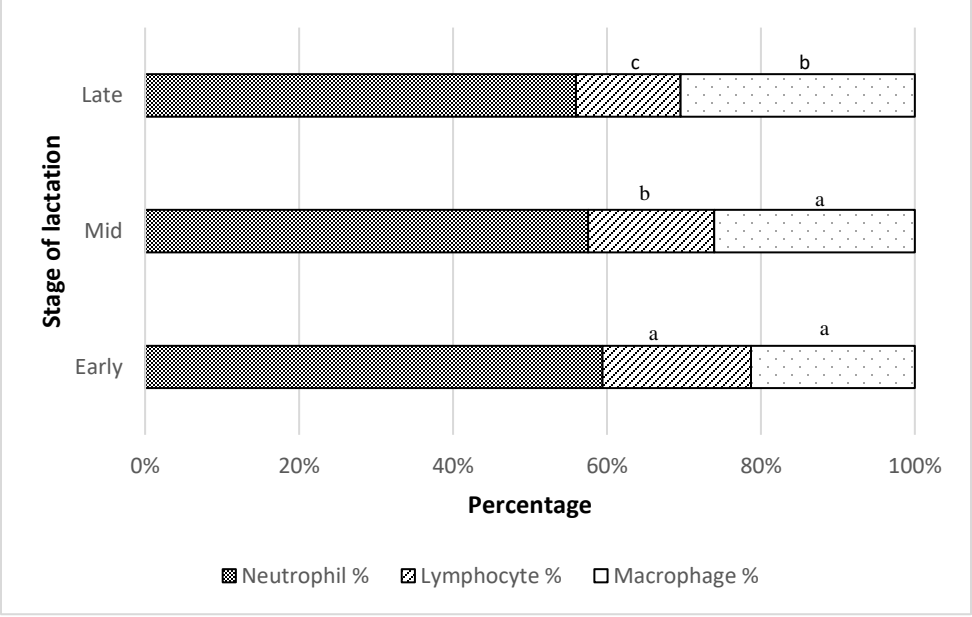
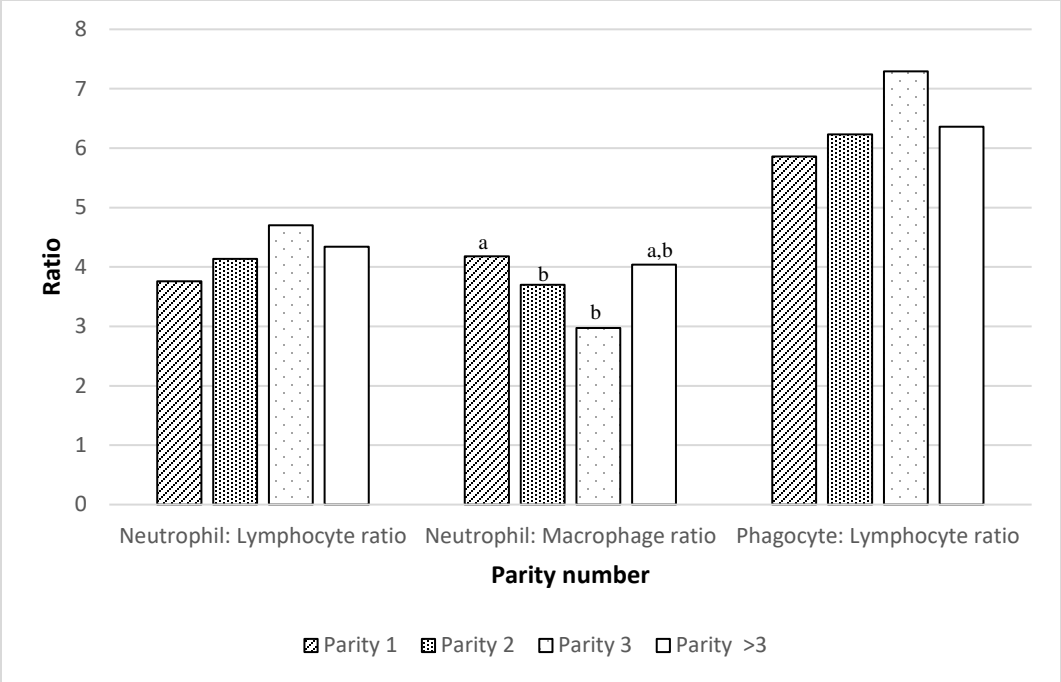


Figure 1.2. Milk leukocyte proportions by (a) parity number (1; 2; 3; >3) and (b) stage of lactation (early [<50 DIM], middle [50-250 DIM]; and late [>250 DIM]). Categories within same leukocyte type with different letters indicate statistically significant difference at $P < 0.05$.



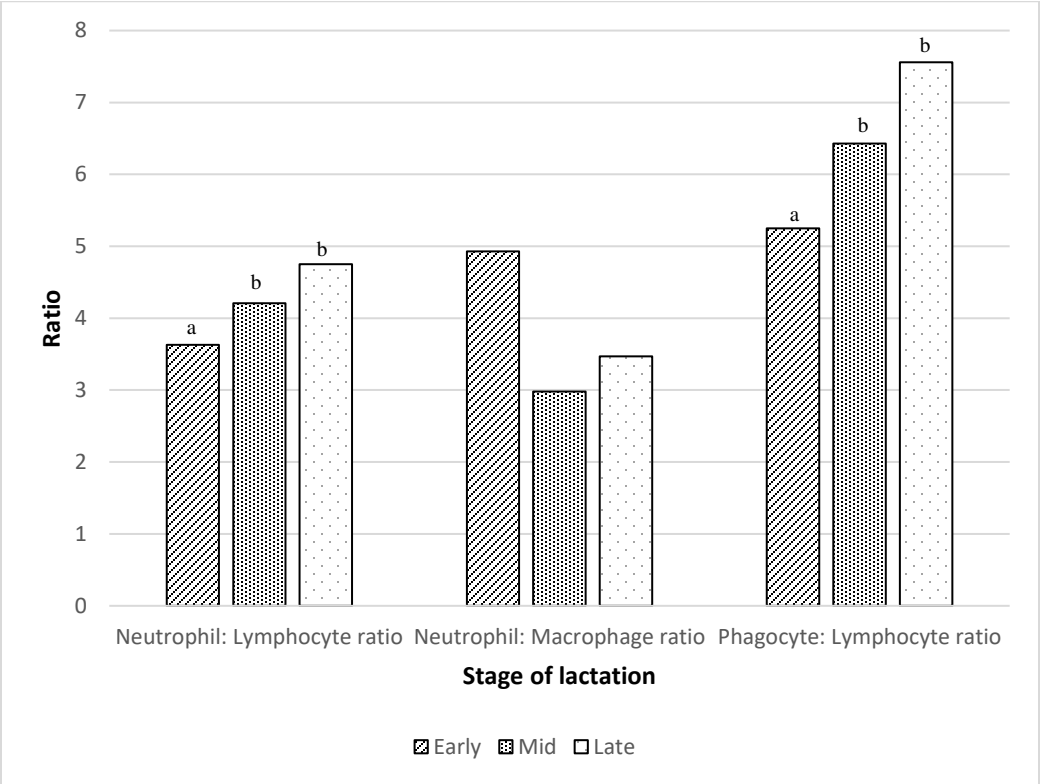
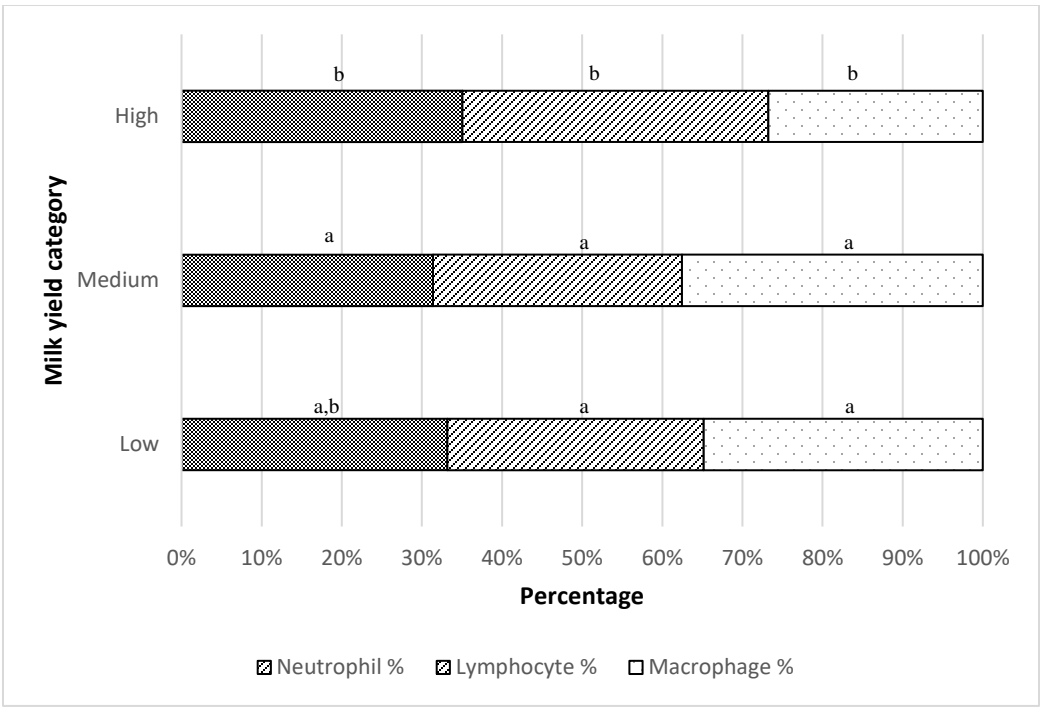


Figure 1.3. Milk leukocyte ratios by (a) parity number (1; 2; 3; >3) and (b) stage of lactation (early [<50 DIM], middle [50-250 DIM]; and late [>250 DIM]). Categories within same leukocyte ratio with different letters indicate statistically significant difference at $P < 0.05$



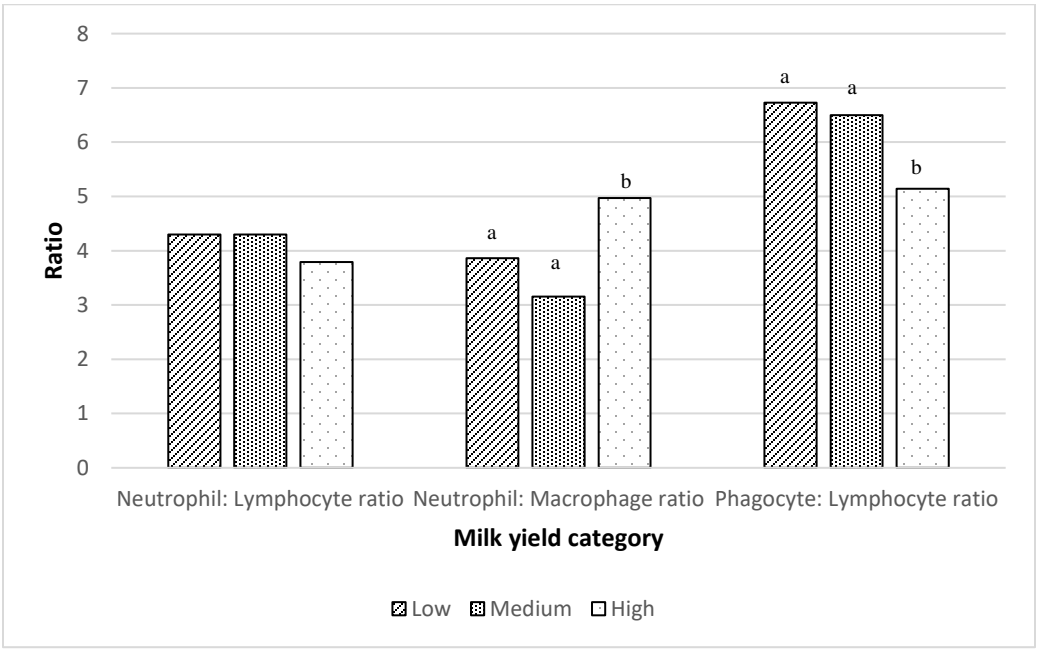


Figure 1.4. Milk leukocyte proportions (a) and milk leukocyte ratios (b) by milk yield category. Categories within same group (leukocyte type or leukocyte ratio) with different letters indicate statistically significant differences at $P < 0.05$. [1]Milk-yield categories: Low = <20 kg, medium = 20.1–29.0 kg, and High = >29.0 kg)

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CHAPTER 2 - USE OF MILK ELECTRICAL CONDUCTIVITY FOR THE DIFFERENTIATION OF MASTITIS CAUSING PATHOGENS

Introduction

Despite decades of control efforts, mastitis remains a high prevalence health disorder affecting animal wellbeing and profitability of dairy operations (Halasa et al., 2007; Payorala, S. 2003). Intramammary infection (IMI) results in changes in the composition of milk, including an increase in salinity levels. This is demonstrated by changes in electrical conductivity (EC) that is determined by the concentration of anions and cations, especially elevated concentrations of sodium and chloride ions (Kitchen B., 1981; Payorala et al., 2003).

In-line conductivity analyzers in milking systems monitor levels of ions in milk during the milking process (Maatje et al., 1992; Khatun et al., 2017) and combine measurements of EC and milk yield (MY) to improve the ability to detect mastitis (Fernando et al., 1982; Milner et al., 1996; Milner et al., 1997).

Timely information regarding the involvement of specific mastitis-causing pathogens during IMI has significant effects on the design of mastitis control programs and improves treatment outcomes (Lago et al., 2011). However, the cost and time requirements associated with milk culture, together with the risk for contamination and mishandling of samples limits this approach as a routine diagnostic procedure in commercial farms (Godden et al., 2017).

Although the use of milk EC on mastitis detection is widespread, there is scarce research on the potential of EC to identify mastitis pathogens causing IMI. Moreover, although milk EC is used to detect mastitis based on specific cutoff values (Nielen et al., 1995), the temporal dynamics of these deviations around the time of IMI detection by type of pathogen involved have not been extensively explored.

We hypothesized that specific temporal patterns of milk EC combined with other established parameters, such as MY changes, may be indicative of particular groups of pathogens involved in IMI. Therefore, our objective was to characterize the pattern of milk EC provided by an in-line mastitis detection system during IMI, considering specific mastitis-causing pathogen group involvement.

Materials and Methods

Animals and housing

All the animal related procedures in this study were reviewed and approved by the Institutional Animal Care and Use Committee at Colorado State University (Protocol ID: 16-6775A). The study population consisted of milking cows from two commercial farms located in Texas and Colorado, USA, milking 3,500 and 1,500 cows, respectively. Cows in both farms were housed in free stall barns with sand bedded stalls and subjected to 3 daily milkings in rotatory milking parlors equipped with Afimilk® milk analysis system (Afimilk Ltd., Kibbutz Afikim, Israel) that provided individual MY and milk EC for each milking. Animals in both farms did not receive any vaccinations against mastitis pathogens. Cows in the study with mastitis did not receive any antibiotic treatment within the 14 days of observation.

Experimental Procedures

A total of 400 lactating Holstein cows from both farms (100 cases and 100 controls from each farm) were enrolled in the study and monitored for milk EC (reported in milliSiemens [mS/cm]) and MY (kg) by the Afimilk® system. Cows identified as presenting high electrical conductivity (HEC) following the manufacturer's proprietary algorithm were enrolled as cases in the study. The HEC category included cows presenting a deviation in milk EC > 15% relative to their rolling average from the previous 7 corresponding milkings (same period of the day).

A sterile composite milk sample was collected from the 4 quarters from each of the study cows in a sterile tube using aseptic technique. Briefly, the teats were cleaned and disinfected using 70% alcohol in a cotton gauze. The first few streams of milk were discarded and 10 ml of foremilk was aseptically collected in a sterile tube as a composite sample from each of the 4 quarters. Samples were immediately refrigerated before transportation to a commercial laboratory for bacteriological culture and speciation.

All milk samples were submitted to and received by The Dairy Authority, LLC (Greely, CO, USA). Approximately 0.01 ml of each milk sample was inoculated using a disposable inoculation loop (Hardy Diagnostics, Santa Maria, CA, USA) onto blood agar plates containing 4% washed bovine blood (Quad Five, Ryegate, MT, USA) and 0.1% esculin (Sigma-Aldrich, St. Louis, MO, USA) and MacConkey agar plates (Oxoid, ThermoFisher Scientific, Waltham, MA, USA) and incubated aerobically at 37°C. Bacteriological growth was identified after 24 and 48 h of incubation according to National Mastitis Council (1999). Briefly, *Staphylococcus aureus* and *Staphylococcus* spp. were identified by hemolytic pattern and tube coagulase test. *Streptococcus* spp. was identified by a catalase test (Hydrogen Peroxide) and Gram stain (JorVet gram stain kit, Sigma-Aldrich). *Escherichia coli* and *Klebsiella* spp. were identified using morphologic characteristics of colonies on MacConkey agar, production of indole, motility, and utilization of citrate. Approximately 0.1 ml of each milk sample was also inoculated onto a Modified Eaton's Mycoplasma Agar (The Dairy Authority Labs) with a polyester swab (Hardy Diagnostics). Mycoplasma agar plates were incubated in an 8-10% CO2 incubator at 37˚C for 10 days. Mycoplasma agar plates were examined under a dissecting microscope at 3, 7 and 10 days.

One control (CON) cow, matching the stage of lactation, parity number, and housing pen was simultaneously selected for each case cow and a milk sample was collected. Bacteriological culture

results were grouped into four general categories: Gram positive (GP), Gram negative (GN), other (OTH), and no growth (NOG; Table 1).

Based on conductivity status and pathogen growth cows were categorized into 4 groups as presented by Goncalves et al. (2017): 1) Specific mastitis (n = 96; HEC and culture positive); 2) Non-Specific mastitis (n = 84; HEC and no growth in culture); 3) Latent mastitis (CON and culture positive; n = 57); and 4) Healthy (CON and NOG; n = 110).

Statistical Analysis

Repeated measures analyses were conducted using average daily EC data from 7 d before to 7 d after enrollment in SAS ver. 9.3. Mixed models using compound symmetry variance structure were created with EC as a dependent variable and treatment group, bacteriological culture category, EC status at d -1, and days relative to sample collection in the model as independent variables. In addition, stage of lactation (early [≤60 DIM], mid [61-200 DIM], and late [>200 DIM]) and parity groups (1, 2, 3, >3) were included in the models and tested for significance.

In general, depending on the analysis, the models were defined as follows:

$$Y_{ijklm} = \mu + Path_i + Day_n + Par_j + St_k + ECSt_l + Mast_m + e_{ijklm}$$

Where:

Y_{ijklm} = dependent variable (EC, MY)

μ = overall population mean

$Path_i$ = effect of pathogen category (NOG, GN, GP, or OTH)

Day_n = effect of day relative to sample collection (-7 d to 7 d)

Par_j = effect of parity number (1, 2, 3, or >3)

St_k = effect of stage of lactation (early, mid, or late)

$ECSt_l$ = effect of EC status at d -1 (HEC or CON)

$Mast_m$ = effect of mastitis category (healthy or specific or non-specific or latent)

e_{ijklm} = error term

For all the analyses, statistical significance was defined at P-value < 0.05.

Results and Discussion

A total of 400 lactating Holstein cows (100 cases and 100 controls from each farm) were enrolled in the study. Forty (10%) samples were considered contaminated due to multiple pathogens growth (>2 and/or not related to mastitis) and were removed from the study. A group of 13 animals, irrespective of pathogen growth and identification were removed from the analyses due to a pattern suggestive of chronic mastitis episodes, as determined by farm records. The 347 remaining cows were categorized in early lactation (n = 88), mid lactation (101), and late lactation (158). A total of 150, 99, 54, and 44 animals were first parity, second parity, third parity, and parity ≥ 4, respectively.

Average EC on d -1 was 12.5 ± 0.51 mS/cm in HEC and 10.8 ± 0.49 mS/cm in CON animals (P < 0.0001; Table 2). Average milk yield on the day of identification was greater in CON than on the HEC (37.6 ± 5.12 kg vs. 33.5 ± 5.19 kg; P = 0.003). However, animals selected in the two groups were not different in terms of DIM and parity.

Pathogens identified by milk culture included: Coagulase Negative *Staphylococcus aureus* (n = 52), *Pseudomonas* spp. (33), *Corynebacterium* spp. (28), *Streptococcus* spp. (16), *Escherichia coli* (5), *Staphylococcus aureus* (5), *Mycoplasma* spp. (4), *Trueperella pyogenes* (4) *Prototheca* spp. (2), *Acinetobacter* spp. (2), and *Pasteurella* spp. (1). Consequently, the remaining 347 samples were classified as GP (n = 105), GN (41), OTH (7), and NOG (194).

Average EC on d -1 were similar for the different bacteriological culture categories: 11.4 ± 0.57 mS/cm, 11.7 ± 0.52 mS/cm, 12.3 ± 0.82 mS/cm, and 11.7 ± 0.51 mS/cm in GN, GP, OTH, and NOG, respectively ($P > 0.05$). Average milk yield on d -1 in GN, GP, OTH and NOG were 36.2 ± 5.65 kg, 36.2 ± 5.33 kg, 45.6 ± 7.52 kg, and 35.0 ± 5.23 kg, respectively ($P > 0.05$).

Parity number was only associated with EC on d -1 in HEC cows ($P = 0.001$); for animals in this category the greatest EC was observed in parity 3 (12.3 ± 0.25 mS/cm), followed by parity 2 (11.9 ± 0.21 mS/cm), parity >3 (11.6 ± 0.46 mS/cm), and primiparous cows (11.2 ± 0.2 mS/cm). Milk EC in CON animals was similar for different parity numbers (Figure 1).

Stage of lactation was associated with EC on d -1 ($P < 0.05$). Overall, cows in mid-lactation had lower EC than animals in early and late lactation (11.3 ± 0.17 mS/cm, 10.6 ± 0.16 mS/cm, and 11.1 ± 0.13 mS/cm, respectively). For animals in HEC, EC was smaller in mid lactation (11.3 ± 0.23 mS/cm) than in early (12.4 ± 0.21 mS/cm) and late-lactation (12.0 ± 0.18 mS; $P < 0.05$). On the other hand, in CON animals EC was not associated with stage of lactation (Figure 2). Milk EC and milk yield on d -1 were not associated with pathogen group (Figure 3).

In cows categorized as healthy, average daily milk EC remained similar (about 10 mS/cm) during all the monitoring period (Figure 4). However, MY increased in the days subsequent to sampling ($P < 0.0001$).

In mastitis cases categorized as specific (HEC and culture positive), a significant interaction effect on EC for day by bacteriological category was observed ($P = 0.029$; Figure 5). On d -1 EC was greater in GP compared with GN (11.4 ± 0.29 mS/cm vs. 10.8 ± 0.34 mS/cm; $P = 0.03$). In GP, milk EC on day -1 was the highest for the whole period (±7 d). For GN cultures, EC values were significantly higher throughout the entire monitoring period as compared to d -1, with exception of d5. In OTH cows, no specific trend across days was identified.

Interestingly, a significant interaction effect was also observed for day by pathogen category (GP vs. GN) for daily milk yield ($P < 0.0001$; Figure 5). Average MY remained constantly smaller in GP than in GN animals before and after sampling. Milk yield on d -1 was significantly smaller in GP than in GN (30.3 ± 1.02 kg vs. 34.1 ± 1.6 kg, respectively; $P = 0.04$). In GP cows, milk yield on d -1 was different from that of d -7, -6, -5, -3, -2, 2, 3, 4, 5, 6, and 7. However, for GN pathogens no statistical difference in MY across days was observed.

In the non-specific mastitis category cases (HEC and culture negative), EC was elevated during the entire study period, as compared to the healthy group. Milk EC on day -1 was the greatest ($P < 0.05$; Figure 6). Milk yield in this mastitis category was the lowest when compared with other mastitis categories and MY at days 2, 5, and 6 after sampling was greater than MY at d -1 ($P < 0.05$).

For animals in the latent mastitis category (positive culture and not flagged), EC and milk yield did not demonstrate statistical differences across days when compared with d -1 (Figure 7). A constant EC and undulating milk yield was observed which failed to provide specific pattern of EC or MY for this category.

Discussion

We evaluated the association of temporal dynamics of EC and different groups of mastitis pathogens in four different mastitis event categories. In addition, potential associations between EC and stages of lactation and parity number were evaluated. Previous reports concluded that 15% increase in conductivity from the rolling average was an optimum cut point for mastitis detection (Maatje et al., 1992; Khatun et al., 2017), which is in agreement with the algorithm used to determine cases for this experiment. The average conductivity obtained for healthy animals in this study (10.8 mS/cm) was similar to that reported by Gaspardy et al. (2012) who observed the

average EC to be 9.4 mS/cm. Milk EC for the associated pathogen groups in affected cows in this study (12.5 mS/cm) was greater than that of previous reports indicating that EC in infected quarters was greater than 7.2 mS/cm (Maatje et al., 1992) and greater than 5.6 mS/cm (Hillerton et al, 1991).

Woolford et al (1998) reported that parity and stage of lactation were associated with different levels of EC, which was supported by our study, where we observed that EC was greater in early and late lactation. This finding could be attributed to variable solids content in milk across lactation stages (Auldist et al.,1995). However, when the total population was categorized into HEC and CON, the statistical difference was only observed among HEC animals, suggesting that affected animals with high EC may behave differently through the lactation. In addition, we observed that in CON animals parity number was not associated with EC. This result is supported by Janzekovic et al. (2009) who observed non-significant differences in EC among parity groups, with 6.0 mS/cm, 6.4 mS/cm and 6.3 mS/cm in < 2; 3rd and 4th; ≥ 5 lactation, respectively. Contrary, in HEC animals second and third parity had elevated levels of EC, which may be related to higher susceptibility to mastitis in older cows, as observed by Woolford et al (1998). Thus, differences in parity and stage of lactation should be accounted for in mastitis detection models that are based on EC.

We observed that animals in the OTH category demonstrated an inconsistent behavior on the day of identification (d -1); although they had high EC, MY was also observed to be high, which may be due to the small number of animals in this group, indicating that the results are not a representation of the actual behavior of this category.

We detected pathogen growth in both HEC and CON groups, which was a limitation for making a clear identification of the affected cows. Therefore, based on the report by Goncalves et al. (2017), we further categorized the animals in this study into healthy or affected by specific mastitis events, non-specific mastitis, and by latent mastitis. As expected, in animals classified in the healthy category EC was constantly lower and exhibiting minor deviations.

As stated by Hass et al. (2005) the effect and severity of IMI depends on the specific causal pathogens and differences in the recovery patterns should be expected among the pathogen group. Milner et al. (1996) infused the mammary gland with different pathogens and, based on EC, they were able to detect *S. aureus* two milkings prior to the appearance of clots. Remarkably, *Streptococcus uberis* was not detected based on EC. In a different report quarters with *S. aureus* and S. *agalatiae* had lower conductivity values than quarters infected with environmental *Streptococci* (Mansfeld et al., 2001).

Similar variation by pathogen group was observed in animals with specific mastitis events in our study. Gram positive pathogens demonstrated the most notorious EC deviations across time, with greatest increments in EC and greatest reductions in MY on the day of identification. Contrary, GN pathogens evidenced both a small increase in EC and a small decrease in milk yield on the day of detection.

Hassan et al. (2009) used neural networks to detect mastitis categories including minor and major mastitis causing pathogens and observed that SCC and electrical resistance had the greatest discriminating power. However, in a separate study Kamphuis et al. (2011) failed to provide evidence to predict the Gram classification of clinical mastitis causal pathogens when EC values were used for only one day, suggesting that considering the temporal pattern of the EC, as opposed to the values for a single day, may be beneficial. In agreement, results from our study indicate that

the temporal pattern of milk yield and EC from 7 day before identification may be suggestive of the pathogen involved, which may be a suitable tool in developing algorithms for detection of mastitis pathogens.

The non-specific mastitis category also demonstrated increased EC and decreased MY around d -1. Non-specific mastitis may be the result of injury or environmental conditions, where no pathogens are involved. Mild infections in which the concentration of the pathogen load in the sample is not high enough for the detection of pathogen growth could also fall in this category. However, considering these mastitis events in control programs is relevant, as they are associated with significant drop in milk yield.

The latent mastitis events demonstrated no abnormal EC deviations in spite of being associated with positive bacteriological cultures and the presence of undulating milk yield. These animals may be in very early stages of infection, where pathogens have not yet caused a significant destruction of epithelial membranes to demonstrate increased EC levels. Interestingly, these cases would escape detection by use of EC and consequently, other parameters would be required in a mastitis control program to address this condition.

Woolford et al. (1998) concluded that MY and EC differed between consecutive milkings, which could affect their use in mastitis detection. Therefore, to account for this variation, average conductivity for the day and total daily MY were used in our study. In addition, we acknowledge that IMI by specific pathogen species results in diverse pathophysiologic events and combining microorganisms in groups may confound their individual behaviors. Another limitation of our study is that animals in a recovery phase from previous infection may have higher conductivity even without the presence of a viable pathogen. In addition, pathogen growth may not have been detected, even in an infected quarter. We partially addressed these issues by removing cows

presenting clinical cases of mastitis within the previous 20 days and using a separate category of non-specific mastitis to study these events.

Conclusion

Characteristic temporal patterns in EC and MY in particular pathogen groups may provide indications for differentiation of groups of mastitis-causing microorganisms. Further research to build detection models using EC, MY, and cow level factors are required for accurate differentiation of mastitis pathogens.

Table 2.1: Distribution of study cows by milk bacteriological culture categories and conductivity status on the day of identification (d -1). [1]HEC = High electrical conductivity cows (n=180) identified by the system with positive deviation >15% in the manufacturer's proprietary algorithm. Control (CON) cows within normal ranges for EC, matched to each case.

	HEC[1]	Control	Total
Gram positive bacteria; n (%)	66(63%)	39(37%)	105
Coagulase Negative *Staphylococcus aureus*	32(62%)	20(38%)	52
***Corynebacterium* sp.**	17(61%)	11(39%)	28
Staphylococcus aureus	2(40%)	3(60%)	5
Streptococcus sp.	11(69%)	5(31%)	16
Trueperella pyogenes	4(100%)	0(0%)	4
Gram negative bacteria	25(61%)	16(39%)	41
***Acinetobacter* sp.**	1(50%)	1(50%)	2
***Escherichia*. coli**	5(100%)	0(0%)	5
***Pasteurella* sp.**	0(0%)	1(100%)	1
***Pseudomona* sp.**	19(58%)	14(42%)	33
OTH pathogens	5(71%)	2(29%)	7
***Mycoplasma* sp.**	3(75%)	1(25%)	4
***Prototheca* sp.**	1(50%)	1(50%)	2
Yeast	1(100%)	0(0%)	1
No growth	110(57%)	84(43%)	194
Total	180(52%)	167(48%)	347

Table 2.2: Descriptive statistics (average ± standard error) for multiple cow parameters by EC categorization on the day of identification (d -1). [1]HEC = High electrical conductivity cows (n=180) identified by the system with positive deviation >15% in the manufacturer's proprietary algorithm. Control (CON) cows (n=167) within normal ranges for EC, matched to each case.

	HEC[1] (n = 180)	**Control (n = 167)**	**P-value**
Electrical conductivity (mS)	12.54±0.5	10.81±0.49	<0.0001
DIM	219.14± 28.5	210.09± 28.48	0.55
Parity number	2.29±0.09	2.04±0.09	0.07
Milk yield (kg)	33.47±5.2	37.61±5.12	0.003

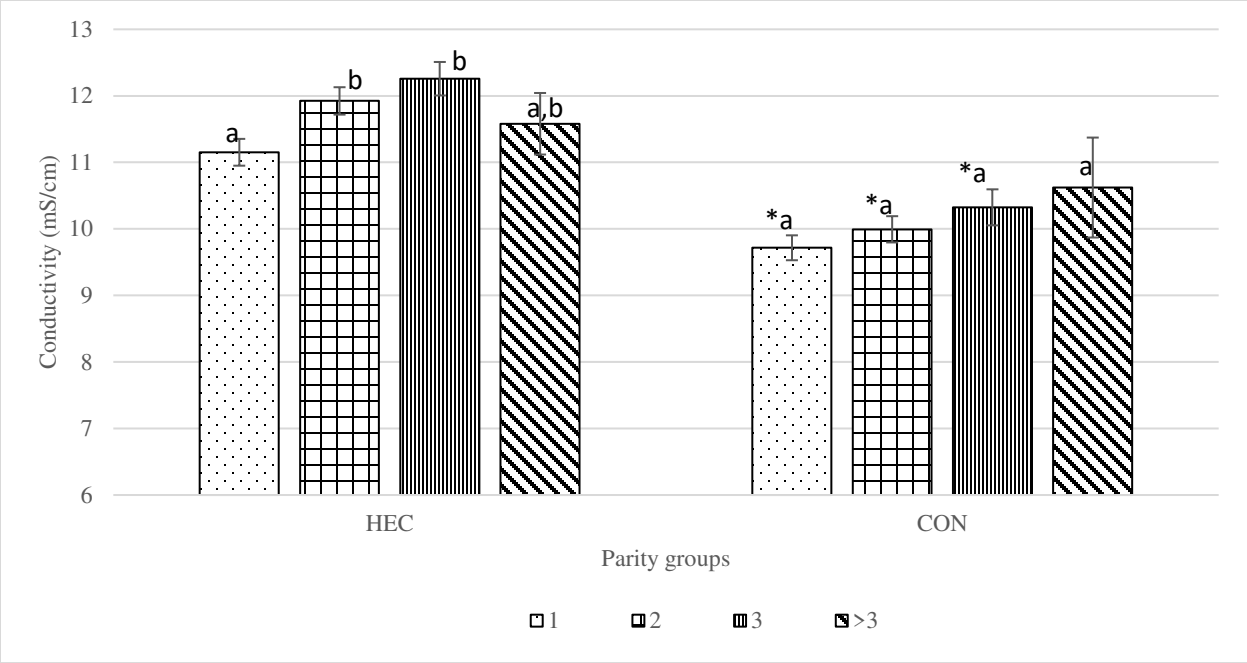

Figure 2.1. Average (SE) electrical conductivity in HEC and CON cows on the day of identification (d -1) by parity group. Different letters within group indicate statistical differences (P-value < 0.05). Asterisk indicate significant differences between groups. HEC = High electrical conductivity cows (n = 180) identified by the system with positive deviation >15% in the manufacturer's proprietary algorithm. Control (CON) cows (n = 167) within normal ranges for EC, matched to each case.

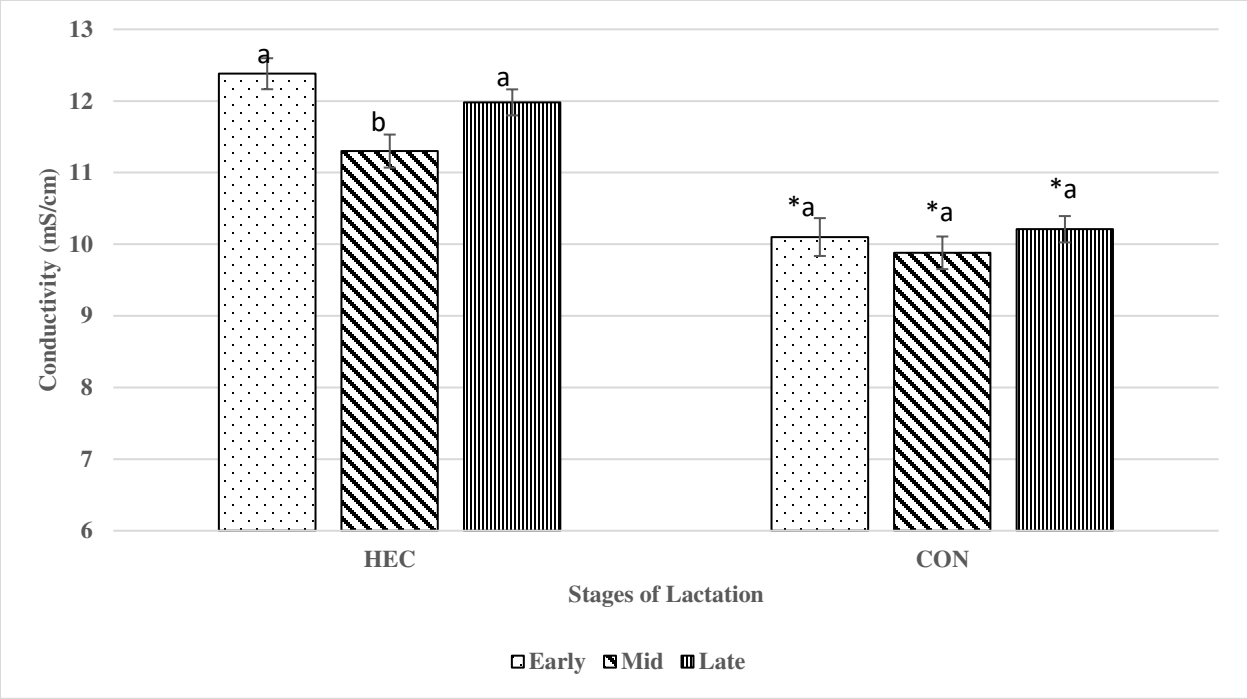

Figure 2.2. Average (SE) electrical conductivity in HEC and CON cows on the day of identification (d -1) by stage of lactation[2]. Different letters within group indicate statistical differences (P-value < 0.05). Asterisks indicate significant differences between groups. HEC = High electrical conductivity cows (n=180) identified by the system with positive deviation >15% in the manufacturer's proprietary algorithm. Control (CON: n= 167) cows within normal ranges for EC, matched to each case. Stage of lactation = Early (DIM ≤ 60; n = 88); mid (DIM = 61-200; n=101); and late (DIM > 200; n=158).

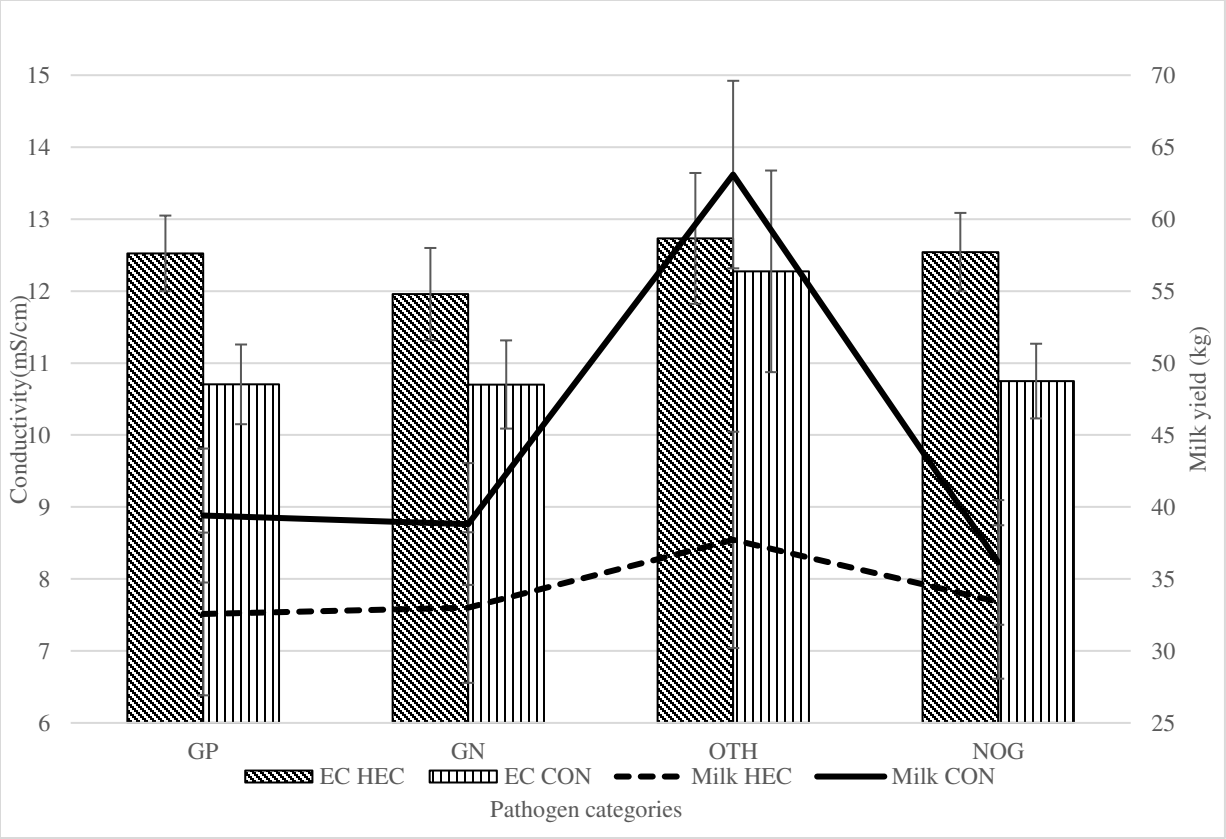

Figure 2.3. Average (SE) electrical conductivity and milk yield in HEC and CON[1] cows on the day of identification (d -1) by pathogen group. [1]HEC = High electrical conductivity cows (n=180) identified by the system with positive deviation >15% in the manufacturer's proprietary algorithm. Control (CON) cows within normal ranges for EC, matched to each case. GP = gram positive; GN = gram negative; OTH = other pathogen; NOG = No growth.

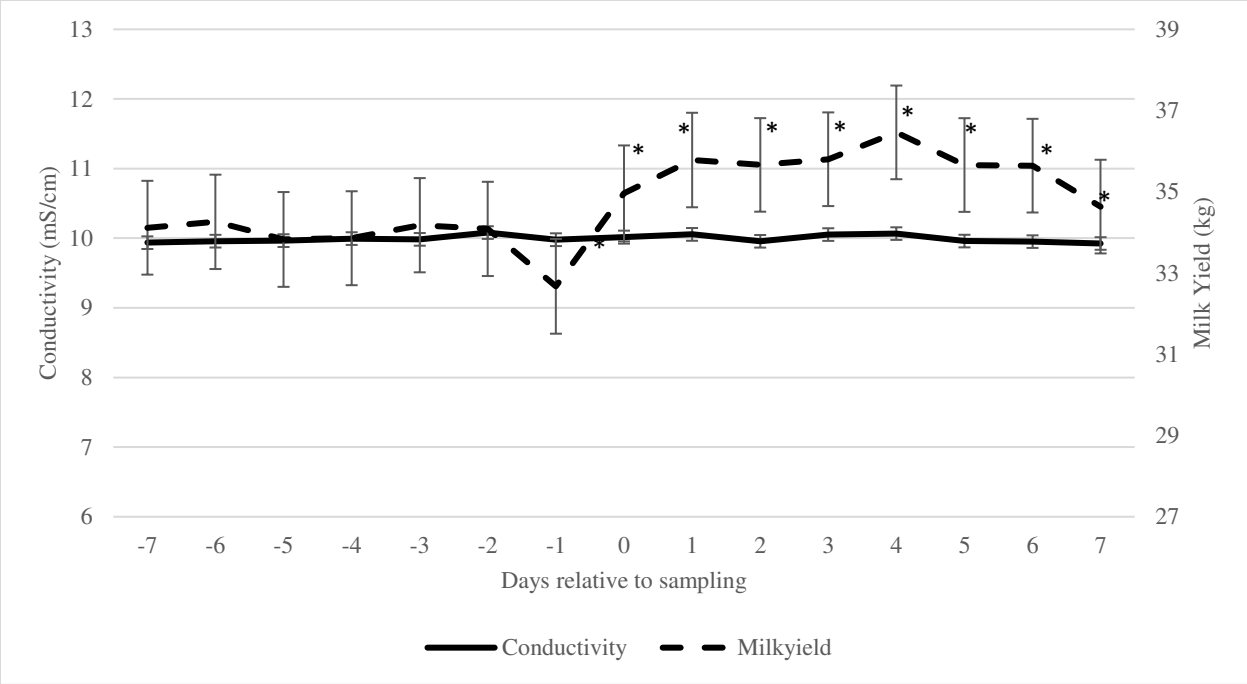

Figure 2.4. Average (SE) milk yield (broken line) and conductivity (solid line) in healthy cows from d -7 to d 7 relative to sampling. Similar symbols (*) across time points within each parameter indicate significant difference from day -1.

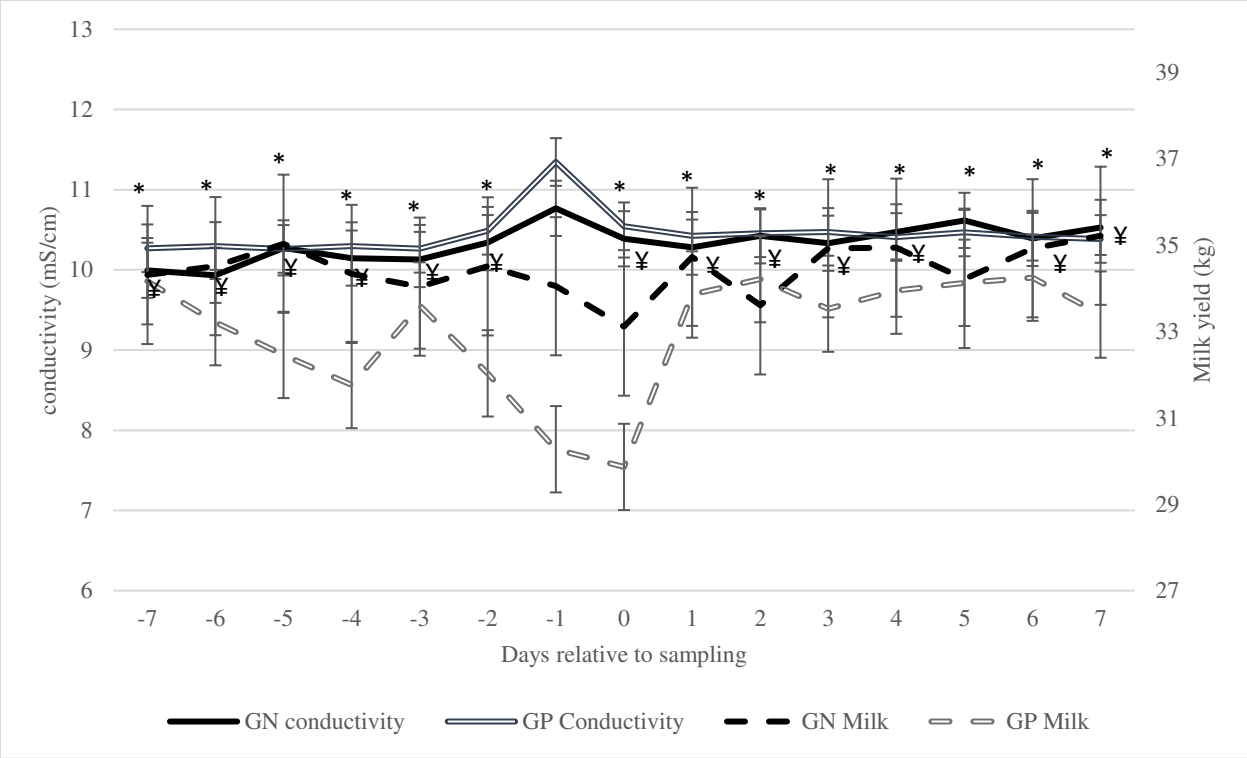

Figure 2.5. Average (SE) milk yield (d^{-1}) and conductivity in specific mastitis (high EC and culture positive) from d -7 to d 7 relative to sampling. Similar symbols across time points within each parameter indicate significant difference from day -1.

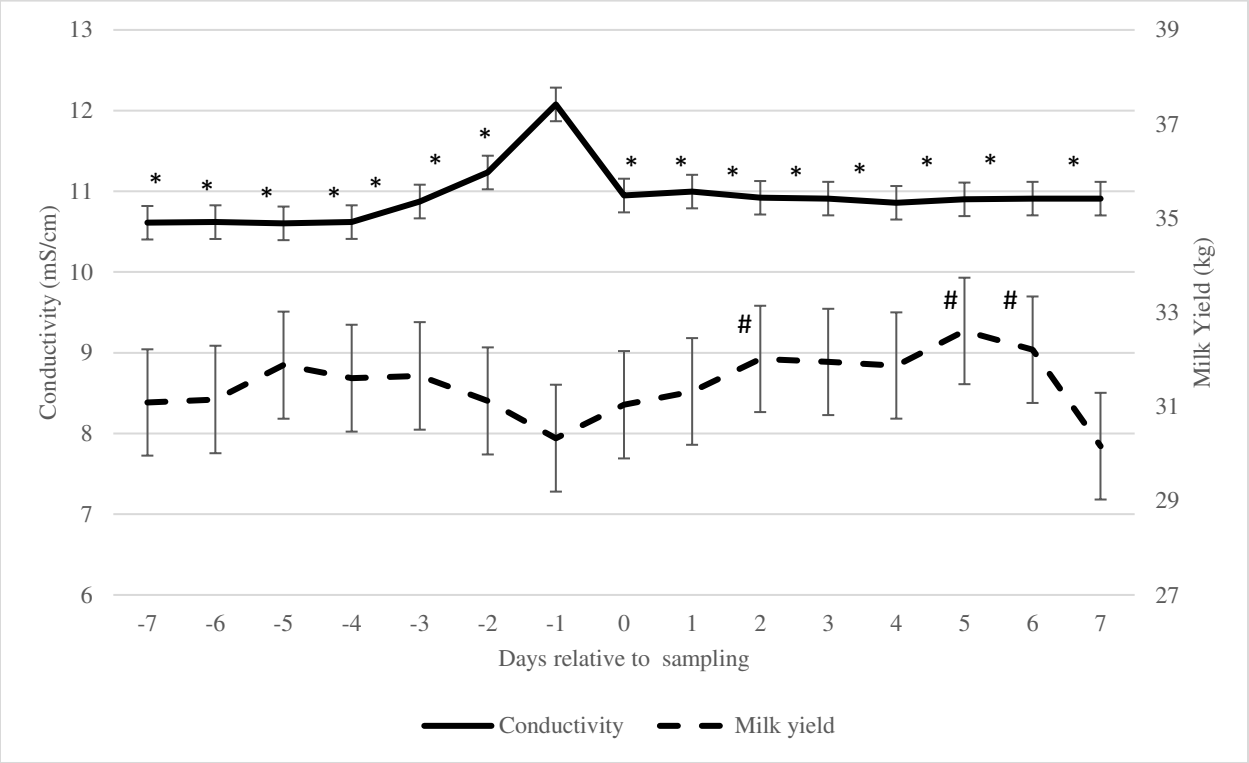

Figure 2.6. Average (SE) milk yield (d-1; broken line) and conductivity (solid line) in non-specific mastitis cases from d -7 to d 7 relative to sampling. Similar symbols across time points within each parameter indicate significant difference from day -1.

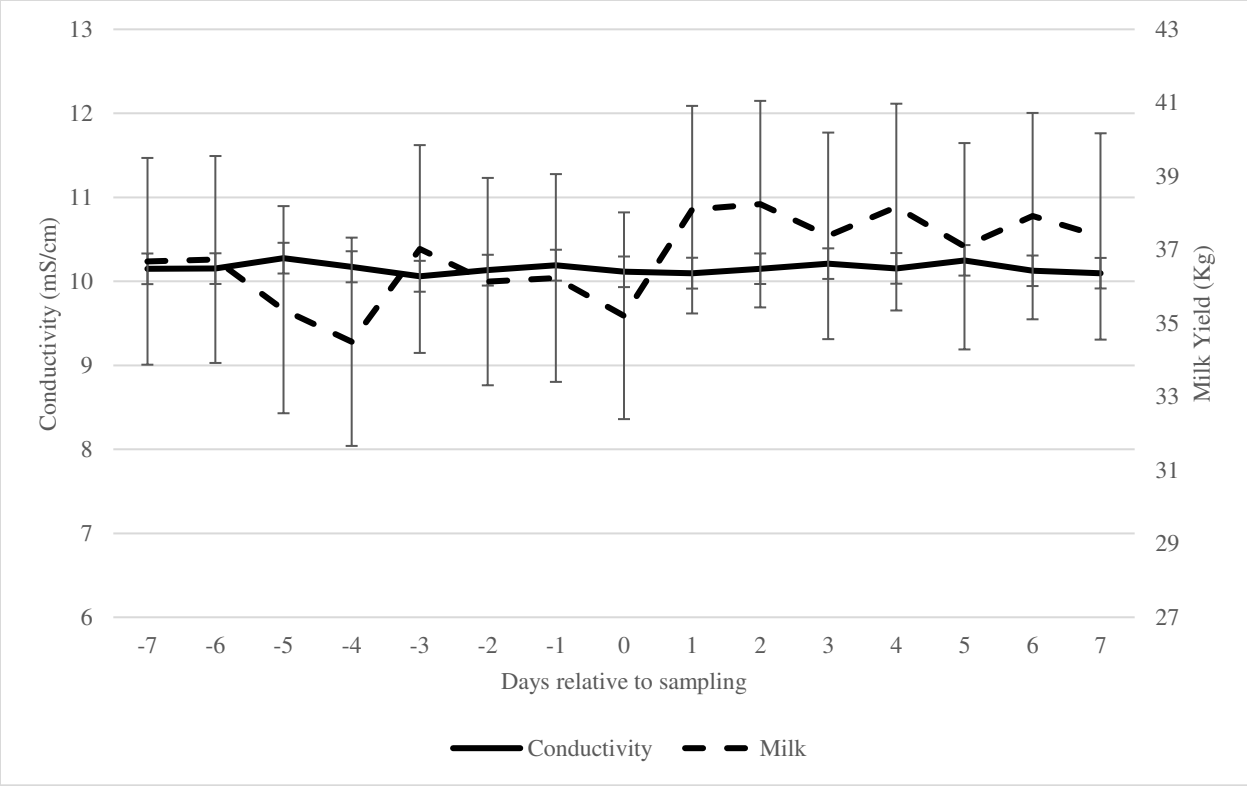

Figure 2.7. Average (SE) milk yield (d^{-1}; broken line) and conductivity (solid line) in latent mastitis cases from d -7 to d 7 relative to sampling. Similar symbols across time points within each parameter indicate significant difference from day -1.

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CHAPTER 3 – EVALUATION OF MILK COMPONENTS RATIOS FOR MONITORING OF HEALTH DISORDERS DURING EARLY LACTATION

Introduction

Monitoring of health disorders during early lactation is a key component in the management of dairy farms. Precision dairy technologies, including daily milk yield recording and milk component monitoring, are already in use in dairy farms and provide opportunity for continuous herd health monitoring. Modern milking systems allow for constant monitoring of milk yield and components in individual cows helping to detect disease at an early stage, before farmers are able to visually identify clinical signs, avoiding the substantial loss in production and health. These inline methods of measuring milk components have been proposed to replace the labor intensive, time consuming, and expensive laboratory methods (Kaniyamattam and De Vries, 2014).

As feed intake is reduced during disease, a shortage of available energy followed by fat mobilization will result in increased fat and decreased protein content in milk (Bauman and Griinari, 2003; Friggens et al., 2007). The fat content increases due to increased amount of free fatty acids in blood, while decreased protein results from a delay in the protein production process due to shortage of energy (Bauman and Griinari, 2003). There is a decrease in milk lactose percentage in animals with health disorders, which can be attributed to reduced feed intake and rumen function leading to an insufficient level of energy substrates absorbed by the rumen. This ultimately results in a lack of blood glucose for lactose synthesis (Schmidt GH., 1966). Because of this antidromic behavior of individual milk components, ratio of these milk components are suggested as potential indicator of health status in dairy cows (Duffield et al., 1997; Heuer et al., 1999; Toni et al., 2011).

The ratios of milk components (fat to protein ratio (FPR), fat to lactose ratio (FLR), and protein to lactose ratio (PLR) have been considered better indicators of health disorders than the individual parameters used alone (Grieve et al., 1986; Reist et al., 2002). Fat to protein ratio is conventionally considered indicator of energy balance and therefore has been used as tool for monitoring energy balance in the feed ration (Grieve et al., 1986, Duffield et al., 1997, Heuer et al., 1999). Fat to protein ratio is observed to be helpful to detect ketosis (Duffield et al., 1997), displaced abomasum (Geishauser et al, 1998), and has prognostic value for retained placenta, metritis, lameness, mastitis, ovarian cysts and clinical endometritis (Geishauser et al, 1998, Heuer et al., 1999, and Toni et al., 2011). Fat to lactose ratio has also been used to detect animals in negative energy balance (Reist et al., 2002) and rumen acidosis (Kirchmann et al., 2016) with satisfactory efficiency. Despite multiple research reports on the usefulness of these parameters, sufficient interpretation of these data as a guide for herd health management has been limited (Hamann and Kormker, 1997).

Therefore, our hypothesis was that ratios of milk components are affected by health disorders in a magnitude that is large enough to be detected by the use of mathematical algorithms. Therefore, the objective of the study was to evaluate the potential of milk fat to protein (FPR), milk protein to lactose (PLR), and milk fat to lactose (FLR) ratios for detection of disease prior to evident clinical signs. In addition, the performance of two proposed indexes, based on changes in ratio of components were evaluated.

Materials and Methods

Climatic data

Weather variables were obtained from Weather Underground® at a station near the dairy unit. Daily THI values during the study period ranged from 41.0 units to 81.7 units. The highest

THI was recorded in August and the lowest in January. During the overall study period, daily THI were higher than 76.2 for 97 days. The monthly average THI was higher than 76.2 during 4 months of the study period (June, July, August, and September).

Study animals and management

This study was conducted at the University of Florida Dairy Unit (Gainesville, FL, USA) with the approval of the Institutional Animal Care and Use Committee from University of Florida. The study population was the same population presented in Paudyal et al. (2018) that consisted of 210 multiparous Holstein cows enrolled at 21 day before estimated due date from November 2013 to August 2014. The Dairy Unit milked about 500 Holstein cows twice daily, with a rolling herd average of 10,000 kg/cow. Details on animals and management procedures are reported in a previous study (Paudyal et al., 2018). In brief, cows were moved out of the maternity pen within 3h after calving and housed in a hospital barn from day 0 to 2 postpartum. Cows in the hospital were fed the postpartum total mixed ration. At 3 days postpartum, healthy cows were moved to the main lactating herd and kept in 2-row sand-bedded freestall barns equipped with head locks. Freestall barns were also equipped with fans with misters and sprinklers over the feed bunk. Stocking density in the freestall barns that housed early lactation cows ranged from 80% to 92%.

Milk components monitoring

A real time individual cow milk analyzer manufactured by the AfiLab (AfiMilk Inc, Afikim, Israel) was installed per parlor stall. AfiLab uses the principles of near-infrared spectroscopy (NIR) for online milk analysis based on light scattering principles as described by Tenskova et al. (1999). The advantages of NIR system over other systems were that results were quickly available and were determined using non-destructive methods of on-line measurements. Afilab system provided observations of milk fat, protein and lactose and amount of blood in the

milk per cow per milking and were automatically stored in Afifarm software. Accuracy estimates of Afilab were in the range of 2 to 6% fat and 2 to 5% protein (Kaniyamattam et al, 2014) but the accuracy is not available for range of lactose. During milking, the Afilab equipment measured milk components per 200 ml of milk flowing through the machine and provided results as an average of about 70 observations per cow per milking. Each Afilab unit was set to zero (calibrated) monthly and remotely by Afimilk using DHIA milk test as part of routine maintenance to fix internal problems and to eliminate bias in the farm (Kaniyamattam et al, 2014). Milk components after 72 hours of calving was used for the analysis to avoid altered components of colostrum.

Herd health monitoring

Herd health was monitored by the University of Florida veterinarians and trained farm staff. All health events including calving-related events such as, dystocia and delivering twins, were recorded by farm personnel. According to the farm standard operating procedures, all cows received a routine postpartum health evaluation at days 4, 7 and 12 after calving. This evaluation was performed immediately after the morning milking and included assessment of attitude (stance/posture, how the cow holds her head, and alertness), rectal temperature, rectal palpation and examination of vaginal discharge, udder inspection, assessment of urine ketone bodies (Ketostix, Bayer Corporation, Elkhart, IN, USA) and investigation of displacement of the abomasum. In addition, automatic health reports were created for every milking event based on individual milk production and milk component levels provided by the AfiFarm system. Specific health disorders considered in our analyses included mastitis, metritis, clinical hypocalcemia, digestive conditions, lameness and clinical ketosis, and were assessed until 60 days in milk (DIM). The definite diagnosis for the diseases in study was made by UF veterinarians or trained farm personnel based on the Dairy Unit standard operating procedure definitions developed at the

University of Florida, College of Veterinary Medicine. In brief, these disorders were defined as follows: Mastitis [MAS], presence of significant flakes and clumps in milk, udder quarter may be hot, swollen, hard and painful with watery or serous secretion; metritis [MET], cows off feed, depressed, presenting watery, fetid or non-fetid vaginal discharge that is dark red to brown in color within 21 DIM; clinical hypocalcemia [HYC], down cow or cow unsteady before calving to 1 or 2 days after calving with no other abnormal physical exam findings (especially MAS or MET) head may be turned into the flank or may be extended, ears are cold and the nose is dry; digestive conditions [DIG] included indigestion and diarrhea; lameness [LAM], presence of arched back or favoring one leg or abnormal gait; and clinical ketosis [KET], negative milk deviation, off milk, licking pipes, unsteady, nervous attitude, strong positive in the urinalysis (Ketostix, Bayer Corporation Elkhart, IN, USA). Cows were considered under the category sick at the time of diagnosis if any of the previous disorders were diagnosed.

Predictive models and statistical analysis

During this study two indices similar to Paudyal et al. (2018) were developed to explore the association between milk components ratio and disease events considering specific disease: (i) Cow index (CIx) based on changes in the parameter occurring in the affected cow at the day of disease diagnosis, relative to the previous days; and (ii) Mates index (MIx) based on deviations in the parameters in the affected cow relative to healthy pen mates. Daily milk fat %, milk protein %, and milk lactose % were calculated as an average of two milkings in a day. CIx was calculated as the difference in d0 minus the average ratios during −3 day to −5 day relative to d0 (avg −3 to −5 days), divided by avg −3 to −5 days. Therefore, CIx = (d0 – {avg −3 to −5 days})/ (avg −3 to −5 days). The time of occurrence of a disease event was set at the time of diagnosis by the attending veterinarian or the farm personnel. Animals were considered affected

by the condition for different period according to disease condition as suggested by a previous report (Sanguensa, 2017). Animals affected by mastitis were considered sick for 5 days post diagnosis, metritis for 4 days, milk fever for 1 day, digestive disorder for 2 days, lameness for 5 days, and ketosis for 2 days post diagnosis.

The mates index (MIx) was calculated as a difference between the affected cow and healthy pen mates for the value (d0 – {avg −3 to −5 days}) divided by d0 value of the ratio in healthy pen mates. Therefore, MIx = [(affected cow d0 – {avg −3 to −5 days}) − (pen mates d0 – {avg −3 to −5 days})]/pen mates d0. Subsequently, binary alerts for diagnosis were tested based on multiple CIx and MIx indices (CAV and MAV) values. Multiple cut-off values were evaluated by use of receiver operating characteristic (ROC) curves generated with the LOGISTIC procedure of SAS (Version 9.4; SAS Institute Inc., Cary, NC, USA). Subsequently, an alert value (CAV and MAV) was determined for both indices when the corresponding index value was less than –0.1 or more than +0.1. A ROC curve is a graphical method to determine the performance of a diagnostic test and represents a plot of the sensitivity of a test versus the false positive rate (1 – specificity) computed for various cut-off points to select the optimum point for distinguishing between affected and non-affected individuals. The null standard also called the 'chance diagonal' represents the ROC curve of a diagnostic test with no ability to distinguish between patients with or without the disease. A ROC curve that lies above the chance diagonal has some diagnostic ability, which becomes more accurate the closer it is to the upper left hand corner of the plot (Obuchowski, 2005).

Sensitivity and specificity for each cut-off value were calculated by use of the FREQ procedure (SAS), considering the agreement between the reported disease event and the value of the corresponding alert index (0; 1). Cow-days were considered for the calculation of both Se and Sp. The Se calculations were performed considering the proportion of cow-days deemed as sick

that had a concurrent positive alert. Specificity was calculated as the proportion of healthy cow-days that had negative tests. It was expected that some cows would present with concomitant health conditions (Ospina et al., 2010), but to maintain a smaller number of disease categories, each condition was analyzed separately. A combined alert (CmbA) was created using the parallel interpretation for CAV and MAV; that is, cows that tested positive to any of the two alerts were considered as affected (Dohoo et al., 2009).

All the analyses were performed using SAS® (SAS Inst. Inc., Cary, NC). CORR procedure was used to evaluate the correlation between different ratios. LOGISTIC procedure was used to estimate the AUC values for the Receiver Operating Characteristic (ROC) analysis.

Results and Discussion

Disease presentation

The initial study population consisted of 210 multiparous Holstein cows. A total of 198 cows were followed through 60 DIM and successfully completed the study. Overall, 136 cows developed at least one disorder. Specifically, 43 cows were diagnosed with mastitis, 32 were diagnosed with metritis, 7 with milk fever, 32 with signs of obtundation, dehydration and fever, 64 with digestive problems, 25 with foot problems, and 45 with ketosis.

Milk components

Overall the study cows had on average (+SD) daily fat % of 3.74 (± 0.58), protein % of 3.17 (± 0.31) and lactose % of 4.64 (± 0.41). The average daily milk yield of the herd was 38.5 (± 11.4) kg during the study period. Ratios of milk components demonstrated different trends depending on specific health disorders. On the day of detection, FPR was greatest in animals affected with KET followed by MET, DIG, LAM, and HYC. We observed that the FPR in d0 tended to increase from 5 days before disease diagnosis except for HYC, where the ratio decreased

from day -2 to day 0 (Figure 1). However, we observed increased FLR on day 0 from 5 days before the disease for all the conditions except MET (Fig 1).

An interesting pattern was observed for PLR that the ratio decreased on day 0 as compared to day -5 observation for animals affected with MET, DIG, and KET but increased for animals affected with HYC, LAM, and MAS (Fig 1).

Except for FPR in LAM, odds ratios were statistically significant in specific disease conditions for all three ratios. Unit decimal point increase in FLR had greater odds for MAS than similar increase in FPR and PLR. Similarly, unit decimal point increase in PLR had greater odds for MET than similar increase in FPR and FLR. In addition, unit decimal point increase in FPR had greater odds for DIG and KET than similar increase in FLR and PLR (Table 4). Interestingly, unit decimal point increase in FPR was associated with protective effect on odds for HYC whereas similar increase in FLR and PLR had greater odds for HYC.

A high correlation was observed between PLR and FLR, however the correlation was greater in sick cows (r = 0.7) than in healthy cows (r = 0.6) (Fig 2). A moderate correlation was observed between FLR and FPR with 0.52 in healthy and 0.48 in sick cows. Protein to lactose ratio and FPR had small negative correlation(r) in healthy and sick animals (-0.28 and -0.27 respectively).

The AUC values from the ROC analysis for CAV based on FPR ranged from 0.6 (LAM) to 0.81 (HYC) for specific diseases (Table 1). Cow alert value (CAV) was most effective in detecting HYC (Se = 100%, Sp = 61.9%), followed by MET (78.5% , 62.2%), MAS (65.2%, 62.2%), DIG (62.3% and 62.1%), LAM (57.9%, 62%), and KET (61.8%, 62%). For CAV based on FLR, AUC values ranged from 0.61 (DIG) to 0.82 (HYC). Cow alert value (CAV) based on FLR was most effective in detecting HYC (Se= 100% and Sp = 63%), followed by MAS (71.2%,

63.4%), MET (68.1%, 63.2%), KET (67.1%, 63.1%), LAM (60%, 63.1%), and DIG (59.5%, 63.1%). For CAV based on PLR, AUC values ranged from 0.59 (LAM) to 0.86 (HYC). Cow alert value (CAV) alert based on PLR was most effective in detecting HYC (Se =100%, Sp = 72.5%), followed by MET (81.1%, 72.8%), MAS (60.5%, 72.9%), KET (56.8%, 72.6%), DIG (52.9%, 72.6%), and LAM (44.8%, 72.5%).

Area under the curve (AUC) values for the MAV based on FPR ranged from 0.81 (HYC) to 0.6(LAM) (Table 2). The MAV based on FPR was most effective in detecting HYC (Se =100%, Sp = 62.8%), followed by MET (77.6%, 63.1%), MAS (68.6%, 63.2%), KET (68.5%, 62.9%), DIG (63.9%, 62.9%), and LAM (57.01, 62.9%). For the MAV based on FLR, AUC values ranged from 0.83 (HYC) to 0.6 (LAM). The MAV based on FLR was most effective in detecting HYC (Se =100%, Sp = 65.5%), followed by MAS (74.6%, 65.9%), MET (73.3%, 65.7%), KET (72.7%, 65.7%), DIG (65.3%, 65.7%), and LAM (55.2%, 65.6%). The AUC values for MAV based on PLR, ranged from 0.59 (LAM) to 0.87(HYC). The MAV based on PLR was most effective in detecting HYC (Se = 100%, Sp =73.2%), MET (81.1%, 73.6%), MAS (60%, 73.6%), KET (55.7%, 73.3%), DIG (54.6%, 73.6%), LAM (44.8%, 73.3%).

Discussion

Inline devices for determination of milk components based on modern technologies such as NIR are commercially available and provide opportunity for use in health monitoring of dairy cows (Kaniyamattam and De Vries, 2014).

Due to reduced feed intake during health disorders, there is increase in milk fat percentage (Bauman and Griinari, 2003; Friggens et al., 2007) along with decrease in protein and lactose percentages (Bauman and Griinari, 2003; Friggens et al., 2007; Schmidt GH., 1966). Because similar increase in protein percentage is not observed, as observed in milk fat percentage, this phenomenon cannot be linked to concentration effect in sick cows due to reduced milk yield

(Kirchman et al., 2017). Because of these differences in behavior of individual milk components, milk component ratios are considered more sensitive, consistent and better indicator of disease conditions (Grieve et al., 1986; Duffield et al., 1997, Heuer et al., 1999, Toni et al.,2011).

Results from our study indicate that depending on the health disorders, milk components demonstrate different temporal patterns for FPR, FLR and PLR. Based on higher AUC results for both the CAV and MAV, FPR was more useful in detecting DIG, whereas FLR was more useful in detecting MAS, LAM, and KET. In addition, PLR was more effective in detecting MET and HYC. Therefore, conventional herd management methods that suggest using FPR for all energy related health disorders should be evaluated with caution.

With unit decimal point increases in the ratios, we observed that the odds of a cow being affected with DIG and KET were greater for FPR, odds of a cow being affected with MAS, LAM, and KET were greater for FLR and odds of a cow being affected with MET was greater for PLR. The ratios were more correlated in healthy animals than in sick animals, which suggest us that the ratios are altered when animals become sick. This phenomenon is supported by previous research that suggests utilizing FPR to assess energy balance associated with KET in dairy cows (Krogh et al., 2011; Friggens et al., 2007).

Results from a previous study identifying effects of fasting on Holstein cows demonstrated a similar change in milk composition with a reduction in lactose percentage and milk production, and an increase in milk fat percentage (Gowen and Tobey, 1931). Similarly, cows affected with diseases such as MAST, LAM and KET demonstrate metabolic stress (Buttchereit et al., 2010) and demonstrate a similar change in milk composition. This supports our study hypothesis that animals experiencing stress due to disease demonstrate altered composition of milk.

Previous studies have associated changes in FLR with SARA. In this study, we categorized all digestive disorders into one group and we observed that FLR is associated with DIG. Kirchman et al. (2017) determined milk FLR to be the best measure for diagnosing rumen indigestion among other milk component ratios. Our study used a similar statistical analysis; however DIG disorders were better identified using FPR ratio. LAM was associated with lower values of FLR in this study, which could be associated with high grain ration leading to rumen acidosis, which could be related to both laminitis and milk fat depression.

The study by Kirchman et al. (2017) used a cutoff of (0.1) on individual animal FLR and identified over half of the cows with rumen disorders (Se = 57 %). Our study resulted on higher Se when using CAV (62.3 %) and MAV (69 %). This higher sensitivity in detecting DIG conditions can be attributed to the indices that we developed based on deviation of the parameters rather than using a conventional cutoff for a continuous variable. Using 1.5 cutoff for FPR , Jenkins et al., (2015) obtained 75% Se and 78% Sp to detect subclinical KET and observed that the propylene glycol treatment was effective for these animals. We observed slightly lower Se (68%) based on FPR to detect clinical KET.

The diagnosis of health disorders based on a cutoff of fat to protein value is a challenge because FPR is a continuous variable and is subject to daily fluctuations based on diet and stage of lactation (Buttchereit et al., 2010). As a clear threshold for diagnosis of metabolic problems is not yet defined, an index based on deviation of the parameter could be better utilized to detect sick animals. Another strength of this study is that we were able to compare the deviation of the individual animal with the deviation of the pen-mates. This decreases the error due to changes in management, feeding, or weather. The instrument we used was validated for measuring milk components and observed the correlation between laboratory test and inline milk analyzer at this

farm to be 0.59 for milk fat and 0.67 for milk protein, (Kaniyamattam and De Vries 2014). This provides strong evidence that data we used was near to values present in milk.

There are disease conditions where the milk fat % is expected to decrease and the milk protein % and lactose % are expected to increase. The greater ratio of amino acid and glucose relative to ratio of acetate and long chain fatty acid in blood circulation of animals results in increased rates of synthesis of protein and lactose, leading to less fat in the mammary gland (Sutton, 1989). Consequently, milk protein, milk lactose, and milk yield were increased, while milk fat concentration dropped due to change in the ratio of precursors of milk synthesis (Sutton, 1989). Milk protein concentration was positively correlated with ME intake except with ME provided by digestible lipids (Walker et al., 2004). On the other hand, lactose has been suggested to increase by 2-fold in affected quarters as compared to normal quarters in cows affected by mastitis (Hamann et al., 1997). This suggests that during disease conditions, the deviation of milk components could be either positive or negative depending on the disease. Therefore, we considered both a positive deviation and negative deviation by using the cut point of +0.1 or -0.1 for CIx and MIx when selecting the cutoff for the CAV and MAV alarms.

Vos and Groen (1999) observed a highly negative correlation between protein to fat ratio and fat% ($r = -0.77$) and a slight positive correlation between protein to fat ratio and protein % ($r = 0.18$), suggesting that the ratios could be a good indicator of the changes in milk components. Concentrations of fat, protein and FLR were negatively correlated with energy balance, whereas milk lactose had a positive correlation with the energy balance with FLR having the highest correlation (-0.589) (Buttchereit et al., 2011). Negative correlation between energy supply and FPR in the early stage of lactation (-0.53) was observed by Hamann and Kromker (1997) supporting our use of the parameter for detecting the diseased state.

Toni et al., (2011) observed an increased risk of being culled from the herd with increasing FPR. They also observed that higher odds of MET were associated with increasing FPR ratio, supporting the observation from our study, where we calculated odds of 1.42 for unit decimal increment in FPR. Contrary the study by Toni et al. (2011) did not observe an effect of FPR on the incidence of MAS, opposed to our study where we identified a Se as high as 76%. This discrepancy could be because their conclusion was based on a single milk sample and the use of single cutoff point for the continuous variable whereas we devised indices based on deviations and utilized temporal data from inline milk analyses in our study.

An interesting finding of our study was that PLR was effective in detecting MET. The use of real-time values of milk components differentiates this study from previous studies. Our results are based on larger number of observations per animal and for a longer period to investigate temporal pattern of the parameter

Conclusion

Monitoring ratios of milk components had the potential for detection of different disease conditions in transition dairy cows. The sensitivity and specificity differed depending on specific disease conditions, with MAV being more sensitive than CAV for early detection of health disorders.

Table 3.1: Sensitivity (Se) and specificity (Sp) of the alert based on changes in of the parameter in the affected cow relative to the days previous to diagnosis (CAV) to detect specific health disorders for each of the study parameters (FPR, FLR and PLR).

	FPR[1]			FLR[2]			PLR[3]		
	Se[4]	Sp[5]	AUC[6]	Se[4]	Sp[5]	AUC[6]	Se[4]	Sp[5]	AUC[6]
Mastitis	65.2	62.2	0.64	71.2	63.4	0.67	60.5	72.9	0.67
Metritis	78.5	62.2	0.70	68.1	63.2	0.66	81.1	72.8	0.77
Milk fever	100	61.9	0.81	100	62.9	0.82	100	72.4	0.86
Digestive	62.3	62.1	0.62	59.5	63.1	0.61	52.9	72.6	0.63
Lameness	57.9	62.0	0.60	60.0	63.1	0.62	44.8	72.5	0.59
Ketosis	61.8	62.0	0.62	67.1	63.1	0.65	56.8	72.6	0.65

[1]Daily fat to protein ratio

[2]Daily fat to lactose ratio

[3]Daily protein to lactose ratio

[4]Sensitivity

[5]Specificity

[6]Area under the curve from the receiver operator characteristic curve analyses

Table 3.2: Sensitivity (Se) and specificity (Sp) of the alert based on the deviation of the parameter in the affected cow relative to healthy pen mates (MAV) to detect specific health disorders for each of the study parameters (FPR, FLR and PLR).

	FPR[1]			FLR[2]			PLR[3]		
Health disorder	Se[4]	Sp[5]	AUC[6]	Se[4]	Sp[5]	AUC[6]	Se[4]	Sp[5]	AUC[6]
Mastitis	68.6	63.2	0.66	74.6	65.9	0.70	60.0	73.6	0.67
Metritis	77.6	63.1	0.70	73.3	65.7	0.70	81.0	73.6	0.77
Milk fever	100	62.8	0.81	100	65.5	0.83	100	73.2	0.87
Digestive	63.9	62.9	0.64	65.3	65.7	0.65	54.6	73.4	0.64
Lameness	57.0	62.9	0.60	55.3	65.6	0.60	44.8	73.3	0.59
Ketosis	68.5	62.9	0.66	72.7	65.7	0.69	55.7	73.3	0.65

[1]Daily fat to protein ratio

[2]Daily fat to lactose ratio

[3]Daily protein to lactose ratio

[4]Sensitivity

[5]Specificity

[6]Area under the curve from the receiver operator curve analyses

Table 3.3: Sensitivity (Se) and specificity (Sp) of the alarm combined for higher sensitivity (CmbA) to detect specific health disorders for each of the study parameters (FPR, FLR and PLR).

Health disorder	FPR[1]			FLR[2]			PLR[3]		
	Se[4]	Sp[5]	AUC[6]	Se[4]	Sp[5]	AUC[6]	Se[4]	Sp[5]	AUC[6]
Mastitis	70.1	57.6	0.64	76.1	58.2	0.67	61.9	68.4	0.65
Metritis	80.2	57.6	0.69	74.1	58.0	0.67	82.8	68.4	0.75
Milk fever	100	57.3	0.79	100	57.8	0.79	100	68.1	0.84
Digestive	68.9	57.5	0.63	66.1	57.9	0.62	57.9	68.2	0.63
Lameness	61.7	57.4	0.60	62.9	57.9	0.60	49.5	68.1	0.59
Ketosis	68.5	57.4	0.63	72.7	57.9	0.65	62.5	68.2	0.65

[1]Daily fat to protein ratio

[2]Daily fat to lactose ratio

[3]Daily protein to lactose ratio

[4]Sensitivity

[5]Specificity

[6]Area under the curve from the receiver operator characteristic curve analyses

Table 3.4. Odds ratio (95% CI) of specific diseases for each decimal point increment in the milk component ratios. [1]Daily fat to protein ratio [2]Daily fat to lactose ratio [3]Daily protein to lactose ratio

Health disorder	FPR[1]	FLR[2]	PLR[3]
Mastitis	1.20 (1.13-1.27)	1.32 (1.27-1.37)	1.27 (1.19-1.29)
Metritis	1.42 (1.34-1.51)	1.32 (1.26-1.38)	1.67 (1.09-1.24)
Milk Fever	0.53 (0.31-0.92)	1.34 (1.15-1.57)	1.36 (1.2-1.55)
Digestive	1.35 (1.27-1.44)	1.29 (1.23-1.35)	1.16 (1.09-1.24)
Lameness	1.04 (0.95-1.15)	1.22 (1.60-1.29)	1.22 (1.15-1.28)
Ketosis	1.52 (1.43-1.62)	1.33 (1.27-1.39)	1.13 (1.04-1.23)

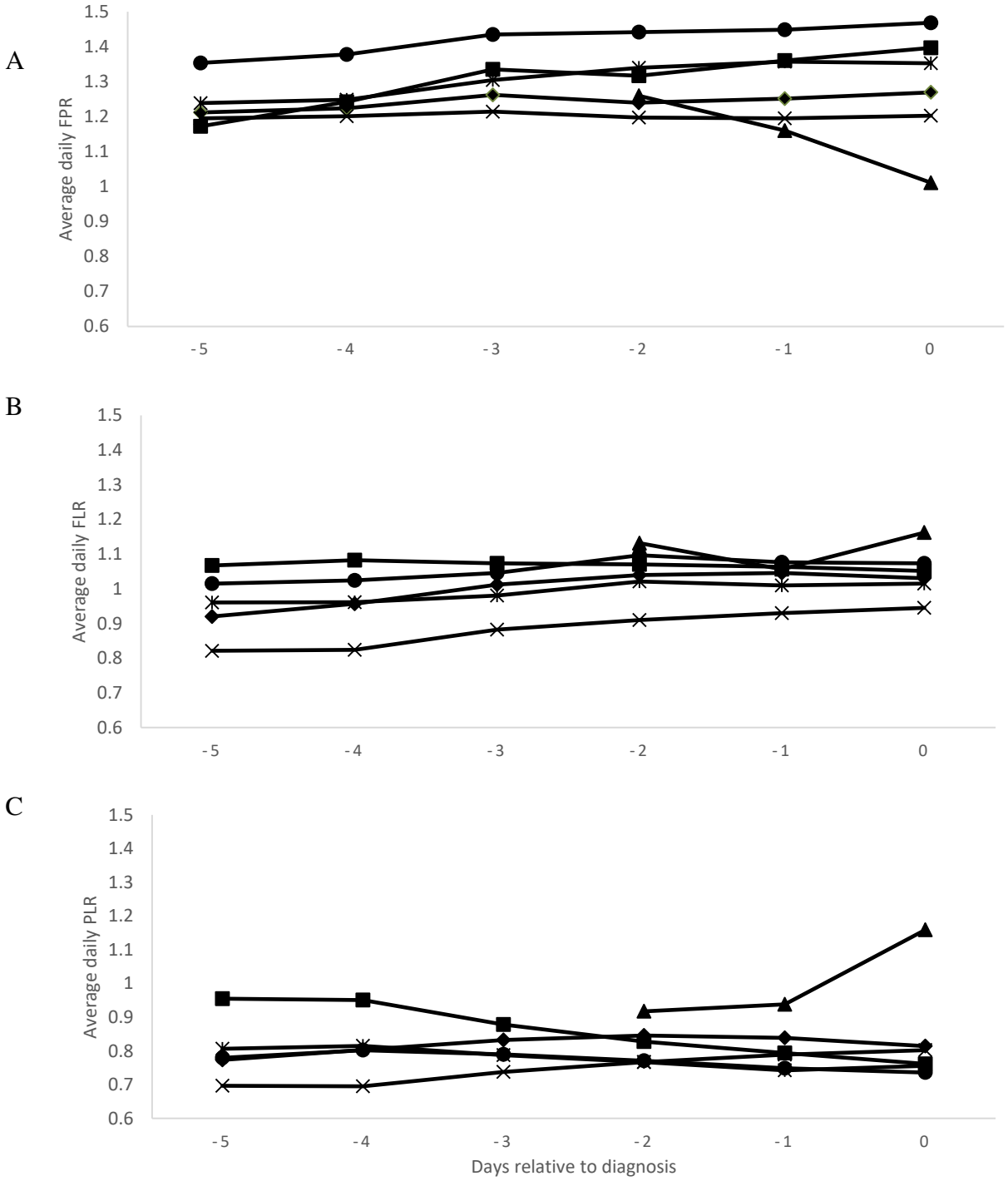

Figure 3.1. Average daily A) fat to protein ratio (FPR); B) fat to lactose (FLR) ratio; C) protein to lactose ratio (PLR); from −5 day to the day of disease diagnosis (d0). (a) Metritis (■), mastitis (♦), digestive (*), clinical Milkfever (▲), lameness (X) and clinical ketosis (●). A total of 43, 32, 7, 32, 64, 25 and 45 cows were diagnosed with mastitis, metritis, clinical hypocalcemia, depression/dehydration/fever, digestive conditions, lameness and clinical ketosis.

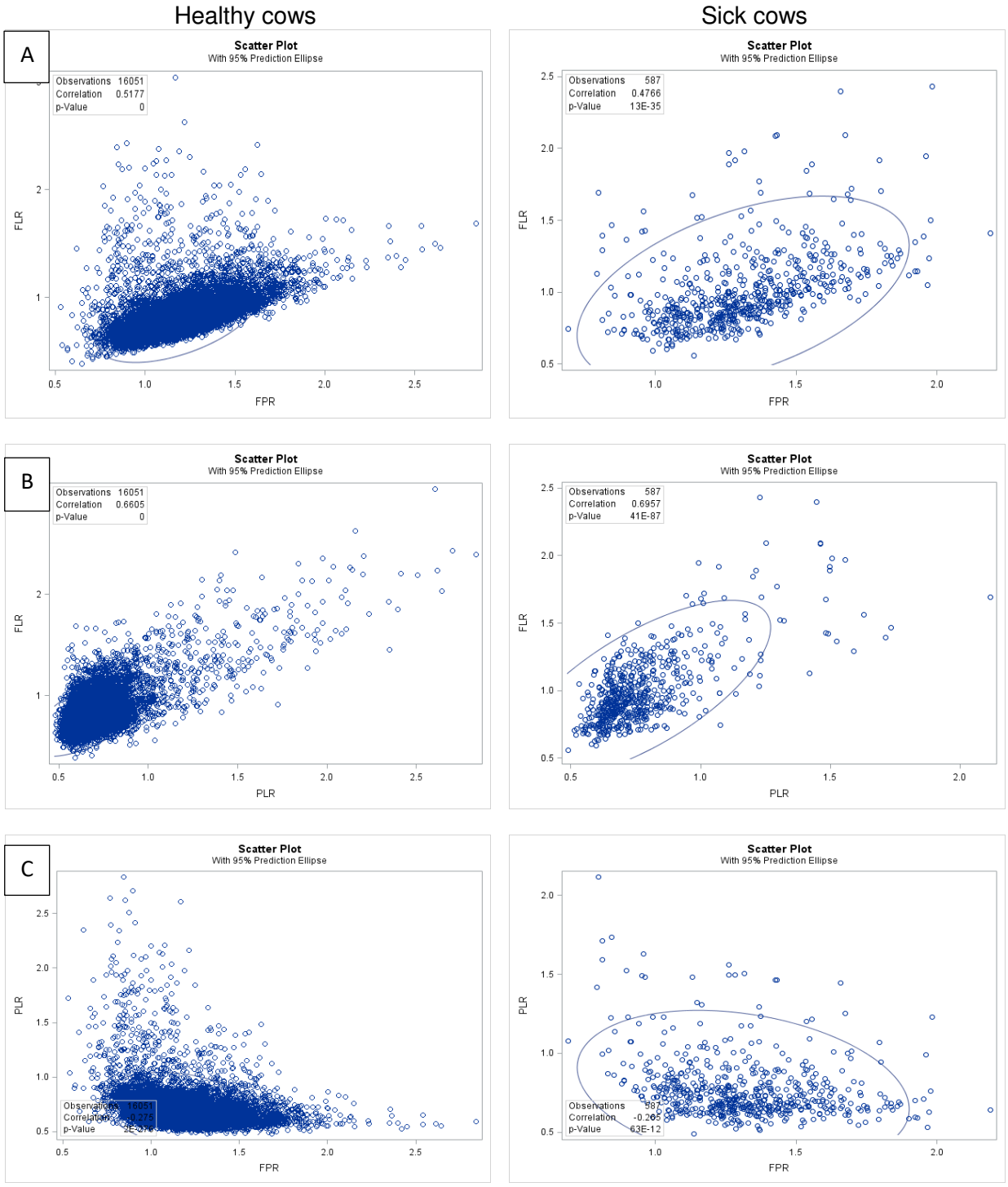

Figure 3.2. Correlations between the three ratios in analysis A) fat to lactose ratio (FLR) and fat to protein ratio (FPR); B) fat to lactose ratio (FLR) and protein to lactose ratio (PLR); C) protein lactose ratio (PLR) and fat protein ratio (FPR) in sick and healthy cows.

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CHAPTER 4 - EVALUATION OF ACTIVITY BEHAVIOR USING A LEG MOUNTED SENSOR TO DETECT SPECIFIC LAMENESS DISORDERS.

Introduction

Lameness is a severe economic and welfare concern for the dairy industry as the ability of cattle to perform normal activity behaviors and adequate locomotion is impacted during lameness (Dutton-Regester et al., 2018). Therefore, early detection and prompt correction of locomotion disorders are critical aspects of management of lame cows. Traditionally, dairy producers depend on visual observation of feet and leg disorders using locomotion scoring, which is subjective, time-consuming, and prone to individual bias (Weigele et al., 2018).

Recently, various automatic systems have been developed for the identification of lameness disorders (Dutton-Regester et al., 2018) and could potentially replace visual observations (Weigele et al., 2018; Schlageter-Tello et al., 2018; Schlagater-Tello et al., 2014). These systems have resulted on reductions in expenses due to early detection and treatment (Van De Gucht et al., 2018) and the number of producers adopting these technologies is increasing (Van De Gucht et al., 2017).

There is evidence indicating altered behavioral patterns including rumination, activity, lying time, and other behavioral variables in lame animals (Weigele et al., 2018; Thorup et al., 2016; Westin et al., 2016). The dynamics of these behavioral variables may be different for animals affected with specific lameness disorders as compared to animals requiring corrective hoof trimming. However, research evaluating their potential for disorder differentiation is scarce. Our hypothesis was that cows requiring corrective hoof trimming (TRM) could be differentiated from cows with identifiable lameness disorders (DIS) using behavioral variables based on activity. Consequently, the objective of this study was to characterize lying time, bouts frequency, number

of steps, and milk yield in cows that were subjected to corrective hoof trimming or that were treated for specific lameness disorders.

Materials and Methods

Animals and Animal Housing

This study was conducted at a commercial USDA certified organic dairy herd in northern Colorado with the approval from the Institutional Animal Care and Use Committee at Colorado State University (protocol ID: 17-7665A). The study population consisted of 310 primiparous and multiparous Holstein cows enrolled within 20 days in milk (DIM). Cows were enrolled between December 2017 and January 2018, and monitored until June 2018 for lameness disorders. The dairy milked approximately 3500 Holstein cows three times daily, with a rolling herd average of 8,600 kg/cow. All cows were housed in free stall barns with sand bedded stalls and had free access to a contiguous dry lot. Cows were fed total mixed ration (TMR) twice a day to meet or exceed the nutritional requirement for a lactating Holstein cow producing 30 kg/d milk with 3.5% fat and 3.1% true protein (NRC, 2001). Throughout the study period the diet consisted of corn silage (14 to 17.5%); wheat silage (13 to 20%); a premix containing soybean, soy hulls, corn, wheat, and minerals and vitamins (47.5 to 50.5%); sorghum silage (3.0 to 4.5%); alfalfa hay (12 to 16%); and grass hay (0 to 3%). Trace mineral salt and water were provided ad libitum. During the grazing season (May-September), cows had access to pasture and grazing (started April 23, 2018 cows in the study) which provided a significant portion of the total ration.

Lameness management in the farm

Cows were regularly monitored for lameness. Animals demonstrating signs of lameness were taken to a chute and examined for the presence of specific lameness disorders according to farm standard operating procedure. The whole herd was surveyed for lameness scores at least once

every three months. All cows received preventative hoof trimming at least once every six months. The selected herd had a history of lameness associated with footrot (FRT), white line disease and digital dermatitis (DID). The whole herd went through preventive footbath with acidified 5% copper sulfate solution twice weekly. Animals diagnosed with DIS were treated according to the farm protocol using therapies approved for use in a USDA certified organic dairy farm.

Experimental procedures

On the day of enrollment (d0), cows were afixed with a pedometer (Icecube®, Icerobotics, Edinburgh, UK) below the fetlock of a hind leg using a Velcro band. The pedometer provided activity and steps based on three-dimensional accelerations collected at 16 Hz. The system was previously validated by Brochers et al. (2016) who identified very high correlation ($r > 0.99$, CCC > 0.99) of the system with visual observation. On the day of enrollment, animals were on average 12±5 days in milk. The study cows were monitored for milk yield during each session using Delpro® monitoring system (DeLaval, Sweden). Activity behavior in terms of motion index, total steps, lying time, and daily bouts were provided by the Cowalert® system (Icerobotics, Edinburgh, UK) every 15 minutes and downloaded at a server in milking parlor when the animal arrived for milking. The system provided lying time (minutes), a number of steps (frequency), daily bouts (frequency), and motion index (MOI: the sum of measured net acceleration in the three dimensions minus an offset for gravity, and as such an expression of leg activity). Detailed information about parameters and related calculations can be found in Thorup et al., (2015). Lameness probability values were developed based on manufacturer's proprietary algorithm, based on which the system provided lameness alerts to identify the affected animal. The herd was constantly monitored by an experienced hoof trimmer to identify cows for the presence of lameness disorders that required either trimming intervention (TRM) or a corrective therapy (DIS). The cows were screened based

on altered gait and painful movement and taken to trimming table for the confirmatory diagnosis. One of the authors (SP) conducted locomotion scoring of all cows every 15 days throughout the period of this study. Cows were evaluated for locomotion scores (LS) on a scale of 1 to 5 (LS1 = stands and normally walks with a level back; LS2 = stands with flat back but arches when walks, abnormal gait; LS3 = stands and walks with an arched back and short strides; LS4 = arched back standing and walking, favors one or more limbs but can still bear some weight on them; LS5 = pronounced arching of back, reluctant to move, complete weight transfer off the affected limb (Sprecher et al., 1997).

Ambient temperature and humidity were measured using the HOBO UX100-011 temp/RH 2.5% loggers (Onset Computer Corporation, Bourne, MA, USA) that were installed in 6 different points in the dairy farm to obtain the temperature and relative humidity status of the entire farm. The loggers were set to a sampling rate of 1 reading every hour. These values were used to determine temperature humidity index (THI) for each time period. Daily THI values were obtained as an average of the all readings between the 24 hour period every day.

The THI was calculated using the equation $THI = (1.8 \times T + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T - 26)]$, where T = temperature (°C) and RH = relative humidity (Manriquez et al, 2018). In considering the level of heat stress exposure for the individual across time, days were categorized as presenting low or high THI when THI <68 or ≥ 68 units, respectively. In a previous report, Ravagnolo and Miszal (2002) suggested the use of 68 THI units as the reference value for heat stress evaluation on dairy cow reproduction studies.

Daily values of steps, lying time and MOI were obtained as the sum of the 15-minute values observed for the entire day. Daily THI was obtained as an average THI obtained for the hourly interval of the day. Daily milk yield was obtained as a sum of milk yields from each milking

sessions of the day. Insemination and health records were obtained from the PCDart herd management software (Dairy Records Management Systems, Raleigh, NC, USA).

Statistical analysis

Datasets were managed using Microsoft Excel software. All the statistical analyses were performed using SAS® (SAS Inst. Inc., Cary, NC, USA). The GLM procedure in SAS was used to obtain mean values of the parameters with respect to DIM, parity and locomotion scores of animals. Days in milk and parity were included in all statistical models because of their significant effect on the study parameters. Cows were categorized into transition period (0 -21 DIM), early lactation (21- 150 DIM) and mid lactation (150-212 DIM) based on the days in milk. Mean values of parameters (relative to day of diagnosis) were obtained and checked for difference using the MIXED procedure. A predictive model was constructed using the LOGISTIC procedure that was used to evaluate the probability of DIS and TRM considering parity, days in milk, number of steps, lying time, bout frequency and stage of lactations as explanatory variables. Continuous variables were converted into categorical parameters using the median value as cutoff; high steps vs low steps categories ($>$ 1994 vs $\leq$ 1994 steps/d), high lying time vs low lying time categories ($>$ 585 vs $\leq$ 585 minutes/d), high bout frequency vs low bout frequency ($>$18 vs $\leq$18 bouts/d). Daily MOI and daily number of steps had a significantly high correlation ($r = 0.989$; $P < 0.0001$); therefore, MOI was not used in prediction models. Access to pasture, temperature humidity index and presence of estrus alarm were fitted into the model to account for altered behavior pattern during these events.

In general, depending on the analysis, the models were defined as follows:

logit P ($Y_{ijklmnop}$ = 1) = $\beta 0 + Par_i + DIM_j + Stepscat_k + Lycat_l + Boutcat_m + DIMcat_n + Heatalarm_o$ $+Grazing_p + THIq + \varepsilon_{ijklmnopq}$

Where:

$Y_{ijklmno=}$ dependent variable (DIS, TRM)

$\beta 0$ = intercept

Par_i = effect of parity (primiparous or multiparous)

DIM_j = effect of days in milk

$Stepscat_k$ = effect of steps category (≤ 1994 or > 1994)

$Lycat_l$ = effect of lying time category (≤ 585 or > 585)

$Boutcat_m$ = effect of bout frequency category (≤18 or >18)

$DIMcat_n$ = effect of stage of lactation (transition period (<21 DIM), early (21-150 DIM), or mid (>150 DIM) lactation)

$Heatalarm_o$ = effect of the presence of estrus alarm from the system

$Grazing_p$ = effect of access to graze in pasture

THI_q = effect of temperature humidity index

$\varepsilon_{ijklmnopq}$ = error term

Results and Discussion

Climatic data

Daily THI values during the study period ranged from 14.1 units to 72.4 units. The highest THI was recorded in July, and the lowest THI in February. During the overall study period, daily THI were less than 68 for 182 days. The monthly average THI was higher than 68 in 2 months of the study period (June and July).

Disease presentation

The initial study population consisted of 310 multiparous Holstein cows. Parity number ranged from 1-10 with 69 primiparous and 241 multiparous cows. Cows were enrolled at 12 ±5 DIM. A total of 289 cows successfully completed the study from enrollment to June 30, 2018. Remaining cows left the study due to culling not related to lameness or movements to another unit within the farm. Overall, 75 cows developed at least one of the foot disorders considered in the study and 190 cows received corrective hoof trimming. In specific, 21 cows were diagnosed with foot injury (INJ) and 21, 10, and 23 cows were diagnosed with DID, FRT, and non-specific lameness (LAM) respectively.

For the duration of the study, cows had a median lying time of 585 min/d ranging from 198 to 1,328 min/d. The median number of steps was 1994 per day ranging from 96 to 19,996. The median bout frequency was 18 ranging from 6 to 71. The mean (±SD) milk yield per day during the study period was 36.21 (±9.9) kg.

Activity dynamics across lactation and parity number

Lying behavior was associated with parity and DIM. There was significant effect on lying time for parity number by DIM interaction ($P < 0.001$; Fig 1). Daily lying time was greatest in mid lactation in both parity groups. The primiparous cows lied down more in early lactation compared to the transition period whereas multiparous cows lied down more during transition period compared to early lactation ($P < 0.0001$; Table 1). Lying time was associated with season: greater daily lying time in the cool season and the lying time decreased in the hot season (THI > 68; Fig 2). The temporal distribution of lying time demonstrated a periodic drop in lying time at fixed intervals representing reduced lying time for animals during estrus (Figure 2). A Similar parity by DIM interaction effect was observed on daily bouts ($P< 0.0001$; Table 1). Greater number of bouts

was observed in early lactation in both parity cows. A Greater number of steps was observed in primiparous compared to multiparous cows (P< 0.001; Table 1). The number of steps increased with DIM in both parity cows (P< 0.0001; Table 1). There was a significant effect on motion index for parity by DIM interaction. Primiparous cows had greater MI during transition period and early lactation whereas; multiparous cows had greater MI during mid lactation (Table 1).

Daily lying time decreased when cows went to pasture (566.6 ± 5.1 vs 599.3 ± 4.8; P < 0.0001). A greater number of bouts was observed (19.6 ± 0.23 vs 19.1 ± 0.2; P<0.002) when the cows went to pasture. Grazing was associated with greater number of steps (4253.1±47.6 vs 2065.75±45.2; P< 0.0001). A high motion index was observed when cows went to pasture vs not allowed access to pasture (15523.5±187.2 vs 7134.3±177.8; P < 0.0001). On the other hand, daily milk yield was decreased (30.1±0.4 vs 30.6±0.4; P < 0.0001) when cows had access to pasture.

Activity parameters and Lameness

The severity of lameness demonstrated by locomotion scores was associated with lying time. Lying time was greatest for animals with LS4 and LS5 as compared to animals with LS1, LS2, and LS3(P< 0.05). Cows with LS4 and LS5 had a greater number of bouts per day as compared to animals with LS1, LS2, and LS3 (P < 0.05). Number of steps were reduced in animals with LS4 and LS5 (P < 0.05). The MI was reduced for animals with LS4 and LS5. The lameness probabilities provided by the system were significantly greater for cows with LS4 and LS5 than for cows with LS1, LS2, and LS3 (P < 0.05; Table 2).

Lying time for DIS was greater than TRM for all days before and after the diagnosis. A greater deviation of lying time from 7-day (d) average was observed in DIS than TRM. Average lying time for all specific lameness disorders was decreased until day 0. All disorders lied down

more than TRM and the healthy animals on days before diagnosis (Figure 3). Immediately after the treatment, lying time decreased in animals with LAM, FRT, and INJ.

Bout frequency was greater in DIS than both TRM and healthy cows in d-1 and d0, and started to decrease after treatment on d0. Deviation in daily bouts from rolling average was greater in TRM during d-1 and d0. Specific lameness disorders demonstrated increased daily bouts starting d-3. The LAM, FRT and INJ groups had greater bouts than TRT and healthy cows. The bout frequency decreased immediately after treatment in DID whereas, for FRT and INJ the bouts frequency decreased slowly. All specific lameness disorders demonstrated a negative deviation from 7d average on d-3, d-2, and d-1 (Figure 4).

Daily number of steps were smaller in DIS compared to TRM and healthy cows on all days. Across the days, daily steps in DIS decreased until d0, and cows did not reach the level of healthy animals until d7. A greater negative deviation from 7d average daily steps was observed in TRM for d-3, d-2, and d-1. Specific lameness disorders demonstrated decreased number of steps until d-1. A quick recovery in daily steps after treatment application was observed in DID and FRT starting d4. A greater negative deviation from 7d average daily steps was observed for LAM, and FRT on d-3 to d-1 (Figure 5).

Daily milk yield for DIS dropped on days -5, -3, and -2, whereas milk yield reached the level of healthy animals or above after the treatment on d0 (except on d5). Although TRM demonstrated a drop in milk from rolling average, the deviation for DIS was greater on d -5, d -3 and d -1 (Figure 6). Among specific lameness disorders, DID and FRT demonstrated a greater decrease in milk yield. The milk yield recovery after treatment was slow and remained low in animals with FRT and INJ. The deviation of daily milk yield from 7-day average was pronounced in LAM, DID, and FRT.

Results from logistic models

Lying time and bout frequency categories were not associated with TRM. Cows in the high steps category had 0.4 times odds of TRM than cows in low steps category. Primiparous cows had 0.6 times odds of TRM compared to multiparous cows. Cows in mid lactation had 4.4 times greater odds of TRM compared to early-lactation cows. Presence of estrus and access to pasture were not associated with TRM.

Cows in high lying time category had 2.6 times greater odds of DIS than cows in low lying time category. Cows in higher bout category had 1.8 times greater odds of DIS than cows in low bout category. Cows in high steps category had 0.2 times greater odds of DIS than cows in low steps category. Primiparous cows had 0.8 times odds of DIS than multiparous cows. Cows in early lactation had 1.5 times odds of DIS compared to mid lactation cows. Presence of estrus was associated with reduced odds of DIS and access to pasture was associated with greater odds of DIS.

Discussion

Automatic activity monitoring has been proposed due to labor-intensive and time-consuming methods that required visual observation (Blackie et al., 2011). The sensor devices are effective in measuring cow behavior and useful in herd health monitoring (Borchers et al., 2016; Paudyal et al., 2018). Recognizing the early stages of lameness remains a challenge and producers underestimate the prevalence of lameness leading to delayed treatment of disorders (Leach et al., 2012; Barker et al., 2018). Therefore, there is a need for systems that can automatically detect lameness at an early stage without the need for time-consuming behavioral observations. Thus, automated lameness detection systems are useful in the larger herd that have less time for farmers

to monitor the herds and current systems are identified to be more sensitive than traditional observational methods for lameness detection (Nuffel et al., 2015).

In our study cows, the dynamics of the behavioral variables obtained from the system were different in TRM, DIS, and healthy cows. Lying time and bout frequency increased in DIS whereas number of steps were decreased in DIS. However, the parameters did not demonstrate conclusive patterns to detect specific disorders, likely due to small numbers of animals suffering from each lameness disorders. In average, cows with LS4 and LS5 lied down more time, had a greater number of bouts, took fewer steps, and had greater variability demonstrated by very high standard errors.

Lying behavior is considered very important for the well-being of cattle; when deprived of the opportunity to rest, cattle showed signs of distress and physical exhaustion (Munksgaard et al., 1999). The lower lying time in the early stage lactation observed in this study was similar to Maselyne et al. (2017) where they observed a drop in lying time at 22 DIM followed by a subsequent increase. Motion index and steps in this study demonstrated a similar increase during mid-lactation, as described by Maselyne et al. (2017). Lameness condition is associated with changes in lying behavior, but the results are equivocal, with some reports indicating increased lying time (Singh et al., 1993; Galindo and Broom, 2002; Blackie et al., 2011) no difference (Ito et al., 2010; Yunta et al., 2012), and decreased lying (Cook et al., 2004). This issue was evident in our study: we observed greater lying time in animals affected by the disorders but when we categorized the animals into the transition period, early, and mid-lactation animals, animals in early and mid-lactation had shorter lying times. Our results for parity and lactation stage were similar to those of Westin et al. (2016) that reported that increased lying time was associated with increased parity and later stage of lactation. Older cows (parity $\geq$ 3) spent about 0.5 h/d extra lying

compared with primiparous cows, similar to the observation from our study, where primiparous animals lied down less than multiparous animals.

Early research indicates that locomotion assessments have effectively identified lameness with mobility impairment being correlated with lesion severity (Whay et al. 1997). Chronic foot lesions were associated with higher locomotion scores than acute foot lesions (O'Callaghan et al., 2003). There are reports that demonstrate numerical gait rating system to be able to achieve 92% accuracy in classifying sole ulcers (Flower & Weary, 2006). An advantage of automatic lameness detection is that the measurements are not biased because cows will not try to hide their weakness and pain compared to measurements from visual observations due to guiding behavior of a human (Weary et al., 2009). The locomotion scoring performed in this study demonstrated associations with the altered behavioral parameters including increased lying time and decreased number of daily steps.

Similar to observations from this study, Mazrier et al. (2006) reported that the reduction in activity (e.g., average steps/hour) for lame cows ranged from 9 to 68% and almost half of the lame cows showed a reduction of more than 5% during the 7 to 10 days prior to clinical signs. In 92% of the lameness cases, the decrease in activity was more than 15%. In our study, we observed a deviation of daily steps up to 18% for LAM animals. Yunta et al. (2012) observed that lame cows stand up later and lie down earlier after fresh feed is delivered compared to non-lame cows. Similar to this observation, our study demonstrated a greater number of daily bouts in the affected animals, illustrating that lame cows get up and lie down more than non-lame herd mates. In contrast, Ito et al. (2010) reported that lying bout frequency was not associated with lameness. Alsaaod et al. (2012) observed significant differences in normal daily behavior between individual cows than those caused by lameness and therefore use of deviation was suggested. We observed that these

deviations are different for multiple lameness categories. A 7-d average of the daily mean values was calculated for all variables in our study to compare both groups against each other. The 7-d rolling average used in our study is a commonly used number in farm management software for health and performance monitoring purposes (Van Hertem et al., 2013).

Weigele et al. (2018) observed that even with moderate lameness, cows had a longer lying duration, and lower average neck activity confirming distinct activity behavior for lame and non-lame animals. Thorup et al., (2016) observed that lameness disorders introduced more inter-individual variation in feeding characteristics and rumination behavior was not affected by the lameness, but the feeding behavior was affected.

Miguel-Pacheco et al. (2016) investigated the lying pattern in the animals after treatment was performed. Cows that received both a therapeutic trim and a foot block, observed higher lying times post-treatment as opposed to cows receiving TRM only. This observation may explain the various deviations and trends in lying behavior observed in our study. Given the lack of difference in TRM in our study, this finding suggests that lying time differences is an effect of the block or treatment or combination of these rather than of trimming behavior (Miguel-Pacheco et al., 2016).

In a survey, farmers who already used estrus detection system were more willing to use automatic detection systems instead of visual lameness detection. A sensor attached to a cow was preferred followed by a walkover system and a camera system. After informing farmers about the consequences of lameness, automatic detection was preferred over visual lameness detection systems (Van de Gucht et al., 2017). The results from this study supporting the potential to differentiate animals requiring trimming and treatment may further encourage producers to adopt the technology.

Conclusion

It is concluded that the dynamics of lying time, bout frequency, daily steps and milk yield around hoof trimming are specific and require different recovery times after treatment to reach normal levels. Monitoring behavioral variables have the potential to detect animals needing treatment for lameness disorders or needing trimming. However, detection of specific lameness disorders using these activity monitors in dairy cows should be taken with caution.

Table 4.1: Distribution of lying time, bouts, steps and motion index across the lactation categorized by DIM. **denotes significant interaction effect of DIM category and Parity group at P < 0.001; ## represents significant effect of DIM category (P < 0.001) and Parity (P < 0.001).

DIM	Lying time (min/d)**		Bouts (n/d) **		Steps (n/d)##		Motion Index (units/d) **	
	Primi parous	Multi parous	Primi parous	Multi parous	Primi parous	Multi parous	Primi parous	Multi parous
Transition (0 – 21)	503.2±6.6	579.09±3.2	17.9±0.3	18.0±0.2	1844±71	1705±34	5851±281	5572±135
Early lact (21 – 150)	573.1±1.6	570.6±0.8	19.9±0.1	18.4±0.1	2345±17	2168±8	8123±67	7580±34
Mid lact (>150)	596.8±2.8	584.9±1.5	19.4±0.1	18.2±0.1	4420±31	4327±16	15868±121	16015±65

Table 4.2: Average (± SE) lying time (lying), bouts frequency (bouts), total daily steps (steps), motion index (MOI), milk yield (milk) and probability of demonstrating lameness signs obtained from the system (probability) across the five lameness scores (LS). Different superscripts within a row represent statistically significant differences at $P < 0.05$.

	LS1 (n = 3876)	LS2 (n = 104)	LS3 (n = 44)	LS4 (n = 24)	LS5 (n = 1)
Lying time (min/d)	574.99±2[a]	570.12±13[a]	585.44±20[a]	674.63±27[b]	894.42±121[b]
Bouts (n/d)	18.32±0.11[a]	19.19±0.64[ab]	18.98±0.96[ab]	21.76±1.34[bc]	27.19±5.83[ab]
Steps (n/d)	2760.9±27[a]	2985.11±161[a]	2977.19±245[a]	2079.47±342[b]	700.43±1489[a]
MOI (units/d)	9838.24±106[a]	10902±624[a]	10480±947[a]	7157.73±1321[b]	2194.86±5752[a]
Milk yield (kg/d)	35.85±0.18	34.82±1.06	36.47±1.57	34.96±2.16	38.42±9.6
Probability(%)	15.17±0.2[a]	15.17±0.9[a]	15.15±1.5[a]	25.35±2.1[b]	60.46±7.9[b]

Table 4.3: Average (± SE) of lying time (lying time), bouts frequency (bouts), total daily steps (steps), and milk yield (milk yield) in lameness conditions(Foot disorders- presence of any specific lameness conditions, Hoof trimming- animals that required hoof trimming, Healthy- animals not submitted for treatment of foot disorders or hoof trimming) across different stages of lactation. Different superscripts within a row represent statistically significant differences at P< 0.05.

	Foot disorders	**Hoof trimming**	**Healthy**	**Overall**
Lying time (min/d)				**P< 0.0001**
Transition period (<21 DIM)	745.7±64.05	-	632.9±13.15	630.9±13.4[a]
Early (22- 150 DIM)	429.5±7.07	559.9±13.02	575.5±3.1	572.9±3.4[b]
Mid lactation (> 150 DIM	393.4±8.32	574.8±17.64	566.2±2.93	563.9±3.2[c]
Bouts (n/d)				**P = 0.0005**
Transition period	22.1±2.99	-	19.9±0.64	19.8±0.62[ab]
Early	14.3±0.38	18.5±0.63	18.8±0.23	18.7±0.16[a]
Mid lactation	15.9±0.43	20.3±0.84	19.2±0.23	19.1±0.15[b]
Steps (n/d)				**P< 0.0001**
Transition period	2369.4±632	-	2426.1±135.05	2472±136.24[a]
Early	5522.4±79.13	2793.6±133.68	2488.4±48.25	2543±34[a]
Mid lactation	6345.0±90.2	3480.6±177.95	3164.8±47.32	3206.9±34[b]
Milk yield (kg/d)				**P< 0.0001**
Transition period	29.1±4.61	-	26.7±1.08	26.7±1.08[a]
Early	31.5±0.50	33.7±0.97	31.9±0.23	31.9±0.25[b]
Mid lactation	33.9±0.59	35.2±1.23	34.9±0.22	34.8±0.24[c]

Table 4.4. Odds (95% CI) of presence of hoof disorder and necessity of hoof trimming obtained from the predictive models based on logistic regression. Median value for each parameter was determined as cutoff to obtain categorical variables.

	Odds ratio	Confidence interval (95%)	
Presence of foot disorder			
Lying time category (>585 vs ≤ 585 min/d)	2.6	2.14	3.19
Bout frequency category (> 18/d vs ≤ 18/d)	1.8	1.50	2.19
Daily steps category (>1994/d vs ≤ 1994/d)	0.2	0.10	0.16
DIM	1.0	1.02	1.09
Parity (Parity 1 vs Parity >2)	0.8	0.68	1.01
DIM CAT (Transition vs Mid lactation)	<0.01	<0.001	>999.99
DIM CAT (Early vs Mid lactation)	1.5	1.15	1.90
Presence of estrus	0.13	0.11	0.16
Access to pasture	4.7	3.49	3.36
Temperature Humidity Index	1.0	1.00	1.02
Hoof trimming			
Lying time category (> 585 vs ≤ 585 min/d)	0.9	0.61	1.56
Bout frequency category (> 18/d vs ≤ 18/d)	0.9	0.63	1.22
Daily steps category (> 1994/d vs ≤ 1994/d)	0.4	0.24	0.77
DIM	1.0	0.99	1.01
Parity (Parity 1 vs Parity >2)	0.6	0.38	0.93
DIM CAT (Transition vs Mid lactation)	<0.001	<0.001	>999.99
DIM CAT (Early vs Mid lactation)	4.8	2.13	11.10
Presence of estrus	1.5	0.46	4.7
Access to pasture	0.5	0.21	1.18
Temperature Humidity Index	1.0	0.99	1.05

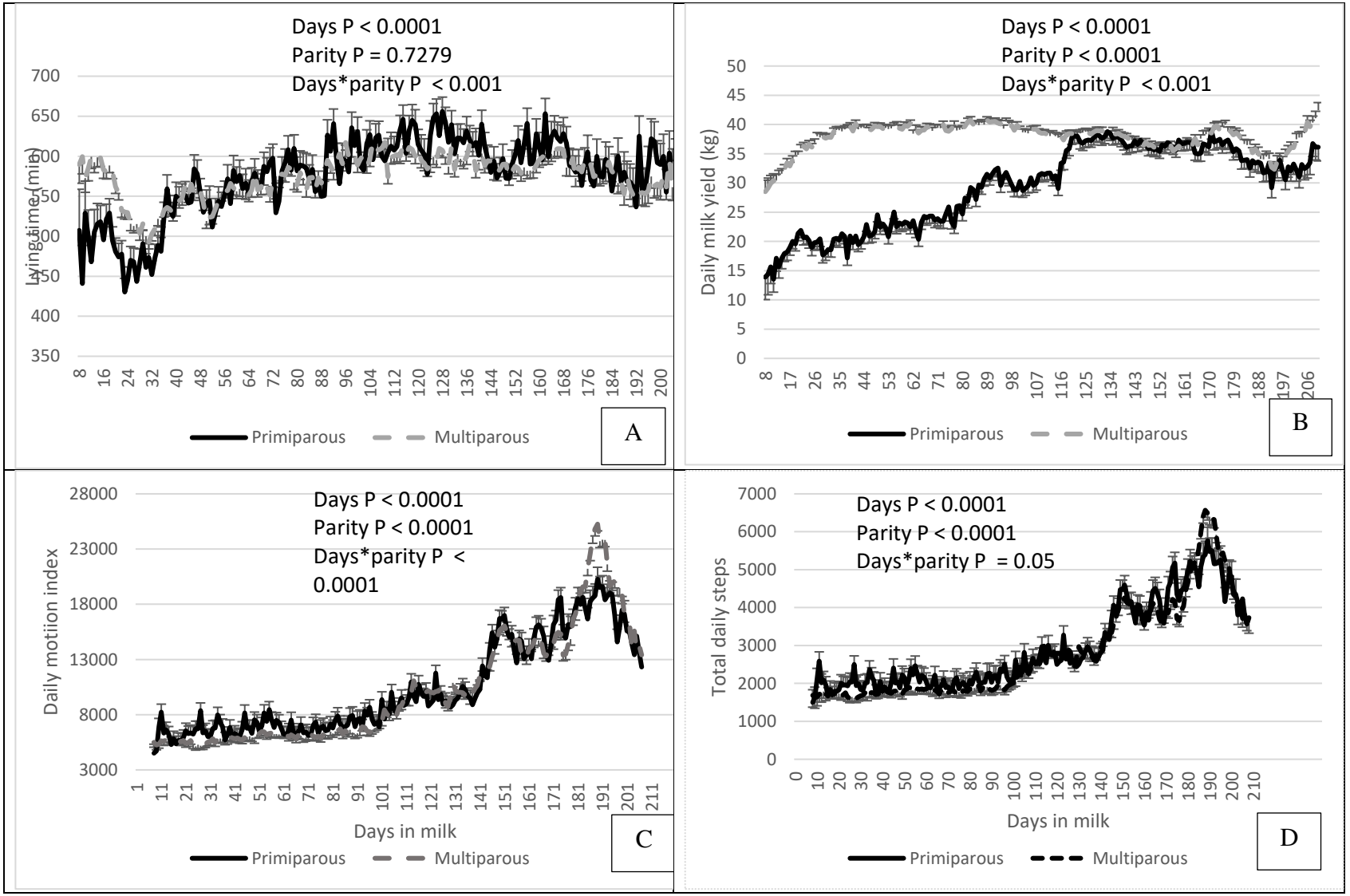

Figure 4.1. Distribution of study parameters across the DIM in the study period: A) lying time, B) daily milk yield, C) daily motion index, and D) total daily steps.

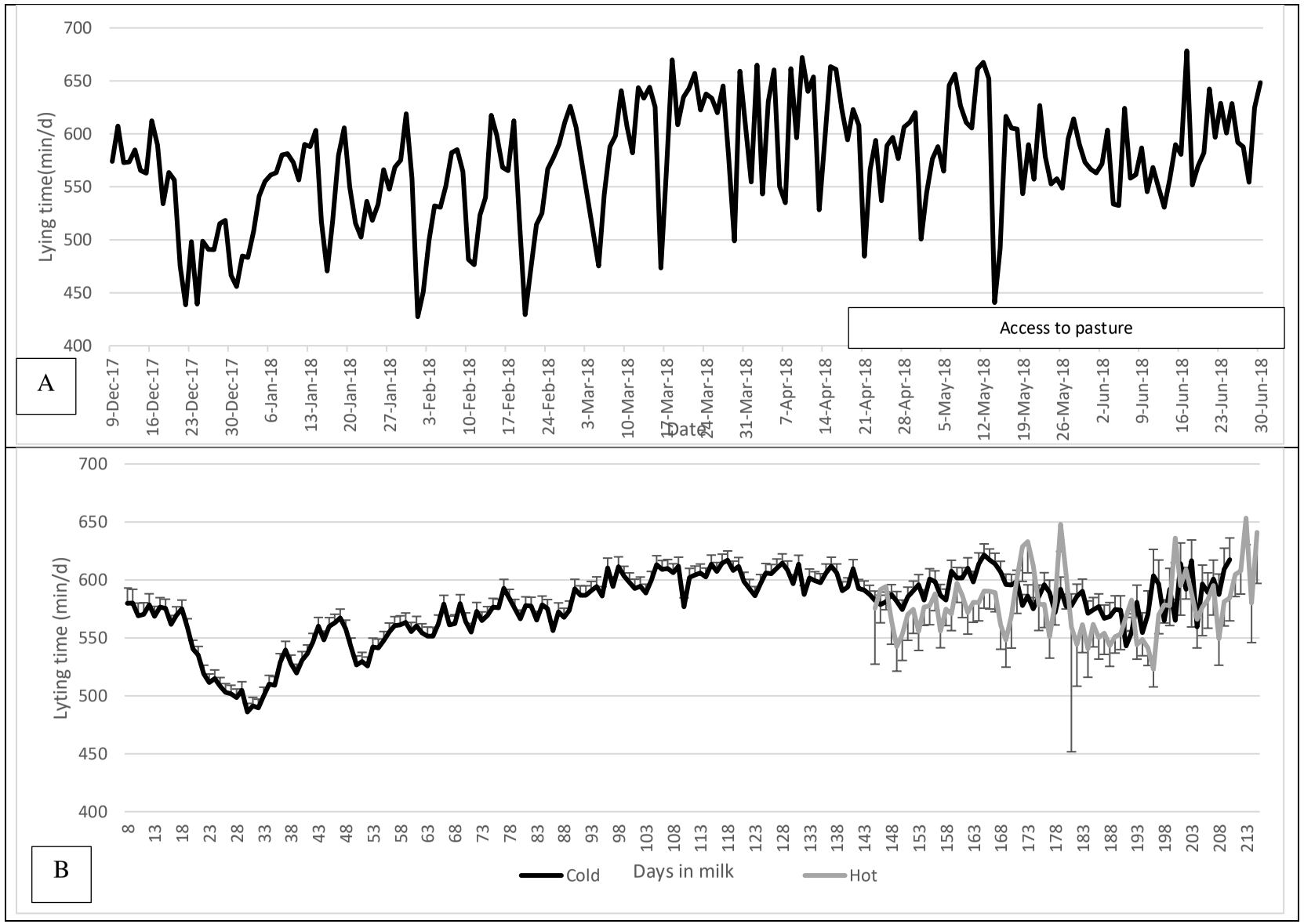

Figure 4.2. Daily lying time by date (A) and by days in milk during the cold and hot season (B).

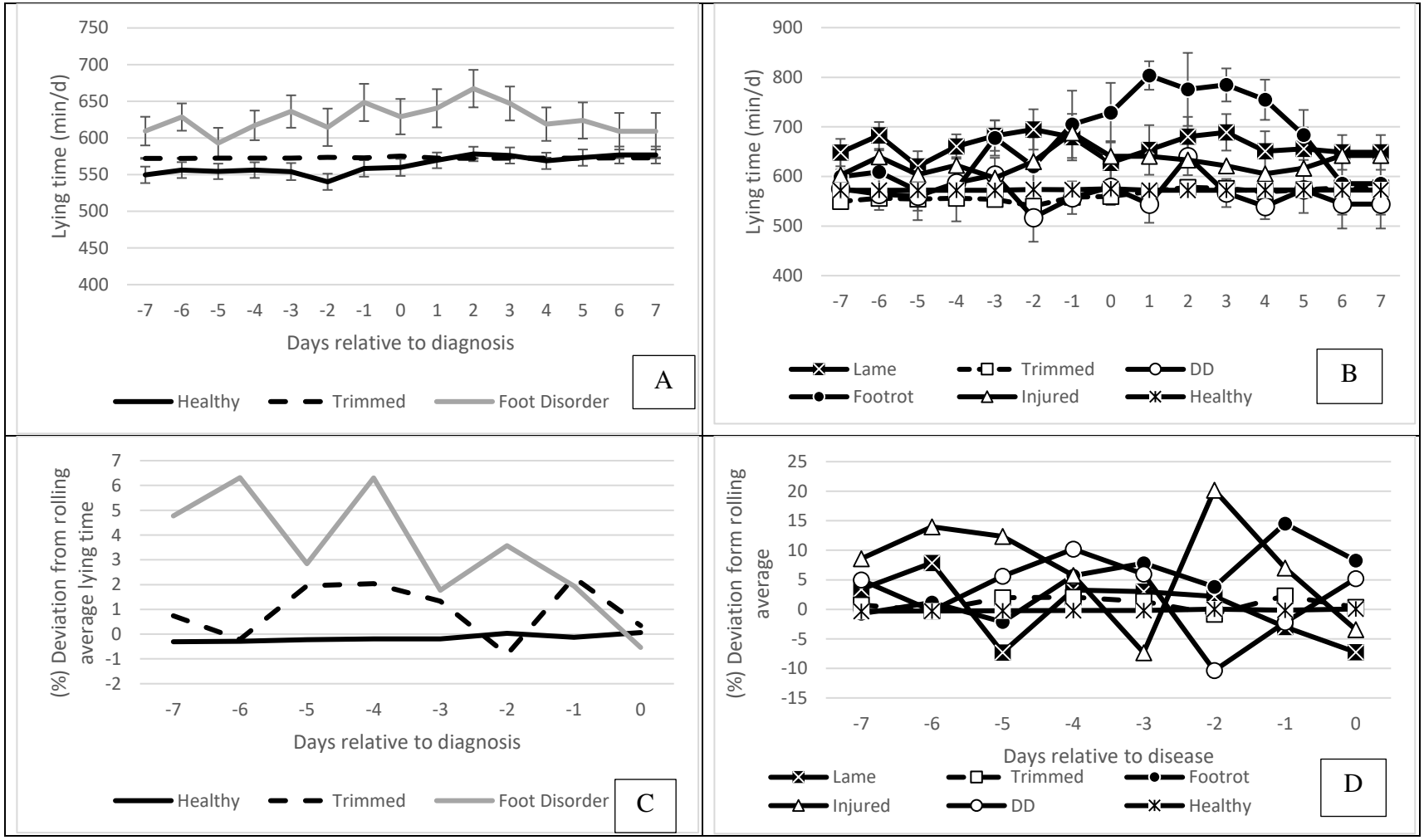

Figure 4.3. Average (±SE) daily lying time from -7 day relative to the diagnosis (d0) to 7-day post treatment in A) animals in three lameness categories; B) animals diagnosed with specific lameness disorders; and C&D) deviation (%) of lying time from the 7 day rolling average for animals divided into three and six foot disorder categories respectively. A total of 21, 21, 10, 190, 23 cows were diagnosed with foot injury (Injured), digital dermatitis (DD), foot rot (Footrot), foot trimming (Trimmed) and nonspecific lameness (Lame) respectively.

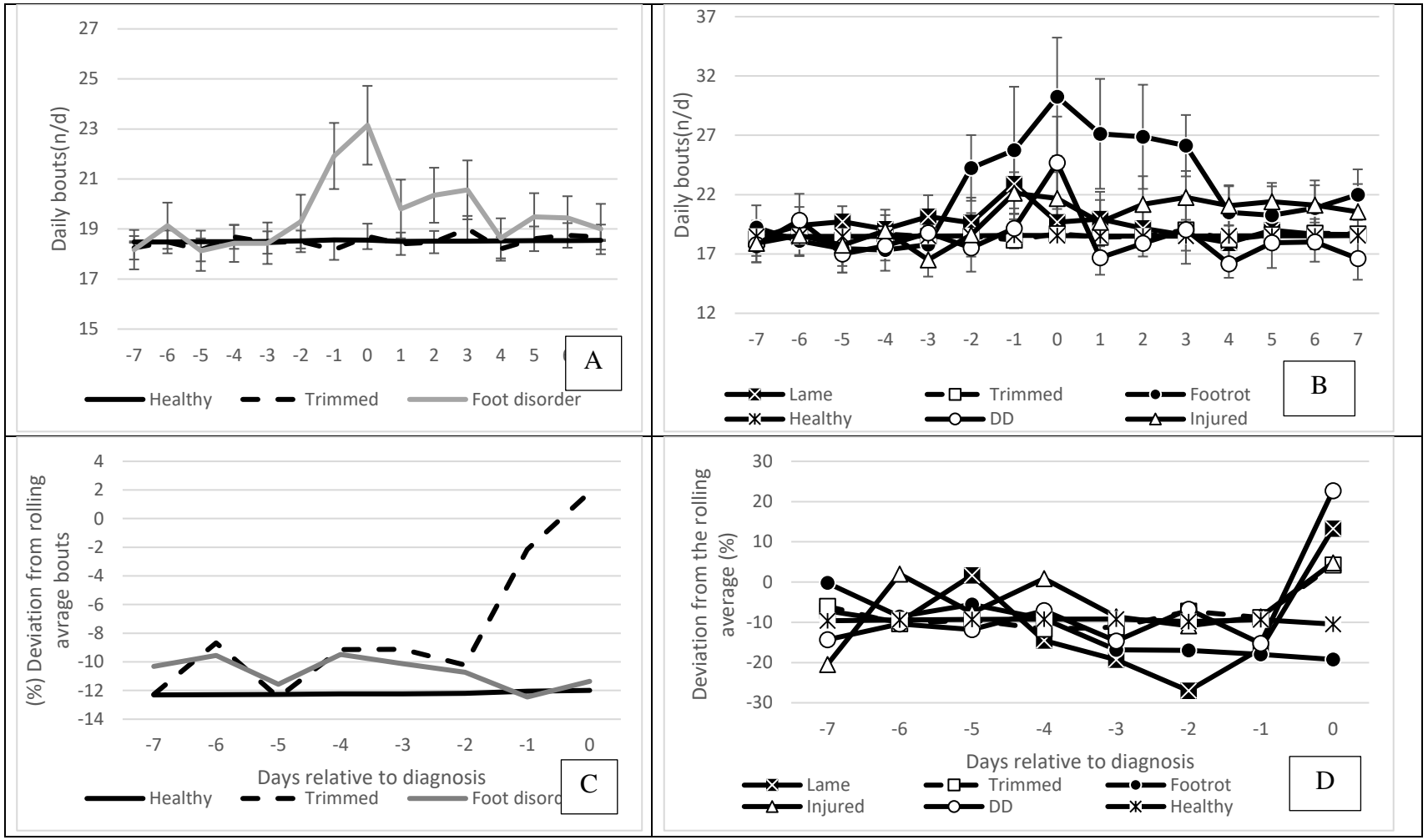

Figure 4.4: Average (±SE) daily bouts from -7 day relative to the diagnosis (d0) to 7-day post treatment in A) animals in three lameness categories; B) animals diagnosed with specific lameness disorders; and C&D) deviation (%) of lying time from the 7 day rolling average for animals divided into three and six foot disorder categories respectively. A total of 21, 21, 10, 190, 23 cows were diagnosed with foot injury (Injured), digital dermatitis (DD), foot rot (Footrot), foot trimming (Trimmed) and nonspecific lameness (Lame) respectively.

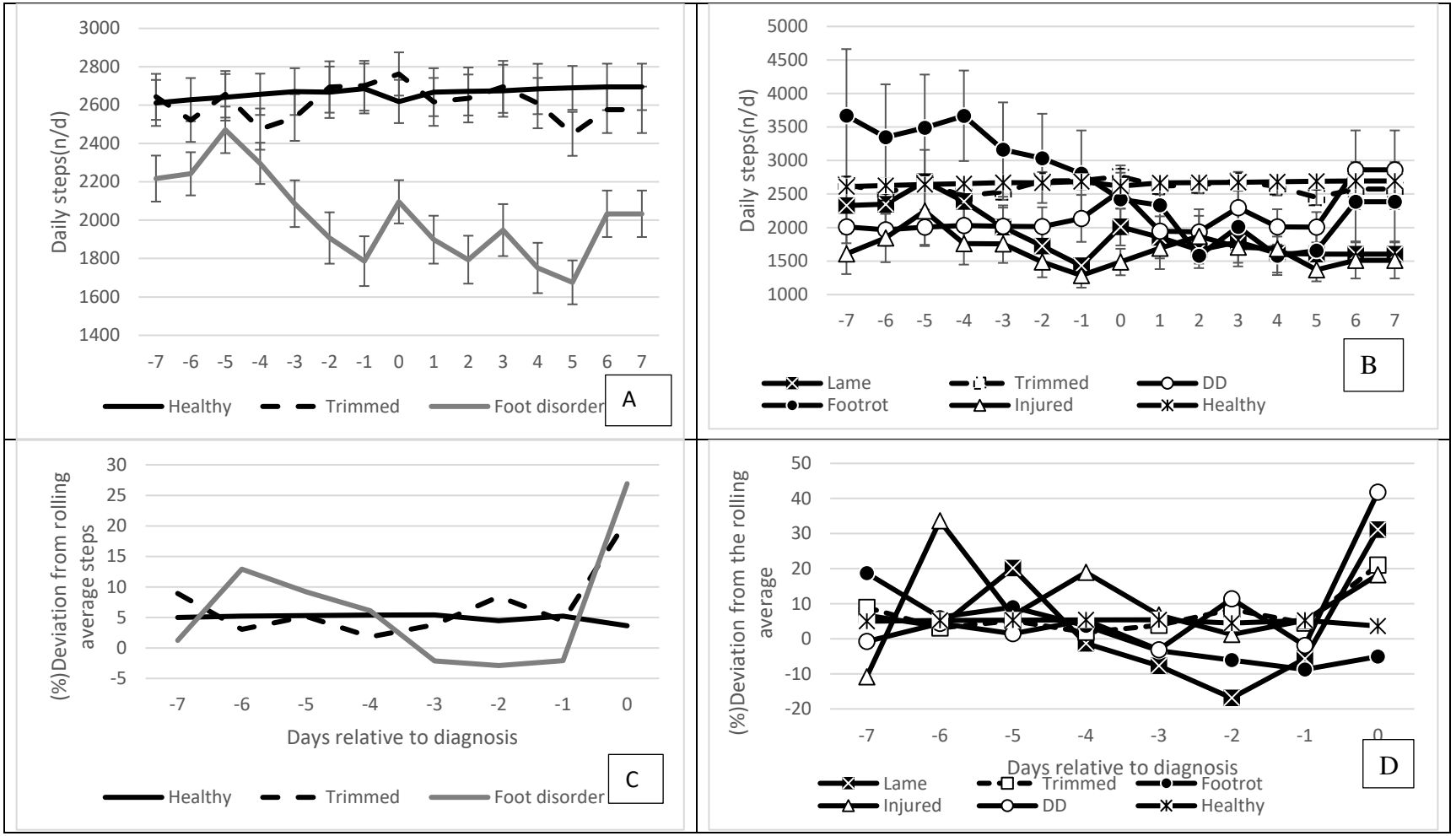

Figure 4.5: Average (±SE) daily steps from -7 day relative to the diagnosis (d0) to 7-day post treatment in A) animals in three lameness categories; B) animals diagnosed with specific lameness disorders; and C&D) deviation (%) of lying time from the 7 day rolling average for animals divided into three and six foot disorder categories respectively. A total of 21, 21, 10, 190, 23 cows were diagnosed with foot injury (Injured), digital dermatitis (DD), foot rot (Footrot), foot trimming (Trimmed) and nonspecific lameness (Lame) respectively.

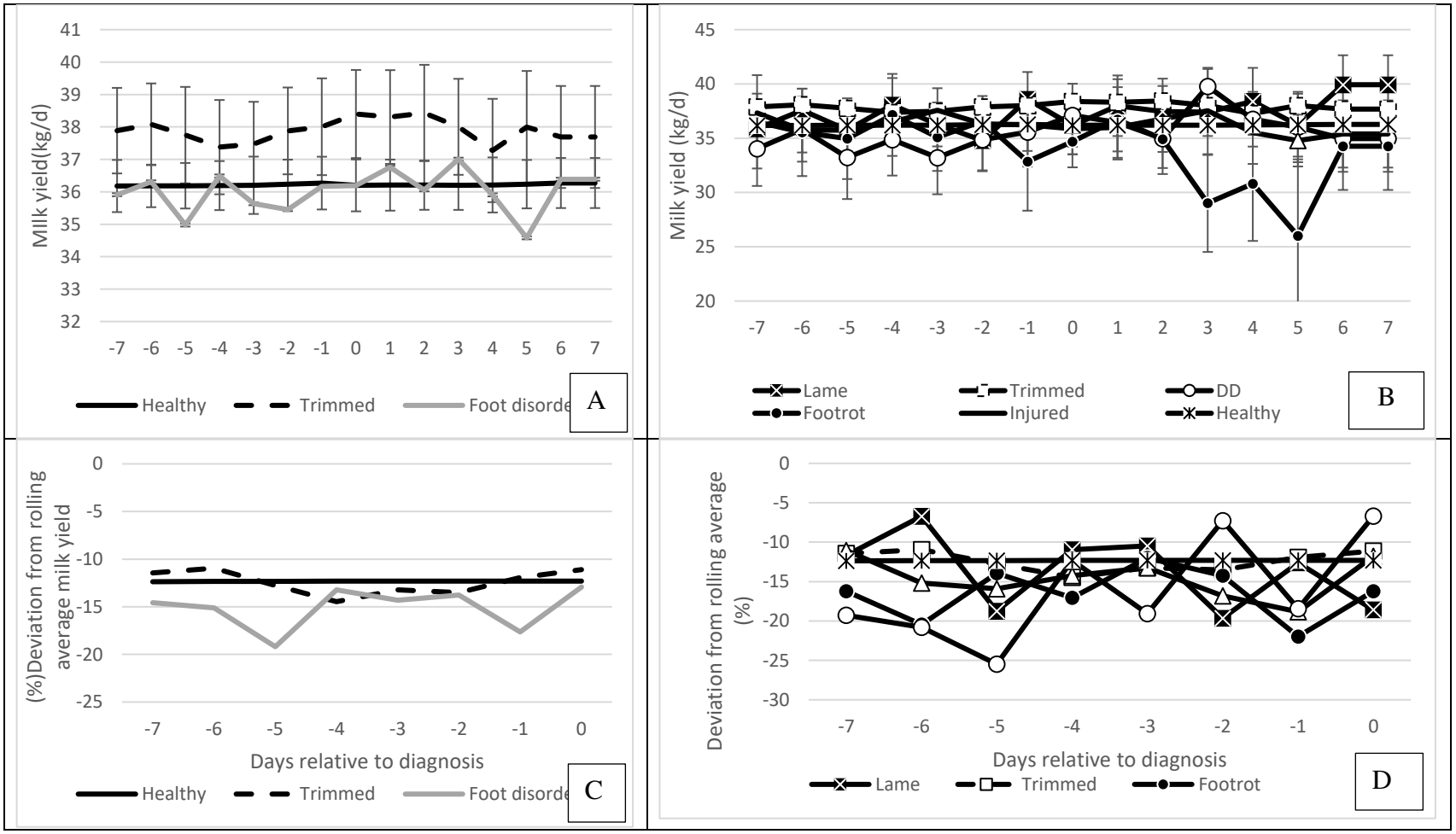

Figure 4.6: Average (±SE) daily milk yield from -7 day relative to the diagnosis (d0) to 7-day post treatment in A) animals in three lameness categories; B) animals diagnosed with specific lameness disorders; and C&D) deviation (%) of lying time from the 7 day rolling average for animals divided into three and six foot disorder categories respectively. A total of 21, 21, 10, 190, 23 cows were diagnosed with foot injury (Injured), digital dermatitis (DD), foot rot (Footrot), foot trimming (Trimmed) and nonspecific lameness (Lame) respectively.

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CHAPTER 5 - EFFICACY OF NON-ANTIBIOTIC TREATMENT OPTIONS FOR DIGITAL DERMATITIS IN AN ORGANIC DAIRY FARM

Introduction

Digital dermatitis (DD) is a contagious, painful, wart-like disorder of the digits of dairy cows, presented as a superficial inflammation of the skin (Berry et al., 2012). Although the precise etiology of this disease is unknown, DD is considered a multifactorial polybacterial condition, with *Treponema* spp. being the most commonly isolated pathogens (Dopfer et al., 2012; Krull et al., 2016). The prevalence of this disorder ranges from 15 to 49% in conventional dairy farms and has been estimated to be about 10% in organic dairy farms (Solano et al., 2017; Pinedo et al., 2017). In a recent USDA survey, 31% of the operations with bred heifers and 75% of the operations with cows reported at least one case of DD with higher percentages in medium and large operations (USDA, 2018). As this is a painful condition, DD results in lameness and represents a serious concern due to decreased milk production and reduced welfare of dairy cows (Shearer et al., 2015; Biagiotti, 2016; Pinedo et al., 2017). Early detection and treatment are crucial for restoring the function of the feet and the well-being of the animal.

Various antimicrobial formulations are available for the treatment of DD (Shearer et al., 1998; Pol et al., 2007; Shearer et al., 2015). However, due to prohibitions in the use of antibiotics, organic dairy farms face serious limitations in the use of therapeutic resources and, consequently, non-antibiotic treatment options have been explored as alternatives for the treatment of DD lesions (Jacobs et al., 2018). The application of topical therapies under a bandage is the most common procedure for DD treatment in organic dairies and poultices or emulsions are used to create an ointment that is applied on the DD lesions (Biagiotti, 2016; Pinedo et al., 2017). However, the efficacy of these preparations has not been fully investigated, and there is a lack of scientific

evidence regarding cure rates of DD lesions. Previous studies that tested products containing mixtures of soluble copper, a peroxide compound, and a cationic agent have demonstrated efficacy in the treatment of DD lesions (Laven et al., 2006) and studies using copper sulfate, iodine, and honey suggest their potential for use in the treatment of DD (Durani et al., 2008; Oelschlaefel et al., 2012; Holzhauer et al., 2012).

Here, we investigated three options for treatment of DD lesions, using formulations made by combining copper sulfate, honey, and iodine. Our hypothesis was that these formulations would range in effectiveness in the cure of DD lesions, as compared to control cows not receiving treatment. Therefore, our objective was to evaluate the short and long-term effect of two treatment formulations on lesion size, lesion score, pain response, and evidence of lameness in organically managed dairy cows.

Materials and Methods

Study animals and housing

This study was conducted in a commercial USDA certified organic dairy herd in Northern Colorado with the approval from the Institutional Animal Care and Use Committee at Colorado State University (protocol ID: 17-7052A). The study population consisted of 70 multiparous Holstein cows identified as having early DD lesions between April and June 2017. The dairy milked approximately 3,500 Holstein cows three times daily, with a rolling herd average of 8,600 kg/cow. All cows were housed in free stall barns with sand bedded stalls and had free access to a contiguous dry lot. Cows were fed a TMR twice a day to meet or exceed the nutritional requirement for a lactating Holstein cow producing 30 kg/d of milk with 3.5% fat and 3.1% true protein according to NRC (2001). Throughout the study period the diet consisted of corn silage (14 to 17.5%); wheat silage (13 to 20%); a premix containing soybean, soy hulls, whole cottonseed, corn,

wheat, and minerals and vitamins (47.5 to 50.5%); sorghum silage (3.0 to 4.5%); alfalfa hay (12 to 16%); and grass hay (0 to 3%).

Lameness management in the farm

The herd was regularly monitored for lameness by screening for lame animals by trained hoof trimmer. Cows showing any lameness sign were moved to the trimming chute and examined for the presence of specific conditions. The whole herd was surveyed for lameness scores at least once every three months and all cows received trimming at least once every six months. The herd had a history of lameness cases associated with DD with an average prevalence of 10%, which was managed without the use of any antibiotics. The whole herd went through preventive footbath with acidified 5% copper sulfate solution twice weekly.

Experimental procedures

A total of 70 cows were enrolled in the study. Cows were screened in the milking parlor using a flashlight and a mirror attached to a spatula to identify DD lesions. Additionally, cows were screened while in the headlocks, looking for pain in the area around the interdigital cleft of the hind feet. Cows with lesions suggestive of DD (n = 155) were taken to the trimming chute and examined. Subsequently, DD lesions were categorized according to M-scoring system (Dopfer et al., 1997; Holzhauer et al., 2011): M0 = normal digit skin without signs of DD; M1= early, small, circumscribed red to grey lesions less than 2cm in diameter; M2 = red or red grey, active, ulcerative lesions > 2 cm in diameter; M3 = healing stage where a firm scab like material has covered the DD lesions; M4 = late chronic lesions that may have thickened epithelium or proliferative filamentous or scab like mass; M4.1 = chronically affected foot that displays both M4 and M1 stages. Only cows with M1 and M2 stage DD lesions in their hind feet were included in the study. On day 0, before application of treatment, all cows were evaluated for pain and lesion

size and received a locomotion and a lesion score. Pain scoring was based on pressure algometry (Millman, 2013) defined as the cow's response to firm pressure with one thumb (approximately 50 N/cm^2) (Holzhauer et al., 2008; Hernandez et al., 1999). The responses were recorded as a number that ranged from 0 to 2 (0 = no signs of pain; 1 = signs of mild pain; 2 = signs of severe pain). Lesion size (cm) was measured as a continuous outcome variable using a standard measuring ruler (Hernandez et al., 1999). Cows were evaluated for locomotion with a scoring system on a scale from 1 to 5 (1 = stands and walks normally with a level back; 2 = stands with flat back but arches when walks, abnormal gait; 3 = stands and walks with an arched back and short strides; 4 = arched back standing and walking, favors one or more limbs but can still bear some weight on them; 5 = pronounced arching of back, reluctant to move, complete weight transfer off the affected limb)(Sprecher et al., 1997). In addition, the type of lesion (early vs mature) was recorded for all study cows; early lesions were round to oval, flat or concave, raw, moist red-yellow to gray, and had tufted or granular strawberry like surfaces, whereas mature lesions were raised, with surfaces covered by small filiform papillae (Read et al., 1998).

Two treatments were prepared at the beginning of the experiment. A total of 30 ml 7% Tincture Iodine (Triodine-7®) was mixed with 940 g copper sulfate to prepare a formulation that was applied to the copper sulphate iodine (CUI) group. Similarly, 30 ml of iodine (Triodine-7®) was mixed with 570 g of honey (Raw and Unfiltered Honey®) to be applied to the honey iodine (HOI) group. The formulations were dispensed using standard measuring spoons. Each animal received an amount equivalent to 10 ml of the formulation in a clean paper towel that was applied topically in the lesion after thorough cleaning of the site with water and drying. Treatment paper towels were fixed in place using a foot wrap that was removed 3 days after treatment application. Control (CON) animals were cleaned and bandaged without any formulations in them.

After initial evaluation on d0, animals were assigned randomly into one of the three treatment groups to receive a one-time treatment on the trimming chute, along with the functional hoof trimming by a trained hoof trimmer. The CON group only received regular footbath after milking, whereas the treatment groups (CUI and HOI) received the treatment formulations wrapped by a bandage for 3 days along with the regular footbath. Cows were evaluated for pain and lesion size and received a locomotion score and a lesion score on day 3 (d3), 12 (d12), 28 (d28), and 120 (d120) by one author that was blinded to the treatment groups. In case of severely deteriorating feet conditions, treatments were executed according to the initial treatment group assigned to the animal (n = 8). Throughout the study period, all treatment groups were maintained under the same management and housing conditions and were fed identical rations. A subsample of 45 cows were followed up until 120 days (d120) due to animals being removed from the farm due to culling or movement to another unit. Clinical cure rate was defined as the transition from active to non-active stages (M1/M2 to M0 or M4) (Holzhauer et al., 2011; Jacobs et al., 2018). The difference in lesion size relative to d0 was obtained as an outcome variable that provided an evaluation of the lesion progression relative to the day of enrollment to account for different proportion of M1/M2 lesions in each treatment groups at enrollment.

Statistical analysis

Data were entered into a Microsoft Excel spreadsheet and all analyses were conducted using statistical software. Due to low number of animals in some response variable categories, categories were collapsed into binary outcomes; either presence or absence of pain (pain scores $\geq$ 1 considered painful (1), pain scores = 0 considered not painful (0)) and either lame or non-lame cows (locomotion scores > 2 considered lame (1), locomotion score $\leq$ 2 considered not lame (0)). The FREQ procedure in SAS was used to compare proportions of categorical outcomes by

treatment group, using chi squared test and Fisher's exact test. Repeated measures analyses using MIXED procedure was used to evaluate continuous responses of lesion size and lesion difference. GENMOD procedure was used to model the categorical responses of pain and lameness.

Results and Discussion

Baseline comparisons

A total of 70 cows were enrolled and allocated into CUI (n = 24), HOI (n = 23), and CON (n = 20). Overall, 55% of the lesions were on the right rear foot and cows in CUI, HOI, and CON had 54%, 64% and 48% of the lesions in their right rear foot respectively. Cows in CUI, HOI, and CON were on average (±SD) 105±72 DIM, 128±57 DIM and 126±53 DIM respectively. Cows in the study were on average 4.5 years old and were in 2nd parity (54.7%), 3rd parity (20.3%), and in parity ≥ 4 (24.9%).

Response to treatment

The proportion of animals with M0 and M1 lesions decreased with follow up days for all treatment groups. Lesions classified as M4.1 stage appeared on d28 for cows in HOI group, whereas in CON group M4.1 lesions appeared only on d120 (Figure 1). Early lesions at the start of the study progressed to more mature lesions in all treatment groups. However, on d120, HOI group had a greater proportion of early than mature lesions (Figure 2).

A significant effect for the interaction between treatment and day on the size of the lesion was observed (P = 0.0016). The average (±SE) lesion diameter for animals in CUI was 2.38 (±0.22) cm on the day of enrollment (d0) and decreased to 2.25 (±0.23) on d3, 2.04 (±0.22) on d12, 2.27 (±0.23) on d28, and 1.19 (±0.28) on day 120. Animals in HOI group started the study with 2.02 (±0.22) cm lesion on average and the lesion reduced to 1.69 (±0.23) on d3, 1.79 (±0.24) cm on d12, 2.23 (±0.23) cm on d28, and 2.024 (±0.223) on d120. Animals in CON group had average

lesion size of 1.7 (±0.22) cm on d0 that increased to 2.25 (±0.23) on d3, 2.21 (±0.226) on d12, 1.97 (± 0.226) on d28, 1.27 (±0.286) on d120 (Figure 3).

The difference in lesion size relative to d0 was associated with both treatment groups and follow up days ($P < 0.05$). For CUI group the average decrease in lesion size was -0.245 cm on d3, -0.398 cm on d12, -0.0874 cm on d28 and -1.19 cm on d120. For the HOI group, the average decrease in lesion size was -0.39 cm on d3, -0.23 cm on d12, but size increased on average by 0.19 cm on d28 and by 0.758 cm on d120. For CON group, the average increase in lesion was 0.59 cm on d3, 0.57 cm on d12, and 0.33 cm on d28 whereas the lesion decreased on average by -0.39 cm on d120 (Figure 4).

When evaluating the pain response on d3, a greater proportion of animals experienced pain in CON followed by HOI and CUI ($P = 0.011$). However, on d12 a greater proportion of animals in HOI group demonstrated pain, followed by CON and CUI. The trend continued until d28 and was significantly different on d120 ($P = 0.0003$; Table 2). On d3, the odds of an animal showing pain were greatest in CON followed by HOI and CUI. The odds of pain were increased for HOI and CUI groups in the corresponding follow up days. HOI group had greater odds of showing pain than CON starting d12 whereas CUI had greater odds of pain only on d120. On comparing the topical treatments, CUI always demonstrated lower odds of experiencing pain than HOI (Table 2).

On d3, a greater percentage of animals in CON experienced lameness followed by HOI and CUI groups ($P = 0.032$). A similar trend was observed on d12 ($P = 0.14$), and d28 ($P = 0.027$), whereas on d120 HOI had greater proportion of animals demonstrating lameness, followed by CON and CUI ($P = 0.01$). On d3, the odds of lameness were greater in CON followed by HOI and CUI (Table 3). The odds were increased for HOI and CUI in the corresponding follow up days. Interestingly, HOI had greater odds of demonstrating lameness than CON on d28 and d120,

whereas CUI had greater odds than CON only on d120. Comparing among the treatment groups, HOI had greater odds of lameness than CUI on all follow up days (Table 3).

The cure rate of DD lesions, defined as the % of M1 and M2 lesions identified at enrollment that transitioned to M4 or M0 stage on the day of follow up, was greatest in CUI on all follow up days, followed by HOI on d3 and d12 and CON on d28 and d120 (Table 4). On d3, the odds of cure were greatest in CUI followed by HOI and CON (Table 4). The odds of cure in CUI and HOI was greater than CON until d28 and d12 respectively after which odds of cure was greater in CON. Comparing the two topical treatments, CUI had greater odds of the lesion being cured than HOI on all follow up days (Table 4).

Discussion

Our results indicated that the non-antibiotic formulations tested in this study have variable effects on the treatment of DD lesions. A smaller percentage of cows experienced pain after treatment with CUI until d12, but on d28 and d120, CON had smaller percentages of animals evidencing pain. When compared to CON, where the percentage of animals experiencing pain constantly decreased across follow up days, HOI did not perform well in decreasing the pain response. CUI had the smallest percentage of lame cows on all follow up days and the cure rate was greatest in this group on all follow up days. The average lesion size in CUI decreased from d0 across the follow up days except on d28, whereas in HOI group average lesion size decreased on d3 and constantly increased on other follow up days. On the other hand, average lesion size constantly decreased in CON group starting d12.

Regarding regression of lesions, CUI performed better than HOI and CON. Lesion size differences for CUI were always negative, indicating that lesion size on all follow up days was smaller than on d0. For HOI, on average, the lesion was smaller on d3 and d12 but was greater

than d0 on d28 and d120. However, for CON the lesion difference was negative on d120 demonstrating the decreased size of the lesion only on d120.

Digital dermatitis lesions in bovines heal by second intention with granulation tissue formation[7]. Previous studies using one-time treatment with topical formulations have associated the bandage with a significant decrease in DD lesion score (Krull et al., 2016). Other studies have used gel paint (Holzhauer et al., 2011), and spray (Jacobs et al., 2018) as treatment application methods. The technique used in our study, including the application of a bandage provided protection from the contamination and washing away of the treatment pharmaceuticals as reported in a previous study (Moore et al., 2001).

Copper sulfate is a bacteriostatic agent that acts by reaction of Cu^{++} with protein thiol groups of pathogens and should be applied in a dilution to minimize the risk of burning the skin of animals[24]. We combined copper sulfate with iodine, which is an antiseptic agent that in itself provides free iodine radicals with bactericidal properties (Durani et al., 2008). The antibacterial benefits of honey are related to its low pH, high osmolarity, and high peroxide activity (Oelschlaefel et al., 2012; Shearer et al., 2015). Since antibacterial activity in honey is primarily due to hydrogen peroxide that is produced by the action glucose oxidase in honey, a higher concentration of honey causes cellular and protein damage by giving rise to oxygen radicals (Bang et al., 2003). To minimize this effect, we used the treatment formulation containing a combination of honey and iodine. Shearer and Hernandez (2000) tested a modified product that contained 2% of original peroxide concentration, 75% of the original concentration of copper and 200% of original cationic concentration. They reported this product to be more effective than commercial products and oxytetracycline in treating DD lesions. Commercial non-antibiotic pastes containing copper and zinc sulfates, and sodium chlorides as an active ingredient have frequently been

reported to be effective in the treatment of DD (Britt et al., 1996; Shearer and Hernandez, 2000; Moore et al., 2001).

In another study conducted in 70 DD hoofs, the honey treated group demonstrated greater cure of lesions (40%) than the group that did not receive any treatment (8%) (Oelschlaefel et al., 2012). The study also concluded that using the honey product was associated with faster healing of lesions. This result is in agreement with our results where we observed a similar cure rate for HOI (50% on d12). However, we observed greater (31%) cured lesions in the CON group, which may be due to the footbath treatments.

Clinical cure rate was defined as the transition from active to non-active stages (M1/M2 to M0 or M4) (Holzhauer et al., 2011; Jacobs et al., 2018) as opposed to a complete lesion healing that implies foot skin returning to an unaffected state (M0). Resolution to healthy foot skin is difficult to obtain (Krull et al., 2016); therefore a treatment option should be able to achieve a manageable state of disease by limiting the presence of an active lesion (Dopfer, 2009). Consequently, the transition of the lesion score and the reduction of the lesion size should be considered relevant outcomes of DD control strategies.

Other non-antimicrobial formulations have been found to perform equally well as compared to the antibiotic options (Shearer et al., 2000). Apley (2015) reported that 5% copper sulfate solution healed 20% of the lesions. This formulation was received by CON in this experiment, and the d28 outcome revealed 73% cured lesions in our study. This discrepancy may be due to the difference in definition of healing and curing, and to acidification of the $CuSO_4$ footbath used in our study herd leading to a higher cure rate. Iodine used in our study has previously been used in the treatment of DD lesions as a spray and observed no effect on the prevalence of DD (Esch et al., 2000). However, we applied the formulation directly on the lesion

that was covered with a bandage, which may have resulted in better outcomes in our study. Similar to our observation in this study, twenty-eight days after single treatment CUI had significantly reduced scores for signs of pain and lesion size as observed by Moore (2001).

Most of the conventional DD treatment studies commonly use Tetracycline and Oxytetracycline and consider positive control in DD treatment evaluation studies. Britt (1996) and Shearer (2000) used an antibiotic treatment as standard therapy for comparison of the efficacy of non-antibiotic formulations. However, as this study was conducted in a USDA certified commercial dairy herd, there was no opportunity for a positive control group. Britt (1996) also investigated a placebo group treated with tap water spray, but due to ethical and animal welfare regulations, we provided the regular footbath to all treatment groups. Although this study is set in the organic dairy farm, we observed up to 76% cure rates that are similar to improvement rates obtained for either chlortetracycline or comparable oxytetracycline treatments (58% to 87%) (Berry et al., 2010).

Conclusions

In conclusion, according to the results of this study, the CUI may be a valuable treatment option for acute digital dermatitis in organic dairy farms. However, the effect of HOI was not established for long-term treatment and control.

Table 5.1**:** Demographic and physical characteristics at the time of study enrollment (baseline) for 70 Holstein cows that received copper sulfate and iodine (CUI), honey and iodine (HOI) and no treatment (CON). Values represent the mean (±SD) or numbers. CUI group received formulation containing copper sulfate and iodine. HOI group received formulation containing honey and iodine. CON group did not receive any treatment. [1]CUI = Copper sulfate and iodine, [2]HOI= Honey and iodine, [3]CON = Control, [4]DIM = Days in milk subsequent to calving.

	Treatment groups			
Parameters	**CUI[1]**	**HOI[2]**	**CON[3]**	***P* value**
N	24	23	23	
DIM[4]	105.23±72	127.71±57	126.21±53	0.47
Age at enrollment	4.57±1.5	4.58±1.2	4.44±1.3	0.93
Lactation number	2.95±1.4	2.73±0.9	2.75±1.2	0.81
Lesion on right claw(n)	14	15	11	
Lesion on left claw(n)	10	8	12	

Table 5.2. Proportion of cows that had signs of pain after application of treatment, and the ratio of odds of cows demonstrating pain on different follow up days Values represent the proportion of cows demonstrating pain (percentage) or the ratio of odds of cows demonstrating pain in two treatment groups (95% confidence interval). The study animals were followed until 120 days after treatment application, and pain response was evaluated. Pain score was based on cow's response to firm pressure with one thumb (approximately 50 N/cm^2) evaluated as presence (1) or absence (0) of pain. The proportion of animals was evaluated using the chi-squared test and odds ratio was obtained using logistic regression analysis for the study participants in Table 1. [1]CUI = Cows that received formulation containing copper sulfate and iodine. [2]HOI= Cows that received formulation containing honey and iodine. [3]CON = Cows that received no treatment.

	Follow up days			
Treatment	**Day 3**	**Day 12**	**Day 28**	**Day 120**
CUI[1] (%)	4/22 (18.2%)	4/23 (17.4%)	4/21 (19.1%)	1/14 (7.1%)
HOI[2] (%)	11/22 (50%)	10/20 (50%)	9/22 (40.9%)	10/18 (55.6%)
CON[3] (%)	13/21 (61.9%)	10/22 (45.5%)	4/22 (18.2%)	1/13 (7.7%)
P-value	0.011	0.05	0.15	0.0003
Odds ratio				
CUI[1] vs CON[3]	0.04 (0.01-0.46)	0.11 (0.01-0.95)	0.90 (0.09-8.39)	1.31 (0.03-61.33)
HOI[2] vs CON[3]	0.91 (0.14-6.19)	3.06 (0.39-23.69)	12.21 (1.11-134.91)	102.20 (2.76 -999)
CUI[1] vs HOI[2]	0.05 (0.01-0.59)	0.04 (0.01-0.49)	0.07 (0.01-0.93)	0.01 (0.01-0.43)

Table 5.3- Proportion of cows that had that had signs of lameness (locomotion score≤ 2 are not lame; locomotion score > 2 are lame) after application of treatment and odds ratio of cows exhibiting lameness on different follow up days. Values represent the proportion of cows demonstrating lameness (percentage) or the ratio of odds of cows demonstrating lameness in two treatment groups (95% confidence interval). The study animals were followed until 120 days after treatment application, and lameness condition was evaluated. Cows were evaluated for locomotion scoring on a scale of 1 to 5 (1 = stands and walks normally with a level back; 2 = stands with flat back but arches when walks, abnormal gait; 3 = stands and walks with an arched back and short strides; 4 = arched back standing and walking, favors one or more limbs but can still bear some weight on them; 5 = pronounced arching of back, reluctant to move, complete weight transfer off the affected limb). Cows were categorized as not lame (0) if locomotion score was less than or equal to 2 and categorized as lame (1) if locomotion score was greater than 3.

	Follow up days			
Treatment	**Day 3**	**Day 12**	**Day 28**	**Day 120**
CUI[1] (%)	4/22 (18.2%)	5/23 (21.7%)	3/21 (14.2%)	2/14 (14.3%)
HOI[2] (%)	6/22 (27.3%)	7/20 (35%)	7/22 (31.8%)	8/18 (44.4%)
CON[3] (%)	11/21 (52.3%)	11/22 (50%)	4/22 (18.2%)	2/13 (15.4%)
P-value	0.032	0.14	0.027	0.011
Odds ratio				
CUI[1] vs CON[3]	0.22 (0.07-0.75)	0.37 (0.13-1.06)	0.99 (0.23-4.26)	1.63 (0.19-14.17)
HOI[2] vs CON[3]	0.35 (0.12-1.04)	0.62 (0.22-1.71)	2.45 (0.74-8.05)	5.23 (0.76-36.17)
CUI[1] vs HOI[2]	0.63 (0.16-2.5)	0.59 (0.18-1.97)	0.41 (0.09-1.69)	0.31 (0.08-1.22)

Table 5.4**-** Proportion of cows that demonstrated cure of the lesions after application of treatment and ratio of odds of cows demonstrating cure on different follow up days. Values represent the proportion of cows demonstrating cure of lesions (percentage) or the ratio of odds of cows demonstrating cure of lesions in two treatment groups (95% confidence interval). The study animals were followed until 120 days after treatment application, and cure rate was evaluated. The clinical cure rate was defined as the transition from active to non-active stages (M1/M2 to M0 or M4) of lesions. DD lesions were categorized as cured (1) and not cured (0). The proportion of animals cured was evaluated using the chi-squared test and odds ratio was obtained using logistic regression analysis for the study participants in Table 1.

	Follow up days			
Treatment	**Day 3**	**Day 12**	**Day 28**	**Day 120**
CUI[1] (%)	7/22 (31.8%)	15/23 (65.2%)	16/21 (76.2%)	11/14 (78.6%)
HOI[2] (%)	6/22 (27.3%)	10/20 (50%)	14/22 (63.6%)	8/18 (44.4%)
CON[3] (%)	3/21 (14.3%)	7/22 (31.8%)	16/22 (72.7%)	10/13 (76.9%)
P-value	0.38	0.08	0.64	0.005
Odds ratio				
CUI[1] vs CON[3]	2.77 (0.61-12.59)	4.13 (1.19-14.38)	1.26 (0.31-5.07)	0.62 (0.08-4.58)
HOI[2] vs CON[3]	2.28 (0.49-10.57)	2.01 (0.57-7.03)	0.65 (0.18-2.33)	0.14 (0.02-0.30)
CUI[1] vs HOI[2]	1.21 (0.33-4.43)	2.06 (0.59-7.06)	1.93 (0.50-7.43)	4.33 (0.95-19.63)

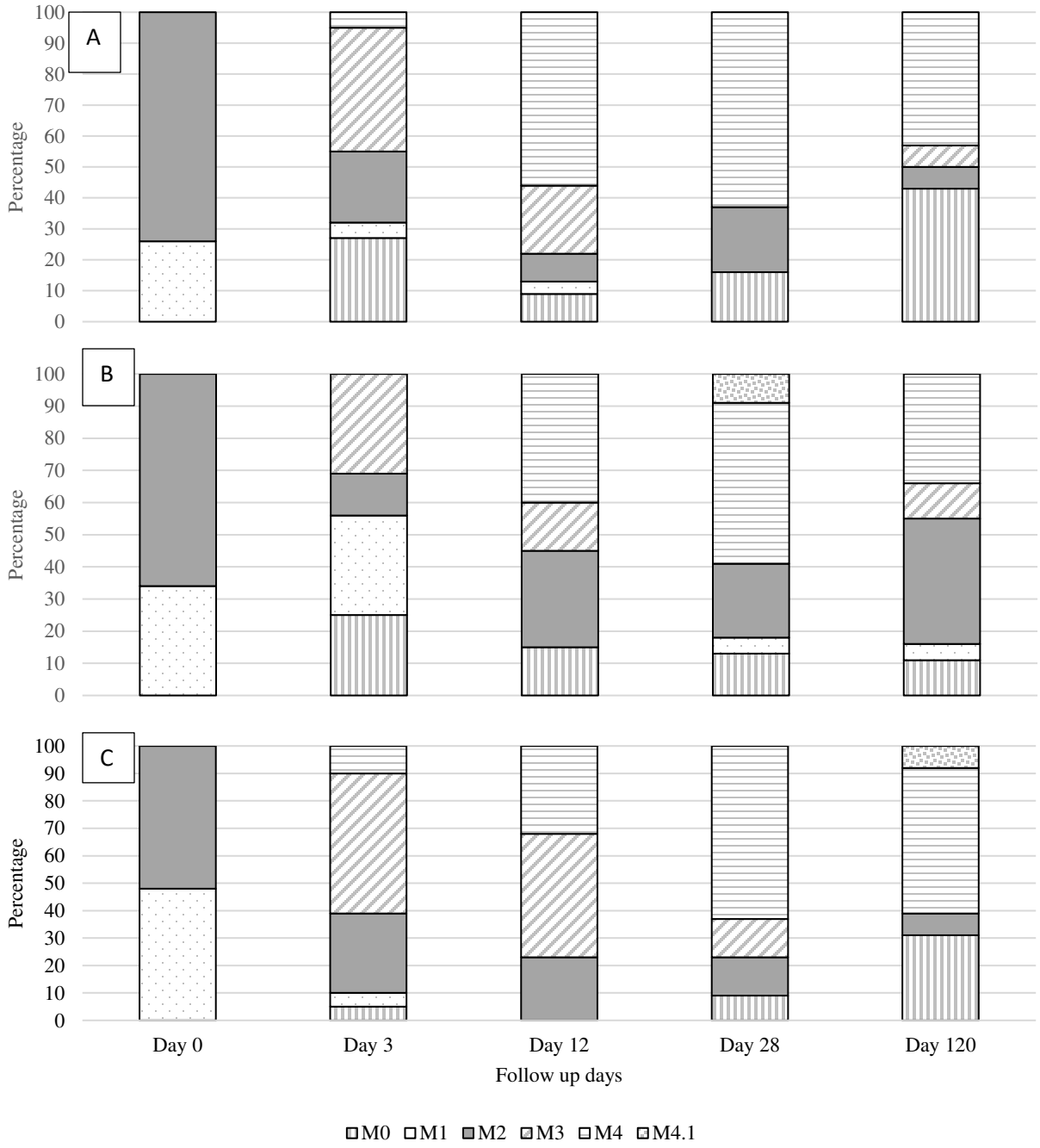

Figure 5.1- Percentage of digital dermatitis (DD) lesion scores across different follow up days after one time treatment with: A) formulation containing copper sulfate and iodine (CUI), B) honey and iodine (HOI), and C) non-treated control (CON). DD lesions were categorized according to M-scoring system: M0 = normal digit skin without signs of DD; M1= early, small, circumscribed red to grey lesions less than 2cm in diameter; M2 = red or red grey, active, ulcerative lesions > 2cm in diameter; M3 = healing stage where a firm scab like material has covered the DD lesions; M4 = late chronic lesions that may have thickened epithelium or proliferative filamentous or scab like mass; M4.1 = chronically affected foot that displays both M4 and M1 stages.

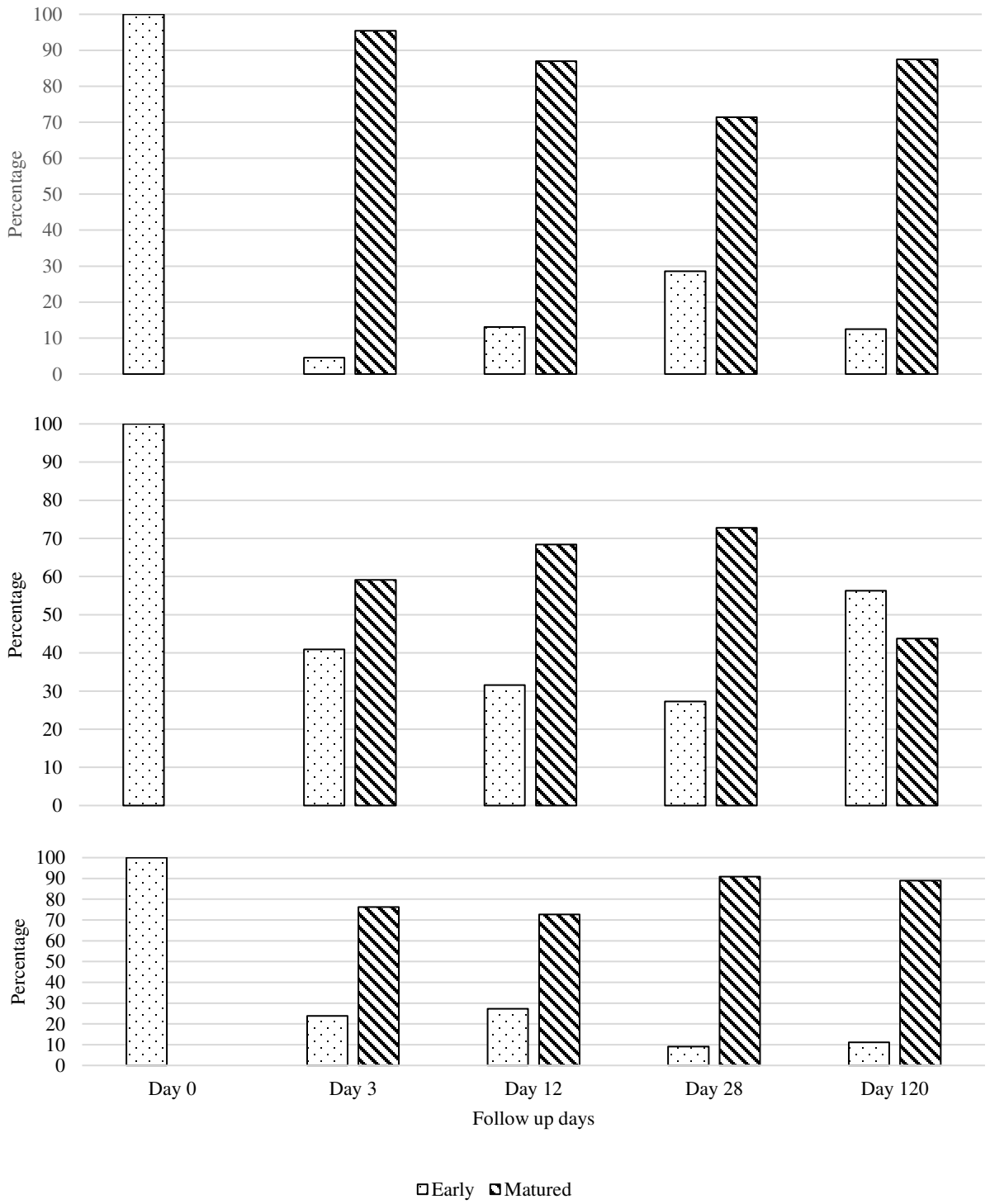

Figure 5.2**.** Percentage of digital dermatitis (DD) lesion types across different follow up days (day 0, day 3, day 12, day 28, day 120) after one time treatment with formulation containing: A) copper sulfate and iodine (CUI), B) honey and iodine (HOI), and C) non-treated control (CON). DD lesions were categorized as early and matured lesions: early lesions were round to oval, flat or concave, raw, moist red-yellow to gray, and had tufted or granular strawberry like surfaces whereas mature lesions were raised, with surfaces covered by small filiform papillae.

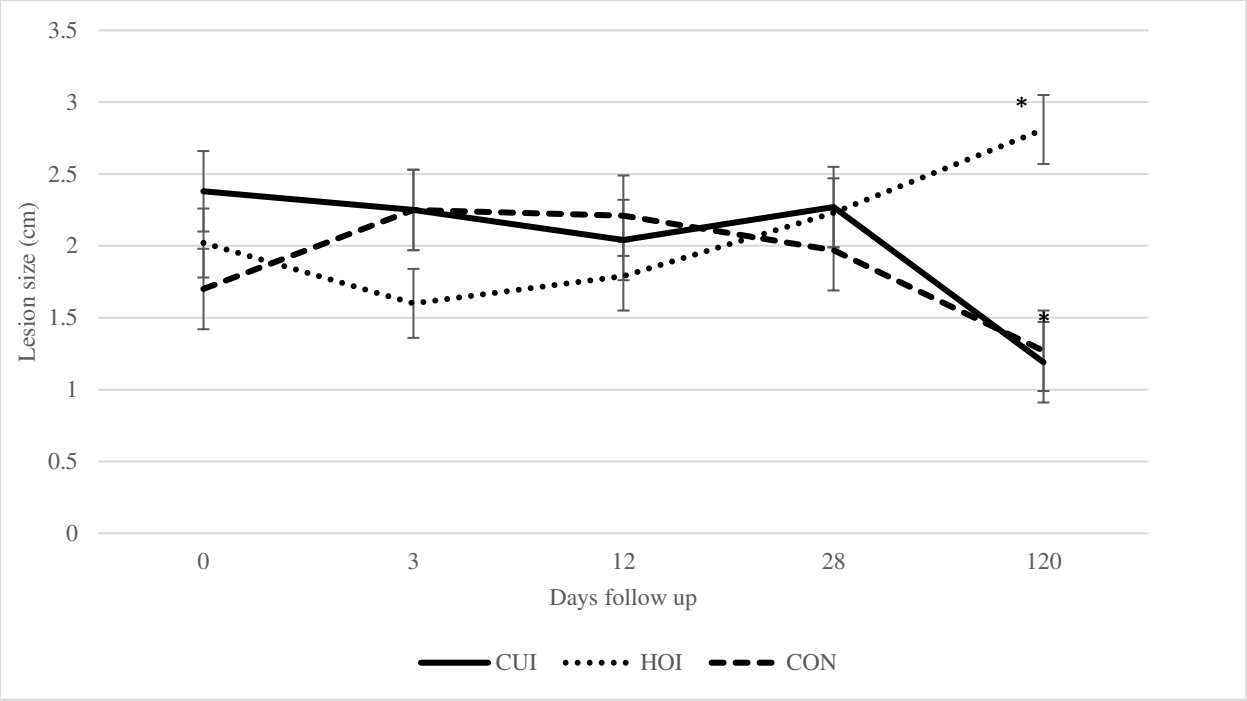

Figure 5.3**.** Mean ± SE lesion size (cm) by treatment group across the follow up days. The solid line represents a group that received formulation containing copper sulfate and iodine (CUI; n = 24); the dotted line represents a group that received formulation containing honey and iodine (HOI; n = 23); broken line represents non-treated control group (CON; n = 23). Animals were followed on day 3, day 12, day 28 and day 120 after application of treatment. *significantly ($p < 0.05$) different values on the day of follow up.

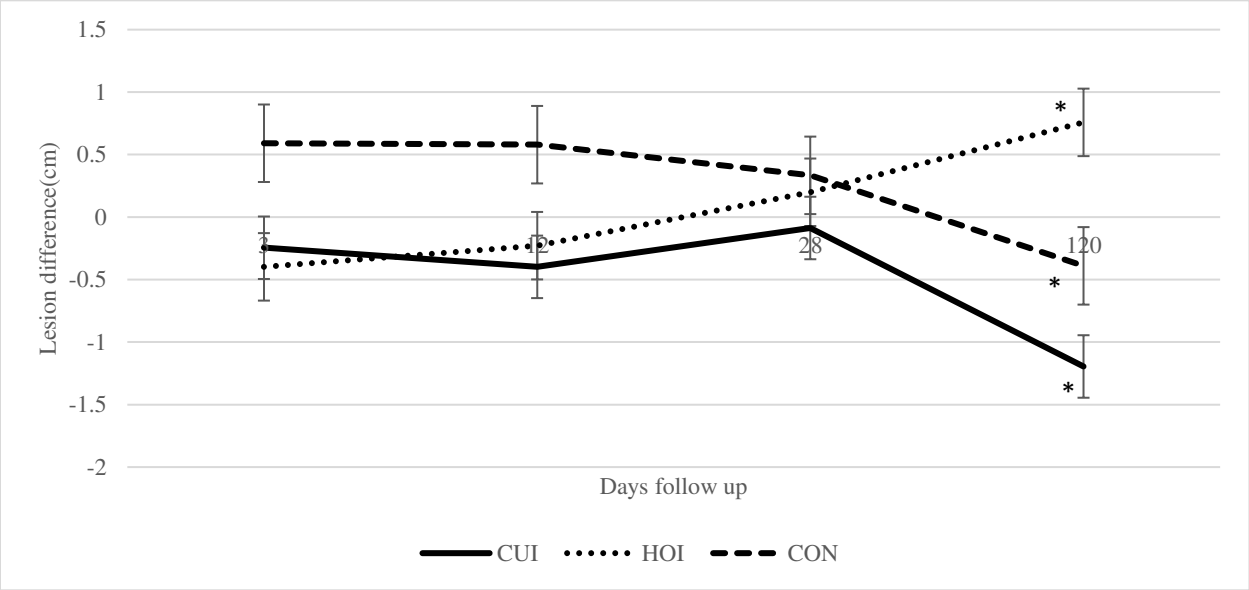

Figure 5.4. Mean ± SE difference in size of lesions from the day of enrollment by treatment groups across the follow up days. The solid line represents a group that received formulation containing copper sulfate and iodine (CUI; n = 24); the dotted line represents a group that received formulation containing honey and iodine (HOI; n = 23); broken line represents control group (CON; n = 23). Animals were followed on day 3, day 12, day 28 and day 120 after application of treatment. *significantly ($p < 0.05$) different values on the day of follow up.

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CONCLUSIONS

Early detection of disease is possible

Our findings indicate that there is potential for detection of subclinical mastitis and lameness disorders before evident clinical signs by the use of sensor technologies. Based on observations of variables under study, we identified that altered electrical conductivity patterns begin 3 days before a mastitis event and milk component patterns begin 4 days before different transition disorders. Diseases during early lactation could be detected with satisfactory sensitivity and specificity based on milk component analyses. Similarly, using lying time and bout frequency, cows requiring treatment of foot disorders or a corrective trimming could be distinguished as early as 3 days before the evident signs.

Milk analysis leads to improved mastitis detection

Subclinical mastitis could be detected using milk leucocyte differential and electrical conductivity with satisfactory precision. The focus of this dissertation was placed on identifying categories of mastitis causing pathogens based on these parameters. Our results identified significant differences in white cell proportions in milk between specific pathogen groups, but the magnitude of the changes was small. This research also reported higher macrophage % in healthy quarters than in quarters infected by any pathogen. The proportional decrease of macrophage % with increases in neutrophil% was evident in sick quarters. We also perceived that Gram-positive pathogens demonstrated the greatest electrical conductivity deviations across time, with greatest increments in electrical conductivity and greatest reductions in milk yield on the day of identification. Contrary, Gram-negative pathogens evidenced both a small increase in electrical conductivity and a small decrease in milk yield on the day of detection. This ability for mastitis

pathogen differentiation would help on targeting specific treatments for pathogens involved early in the disease process.

Milk components are related to transition cow diseases

Results from our study indicated that depending on the health disorder, milk components demonstrated different temporal patterns for fat protein ratio, fat lactose ratio, and protein lactose ratio. Based on the highest AUC results for both the cow and pen level variables, fat protein ratio was more useful in detecting digestive disorders, whereas fat lactose ratio was more useful in detecting mastitis, lameness, and ketosis. In addition, protein lactose ratio was more effective in detecting metritis and hypocalcemia. Therefore, based on disease of interest, different monitored parameters could be utilized for detection of specific health disorders.

Activity behavior can identify lameness disorders

The results from this dissertation demonstrate potential to differentiate animals requiring hoof trimming and treatment of foot disorders which may further encourage producers to adopt this technology. It was concluded that the dynamics of lying time, bout frequency, daily steps and milk yield around hoof trimming are specific and require different recovery times after treatment application to come reach normal levels.

Cupper sulphate is better treatment of digital dermatitis

We evaluated two combinations of honey, iodine, and copper sulphate for their efficiency on digital dermatitis treatment. We observed that the copper sulphate and iodine combination was the most effective in treatment of the disorder in long and short term based on observed cure rate, reduced pain, and reduced lesion size.

New perspectives in animal well being

As research provide new knowledge on the use of precision dairy technologies, more opportunities for improving management strategies to enhance animal health, production and reproductive performance are available. Additionally, these novel therapeutic strategies for use will prove beneficial in controlling the infection in organic herds as well as reducing the antibiotic use in conventional herds. Thus, the focus of dissertation on early detection combined with novel treatment strategy brings new perspectives in animal health and veterinary medicine research.

Potential future directions

For farmers, the investment decision in precision dairy technologies to support cow health management will depend on the profitability of a sensor system. An increase in gross margin for an infected animal treated determines the financial viability and investment potential. Results supporting the effective identification of health disorders using precision technologies and effective treatment of disorders using novel strategy does not guarantee the adoption of these methods by farmers. A systems approach to the analysis of these strategies is necessary to elaborate the potential benefits. There are limited studies that evaluate the economic benefit by adopting technology, and economic and environmental benefits from using the non-antibiotic therapy, which leaves opportunity for the future research. Future research efforts on sensor technologies should be directed towards considering net present value of the investment, effect on labor requirement of dairy farm, expected development of the cow's conditions, the expected effect of an action, and the costs of not taking a decision. A comparison of economic and technical (production & reproduction) performance of farms using sensor technology for health management and farms that do not use these sensors is another necessity. There are studies on decision support and economic considerations of dairy farm management decisions, but they are not integrated in a

sensor system. A study using holistic approach to include all aspects of the dairy management decision utilizing the sensor system is the present necessity.