Data-Based Approaches to Improve Accuracy and Timing of Mastitis Detection in Automatic Milking Systems

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DECLARATION

I certify that the work presented in this thesis is my sole original work and this thesis has not been submitted to any other University for any other degree. All sources of information and assistance during the research work are acknowledged accordingly.

This thesis is presented as a collection of manuscripts. All the research chapters have been published in different peer-reviewed journals. Additionally, some of this thesis work has been presented in different seminars, symposiums and conferences. All the chapters within this thesis have been written in publication style with Australian English as preferred language.

Momena Khatun

September 30, 2019

AUTHORSHIP ATTRIBUTION STATEMENT

This thesis includes four research papers, all of them are published in peer-reviewed journals. I, Momena Khatun, was primarily responsible for the design, implementation, data collection, data analysis, and writing up of each of the research studies under the supervision of Professor Sergio Carlos Garcia and Assoc. Professor Peter Thomson (School of Life and Environmental Sciences and Sydney Institute of Agriculture, The University of Sydney, Camden 2570, New South Wales, Australia). All the statistical analyses were conducted primarily in consultation with Assoc. Professor Peter Thomson. All the co-authors have substantial contribution in co-designing, co-analysis and writing the drafts for the manuscript.

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As supervisor for the candidate upon which, this thesis is based, I can confirm that the authorship attribution statements above are correct.

Professor Sergio C Garcia

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Dedicated to my family

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ABBREVIATIONS

The following is a complete list of abbreviations used throughout this thesis. Abbreviations are also defined when first used in each chapter.

$	Australian dollar
%	Percent
°C	Degree Celsius
Ω	Ohm
Ɛ	Random error
Activity	Daily activity (arbitrary unit/day)
AMR	Automatic milking rotary
AMS	Automatic milking system(s)
AUC	Area under the curve
cm	Centimetres
C^+	Upper cusum
C^-	Lower cusum
CI	Confidence interval
Cl^-	Anions
CC	Cumulative sum (cusum) control
CM	Clinical mastitis
d	Day(s)
D 0	Actual day of clinical mastitis
DIM	Days in milk
DM	Dry matter
EC	Electrical conductivity
ECH	Electrical conductivity per hour
ER	Electrical resistance (ohm)
FN	False negative
FP	False positive
GBC	Grain-based concentrate
GLMM	Generalised linear mixed model
h	Hour(s)
IM	Incomplete milking in each milking session

ISO	International Organization for Standardization
K	Reference value
kg	Kilogram(s)
LCL	Lower control limit
LDH	Lactate dehydrogenase
LP	Linear predictor
MDi	Mastitis detection index
MDQ	Draminski mastitis detector 4 quarters
MF	Average milk flow rate (kg/min)
MI	Model implementation
ml	Millilitre
min	Minute(s)
mo	Month(s)
mS/cm	Millisiemens/centimetre
MA	Moving average
MY	Milk yield per milking (kg)
MYH	Milk yield per hour (kg/h)
n	Number
Na^+	Sodium Cations
NSW	New south wales
P	*P*-value
PCA	Principal component analysis
PF	Peak milk flow rate (kg/min)
Q	Quarter
REML	Restricted maximum likelihood procedure
RC	Regression coefficient
ROC	Receiver operating characteristic curve
Rumination	Daily rumination (min/day)
s	Second(s)
SCC	Somatic cell count
SD	Standard deviation
SE	Standard error
Se	Sensitivity
Sp	Specificity
SPC	Statistical process control

Strep.	*Streptococcus*
Staph.	*Staphylococcus*
T	Target
TEM	Milk temperature
TN	True negative
TP	True positive
U	Udder
UCL	Upper control limit
vs.	Versus

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CHAPTER 3 **M. Khatun**, C. E. F. Clark, N. A. Lyons, P. C. Thomson, K. L. Kerrisk, and S. C. García, 2016. Early detection of clinical mastitis from electrical conductivity data in an automatic milking system. *Anim. Prod. Sci.* 57(7) 1226-1232 doi.org/10.1071/AN16707)

CHAPTER 4 **M. Khatun**, R.M. Bruckmaier, P. C. Thomson, J. House, and S. C. García, 2019. Suitability of somatic cell count, electrical conductivity, and lactate dehydrogenase in foremilk before versus after alveolar milk ejection for mastitis detection. *J. Dairy Sci.*, 102 doi.org/10.3168/jds.2018-15752

CHAPTER 5 **M. Khatun**, P. C. Thomson, K. L. Kerrisk, N. A. Lyons, C. E. F. Clark, J. Molfino, and S. C. García, 2018. Development of a new clinical mastitis detection method for automatic milking systems. *J. Dairy Sci.* 101(10):9385-9395. doi: 10.3168/jds.2017-14310.

CHAPTER 6 **M. Khatun**, P. C. Thomson, and S. C. García, 2019. Prediction of quarter level subclinical mastitis by combining in-line and on-animal sensor data. (*Anim. Prod. Sci.*, in press).

Peer-reviewed conference papers

M. Khatun, C. F. F. Clark, N. A. Lyons, K. L. Kerrsik, and S. C. García, 2016. Early detection of clinical mastitis from electrical conductivity data in an automatic milking system. Australasian Dairy Science Symposium on 16th to 18th November, 2016, Camperdown, NSW, Australia. (paper and oral presentation)

M. Khatun, C. F. F. Clark, P. C. Thomson, K. L. Kerrisk, J. Molfino, and S. C. García, 2017. Improving mastitis detection with automatic milking systems: Using electronic data in an automatic milking system to identify clinical mastitis. Australian Dairy Conference on 14th to 16th February, 2017, National Wine Centre of Australia, Adelaide, South Australia. (Paper and oral presentation)

M. Khatun, P. C. Thomson, K. L. Kerrisk, J. Molfino, and S. C. García, 2017. Clinical mastitis detection–development of an accurate detection method for automatic milking systems. American Dairy Science Association Annual Meeting on 25th to 28Th June, 2017, Pittsburgh, Pennsylvania, USA. (Abstract and poster)

M. Khatun, P. C. Thomson, and S. C. García, 2018. Electrical conductivity, daily activity and rumination for early detection of mastitis in pasture based automatic milking systems. Australasian Dairy Science Symposium on 21th to 23rd November, 2018, Palmerston North, New Zealand. (Paper and oral presentation)

Non peer-reviewed conference papers

M. Khatun, C. F. F. Clark, N. A Lyons, L.K Kerrisk, S. C García, 2016. Early detection of mastitis in pasture based automatic milking system (AMS) using new indexes of electrical conductivity. Spatially enabled livestock management symposium on March 31 to 1st April, 2016. Camden, NSW, Australia. (Paper and oral presentation)

M. Khatun, P. C. Thomson, R. M. Bruckmaier, J. House, and S. C. García, 2018. Comparison of cisternal and alveolar milk for mastitis detection. Dairy Research Foundation 2018 Symposium (Revitalising Dairy Production) 17th to 18th July, Camden, NSW, Australia. (Oral presentation)

M. Khatun, R.M. Bruckmaier, P. C. Thomson, J. House, and S. C. García, 2019. Suitability of foremilk before versus after ejection for detection of mastitis in automatic milking systems. 2019 Sydney Institute of Agriculture Research Showcase on 3rd July, 2019. Camperdown, NSW, Australia. (poster)

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ABSTRACT

Bovine mastitis is the inflammation of the entire udder or individual mammary glands. The pain associated with mastitis is a serious welfare issue, and the negative effects on milk production and quality cost millions of dollars to the Australian dairy industry every year. Automatic milking systems (**AMS**) are becoming increasingly popular to minimise labour and labour cost without compromising milk production. Because of lower farmer-cow contact in AMS, farmers or herd managers are dependent on the AMS-incorporated inline sensors for automatic mastitis detection. The International Organization for Standardization (**ISO**) recommended at least 70% sensitivity (**Se**) and at least > 99% specificity (**Sp**) for automatic detection of abnormal milk and currently there are gaps to achieve this ISO-standard by the inline sensors. Hence, we developed and implemented a research program with the overarching goal of improving the accuracy and timing of mastitis detection in AMS by using multiple sources of inline sensor-derived information related to milking, and also animal behavioural changes. The research was largely based on Se and Sp of mastitis detection and quarter-level inline sensor data in AMS. The literature review (Chapter 2) identified the current knowledge gaps and the opportunities to improve the Se and Sp of mastitis detection through new and innovative data-based research in AMS. The electrical conductivity (**EC**) inline sensor data analysis (3-year historic database) focussed on developing new indexes from the available EC data to fulfil ISO standard (Chapter 3). Chapter 3 concluded that EC data alone cannot provide the required accuracy to detect infected quarters, leading us to hypothesise that by incorporating other data, early detection of mastitis in AMS herds could be improved. Moreover, the sensitivity of the EC measuring sensor could also be improved by measuring the most informative milk samples like strict foremilk, which is currently discarded in AMS (Chapter 4). We found that foremilk sampled before milk ejection was

more sensitive for detection of mastitis than foremilk harvested after milk ejection, and that indicators like lactate dehydrogenase (**LDH**) have potential to differentiate mastitis originated from Gram-negative versus Gram-positive pathogens. The hypothesis that multiple milking-related inline sensor data (e.g., milk yield, milk flow rate, number of incomplete milkings) provided better Se and Sp than single inline sensor data was tested in the study reported in Chapter 5. This study demonstrated that by combining multiple measurements with adequate statistical models, mastitis status prediction can be improved. In addition, behavioural changes such as daily activity and daily rumination time captured by activity and rumination sensors (SRC collars) were also useful for better mastitis prediction when combined with EC data (Chapter 6). In summary, better mastitis detection is possible by looking at multiple inline sensor data as well as animal behavioural changes. This thesis provides innovative approaches and scientifically-based possibilities to utilise multiple sources of data for improvement of the Se and Sp of automatic mastitis detection in AMS in the future. The research makes original and innovative contributions to knowledge and sets the basis for future integration of its findings and models into practical tools for herd managers.

CHAPTER 1: GENERAL INTRODUCTION

INTRODUCTION

Mastitis is arguably the biggest health related issue in modern dairy production. In Australia, it is estimated to cost AU$200/clinical infection/year (Dairy Australia, 2017). Because of the health issue and economic loss, early and accurate detection of mastitis has been always a key goal. Recent advances in technology such as automatic milking systems (**AMS**) have included inline sensors that measure milk characteristics like electrical conductivity (**EC**) for automatic detection of mastitis. However, EC alone has been shown to lack the sensitivity and specificity required by the International Organization for Standardization (**ISO,** 2007).

Since the first installation in the Netherlands in 1992, AMS are rapidly increasing in popularity among dairy farmers (Hovinen and Pyörälä, 2011). In Europe, the AMS installation rate exceeds 8% per year; and in 2017 there were over 35,000 AMS units operating in the world (Klimpel, 2016; Salfer et al., 2017). Beyond lifestyle-related factors, a key driver of adoption of AMS is the potential increase in production per cow that can be achieved in AMS by milking a greater number of cows with increased milking frequency (number of milking events/cow/24 hour) and without increasing labour cost (Khanal et al., 2010; Eastwood et al., 2016; Gargiulo et al., 2018).

In Australasian pasture-based systems, AMS were first installed in a research farm in New Zealand in Waikato (DairyNZ) followed by a commercial farm in Victoria, Australia in 2001 (Jago et al., 2002; Chapman et al., 2009; Lyons et al., 2013). At time of writing there are about 78 AMS farms in Australia and New Zealand (N. Lyons, pers. comm.). It is expected that this number will increase substantially in the next decade as the industry adopts this system (Segio C. Garcia, pers. comm). Climatic suitability combined with predominantly export-orientated markets have led the dairy industries in these countries to be primarily pasture-based.

In AMS all milking-related tasks (e.g., animal identification, cup removers, drafting gates) are automated. The milk harvesting system is equipped with a range of sensors (inline, on-animal) that continuously monitor the performance of individual cows and the whole system, coordinated through a management software and generating enormous amount of data (de Koning et al., 2002; Jacobs and Siegford, 2012). Moreover, cows in pasture-based AMS get milked after travelling voluntarily from the paddock to the milking unit without requiring human intervention, which results in fewer opportunities for farmer-cow contact compared to conventional milking systems (Wildridge et al., 2018). This minimal farmer-cow contact in AMS has created more reliance on the recorded data generated by the automated systems to identify any abnormalities like mastitis that need therapeutic or preventive interventions (Kamphuis et al., 2010; Mollenhorst et al., 2012; Steeneveld et al., 2010). These data could be used to potentially increase the accuracy of mastitis detection.

Currently, the most common inline sensor-derived information used for mastitis detection is electrical conductivity (**EC)**, as EC increases for infected quarters due to altered concentration of anions (Na+) and cations (Cl–) (Kitchen, 1981). Despite its common use, measures from EC sensors are renowned for giving variable results, probably because EC is affected by factors such as temperature, fat content, milking interval and milk fractions (Fernando et al., 1982; Nielen et al., 1992; Ontsouka et al., 2003). This suggests that by accounting for some of these other factors accuracy and/or timing of detection could improve. Another possible explanation for the variability in EC could be associated with the origin of the milk being sampled by the inline sensor as the measure of EC varies in milk contained in the teat and gland cisterns (Bruckmaier et al., 2004; Bansal et al., 2005; Lehmann et al., 2015). However, this has not been demonstrated for pasture-based systems yet.

An additional limitation of EC data alone is that the EC measuring sensor cannot differentiate Gram-positive or Gram-negative originated mastitis. The ability to differentiate type of

pathogen, such as Gram-positive or Gram-negative, would allow for rapid and more specific treatment decision, reducing excessive use of antibiotics.

Given that mastitis is associated with multiple changes in the cow's body (Sordillo, 2005), exploiting multi-sensor data recorded during milking could lead to sustainable improvements in detection of mastitis (Brandt et al., 2010; Hogeveen et al., 2010; Steeneveld et al., 2010). Apart from EC, other sensor-derived information could be related to milk yield, milking frequency, milk flow rate, milking pattern and behaviour changes (Fogsgaard et al., 2012; Medrano-Galarza et al., 2012; Kester et al., 2015), as these have been found to be useful for mastitis prediction (Stangaferro et al., 2016). However, most farmers or herd managers have not been provided with multiple inline sensor-derived data presented in an appropriate format to facilitate decision making, in addition, the complexity and time required would make this task difficult. Consequently, there is clearly a demand for an easily interpretable system to identify mastitis, ideally also with identification of pathogen type for rapid treatment decision (Hovinen and Pyörälä, 2011; Mollenhorst et al., 2012; Russell and Bewley, 2013), as antimicrobial resistance becomes a global concern (Hardefeldt et al., 2018). There is potential for better, more accurate, earlier automatic detection of commonly occurring bovine mastitis, but research to fulfil this goal is lacking. Hence, the overall aim of this research program was to investigate the potential of multiple inline sensor-derived data related to milking and animal behavioural changes to improve the accuracy and timing of mastitis detection in AMS.

OBJECTIVES OF THE THESIS

To address this general aim, the specific objectives of the thesis were to:

1. Develop new indexes to improve Se and Sp from EC data, identifying gaps or limiting factors to improve Se and Sp of automatic mastitis detection in AMS (Chapter 3).
2. Compare strict foremilk and milk after ejection stimulus for their suitability to detect mastitis, including the potential to targeting pathogen-specific differentiation in the future (Chapter 4).
3. Investigate different ways to utilise multiple sources of data together with inline sensors to achieve or improve Se and Sp of mastitis detection in AMS (Chapter 5).
4. Evaluate the potential of sensor-derived data related to behavioural changes for improved automatic mastitis detection in AMS (Chapter 6).

THESIS OUTLINE

This thesis is composed of a review of the published literature (Chapter 2), four chapters arising from four independent studies (Chapter 3 to 6) and a general discussion and conclusions (Chapter 7).

The literature review (Chapter 2) gives an overview of bovine mastitis, impact, pathogenesis, and diagnostics; and identifies knowledge gaps and potential ways of increasing diagnostic efficiency in pasture-based AMS, providing justification for, and need of, the research that was subsequently undertaken in this thesis.

Chapter 3 focuses on the current mastitis diagnostic approaches in pasture-based automatic milking systems with the commonly used sensor measure EC, and the potential for improving accuracy and timing of mastitis detection through better use of EC-derived data. This chapter generated a peer-reviewed publication in *Animal Production Science* entitled “Early detection of clinical mastitis from electrical conductivity data in an automatic milking system by M.

Khatun, C. E. F. Clark, N. A. Lyons, P. C. Thomson, K. L. Kerrisk, and S. C. García, 2016, *Anim. Prod. Sci.* 57(7):1226-1232 doi.org /10.1071/AN16707)".

Key gaps identified in the review referred to the loss of foremilk in AMS, as this milk is discarded during the cleaning process in AMS, and the lack of information in reference to the ability of sensor-derived information to detect mastitis originated from different pathogens. These gaps are addressed in Chapter 4, which generated a peer-reviewed article in the *Journal of Dairy Science* entitled "Suitability of somatic cell count, electrical conductivity, and lactate dehydrogenase in foremilk before versus after alveolar milk ejection for mastitis detection by M. Khatun, R.M. Bruckmaier, P. C. Thomson, J. House, and S. C. García, 2019. *J. Dairy Sci.* 102(10):9200-9212 doi.org/10.3168/jds.2018-15752".

Apart from increasing the efficacy of single sensor-derived data for mastitis detection ability (i.e., measuring the most informative milk sample), mastitis detection could also be improved by utilising multiple sensor-derived data as discussed in Chapter 5. The approach developed from the data generated from a pasture-based dairy farm of the University of Sydney in New South Wales, was also validated with independent data from a commercial pasture-based dairy farm in Tasmania. Chapter 5 was published as a peer-reviewed article in the *Journal of Dairy Science* entitled "Development of a new clinical mastitis detection method for automatic milking systems by M. Khatun, P. C. Thomson, K. L. Kerrisk, N. A. Lyons, C. E. F. Clark, J. Molfino, and S. C. García, 2018. *J. Dairy Sci.* 101(10):9385-9395. doi: 10.3168/jds.2017-14310".

As mastitis also changes cows' behavioural pattern, Chapter 6 reports an original investigation into the mastitis prediction ability of some animal behavioural changes. In this chapter, the mastitis prediction ability of daily activity and daily rumination was tested individually and in combination with commonly used EC data. This chapter also generated a peer-reviewed publication in Animal Production Science entitled "Prediction of quarter level

subclinical mastitis by combining in-line and on-animal sensor data by M. Khatun, P. C. Thomson, and S. C. García, 2019 (*Animal Production Science*, in press)".

Lastly, Chapter 7 provides a general discussion of the results on the studies included in this thesis, integrating the generated knowledge, identifying existing gaps, and providing direction for future research to address these gaps.

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CHAPTER 2: LITERATURE REVIEW

OVERVIEW OF CHAPTER 2

To increase the sensitivity and specificity of mastitis detection in pasture-based automatic milking systems, it is necessary to, firstly, identify the specific reasons for the imperfect Se and Sp; and secondly, identify the knowledge gaps that would need to be addressed to overcome such limitations. Chapter 2 identifies and review these limiting factors and suggests possible ways of improving the sensitivity and specificity of automatic mastitis detection.

ABSTRACT

Because of increased adoption of automatic milking systems (**AMS**), inline sensor generated data for automatic detection of mastitis is at the cutting edge of technology development. However, currently there is a gap to achieve the International Organization for Standardization (**ISO**) recommended sensitivity (**Se**, >70%) and specificity (**Sp**, > 99%) for automatic detection of abnormal milk or mastitis. Before proceeding to improve Se and Sp there is a need to identify the specific reasons for the limited Se and Sp within the available published literature. Therefore, the objective of this review was to bring together, analyse and summarise relevant scientific literature about mastitis diagnostics, particularly automatic mastitis detection in AMS, and to identify knowledge gaps. Based on 29 studies in AMS-based farms, this review has identified: a) the limiting factors of using single inline sensor-derived data for automatic mastitis detection in AMS; and b) how the efficiency of single sensor's mastitis detection performance can be improved by measuring most informative milk samples and/or incorporating multiple sensor-derived data in AMS. This review has found the potential merit of new algorithms and strict foremilk samples to improve the Se and Sp of single sensor like electrical conductivity to detect mastitis. Furthermore, this review has also identified that Se and Sp of mastitis detection by inline sensors could improve by incorporating multiple milking-related data and/or behavioural data. Overall, this review has identified key aspects and gaps of inline sensor-data based detection of mastitis that should be taken into account for better and more efficient automatic mastitis detection in AMS in the future.

INTRODUCTION

Mastitis or inflammatory mammary disease is a common problem of the dairy industry worldwide. Mastitis is very painful and often requires special management to minimise milk production loss and maintain milk quality. About 95-98% of dairy farms in Australia are pasture-based due to availability of pasture land and climatic suitability, allowing cows access to cheap pasture feed for grazing over most of the year. Automatic milking systems (**AMS**) is becoming increasingly popular in these pasture-based Australian dairy farming systems. However, farmer-cow contact in AMS has become minimal in pasture-based AMS as the cows spent most of their time in the paddocks with little direct contact by farm staff. Hence, detection of health problems in general, and mastitis in particular, is a challenge in any AMS and even more of a challenge in pasture-based AMS.

The automatic detection of mastitis in AMS are dependent on the incorporated inline sensors. However, the sensitivity (**Se**) or true positive rate and specificity (**Sp**) or true negative rate achieved by those sensors are not satisfactory. Therefore, incorporating multiple inline sensor data to improve the Se and Sp have received increasing attention in the dairy industry. The purpose of this review was therefore to compile current knowledge in automated mastitis detection, identify current limitations and potential ways to improve Se and Sp; and ultimately suggest future strategies for better automatic detection of mastitis in AMS.

Bovine Mastitis

Bovine mastitis is an inflammation of the individual mammary gland quarter or entire mammary gland (udder) of female cattle, characterised by swelling, heat, redness, hardness and pain of the affected mammary gland/s coupled with abnormalities in milk (e.g., watery

appearance, flakes, clots, or pus) (Murphy et al., 2008). Bovine mastitis is mainly caused by bacteria belonging to the Gram-negative *Enterobacteriaceae* and Gram-positive *Staphylococcaceae*, and *Streptococcaceae* families (Bradley, 2002; Pyörälä, 2003). The major mastitis pathogens are *Strep. uberis*, *Mycoplasma bovis*, *Staph. aureus*, *Strep. agalactiae*, *Strep. dysgalactiae* (Ghadersohi et al., 1999; Shum et al., 2009) and minor pathogen including *Corynebacterium* sp. (*C. bovis*), coliforms (*Escherichia coli)*, environmental *Streptococcus*, *Candida* spp., *Aspergillus fimigatus*, algae and many others pathogen (Reyher et al., 2012; Gonçalves et al., 2016). The pathogens could have multiple strains, for example 154 *Staph. aureus* and 62 *Strep. uberis* strains have been fully characterised from the states of Victoria and Queensland in Australia (Gogoi-tiwari et al., 2015; Phuektes et al., 2001). Mastitis can be classified as Gram-positive mastitis caused by Gram-positive bacteria and Gram-negative mastitis when caused by Gram-negative bacteria. This is important due to differences in therapeutic approach to treatment (Schukken et al., 2012; Lehmann et al., 2015). Depending on the severity of inflammation, bovine mastitis can be classified as clinical, subclinical or chronic form. Clinical mastitis can be visually detected by observing the inflammatory signs (e.g., swelling, heat, redness, hardness, pain) of the udder. The pain associated particularly with the clinical form of mastitis is a serious welfare issue. The subclinical and chronic forms of mastitis are difficult to detect due to absence of visible signs but mostly associated with reduced milk production. The reduced milk production and losses from the deterioration of milk quality by different forms of mastitis (e.g., subclinial, clinical and chromic) are also a serious economic burden for the dairy industry (Hammer, 2005; Peters et al., 2015). The estimated cost for prevention and treatment of mastitis and production loss ranges from AU$50 to AU$467 per cow per year with differences between farms arising from different management situations (Halasa et al., 2007; Huijps et al., 2008).

Pathogenesis and Major Changes During Mastitis

After entering the mammary gland (increased risk associated with dilatation during parturition or open teat canal after milking or due to udder conformation), pathogens start to multiply by escaping the cellular and humoral defense mechanisms of the udder (Sordillo and Streicher, 2002; Paulrud, 2005; Rainard and Riollet, 2006). Their metabolites and toxins induce local immune cells (leukocytes) to release chemo-attractants for the circulatory immune cells (mainly circulatory neutrophils) to the site of infection (Paape et al., 2003; Zhao and Lacasse, 2008). After migration, circulatory neutrophils release bactericidal peptides, proteins, enzymes, proteases and oxidants to destroy the bacteria (Owen and Campbell, 1999; Bank and Ansorge, 2001). At the process of bacterial destruction some of the epithelial cells are also destroyed resulting in release of enzymes, such as N-acetyl-β-D-glucosaminidase, l dehydrogenase (**LDH**), alkaline phosphatase, proteases, among others. The destroyed leukocytes (by apoptosis or engulfed by macrophages) and sloughed off mammary epithelial cells are secreted into the milk resulting in high milk somatic cell count (**SCC**) (Paape et al., 2002, 2003; Zhao and Lacasse, 2008). Once the SCC exceeds 200,000 cells/mL, the quarter is classified as subclinically mastitic, and when 99% of the somatic cells are leukocytes, (with about 90% of these being neutrophils) it is considered as an infected quarter (Harmon, 1994; Schukken et al., 2003). Subclinical mastitis can be converted to the clinical form after severe challenging of the cow's immune system by the pathogen. Clinical mastitis is characterised by clinical symptoms like pain, redness, swelling of the udder and appearance of visible flakes or clots in milk with several million cells/ml, and with systemic fever (Bleul et al., 2006). Chronic mastitis can result in long-term udder infection with periodic clinical symptoms, accompanied by bulk tank readings with SCC > 500,000 cells/mL and a series of individual cow cell count > 250,000 cells/ml (Schepers et al., 1997).

During the process of mastitis development, different constituents of milk such as minerals (e.g., sodium, chloride, potassium, and calcium), lactose, free fatty acids, milk casein, whey protein, milk pH, may alter in their profiles (Kitchen et al., 1984; Brandt et al., 2010). These changes can be found in different intensities in clinical, subclinical, or chronic forms of mastitis with agalactia. In summary, depending on its form, mastitis is associated with reduced milk production, higher conductivity, higher SCC, increased pH, increased wateriness of the milk, and milk with visible clots and flakes (Viguier et al., 2009). In the case of a serious systemic infection, mastitis can lead to the death of the cow.

Immunity and Treatment Responses to Mastitis

After infection or tissue injury there are varieties of non-specific or innate immune responses called innate immunity; these responses are followed by adaptation over time to recognise specific pathogens called adaptive immunity (Bannerman, 2009). For adaptive immunity the underlying immune mechanism triggered by different types of pathogens, for example Gram-negative (e.g., *E. coli*, *Klebsiella* spp., *Enterobacter* spp., and *Citrobacter* spp) and Gram-positive bacteria (e.g., *Strep. uberis*, *Staph. aureus*) are different due to distinct receptor induced immunoregulatory activities (Tietze et al., 2006; Bannerman, 2009; Wellnitz et al., 2011). The immune responses also differ due to different strains of the same type of bacteria and severity of the infection (Khazandi et al., 2015). Besides immune responses, the therapeutic approach to Gram-positive and Gram-negative mastitis are also different (Schukken et al., 2012; Lehmann et al., 2015). For example, common Gram-positive bacteria (*Staph. aureus, Strep. uberis*) are responsible for subclinical infection with formation of scar tissue with impairment of antibiotic entry to destroy the bacteria, leading to chronic mastitis with elevated SCC (Lehmann et al., 2015). On the other hand, there are limited antibiotic

treatment response to Gram-negative mastitis, which therefore often require reduction of clinical symptoms by prophylactic treatment (Schukken et al., 2012).

Behavioural Changes due to Mastitis

Mastitis is a multifactorial disease that affects both animal's physiological and behavioural responses. Behavioural changes include alteration in feeding time, lying time, standing time, self-grooming, rumination, head turning frequency, kicking, isolation character, preference for lying on one side, and increase of restless behaviour (Cyples et al., 2012; Fogsgaard et al., 2012, 2015; Medrano-Galarza et al., 2012; Proudfoot et al., 2014; Sepúlveda-Varas et al., 2014; Watters et al., 2014; Kester et al., 2015). Existing remote sensing technology allows herd managers and researchers to monitor and record some of the behavioural activity changes like lying time, standing time, rumination **(**e.g., SCR HR-LDn activity sensor**)** while the cow is grazing even in remote pasture land. Such sensor-derived data have been found to be useful in predicting severe clinical mastitis situation with or without other health events (Stangaferro et al., 2016). However, it is possible that such changes could increase the accuracy of mastitis detection if combined with milking related data.

Pasture-based Farming

Pasture-based dairy farms are defined as farms where pasture is the single largest feed component (Garcia and Fulkerson 2005). Typically, dairy cows spend most of the time grazing pasture with or without supplementary feed and minimal or zero housing. However, some pasture-based farms will provide improved feed and housing during calving or periods of disease. Pasture-based dairies may also provide cover for inclement weather when needed.

Pasture-based farming is common in New Zealand (99-100%), Australia (95-98%), Ireland and some South America countries (Argentina, Chile and Uruguay) due to availability of enough pasture land and market-related factors (Little, 2010; Dairy Australia, 2017; Shortall et al., 2018). Automatic milking systems (**AMS**) are becoming increasingly popular in pasture-based farming systems.

Overview of the Milking Systems

Milking systems are important to elicit optimal milk letdown/milk removal from the cow with minimal contamination. It is important to have rapid and efficient removal of milk without damage to the teat or gland and with minimal risk for transmitting mastitis-causing microorganisms. Machine milking was commercially introduced in the dairy industry in 1917 (Piddock, 2017). After this early innovation, machine milking has improved from a partially automated to a fully automated milking system. The decision about installation of milking systems is affected by farming system type (e.g., indoor or pasture-based) and grossly, milking systems can be divided into two: conventional and automatic.

Conventional Milking Systems (CMS): In CMS all milking-related tasks are either partly automated or manually undertaken by the operator.

Automatic Milking Systems: In case of AMS, all milking-related tasks are automatic. The first commercially introduced AMS was in Europe in 1992 and since then more than 35,000 commercial dairies around the world are operating with AMS (Rondenburgh et al., 2017; Salfer et al., 2017). In the pasture-based farming systems, AMS were introduced in 2001 and since than more than 78 pasture-based farms in Australia and New Zealand have successfully

integrated AMS into grazing systems (N. Lyons, pers. comm.). Three different types of AMS are commercially available now, namely single-box (DeLaval, Lely, Daviesway, GEA), multi-box (GEA), and robotic rotaries (DeLaval, GEA. Single-box AMS consist of one robotic arm attached to a single milking crate allowing only one cow to be milked at a time. Single-box AMS are able to perform 150 to 180 milkings/day, therefore suitable for herds of 60 to 80 cows to be milked 2-3 times/day (Lyons, 2013; Molfino, 2018). In multi-box AMS there are 2-5 milking crates served by one robotic arm allowing several cows to be milked at a time (Rotz et al., 2010; Lyons, 2013). The automatic milking rotary is commercialised by DeLaval International (**AMR^{TM}**, Tumba, Sweden). The AMR consists of an internal 24-bail herringbone platform with five robotic arms in the centre (two teat preparation arms, two cup attachment arms and one post-milking teat sanitation arm). The AMR has the capacity to perform 60-90 milkings/hour and 1600 milkings/day, therefore suitable for large herds of > 500 cows (García et al., 2007). Because of the possibility of up to 12% greater milk production with approximately 18% reduced labour cost, AMS or AMR are gradually being adopted in the larger-scale herds (García and Fulkerson, 2005; Jacobs and Siegford, 2012; Gargiulo et al., 2018). The enormous amount of data recorded by the AMS during the milking process are advantageous to monitor the performance or changes of individual cows or the entire herd routinely (Jacobs and Siegford, 2012), and this can in part compensate for the reduced farmer-cow contact.

Milk ejection

Milk ejection is a continuous process that commence in dairy cows at about 1 min after tactile udder stimulation. As milk ejection is continuous process the milk composition changes throughout the milking (Ontsouka et al., 2003). During milking cisternal milk (present in

gland and teat cisterns) also termed strict foremilk is first removed before oxytocin-induced alveolar ejection from alveoli and smaller milk ducts (Bruckmaier and Blum, 1998; Lehmann et al., 2015). Currently in AMS, the available sensors do not measure strict foremilk as initial milk is discarded during the teat-cleaning phase, and alveolar milk ejection occurs during the teat cleaning process. Hence, any sensor measurements in AMS are based on a mixture of cisternal and alveolar milk (Bruckmaier and Hilger, 2001; Bruckmaier et al., 2004; Dzidic et al., 2004). Thus, by discarding and not measuring strict foremilk, AMS may be missing out valuable data from potentially the most informative milk for mastitis detection. However, this hypothesis has not been tested yet.

Overview of Mastitis Diagnostics

To diagnose mastitis, a series of steps have to be taken, namely the identification of the cow followed by restraining the cow to examine her and performing the diagnostic tests. Each step has an associated (and variable) probability of failure. To identify presence of mastitis, various diagnostic tests and sensors are available, as discussed below.

Cow-side Tests: Cow side tests are also termed on-site tests, and are performed in the milking parlour. Cow-side tests include: strip cup test (Souza et al., 2016), blackboard strip test (Thomas, 1949), California mastitis test (Fosgate et al., 2013), bromothymol blue test (Marschke and Kitchen, 1985), sodium lauryl sulphate test (Sharma et al., 2010), chloride test (Anirban et al., 2012), white side test (Muhammad et al., 2010), R-mastitest (Deb et al., 2013), Brabant mastitis test (O'Reilly and Dodd, 1969), white slide + dye test (Iqbal et al., 2006), Mastrip test (Ranaut, 2015), Surf field mastitis test (Muhammad et al., 2010), Surf field mastitis test + dye test (Iqbal et al., 2006), Mast-O-test (Deb et al., 2013), modified Aulendorfer mastitis probe test (Buragohain and Dutta, 1991), Wisconsin mastitis test

(Duarte et al., 2015), protease activity measurement (Koop et al., 2015), among other tests. All these tests are time and labour-consuming. Recent technological advances have provided the industry with many useful online sensors (monitoring mastitis in automatically collected samples) to detect mastitis (cow-side/on-site). Examples of such methods/sensors are DeLaval cell counter (Schepers et al., 1997), portable microscopic somatic cell counter (Moon et al., 2007), PortaSCC/PortaCheck assay (Salvador et al., 2014), Somaticell (Rodrigues et al., 2009), handheld conductivity meter (Musser et al., 1998), infrared thermography camera (Colak et al., 2008), Draminski mastitis detector (Iraguha et al., 2017), microfluidic sedimentation cytometer (Garcia-Cordero et al., 2010). The advantages of cow-side tests are their rapidity, relative low cost, convenience, and user-friendliness, and are even suitable for use by non-technical persons. On the other hand, cow-side tests are mainly qualitative with modest diagnostic test characteristics, non- pathogen-specific and labour-demanding to monitor larger herds routinely. In past decades much improvement has been done in the cow-side test performance, reducing the cost and development to perform the test automatically. As both herd size and the cost of labour increase, the number of full-time equivalent staff (FTE) per cow decreases. As the number of FTEs per cow and the general level of husbandry skill of dairy farm decrease, there is a greater need for automated mastitis detection systems. However, the approach to upgrade the cow-side test to an automatic test was not always successful or not robust enough due to limitation in the calibration process (Neitzel et al., 2014).

Laboratory-based Tests: Laboratory-based tests could be defined as tests performed away from cow-side in a close or remote laboratory equipped with mastitis detection instruments. Mastitis pathogens can be accurately detected by culture tests (Pyörälä, 2003), polymerase chain reaction (Koskinen et al., 2009; Ashraf et al., 2017), and enzyme-linked

immunosorbent assay (Kalorey et al., 2007). However, various other sensors are available for diagnosing mastitis in the laboratory, e.g., Fossomatic SCC (Miller et al., 1986), differential cell counter (Pilla et al., 2013), bioluminescent determination assay sensor (Frundzhyan et al., 2008), Coulter milk cell counter (Miller et al., 1986), biochips or lab-on-a-chip (Fernandes et al., 2014), deoxyribonucleic acid (Wu et al., 2005) sensor, gas sensor arrays or electrical nose (Hettinga et al., 2008), liquid sensor arrays or electrical tongue (Mottram et al., 2007), near infrared spectroscopy (Meilina et al., 2009), among others. The major drawbacks of the laboratory-based tests are higher initial investment, lengthy and labour-intensive tests with multiple-step procedures, limited thresholds for some tests with special test-condition requirements, limitation to test bloody milk (biosensors) and they always require skilled personnel to obtain reliable results. Sometimes, the time associated with transferring samples to a remote laboratory can affect the test results and all those factors have made many laboratory tests impractical for routine screening of cows in dairy farms.

Inline sensors: Inline sensors are capable of monitoring and recording changes continuously inline (as milk flows through the line) or in automatically-collected milk samples. Inline sensors are adapted to be incorporated in AMS or AMR for mastitis detection (Hovinen and Pyörälä, 2011) and some have been incorporated in CMS. Because of increased adoption of AMS, there is also a parallel increased demand of inline sensors for automatic detection of mastitis to compensate with reduced inspection time for identifying mastitic cows (Kamphuis et al., 2010a,b; Mollenhorst et al., 2012; Steeneveld et al., 2010a, b). The advantages of inline sensors are that they allow monitoring of subtle changes in milk non-invasively (with the associated benefits for animal welfare) with remote accessibility to data for multiple diseases, and the ability to store the data for a long time. Inline sensors might be developed considering the pathogenesis and cows' physiological changes during mastitis. The advantages and

limitations of the currently available inline sensors for mastitis detection purposes are described below.

Electrical Conductivity (EC): The sensor measuring EC is the most commonly used inline sensor in AMS (Kamphuis et al., 2010a,b; Mollenhorst et al., 2010). The underlying mechanism behind the EC sensor is the change in milk ion concentration (sodium, potassium, calcium, magnesium, and chloride) and pH due to increased vascular permeability of the mammary gland during mastitis (Fernando and Spahr, 1983; Mucchetti et al., 1994). Electrical conductivity can be measured at the udder or quarter level (e.g., by SmartD-TECT for EC measurement) or as an index generated within brand-specific software that incorporate multiple factors to give an indication of the likelihood or risk of mastitis. DeLaval DelPro software produce a measure termed the Mastitis Detection Index (**MDi**), an index that incorporates EC, blood in milk, and milking interval per quarter. However, although EC is the most commonly used inline sensor for mastitis detection, its results are not always satisfactory (Kamphuis et al., 2008; Khatun et al., 2017). Factors such as temperature, milk composition, milking interval are likely contributors to such variability (Fernando et al., 1982; Nielen et al., 1992; Ontsouka et al., 2003). For example, EC increases with milk sample temperature: when the milk leaves the teat cistern of the cow the temperature is about 38°C, that is higher than the standard measuring temperature (25°C) (Norberg et al., 2004); also different milk fractions with different milk composition can influence the EC measurement (Ontsouka et al., 2003). Based on a meta-analysis of EC data, achieving up to 68% Se and 82% Sp for automatic detection of mastitis in AMS, is possible (Hamann and Zecconi, 1998).

Somatic Cell Count (SCC): Somatic cell count is the most frequently used gold standard for

monitoring udder health as well as diagnosis of mastitis (Jayarao et al., 2004; Mollenhorst et al., 2010). Somatic cells are immune leukocytes, namely lymphocytes, macrophages, neutrophils and some epithelial cells secreted into the milk. Online cell counters are commercially available now for SCC measurement in AMS, namely Dairy SCC app for Apple devices, and Lely MQC-C milk quality control system (Sørensen et al., 2016). Installation of SCC sensors is very costly otherwise applicable in a laboratory situation, considering the time and labour demand for analysis.

Colour: The principle of the colour sensor is light reflection or transmission for direct measurement of the physical characteristics of abnormal milk. Although it is an easy procedure to perform, it is not considered to be better or more accurate than EC (Rasmussen and Bjerring, 2005; Kamphuis et al., 2010b). The normal fat colour affects the colour sensor and to obtain informative values, colour sensors should always be used in combination with other sensors (Rasmussen and Bjerring, 2005).

*L-Lactate Dehydrogenase (**LDH**):* Lactate dehydrogenase enzyme is present in the cytoplasm of the epithelial cells and becomes prominent in the mastitis milk secreted from the migrated somatic cells as a part of the animal's immune response against infection. It is a potent indicator of clinical mastitis (Chagunda et al., 2006). The LDH sensor is now commercially available in DeLaval's Herd Navigator^{TM} system and is being tested in a number of commercial dairy farms in Denmark (Mazeris, 2010; Leonardi et al., 2013). However, it appears that best LDH enzyme-based mastitis marker's results were obtained when infections were originated from Gram-negative *E. coli* infection (Sørensen et al., 2015; Hernández-Castellano et al., 2017); and its real value for early mastitis detection is yet to be determined (Friggens et al., 2007).

Milk Yield: The decline of milk yield is a common phenomenon for mastitis and it can be detected before clinical outbreak (Edwards and Tozer, 2004). Although it is a potent indicator for predicting early clinical mastitis status, a decline in milk yield lacks specificity; therefore, it is most useful for creating an alarm for non-specific health-related problems (Deluyker et al., 1991; Lukas et al., 2009).

Heat and Rumination Long Distance (HR-LD) Sensor: Mastitis-associated daily activity and daily rumination changes can be automatically and continuously monitored by on-cow sensors that measure activity and rumination, e.g., HR-LD sensors located in collars or tags (HR Tags, SCR Dairy, Netanya, Israel). Although the HR-LD sensor is particularly designed for monitoring oestrus cycles, such sensor-derived data have been found to be useful in predicting severe clinical mastitis (Stangaferro et al., 2016). However, Se and Sp achieved by HR-LD are not sufficiently high.

Mastitis Differentiation by Inline Sensors

Earlier inline mastitis differentiation possibilities could provide opportunity to implement early and adequate treatment protocols and to avoid excessive use of antibiotics. Because of variable responses and several influential factors, the inline sensor-measure of EC cannot differentiate the type of mastitis. Though, enzymatic immune response analysis (e.g., Lactate dehydrogenase, **LDH**) has been suggested to differentiate the type of mastitis in combination with SCC > 300,000 cells/ml (Hernández-Castellano et al., 2017), several other studies have reported the efficacy of LDH only against Gram negative *E. coli* infection (Larsen et al., 2010; Sørensen et al., 2015; Wellnitz et al., 2015). Thus, the efficacy of LDH to differentiate Gram-positive infections is more doubtful. Another drawback is that currently the inline

sensors' measurement are based on after-alveolar milk ejection or on composite milk samples of four quarters. However, the milk sample after ejection/composite milk samples differ from foremilk before alveolar ejection as the immune profile varies depending on the timing and stimulation process (Lehmann et al., 2015). Moreover, composite milk dilutes the effect of infected quarters' immune response especially if two to three quarters remain healthy (Chagunda et al., 2006). Hence, it needs to be revealed how the inline measurement of EC, SCC, LDH based on foremilk before ejection varies from milk after ejection to differentiate types of mastitis.

Multiple-sensor Based Index

Although much inline sensor data are available in the AMS, herd managers do not necessarily know how to interpret these data for mastitis detection, since typically they have not been provided with multiple inline sensor-derived data presented in an appropriate format to facilitate decision making (Hovinen and Pyörälä, 2011; Mollenhorst et al., 2012; Russell and Bewley, 2013). Hence, there is a demand from herd managers for an easy interpretable system to recommend (and possibly make) decisions for further action, or at least to compile the muliple data sources into a single or relatively fewer measures for decision making about mastitis treatment. Essentially, as mastitis is associated with multiple changes in cows' milk or body physiology and behaviour, utilising multi-sensor information is the cutting-edge approach to improve mastitis detection performance (Hogeveen et al., 2010; Mollenhorst et al., 2010). Theoritically, there are some multiple sensor-based approches such as degree of infection (Højsgaard and Friggens, 2010), decision-tree induction (Kamphuis et al., 2010a), and naive Bayesian network (Steeneveld et al., 2010a) that have been suggested by previous studies to improve mastitis detection performance in AMS. However, the search for the

'perfect' mastitis detection method is still continuing with particular focus on quarter level mastitis detection (Kamphuis et al., 2008; Hogeveen et al., 2010; Mollenhorst et al., 2010).

Algorithms

An algorithm is a set of steps that takes some value/s as input and produces some value/s as output, allowing a decision to be made. An algorithm that is based on multiple sources of data is reported to be more reliable that those based on a single source (Hogeveen et al., 2010). Such an approach of incorporating multiple mastitis-related data by algorithms has been found to produce promising results for accurate or better detection of mastitis with improved Se and Sp, as presented in Table 2. 1. The range of 32% to 100% Se and 69% to 99.8% Sp requires a meta-analysis to provide summary measures of diagnostic test performance. The research for advancing and simplifying algorithms is still ongoing.

Sensitivity and Specificity

Sensitivity (also sometimes called true positive rate) is the proportion of positive (e.g., truly diseased) cases that are correctly detected by the device or test. Specificity (or the true negative rate) is the proportion of healthy cases that are correctly detected as not having the condition or disease. Solely depending on a single diagnostic test for mastitis detection cause error possibility and to minimise the error Se and Sp are calculated using the following formula (Martin, 1984; Schepers et al., 1997).

$$\text{Se}\% = \frac{\text{Number of true positive samples} \times 100}{\text{Number of true positive samples} + \text{Number of false negative samples}}$$

$$\text{Sp}\% = \frac{\text{Number of true negative samples} \times 100}{\text{Number of true negative samples} + \text{Number of false positive samples}}$$

Reliable tests demand a particularly high level of detection of true positive cases and low level of false alerts. The ISO has recommended to have Se > 70% and Sp ≥ 99% for automatic discarding of abnormal milk in AMS (Mein and Rasmussen, 2008; Sherlock et al., 2008). That means that any test should have the capability to misclassify a maximum of one in 100 non-mastitic cows as a case, i.e. a false positive alert. However, the recommended > 70% Se comes at a production and welfare cost by missing up to 30% of true positive mastitis cases. Nevertheless, Se and Sp do not perform well for evaluating time-related changes and a progressive scale of predicted mastitis state (Friggens et al., 2007).

Techniques/Concepts

Apart from Se and Sp calculated by algorithm models several non-model-based techniques or concepts for mastitis detection have been suggested by different groups previously. Example of such approaches include "knowledge-based system" (Hogeveen et al., 1995), "expert opinion" (van Asseldonk et al., 1998), "tracking signal" (Mele et al., 2001), "near-infrared spectroscopic sensing based milk quality assessment" (Kawasaki et al., 2008; Meilina et al., 2009), "temperature assessment" (Hovinen et al., 2008; Polat et al., 2010), "cow-specific prior mastitis probabilities based on non-AMS data" (Steeneveld et al., 2010a), "knowledge-transfer programmes, herd health advisory programmes, cow-based ambulatory work" (Lam et al., 2009, 2013), and "standardised protocol" (Kamphuis et al., 2013), among others. Although such techniques or concepts have been found to be useful for mastitis detection in AMS, the concept of generating single indexes by incorporating multiple data sources is forward-looking and attractive, given its potential to increase accuracy and timing of mastitis detection.

CONCLUSION AND FUTURE RESEARCH POSSIBILITIES

This review has brought together aspects of the most modern dairy farming system, AMS, with automated mastitis diagnostics with current technologies and what might be advanced in the future. In this thesis, inline sensors and sensor-derived data are the main focus to improve the Se and Sp of automatic mastitis detection in AMS as discussed in subsequent chapters. Hence, the knowledge generated from this review has helped to identify the key limitings factors of inline sensor technology for automatic mastitis diagnosis and benefits in exploring the possibilities for future improvement. Overall, from this review we can draw several conclusions that have generated individual chapters' hypotheses for detailed discussion later.

First, single sensor-derived measurement of EC (i.e., the most commonly used inline sensor) does not provide adequate signals for accurate mastitis detection in AMS. Hence, future improvement beyond the accuracy of a single sensor might be possible by applying different algorithms or data to generate a strong mastitis signal to track. Moreover, adjustment is also necessary to capture quarter-level data, which is reported to be more accurate for several sensors (Kamphuis et al., 2008; Mollenhorst et al., 2010). This is the focus of Chapter 3.

Second, the reasons for inadequate Se and Sp by single-sensor methods can be explained by the influence of other factors, such as milk temperature, milking interval, milk composition variations during the milking process. Hence, identification of the most informative milk fraction and possibility of the inline sensors to differentiate types of mastitis pathogen for rapid treatment decision might be a breakthrough in AMS technology for efficient mastitis detection. These two objectives are addressed in Chapter 4.

Third, apart from EC measuring sensor there are many other milking-related inline sensors with potential to contribute to a timing and accurate detection of mastitis. Exploring and evaluating the mastitis detection ability of other milking-related sensors and combining

multiple sensor data into a single signal or single index might be another possibility to apply in AMS in the future. This is dealt with in Chapter 5.

Finally, mastitis also can affect cows' behavioural responses. As sensors such as collar-mounted accelerometers and heat detectors are becoming readily available to monitor behavioural changes automatically, such data might be of use for further enhancement of automatic detection of mastitis in AMS. This hypothesis is tested in Chapter 6.

Table 2. 1. Inline sensor and non-sensor related information for detection of mastitis in automatic milking systems from published literature.

Milking sensor	Other information	Mastitis status	Se (%)	Sp (%)	Analysis methods	References
EC, MY	TEM	clinical	100%	--	MA, threshold	Maatje (1992)
EC, MY	TEM	clinical	75	90	PCA	Nielen (1995b)
EC	milk yield, parity, DIM	subclinical	55	90	logistic mixed model	Nielen (1995a)
EC	--	clinical	77	69	neural network	Nielen (1995c)
EC, MY	TEM, activity	clinical	90	98.2	MA, threshold	Maatje (1997)
EC, MY	TEM, activity	subclinical	76	98.2	MA, threshold	Maatje (1997)
EC, MY	TEM, activity, calving date, estrus date	clinical, subclinical	57-100	95.3-99.4	multivariate time-series with Kalman filter	de Mol (1997)
EC, MY	activity, TEM, automated concentrate feeder	clinical, subclinical	58-73	82-87	conjoint-analysis and expert knowledge	van Asseldonk (1998)
EC	activity	clinical, subclinical	84	97	tracking signal	Mele (2001)
EC, MY	TEM, activity	clinical	100	99.8	fuzzy logic	de Mol and Woldt (2001)
EC, MY	TEM	clinical	100	95.1	time-series with Kalman filter	de Mol and Ouweltjes (2001)
EC	--	clinical, subclinical	45 to 80	74.8	discriminant function analysis	Norberg (2004)
EC, MY, milk flow rate	--	clinical	92.9	93.9	fuzzy logic	Cavero (2006)
LDH, EC, MY	days from calving, breed, parity, other diseases, udder character	clinical	82	99	dynamic deterministic biological model	Chagunda (2006)
MY, LDH	breed, lactation number, health status	clinical	--	--	dynamic and deterministic time series measurement	Friggens (2007)
EC	--	subclinical	84.7	73	MA	Cavero (2007)

Table 2.1. (Cont.)

EC, SCC	--	clinical	80	92.2	fuzzy logic	Kamphuis (2008)
EC	--	clinical	83	99.8	threshold	Claycomb (2009)
EC, MY	DIM, parity, calving difficulty, day relative to breeding, weather	non-specific	48.1	98	statistical process control	Lukas (2009)
EC, SCC, LDH	breed, parity, days from mastitis	clinical	--	--	degree of infection	Højsgaard and Friggens (2010)
EC, MY	color	clinical	32	98.7	decision-tree induction	Kamphuis (2010a)
EC, SCC	--	clinical with abnormal milk	47.4	99	receiver operating char-acteristic curve	Mollenhorst (2010)
EC, MY	--	clinical	86.9	91.4	artificial neural network	Sun (2010)
EC, color	parity, DIM, season, SCC history, mastitis history	clinical	70	97.8	Naive Bayesian network	Steeneveld (2010b)
EC, MY, peak flow, blood, milking number, SCC	lactation no, DIM	clinical	80	99.5	mixed model and threshold	Hammer (2012)
Milking number	clots	clinical	100	--	--	Kamphuis (2013)
MY	--	clinical	63	--	online synergistic control process	Huybrechts (2014)
Online cell count	--	clinical	90.8	99.4	time-series model and threshold	Sørensen (2016)
MY, peak milk flow	Parity, quarter position, DIM, milking interval	clinical	--	--	logistic mixed model	Penry (2017)

DIM = days in milk; EC = electrical conductivity; LDH = lactate dehydrogenase; MA= moving average; MY= milk yield; PCA = principal component analysis; SCC = somatic cell count; TEM = milk temperature.

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CHAPTER 3: Early detection of clinical mastitis from electrical conductivity data in an automatic milking system

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OVERVIEW OF CHAPTER 3

One of the main findings from Chapter 2, was the advantage of using algorithms to increase the sensitivity (Se) and specificity (Sp) of single inline sensor mastitis detection ability. Extending on this work, Chapter 3 investigates different algorithms using a single sensor (electrical conductivity) to improve the Se and Sp and timing of mastitis detection in automatic milking systems, to detect mastitis at the earliest possible day. Retrospective data from the University of Sydney pasture-based dairy farm were used to compare different algorithms based on rolling averages and statistical process control to improve the Se and Sp of electrical conductivity measuring sensor.

ABSTRACT

Mastitis adversely impacts on profit and animal welfare in the Australian dairy industry. Electrical conductivity (**EC**) is increasingly used to detect mastitis, but with variable results. The aim of this study was to develop and evaluate a range of indexes and algorithms created from quarter-level EC data for the early detection of clinical mastitis (**CM**) at 4 different time windows (7 days, 14 days, 21 days, 27 days). Historical longitudinal data collected (4-week period) for 33 infected and 139 healthy quarters were used to compare the sensitivity (Se; target > 80%), specificity (Sp; target > 99%), accuracy (target > 90%) and timing of 'alert' by 3 different approaches. These approaches involved either the use of EC thresholds (range 7.5 to 10 mS/cm); testing of over 250 indexes (created *ad hoc*); and a statistical process control (**SPC**) method. The indexes were developed by combining factors (and levels within each factor), such as conditional rolling average increase, % of variation; mean absolute deviation; mean error %; infected to non-infected ratio; all relative to the rolling average (3 to 9 data points) of either the affected quarter or the average of the four quarters. Using EC thresholds resulted in Se, Sp and accuracy ranging between 47% to 92%, 39% to 92% and 51% to 82%, respectively (threshold 7.5 mS/cm performed best). The six highest performing indexes achieved Se, Sp and accuracy ranging between 68% and 84%, 60 and 85% and 56% and 81%, respectively. The SPC approach did not generate accurate predictions for early detection of CM on the basis of EC data. Improved Sp was achieved when the time window before treatment was reduced regardless of the test approach. We concluded that EC alone cannot provide the accuracy required to detect infected quarters. Incorporating other data (e.g., milk yield, milk flow, number of incomplete milking) may increase accuracy of detection and ability to determine early onset of mastitis.

Keywords: Dairy cows, indexes, statistical process control, thresholds.

INTRODUCTION

Bovine mastitis is an inflammation of the mammary gland, typically caused by bacteria entering into the gland through the teat canal (Pyörälä, 2003). Mastitis adversely impacts profit and animal well-being. It is responsible for significant economic losses to the industry due to reduce milk yield and discarded milk, the costs associated with diagnosis, treatment, culling, increased labour, milk-quality penalties, cow replacement and potential long-term damage to the mammary gland (Bar et al., 2008; Halasa et al., 2009; Hertl et al., 2011a). Mastitis costs can range from $47 to $427 per cow per year, with large differences among farms (Huijps et al., 2008; Steeneveld et al., 2011; Dairy Australia, 2017).

Automatic milking systems (**AMS**) are increasing in popularity as they minimise labour associated with milk harvesting, can reduce labour cost and increase milking frequency, that is often associated with higher milk production per cow (Hovinen and Pyörälä, 2011). In AMS herds, the majority of cows are not visually inspected during milk harvesting, which creates a reliance on fully automated systems to identify those cows that need therapeutic or preventive interventions (Mollenhorst et al., 2012). To ensure that such systems are reliable and viable, they should have a low false-positive alert rate and allow for early and accurate detection of true positive cases (Kamphuis et al., 2010; Steeneveld et al., 2010; Mollenhorst et al., 2012).

Electrical conductivity (EC), the most commonly used milk indicator, is increased during infection due to altered concentration of cations (Na^+) and anions (Cl^-) during mastitis (Kitchen, 1981). These altered levels of ions typically result from altered milk pH and temperature, as well as leakage (from blood vessels into alveoli) between secretory cells as junctions are disrupted by the infection (Mucchetti et al., 1994). The EC sensors incorporated in AMS can continuously measure EC during the milk harvesting process and are termed

'inline' as they monitor the level of ions in the milk during the milking process, without requiring samples to be collected and analysed.

Sensitivity (**Se**) and specificity (**Sp**) are statistical measures of the performance of a binary classification test. Sensitivity (also sometimes called true positive rate) is the proportion of positive (e.g., truly diseased) cases that are correctly detected by the device or test. Specificity (or the true negative rate) is the proportion of healthy cases that are correctly detected as not having the condition or disease. In the past decades, many researchers have reported various performance levels of inline EC sensors, with ranges in Se (47%-83%) and Sp (91%-99%) (Norberg et al., 2004; Claycomb et al., 2009; Hogeveen et al., 2010). Due to increasing popularity of AMS, the use of EC sensor is increasing and the search for the 'perfect' automated mastitis detection system continues. Moreover, there are currently no guidelines for AMS farmers to suggest EC thresholds that should be considered as an alert point for visual inspection of individual cows or that can be used as an early indicator of the imminent onset of clinical signs. The objective of the present study was to develop and evaluate a range of indexes and algorithms created from EC data, for the purpose of early detection of CM with more than 80% Se and 99% Sp.

MATERIALS AND METHODS

Selection of Cows and Quarters

A retrospective longitudinal cohort study was conducted, with data from 52 dairy cows (recorded during the period October 2014 to March 2016) with single-quarter (n = 47) and double quarter (n = 5) clinical mastitis (**CM**) collected from the pasture-based robotic dairy farm of the University of Sydney located near Camden, New South Wales (34.0544°S,

150.6958°E, rain fall: 764 mm/year). Cows were selected on the basis that they had a mastitis event (1 or 2 quarters) that was not preceded by another CM event within the 27- day period before treatment. Not all quarters of the 52 cows were included in the study; quarters were removed from the analysis if they had any one EC reading that exceeded 7.22 mS/cm (the average EC + 2 × SD of a subset of 1st-lactation healthy cows on the test farm) during the 21-27-day period before treatment. The reason for excluding these quarters from the analyses was to ensure that chronically infected quarters were removed. The exclusion criteria resulted in 172 quarters for the analysis with 33 infected quarters and 139 healthy quarters. Cows were mixed parity (average 3.48 lactations; range 1-9) and averaged 216 days in milk (**DIM**) on Day 0 (the day of mastitis diagnosis or treatment).

Mastitis-monitoring Regime

Normal farm practice was to monitor EC reports at least once daily. Cows that were deemed by farm staff to have elevated EC in one or more quarters (without a strict threshold) were designated to be drafted before the next milking, so as to allow visual inspection of the suspect quarter (for redness, heat, and swelling) and its milk (for the presence of flakes, clots or lumps). In the absence of visual signs of infection, a small sample of milk was collected into a California mastitis-test device. Where a positive California mastitis-test result or visual signs of mastitis were confirmed, and the cow was deemed to have CM and was treated with antibiotics, with milk being discarded. The day of detection was deemed as Day 0 (for both healthy and infected quarters) for the purpose of the analysis. Data were extracted for all quarters (CM mastitis and healthy) for a 4-week period (27 days) before the commencement of treatment for CM (where the day of treatment of CM quarter was considered as Day 0 and

27 days prior was Day -27). Analyses were conducted for 7-, 14-, 21- and 27- day time intervals (all as days before the day of diagnosis).

Method 1 (Threshold Approach)

Each quarter-EC of every milking session was compared with EC at different thresholds (7.5, 8, 8.5, 9, 9.5, 10 mS/cm) where CM quarters with a milking that had a measured EC that exceeded the respective threshold were considered as true positives (**TP**). The CM quarters that did not have any EC that exceeded the respective threshold were considered as false-negatives (**FN**), whereas healthy quarters having any milking with higher EC than the respective threshold were deemed false-positives (**FP**) and healthy quarters that did not have any EC measurements that exceeded the thresholds were deemed true-negatives (**TN**). Then Se, Sp, accuracy and the respective standard error (SE for binomial proportions) were determined as follows:

$$\text{Se} = \frac{\text{TP}}{\text{TP}+\text{FN}};\ \text{SE of Se} = \sqrt{\frac{\text{Se}\times(1-\text{Se})}{\text{TP}+\text{FN}}}$$

$$\text{Sp} = \frac{\text{TN}}{\text{TN}+\text{FP}};\ \text{SE of Sp} = \sqrt{\frac{\text{Sp}\times(1-\text{Sp})}{\text{TN}+\text{FP}}}$$

$$\text{Accuracy} = \frac{\text{TP}+\text{TN}}{\text{TP}+\text{FN}+\text{TN}+\text{FP}};\ \text{SE of Accuracy} = \sqrt{\frac{\text{Accuracy}\times(1-\text{Accuracy})}{\text{TP}+\text{FN}+\text{TN}+\text{FP}}}$$

Any thresholds that resulted in Se of > 80% were investigated further in time intervals of 7 days, 14 days, 21 days and 27 days, to determine whether Se, Sp and accuracy could be improved by using only data closer to the time of diagnosis. In addition the first threshold breach or alarm day (close to last observation day) was recorded for each quarter, followed by accounting average early detection days (mean ± SD) for all cows (n = 52) at four time windows (7 days, 14 days, 21 days and 27 days) separately.

Method 2 (Index Approach)

Over 250 indexes were created *ad hoc*, with each index involving an EC manipulation, an EC combination and an EC statistic. The EC manipulations were absolute EC, EC/kg milk harvested at the given milking, EC/hour since previous milking and current EC – EC of the previous milking. The EC combinations were rolling averages (previous 3-9 milkings) of EC data of either the clinical quarter or of the four quarters combined. The EC statistics were conditional rolling average increase of EC, % of variation of EC, mean absolute deviation of EC, mean error % of EC and EC ratio. The statistics were calculated using the following formula(s):

$$\text{Conditional rolling average increase of EC} =$$
$$\text{EC of quarter of interest} \rangle \text{ or}$$
$$\geq \%\text{ increase than previous milkings rolling average EC of 4 quarters,}$$
$$\text{EC of quarter of interest} \rangle \text{ or}$$
$$\geq \%\text{ increase than previous milkings rolling average EC of quarter of interest,}$$
$$\text{EC of quarter of interest} \rangle \text{ or}$$
$$\geq \%\text{ increase of previous milkings rolling averages EC of (4 quarters + quarter of interest)}$$

$$\%\text{ of variation of EC} =$$
$$\frac{(\text{EC of quarter of interest} - \text{Average EC of 4 quarters})*100}{\text{Average EC of 4 quarters}},$$
$$\frac{(\text{EC of quarter of interest} - \text{Average EC of other 3 quarters})*100}{\text{Average EC of 4 quarters}}$$

$$\text{Mean error \% of EC} =$$
$$\frac{(\text{Rolling average EC of previous milkings of quarter of interest} - \text{EC of quarter of interest})^2}{\text{Rolling average EC of previous milkings of quarter of interest}},$$
$$\frac{(\text{Rolling average EC of previous milkings of 4 quarters} - \text{EC of quarter of interest})^2}{\text{Rolling average EC of previous milkings of 4 quarters}},$$
$$\frac{(\text{Average EC of 4 quarters} - \text{EC of quarter of interest})^2}{\text{Average EC of other 3 quarters}}$$

$$\text{Mean absolute deviation} = \frac{\text{Averages of several milkings (Average of 4 quarters} - \text{EC of quarter of interest)'}}{\text{Averages of several milkings (Rolling averages EC} - \text{EC of quarter of interest)}}$$

$$\text{EC ratio} = \frac{\text{(EC of infected quarter)}}{\text{(Averages of healthy quarters EC)}}$$

Similarly to the threshold approach (described in previous section), in the index approach the threshold generated by the indexes acted as a baseline threshold to compare each quarter ECs. Any EC measurements that exceeded the baseline threshold were considered as a mastitis alarm, and TP, FN, FP and TN quarters were categorised on this basis; for example, the CM quarters with any EC measurement that exceeded the baseline threshold were classified as TP; the CM quarters with all EC measurements lower than the baseline threshold were classified as FN; healthy quarters with any EC measurements that exceeded the baseline threshold were classified as FP; and healthy quarters with all EC measurements lower than the baseline thresholds were TN. In addition, the consistency and frequency of threshold breaches were taken into account; breaches were categorised as a (1) single alarm, (2) two, three, or five consecutive alarms, (3) three to five alarms within six consecutive milkings and (4) three to five alarms within 10 consecutive milkings, before the determination of the TP, TN, FN and FP quarters. Taking into account all of the possible EC manipulations, EC combinations, EC statistics and alarm frequency resulted in over 250 indexes that were tested in Microsoft Excel 2010. Then the Se, Sp, accuracy and SE were calculated as per Method 1. Any indexes that resulted in Se of >80% were investigated further in time intervals of 7 days, 14 days and 27 days to determine whether Se, Sp and accuracy could be improved by using only data closer to the time of diagnosis. In addition, the first threshold breach or alarm day (close to last observation day) was recorded for each quarter, followed by accounting average

early detection days (mean ± SD) for all cows (n = 52) at four time windows (7 days, 14 days, 21 days and 27 days) separately.

Method 3 (Statistical Process Control)

A cumulative sum (cusum) control chart (**CC** chart) calculated using QI macros (https://www.qimacros.com/) for Microsoft Excel® was the third method used to identify mastitis alerts. The CC chart consists of 2 calculations called the upper (C^+) and lower (C^-) cusum. The C^+ accumulates deviations above the target (T) value that exceed a value called the reference value indicated as K and C^- accumulates the deviations below the T value that exceed K (Wachs, 2010; Huybrechts et al., 2014). The definitions for C^+ and C^- are as follows:

$$C_t^+ = \max\{0, x_t - (T + K) + C^+_{t-1})\}$$

$$C_t^- = \max\{0, (T - K) - x_t + C^-_{t-1}\}$$

with starting values $C_0^+ = C_0^- = 0$; x_t is the observation at Time t; T is the target value (mean of the observation data); K = 0.5 ×SD, where SD is the standard deviation of the observation data. The process was deemed to be 'out of control' when the C^+ or C^- was outside of the control limits, where upper control limit (UCL) and lower control limit (LCL) were calculated as below.

$$UCL = h \times SD, LCL = -h \times SD,$$

Here, h is the parameter determining the decision interval (in the present study, different values of h were tested arbitrarily: h = 1.5, h = 3 and h = 4) and SD is the standard deviation of the observation data. Data were analysed in four time-windows (7 days, 14 days, 21 days, 27 days, all as days before Day 0). In total, there was about ~13% (941 out of 7249) missing values, which were replaced by the average of previous and subsequent milking ECs, as the

SPC method cannot cater for missing data. Here, the mastitis alert was based on whether or not each quarter had any C^+ and C^- values that fell outside of the UCL or LCL or UCL and LCL or not. Then, the Se, Sp, accuracy and the respective standard errors were calculated as per Method 1.

Statistical Process Control on Model Residuals

A linear mixed model was fitted with the EC data across all milking sessions, using a restricted likelihood (REML) procedure of Genstat for Windows 14 (VSN International Ltd, Hemel Hempstead, Hertfordshire, UK). The mixed model was used to remove the effect of stage of lactation and parity (fixed effects), as well as cow and quarter (random effects) from the observed EC data and to obtain residuals for EC on each milking. A subsequent CC chart was run with these model residual EC values, using the CC chart formulae mentioned above, followed by calculation of the Se, Sp, accuracy and the respective standard errors as per Method 1. Again, all missing values were replaced by the average of previous and later milking EC residual values and the decision interval (h) was varied between 1.5, 3 and 4 standard deviations. Data were analysed in four time-windows (7 days, 14 days, 21 days, 27 days, all as days before Day 0).

RESULTS

Method 1 (Thresholds Approach)

Using only EC thresholds resulted in Se, Sp ranging between 47% and 92% and 39% and 92% respectively (Table 3. 1). The Se was highest at the lower thresholds, but this coincided

with poor Sp. The accuracy of the threshold approach improved as the threshold was increased, but this was associated with significantly lower Se (albeit with much improved Sp). The only threshold that performed with a Se of > 80% was EC of 7.5 mS/cm. Further investigation of this threshold to determine its potential for mastitis detection was conducted by limiting the data to 7-, 14-, 21- and 27-day intervals (all as days before Day 0; Table 3. 2). When only the 7 days before treatment were included in the analysis, Se dropped to 87% but Sp increased from 39% to 63% (accuracy 68%). More accurate early alarms (early detection) were recorded (4.4 ± 2.4) days before antibiotic treatment, and even earlier alarms 16.3 ± 9.5 days were visible but they were dominated by FP alarms (lower Sp) as shown at 27-day time period in Table 3.2.

Method 2 (Index Approach)

The Se, Sp and accuracy achieved by the top six indexes are presented in Table 3. 1. The best- performing indexes were all based on the rolling averages of six milkings. The index that achieved the highest Se (84%) was based on a 15% increase in EC compared with rolling average of six previous milkings of the four quarters and a 15% increase in the rolling average of six previous milkings of the quarter of interest ('15U_15Q'). Limiting the data for this index to the 7 days before the day of treatment reduced the Se from 84% to 79% but increased the Sp from 47% to 70%. Taking the detection period out to the 14-day period leading up to Day 0 increased the Se (84%) but reduced the Sp to 58%. More accurate early alarms (early detection) were recorded 3.6 ± 2.5 days before antibiotic treatment, and even earlier alarms (13.3 ± 8 days before) were visible but they were subjected to higher FP alarms as presented as lower Sp at 27-day time period in Table 3. 2.

Table 3. 1. Sensitivity, specificity and accuracy (means ± SE) achieved by different thresholds and top indexes created *ad hoc* based on electrical conductivity per day of each milking (*n* = 52 cows)

	Item[2]		
Parameter[1]	Se ± SE (%)	Sp ± SE (%)	Accuracy ± SE (%)
Threshold		Threshold approach	
T_7.5	92 ± 4	39 ± 4	51 ± 4
T_8	76 ± 7	66 ± 4	69 ± 4
T_8.5	71 ± 7	79 ± 4	78 ± 3
T_9	58 ± 8	86 ± 3	80 ± 3
T_9.5	53 ± 8	89 ± 3	80 ± 3
T_10	47 ± 8	92 ± 2	82 ± 3
Index_6RA		Index approach	
10U_30Q	71 ± 7	79 ± 4	77 ± 3
15U_15Q	84 ± 6	47 ± 4	56 ± 4
30U_15Q	74 ± 7	82 ± 3	80 ± 3
15U_20Q	79 ± 7	60 ± 4	64 ± 4
15U_25Q	68 ± 8	73 ± 4	72 ± 3
35U_20Q	68 ± 8	85 ± 3	81 ± 3

[1]T = single threshold to compare each milking electrical conductivity; 6RA = rolling average of six previous milking; U = percentage increase in electrical conductivity (EC) compared to rolling average EC of all quarters and corresponding number in column one indicate the threshold levels; Q = percentage increase in EC compared to rolling average EC of the same quarter and corresponding number in column one indicate the threshold levels.
[2]Se = sensitivity; Sp = specificity.

Table 3. 2. Sensitivity, specificity, accuracy and early detection (alarm) days achieved by the best threshold (7.5 mS/cm) and best index (15U + 15Q) at different time widows based on electrical conductivity per day of each milking (*n* = 52 cows)

	Item[1]			
Time window (days)	Se ± SE (%)	Sp ± SE (%)	Accuracy ± SE (%)	ED ± SD (days)
	Best threshold, 7.5 (mS/cm)			
7	87 ± 5	63 ± 4	68 ± 4	4.4 ± 2.4
14	87 ± 5	51 ± 4	59 ± 4	8.0 ± 4.6
21	92 ± 4	45 ± 4	56 ± 4	12.0 ±7.0
27	92 ± 4	39 ± 4	51 ± 4	16.3 ± 9.5
	Best index (15U + 15Q)			
7	79 ± 7	70 ± 4	72 ± 3	3.6 ± 2.5
14	84 ± 6	58 ± 4	64 ± 4	7.1 ± 4.7
21	84 ± 6	51 ± 4	59 ± 4	11. 2 ± 6.4
27	84 ± 6	47 ± 4	56 ± 4	13.3 ± 8.0

[1]Se = sensitivity; Sp = specificity; ED = early detection; 15U + 15Q = 15% increase in electrical conductivity compared to the 6 previous milking rolling average of the 4 quarters and 15% increase in the 6 previous milking rolling average of the quarter of interest.

Table 3. 3. Sensitivity, specificity and accuracy (means ± SE) achieved by cumulative sum (cusum) statistical process-control chart based on electrical conductivity per day of each milking (n = 52 cows)

Decision interval (h)	Item[1]		
	Se ± SE (%)	Sp ± SE (%)	Accuracy ± SE (%)
		7-d time window	
1.5	85 ± 6	18 ± 3	34 ± 4
3	22 ± 6	85 ± 3	70 ± 4
4	5 ± 3	97 ± 2	75 ± 3
		14-d time window	
1.5	98 ± 2	1 ± 1	24 ± 3
3	61 ± 8	41 ± 4	46 ± 4
4	37 ± 8	57 ± 4	52 ± 4
		21-d time window	
1.5	100 ± 0	0 ± 0	24 ± 3
3	88 ± 5	24 ± 4	40 ± 4
4	68 ± 7	35 ± 4	43 ± 4
		27-d time window	
1.5	100 ± 0	1 ± 1	24 ± 3
3	90 ± 5	13 ± 3	31 ± 4
4	83 ± 6	26 ± 4	40 ± 4

[1]Se = Sensitivity, Sp = Specificity, d = Time window included in the control chart; h = Decision interval chosen arbitrary to determine the control limit.
The Se between 7 day and 27-day time windows differ significantly ($P < 0.05$) but Sp and accuracy for all time windows were not significant ($P > 0.05$).

Table 3. 4. Sensitivity, specificity and accuracy achieved by cumulative sum (cusum) statistical process control chart based on residual values of each milking electrical conductivity per day estimated from restricted maximum likelihood procedure (n = 52 cows)

Decision interval (h)	Item[1]		
	Se ± SE (%)	Sp ± SE (%)	Accuracy ± SE (%)
		7-d time window	
1.5	88 ± 5	10 ± 3	28 ± 3
3	22 ± 6	80 ± 3	66 ± 4
4	7 ± 4	98 ± 1	76 ± 3
		14-d time window	
1.5	100 ± 0	0 ± 0	24 ± 3
3	44 ± 8	56 ± 4	53 ± 4
4	15 ± 6	84 ± 3	67 ± 4
		21-d time window	
1.5	100 ± 0	0 ± 0	24 ± 3
3	63 ± 8	31 ± 4	39 ± 4
4	32 ± 7	63 ± 4	56 ± 4
		27-d time window	
1.5	100 ± 0	0 ± 0	24 ± 3
3	76 ± 7	24 ± 4	36 ± 4
4	51 ± 8	51 ± 4	51 ± 4

[1]Se = Sensitivity, Se = Specificity, d = Time window included in the control chart; h = decision interval chosen arbitrary to determine the control limit.
There was no significant difference ($P > 0.05$) for Se, Se and accuracy across the different time windows.

Method 3 (Statistical Process Control, SPC)

In general, SPC did not perform as well as either the threshold or index approaches, regardless of whether each milking EC per day was used (Table 3.3) or residual (mixed model) of each milking EC per day was used (Table 3. 4). Although the Se, Sp and accuracy achieved by the SPC approach based on each milking EC and the residual of each milking EC were numerically different at different decision intervals (e.g., h = 1.5, 3, 4 SD) across four time windows (7, 14, 21, 27 days), they were not statistically significant ($P > 0.05$). The Se based on each milking EC differed significantly ($P < 0.05$) between 7-day and 27-day time windows, but Sp and accuracy were not significant ($P > 0.05$) at any time window (Table 3.3). The Se, Sp and accuracy based on the residual of each milking EC were not significant ($P > 0.05$) across different time intervals (Table 3. 4).

DISCUSSION

Farmers operating with AMS have dramatically reduced contact time with individual cows, and this affects their ability to visually inspect cows (or their milk) so as to identify cows that require therapeutic or preventive interventions (Mollenhorst et al., 2012). Thus, these farmers rely heavily on inline sensors based on recorded data to identify sick cows. To be deemed valuable by the farmers, such systems should have a low FP alert (e.g., high Sp) rate and should accurately lead to early detection of TP (e.g., high Se) cases (Kamphuis et al., 2010; Mollenhorst et al., 2012). Electrical conductivity is the most commonly used inline sensor for detecting mastitis, although it is renowned for giving variable results (Norberg et al., 2004b; Kamphuis et al., 2010; Mollenhorst et al., 2012), probably because it is affected by temperature, fat content, and milk fraction (Nielen et al., 1992; Norberg et al., 2004). In the

field, farmers usually implement EC thresholds of 7-8 mS/cm (sometimes greater), as there are currently no robust best-practice guidelines for EC in AMS herds. The current study found that the optimal EC for minimising the number of clinical cows that would go undetected was 7.5 mS/cm. Unfortunately, the Sp of this threshold was only 39%, that indicates that for each clinical cow alerted there would be an additional two or three cows on the alert list that would not be clinical. This high level of FPs was reduced when the time interval of interest was reduced to the 7-day period leading up to treatment date; however, the farmers does not know the treatment date in advance and is reliant on high Se and Sp to develop robust standard operating procedures for mastitis detection. The variation in Se, Sp and early detection across the different time intervals (7, 14, 21 and 27 days) suggest that there were some individual EC spikes (causing threshold breaches) well before the clinical event, but these were most likely to be FPs rather than an indication that the onset of mastitis was imminent. Minimising the observation window to a short time frame (preferably 48 h) has been recommended to generate CM detection models with ~80% Se and 99% Sp (Hogeveen et al., 2010). Our findings with reduced time windows support the idea that the detection accuracy can be improved; however the challenge remains that the farmers does not know that mastitis is imminent until clinical signs are evident. Filtering through the false alerts would be both laborious, time consuming and prone to some error. The authors recognise that the Se of the tests used in this study is likely to be underestimated due to the unknown timing of exact clinical infection in this retrospective data analysis, making results somewhat 'conservative'. However, despite this, there is some confidence that controlled mastitis-challenge trials would be warranted to further investigate the potential of EC thresholds as an early and accurate indicator of imminent CM. Whilst, other authors have published some encouraging results using complex single algorithms (Claycomb et al., 2009), unfortunately, our comprehensive *ad hoc* approach to index development did not generate a

highly effect mastitis detection index. The six most promising indexes all involved the rolling average of six milkings, indicating that a reasonable number of rolling averages was needed to reduce the influence of false alerts or spikes in the EC. The only index that had a Se exceeding 80% was the index that involved a 15% increase in EC across the udder (all 4 quarters combined), combined with a 15% increase in EC within a given quarter. Reducing the time frame of interest down to the 7-day period before diagnosis reduced the Se slightly to 79%, but dramatically increased the Sp from 47% to 70%, resulting in an accuracy of 72%. This suggests that, with this index, almost one in five clinical cases will go undetected and that for every positive alert, there is ~30% chance that the cow will not be clinical. With only a small change in Sp in the 14-, 21- and 27-day windows, we could surmise that the number of index breaches more than 7 days before diagnosis was very small. Interestingly, on average, the index alerts were occurring 3.6 ± 2.5 days before the day of diagnosis. In contract, the threshold alerts (where EC exceeded 7.5 mS/cm) were occurring 4.4 ± 2.4 days before the date of diagnosis.

The SPC approach has reportedly been successful for the determination of animal health status (Lukas et al., 2008), growth rates, water consumption, dry matter intake (Lukas et al. 2008), and oestrus detection (de Vries and Conlin, 2003; Lukas et al., 2008). However, in the current study, the SPC approach was not as promising in terms Se, Sp and accuracy for mastitis detection, in comparison with the threshold and the index approaches discussed previously. This was the case in both scenarios, regardless of whether or not the cusum was based on the EC values of each milking (each day) or the residual EC values estimated from REML. Most commonly, SPC is used for detection of smaller process shifts (<1.5 SD), whereas the Shewhart control chart is used for large process shifts (> 3 SD, Huybrechts et al., 2014). Due to nature of the data (and the lack of previous reports on using this approach for mastitis detection), we tested a range (1.5 SD, 3 SD, 4 SD) of process shifts in the present

study. Lukas et al. (2009) reported 40-48% Se targeting 98-99.5% Sp on the basis of residuals (mixed model) of combined EC and milk yield measurements, in their investigations into monitoring abnormal udder health status. Whilst our study reported high Se, particularly when the decision interval was low (1.5 SD) or when the observation period was long (21 or 27 days before diagnosis), the corresponding Sp values were very low. Hence, SPC (either of EC or residual EC) is unlikely to be a good tool to aid the early detection of CM. The reasons for the poor performance of SPC might be due to inclusion of prior data for the calculation of running standard deviation and control limits, which make the control limits (upper and lower) less sensitive to the increased number of extreme values of running data (Lukas et al., 2009). The significant difference in terms of Se between time windows 7 and 27 days is mainly due to higher percentage of FP cases (25% vs. 66%), which further support the concept that a small time window is preferable if accuracy of detection is to be maximised. However, as previously mentioned, the farmer cannot foresee the clinical signs of mastitis before the event and will, therefore, have difficulty in differentiating between FPs (that can occur at any time) and similar alerts that are truly leading up to an imminent mastitis case. Essentially, since mastitis is associated with multiple changes in the body and milk, utilising multi-sensor data is likely to provide the best opportunity for the break-through in accurate automated mastitis detection that is required to ensure that animal welfare is not compromised and that wasted milk and wasted production potential are minimised (Mollenhorst et al., 2010). Previous reports, whereby sensor data such as EC, colour, somatic cell count, milk yield, lactate dehydrogenase, activity and milk composition have been combined with other biological risk factors have indicated promising results to reduce false alerts (de Mol and Ouweltjes, 2001; Rasmussen and Bjerring, 2005; Chagunda et al., 2006; Kamphuis et al., 2010b). The drawbacks are that not all brands of AMS have inline somatic cell count equipment available, thereby making owners of those brands reliant on costly (and

less timely) off-farm laboratory analysis. Milk-yield fluctuations are often associated with alarming non-specific health problem (Lukas et al., 2009), the normal fat colour, which may vary by breed, can impede the accuracy of the colour sensor for the detection of blood (Rasmussen and Bjerring, 2005) and lactate dehydrogenase requires installation of equipment such as DeLaval's Herd Navigator™, which is not available to all brands of AMS or even all models of DeLaval's robots. The authors believe that we have exhausted the potential options for EC as a stand-alone inline indicator of mastitis. Future investigations will likely require the incorporation of multiple data sources from both milk and animals, perhaps including behavioural changes. Biological indicators beyond what is currently available should be evaluated, as inline and relatively low-cost sensors deserve investigation without the limitation of what is currently available and possible.

CONCLUSIONS

Although this study demonstrated that there is some potential to detect mastitis by using EC data, we conclude that EC data alone cannot provide the required accuracy to detect infected quarters. Incorporating other information with different approaches is required for the early detection of mastitis in AMS herds.

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Supplementary material

As well as avoiding economic loss, early detection of mastitis reduces on-farm antibiotic usage and thereby reduces the risk for antimicrobial resistance and associated hazards to humans (Hardefeldt et al., 2018).

After conversion of the conventional herring bone milking parlour into automatic milking rotary into 2014, the incidence of mastitis at the University of Sydney's dairy farm increased, but due to several other changes that occurred in parallel (staff, governance, operational management) it was impossible to establish cause-consequence relationships.

Cows were selected on the basis that they had a mastitis event (1 or 2 quarters) that was not preceded by another CM event within the 27- day period before treatment (by trained farm staff or assigned veterinarian).

The exclusion criteria resulted in 172 quarters for the analysis with 33 infected quarters and 139 healthy quarters and exclusion of five cows in the analysis.

Conclusions

Although this study demonstrated that there is some potential to detect mastitis by using EC data, we conclude that EC data alone cannot provide the required accuracy to detect infected quarters. Incorporating other information with different approaches is required for the early detection of mastitis in AMS herds

Reference

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CHAPTER 4: Suitability of somatic cell count, electrical conductivity, and lactate dehydrogenase activity in foremilk before versus after alveolar milk ejection for mastitis detection

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OVERVIEW OF CHAPTER 4

One of the major limiting factors addressed in Chapters 2 and 3, for poor performance of a single sensor to detect mastitis, was the influence of milk composition or compositional variation. Hence, Chapter 4 investigates the suitability of different milk fractions in milk composition to detect mastitis. This chapter also investigates the possibility of differentiating between Gram-positive and Gram-negative mastitis to avoid excessive antibiotic use by rapid treatment decision (as highlighted in Chapter 2). This study was conducted in a controlled experimental design to collect two different milk fractions (before and after milk ejection) from individual quarters of 48 Holstein-Friesian cows.

ABSTRACT

Mastitis is responsible for substantial economic loss and significant animal welfare concerns for the dairy industry. Sensors that measure electrical conductivity (**EC**) and enzyme concentrations of lactate dehydrogenase (**LDH**) are presently used for automatic detection of mastitis. However, EC is not sensitive enough to detect mastitis, and the ability of LDH activity to identify mastitis caused by different pathogens is a potential option that needs to be investigated. This study was conducted to test the following hypotheses: a) strict foremilk before milk ejection is more informative in detecting mastitis, in general, than foremilk removed after cows were stimulated for milk ejection; and b) the value of LDH activity as a mastitis indicator depends on the type of pathogen associated with the infection. Milk samples (before afternoon milking) from 48 Holstein-Friesian cows at the University of Sydney's dairy farm (Camden, New South Wales, Australia) with EC > 7.5 mS/cm in any of the 4 quarters were collected over a period of 2 mo. Quarter milk samples (n = 343) from 48 cows were collected manually in the automatic milking rotary in 3 steps: foremilk before (strict foremilk) and after milk ejection, followed by an aseptic sample for bacteriological culture. The EC (mS), LDH (U/L), somatic cell count (**SCC**, cells/ml), and milk protein and fat content (%) of foremilk in both sampling times were compared and used as predictors for Gram-positive and Gram-negative mastitis. Quarter (n = 515) observations from 44 cows were analysed using a logistic mixed or linear mixed model, with cow and quarter nested within cow as random effects. Milk from both sampling times was also assessed by producing a receiver operating characteristic (**ROC**) curve and calculating the area under the curve (**AUC**) to determine their ability to detect mastitis. Overall, EC and LDH were greater and milk protein (%) was lower in strict foremilk than in milk fractions obtained after milk ejection. Data from strict foremilk samples had slightly higher AUC values (0.98 to 0.99 vs.

0.97 to 0.98, respectively) than did the after-ejection milk samples. Although Gram-negative coliform mastitis had significantly higher LDH activity than did Gram-positive mastitis (6.19 vs. 5.34 $\log_{10}$ U/L), the robustness of this result is questionable due to limited sample size. We concluded that milk samples taken before ejection can influence major mastitis indicators, suggesting that automatic milking system sensors could be modified to monitor milk before ejection for more efficient mastitis detection.

Keywords: Dairy cow, mastitis, quarter, strict foremilk, milk ejection.

INTRODUCTION

Bovine mastitis is an inflammation of the mammary gland that affects animal welfare and has a huge negative economic effects on the dairy industry (Halasa et al., 2007; Huijps et al., 2008). Efficient mastitis detection provides opportunity to implement early and adequate treatment protocols and to avoid excessive use of antibiotics, maintaining good animal health and welfare by reducing soreness, pain and discomfort; enhancing recovery rate; and improving economic return to the farmers (Milner et al., 1997; Lehmann et al., 2015). Currently, an increasing number of dairy farmers worldwide are choosing automatic milking systems (**AMS**), which allow the farmers to maximise milking frequency (and potentially milk production per cow) and minimise labor costs (García and Fulkerson, 2005; Hovinen and Pyörälä, 2011; John et al., 2017). In AMS, the sensors that measure electrical conductivity (**EC**) are the inline sensors most commonly used to detect mastitis. These sensors can continuously measure concentration of ions in milk during the milk harvesting process, albeit with variable results (Kamphuis et al., 2008; Mollenhorst et al., 2012; Khatun et al., 2017). Currently in AMS, EC sensors do not measure strict foremilk present in gland cistern before oxytocin-induced alveolar ejection from alveoli and smaller milk ducts

(Bruckmaier and Blum, 1998; Lehmann et al., 2015). Hence, measurements are based on a mixture of cisternal and alveolar milk (as initial milk is discarded and alveolar milk ejection occurs during the teat cleaning process; Bruckmaier and Hilger, 2001; Bruckmaier et al., 2004b; Dzidic et al., 2004). Previous studies have revealed that the milk composition after alveolar ejection varies from the composition before ejection, with reduced effectiveness for mastitis indicators such as EC and SCC (Bruckmaier et al., 2004b; Bansal et al., 2005; Lehmann et al., 2015). Thus, by discarding and not measuring strict foremilk, AMS may be missing valuable data from potentially the most informative milk with regard to mastitis detection.

On the other hand, the immune mechanisms triggered by major mastitis-causing Gram-positive (e.g., *Staphylococcaceae, Streptococcaceae*) or Gram-negative (e.g., *Enterobacteriaceae*) families are different due to different receptor-induced immunoregulatory activities (e.g., toll-like receptor, lipopolysaccharide, peptidoglycan, lactoferrin; Bradley, 2002; Tietze et al., 2006; Wellnitz et al., 2011). At present, researchers are focusing on immune profile-based inline monitoring sensors to distinguish specific mastitis pathogens for rapid treatment decision as an alternative to the current time-demanding culture or polymerase chain reaction tests (Nyman et al., 2014). Although enzymes (e.g., lactate dehydrogenase, **LDH**; at present only commercially available in Herd Navigator™, DeLaval) are currently used for automatic detection of mastitis (Chagunda et al., 2006b; Mollenhorst et al., 2012), the ability of LDH activity to identify mastitis originating from different pathogens is uncertain. It appears that the best LDH enzyme-based mastitis marker results were obtained when infections originated from live Gram-negative *E. coli* infection (Sørensen et al., 2015; Hernández-Castellano et al., 2017) or were experimentally induced using dead *E. coli* cell wall (lipopolysaccharide; Larsen et al., 2010; Lehmann et al., 2013; Wellnitz et al., 2015). Additionally, quarter-level mastitis investigation

results in higher sensitivity (**Se**) and specificity (**Sp**) than does cow-level investigation (Kamphuis et al., 2008; Mollenhorst et al., 2010). Hence, this study was conducted to test the hypotheses that: a) strict foremilk ejection is more informative in detecting mastitis at the quarter level than is foremilk removed after milk ejection, regardless of causal pathogen; and b) the value of LDH activity as an indicator of mastitis depends on the type (e. g., Gram-positive, Gram-negative) of pathogen associated with the infection.

MATERIALS AND METHODS

All procedures involving animals were approved by the animal ethics committee of the University of Sydney (Project number: 2017/1141). The study was conducted for a period of about 2 mo (Jun. 21 to Aug. 30, 2017).

Experimental Design

This study included fractionised milk samples to investigate the effectiveness of EC, SCC, LDH activity, milk protein, and fat, individually or in combination, as indicators of mastitis when: a) they were determined from milk collected from individual quarters either before or after ejection, and b) infection originated from Gram-positive versus Gram-negative bacteria. Fractional milk samples (before and after ejection) followed by aseptic samples from individual quarters were taken from 48 Holstein-Friesian cows.

Location

The experiment was conducted at the University of Sydney's Corstorphine pasture-based

dairy farm, located in Camden, New South Wales, Australia. The farm had about 85 ha of effective grazing land, mostly with annual ryegrass (*Lolium multiflorum*) oversown on kikuyu (*Pennisetum clandestinum*) to feed the cows. A partial mixed ration containing primarily brewer's grain, orange pulp and pasture silage (lucerne hay, oaten hay) was supplemented when necessary to cover deficits in true pasture. Additionally, all the lactating cows (approx. 350) were supplemented with approximately 7 kg dry matter of grain-based commercial pelleted concentrate (18% protein) per cow in the postmilking area. A year-round calving system was followed, and an automatic rotary system with 24-unit platform and 5 robotic arms (DeLaval Automatic Milking Rotary, Tumba, Sweden) was used for milking the cows.

Indicators for Mastitis Definition

Two different types of mastitis indicators were used in this study, the first based on bacteriological culture to determine the true infection status of the quarter (Sargeant et al., 2001), and the second based on quarter with mastitis predicted by limiting certain SCC thresholds, as reported by previous studies (Hillerton, 1999; Mollenhorst et al., 2010). We used SCC-based indicators to account for (1) decrease of colony-forming units to below detection levels with active inflammation and (2) the possibility of presence of mastitis pathogens requiring specific culture media (e.g., *Mycoplasma* spp., *Coxiella burnetii*) not used in the present study.

Based on bacteriological culture results, quarters identified with different Gram-positive bacteria were defined as Gram-positive mastitis, and quarters with coliform bacteria were defined as Gram-negative mastitis. Quarters with 2 or more pathogens were classified as mixed mastitis, and quarters with no bacterial growth were classified as negative growth, to be considered as negative control.

In terms of SCC, previous studies classified quarter milk with > 100,000 cells/ml (Hillerton et al., 1999) or > 500,000 cells/ml (Mollenhorst et al., 2010) as mastitis (the latter also considered the presence of abnormal milk). In the present study, due to the potential influence of milk fractions on SCC level (Sarikaya and Bruckmaier, 2006) and weaker mastitis response with < 300,000 cells/ml (Hernández-Castellano et al., 2017), we calculated a threshold based on mean plus 1 standard deviation, calculated on a logarithmic scale, of strict foremilk and after-ejection milk samples to define the mastitis quarter with an abnormal SCC range (Gordon et al., 1980). As a result, quarters (n = 104) having > 530,000 cells/ml in the strict foremilk and > 440,000 cells/ml in the sample after ejection were considered as quarters with mastitis; the remaining quarters (n = 411) were considered as negative controls. Any quarters identified with clinical mastitis during the sampling process were treated immediately after sampling with an intramammary broad-spectrum antibiotic.

Milk Sample Collection

Each day, milk samples were collected manually between 1400h and 1700h in the automatic milking rotary. Quarter samples were collected from each cow selected based on EC at 3 points: before ejection (strict foremilk); after ejection or after udder stimulation (cleaning the teats); and an aseptic sample for culture.

For the first step of sampling, milk samples were collected immediately after the cow entered the milking parlor, before any milking procedure and without any teat cleaning (e.g., before any tactile stimulation occurred). The first 2 to 3 squirts (foremilk) collected separately from each quarter within approximately 60 s of touching the udder were considered to be sampled before milk ejection, or strict foremilk. These samples measured approximately 50 ml each.

In the second step of sampling, after direct tactile stimulation (~ 74 s), the udder was further stimulated by rubbing each teat with a towel soaked in warm water containing iodine solution (Iodophor LF12); teats were subsequently dried with tissue paper. After that, milk samples of about 50 ml each were collected separately from each quarter.

Finally, aseptic samples were collected for bacteriological culture. Thus, for each cow, immediately after the second step of sampling, teats were dipped in iodine solution (Iodophor LF12) and cleaned with a 70% alcohol-soaked gauze (as modified from Hogan et al., 1999). Immediately after the teat was thoroughly disinfected, milk samples of about 10 ml were collected from each quarter, following standard procedures to minimise risk of contamination. During milk collection, we followed the same order of quarter sampling, namely, left hind, left front, right hind, and right front, to minimise risks of sampling error.

Immediately after collection, milk samples were transported to the laboratory, where aseptic milk samples were frozen at -20°C until sent to the culture laboratory. Approximately 5 ml of each sample from strict foremilk and after-ejection milk was separated and frozen at -80°C for LDH activity analysis. The remaining milk samples were tested for EC and then mixed with Protectol preservative (Thor, Specialties Pty. Ltd., Wetherill Park, Australia) before being sent to a commercial laboratory for SCC (cells per ml), milk protein (%), and milk fat (%) content analysis.

Criteria Used for Selection and Post-admission Exclusion

Selection Criteria. Forty-eight Holstein-Friesian cows (out of 350 lactating cows) of first to eighth lactation (average 161 days in milk, **DIM**) and having a relatively high EC (≥ 7.5 mS/cm at milking temperature, 38°C) in any of the 4 quarters were screened for milk sampling (Norberg et al., 2004). Based on detection of a mean difference in EC of 0.5 mS/cm

between the milk fraction samples [based on a previous study by Ontsouka et al. (2003)], with a corresponding standard deviation of 0.5 mS/cm, 11 cows would be required, assuming a power of 80% and threshold significance of 5%, whereas 34 cows would be required to detect a difference of 0.25 mS/cm (http://statulator.com). The study by Ontsouka et al. (2003) used 16 cows. Our study used 48 cows, with single samples from 31 and repeated samples from 17 cows. Because EC reading vary according to milk temperature, we selected 7.5 mS/cm arbitrarily to find mastitis quarters with SCC ranges of at least 425,000 to 531,000 cells/ml, according to Bruckmaier et al. (2004a). Identified cows were separated from the voluntary milking herd and fed in designated paddocks to allow their milking sessions (twice daily) to be controlled for monitoring and sampling purposes.

Post-admission Exclusion Criteria. Any cow treated with antibiotic in single or multiple quarters for clinical mastitis was excluded from sampling. One quarter of a cow with adjacent supernumerary teat was not sampled, due to milking inactivity in the AMS. Four cows were not included in the analysis due to insufficient amount of sample (time restriction to collect strict foremilk or insufficient milk after ejection).

Laboratory Analysis

Milk samples collected before and after ejection were analysed for EC, SCC, LDH activity, and milk protein and fat content.

Electrical Conductivity. Electrical conductivity was measured using a Draminski Model 4 × 4 Q MAST mastitis detector (MDQ, Draminski, Olsztyn, Poland) at the University of Sydney M. C. Franklin Laboratory at the day of sample collection. The MDQ is designed to measure

the electrical resistance (e.g., inverse of conductivity) in the range of 10 Ω to 1000 Ω. The electrical resistance (**ER**) value measured by MDQ was converted into EC as follows:

$$\mathrm{ER}\,[\mathrm{ohm}(\Omega)] = \mathrm{Unit\,shown\,in\,MDQ}/1.944$$

$$\mathrm{EC}\,(1{,}000\,\mathrm{mS}) = \mathrm{EC}\,(1\mathrm{S}) = 1\ \mathrm{reciprocal\,ohm}\,[1/\Omega]$$

Milk samples collected before and after ejection were measured separately, following the same order (right hind, right front, left hind, left front) to match the corresponding vessel of the MDQ, to avoid any intervessel measurement variation. The EC was measured at room temperature (25°C), and milk was not heated to adjust for milking temperature (38°C) EC.

Lactate Dehydrogenase Activity. The activity of LDH was measured at the University of Bern, Switzerland, using a commercial test kit LDH International Federation of Clinical Chemistry (Axon Lab AG, Baden, Switzerland) and an automated analyser (COBAS MIRA, Roche Diagnostics, Basel, Switzerland) with minimum detectable activity of 5 U/L.

Somatic cell count, Protein and Fat. A Bentley 2000 auto-analyser was used to measure SCC (cells × 1,000/ml), milk protein (%) and milk fat (%), following the manufacturer's protocol (Bentley 2000 Instruments, Chaska, MN). The SCC was quantified using the principles of laser-based flow cytometry, whereas milk protein and milk fat were measured by mid-infrared absorption built on a single-beam optical system.

Bacteriological Culture. To identify the specific bacteria responsible for mastitis, sterile milk samples were cultured at the University of Sydney microbiological laboratory. All samples were cultured within 7 d of collection, using standardised procedures consistent with National Mastitis Council (Hogan et al., 1999) guidelines, with modifications as described by Shum et al. (2009). We assumed that freezing had no influence on the viability of the specific bacteria in this study, although freezing could influence the microbial quality (Murdough et al., 2010; Ruegg et al. 2016). Briefly, Gram-positive *Streptococcus, Aerococcus*, and *Enterococcus* spp. were identified by their growth in sheep blood agar (**SBA**), *Enterococcal* agar, and Rambach

agar; and by bile aesculin, Christie, Atkins, and Munch-Peterson, and leukocyte alkaline phosphatase tests. *Staphylococcus* isolates were identified by growth in SBA, coagulase test, and Gram-staining. Gram-negative coliforms were identified by their growth on SBA and MacConkey's agar, Gram staining, and potassium hydroxide (slime) test. We did not further differentiate between species of coliform pathogens. Samples that did not yield microbial growth following 48 h of incubation were classified as negative controls. Isolation of 2 or more bacteria genera from the same sample was considered as nondiagnostic or mixed.

Statistical Analysis

Data were analysed using ASReml-R (Butler et al., 2009) built under R version 3.4.3, (http://www.r-project.org). As the distributions of SCC and LDH activity were positively skewed, they were log (base *e*) transformed before analysis to stabilise the variance and achieve normality of the outcome variables, or to reduce leverage of very large values when used as explanatory variables. In total, 515 quarter observations were included in the analysis of sampling times. Other analyses included SCC and LDH activity from, which 3 observations were excluded due to extreme residual deviation (> 3) between before- and after-ejection sampling times.

Sampling Time Differences. The differences in the response observations in 2 different sampling times collected before versus after ejection were assessed using the following linear mixed model:

$$Y = \text{Constant} + \text{Time} + \text{Quarter} + \text{Time} \times \text{Quarter} + \text{Cow} + \text{Cow.Quarter} + \varepsilon,$$

Here Y is the response variable [EC, ln(SCC), ln(LDH), milk protein, or milk fat], Time = before versus after ejection as fixed effect; Quarter = quarter as fixed effect, Time × Quarter = interaction between before- versus after-ejection time with quarter, Cow and Cow.Quarter

= cow, and quarter nested within cow as random effects, and Ɛ = random error. Predicted means were calculated with corresponding 95% confidence intervals.

Assessment of Sampling Times by Receiver Operating Characteristic Curve (**ROC**). The following logistic generalised linear mixed model (**GLMM**) was fitted before ROC assessment:

$$\ln[\pi/(1-\pi)] = \beta_0 + \beta_{EC}EC + (\beta_{LDH} + \gamma_{LDH.Time})\ln(LDH) + \beta_{MP}MP + \beta_{MF}MF + Time + u_C + u_{CQ}$$

where $\pi = P(Y = 1)$ is the probability that the quarter has mastitis at the specified SCC threshold at a particular test session; EC, ln(LDH), MP (milk protein), MF (milk fat), and Time (sampling times), are predictors with interaction of ln(LDH) with sampling times (specified as $\gamma_{LDH,Time}$), as a fixed effect; Note that β_{EC}, β_{LDH}, β_{MP}, β_{MF} specify the overall linear effects of the 4 variates, and u_C and u_{CQ} are the random Cow and Cow.Quarter effects.

Construction of the ROC curves was performed using the pROC package in R (version 3.4.4; Robin et al., 2011). The ROC assessment graphically illustrates the diagnostic test to present sensitivity (**Se**) versus the complement of specificity (1 – **Sp**) for varying cut points (Hanley and McNeil, 1982; Khatun et al., 2018). The cut points are determined for different probabilities (or linear predictors) of the fitted GLMM. The generated area under the curve (**AUC**) value from the ROC curve is used to measure the diagnostic test performance, classified as excellent (0.9–1), good (0.8–0.9), fair (0.7–0.8), poor (0.6–0.7), or fail (0.5–0.6; Swets, 1988). These ROC curves and accompanying AUC values are evaluated at both sampling times (before and after ejection). Test performance is also evaluated using Youden's index (J = Se + Sp – 1), selecting a cutoff point at, which the index is maximised. (Ruopp et al., 2008).

Effect of Sampling Times on EC Measurement. To estimate the effect of sampling time (before or after ejection) on EC, we initially constructed a multivariable logistic GLMM with 4 predictor variables (EC, LDH activity, milk protein, and milk fat), including interaction

effects with sampling times. Following a manual backwards elimination procedure to drop nonsignificant variables, the final model used for EC association as follows:

$$\ln[\pi/(1-\pi)] = \beta_0 + (\beta_{EC} + \gamma_{EC.Time})EC + (\beta_{LDH} + \gamma_{LDH.Time})\ln(LDH) + \text{Time} + u_C + u_{CQ},$$

where $\pi = P(Y = 1)$ is the probability that the particular quarter had mastitis with > 530,000 cells/ml in the sample before ejection and > 440,000 cells/ml after ejection (as described in 'Indicators for Mastitis Definition') at a particular test session; EC and ln(LDH) were predictors having interaction with sampling times (Time), as fixed effects; and u_C and u_{CQ} were random Cow and Cow.Quarter effects. Note that β_{EC} and β_{LDH} specify the overall linear effect of these 2 variates, and $\gamma_{EC,Type}$ and $\gamma_{LDH,Type}$ are used to specify interactions- for example, a deviation of the linear trend for the particular sampling times, before versus after ejection.

Difference Between Gram-positive and Gram-negative Mastitis. The differences in the response observations in Gram-positive and Gram-negative mastitis were assessed using the following linear mixed model:

$$Y = \beta_0 + \text{Culture} + (\beta_{DIM} + \gamma_{Culture.DIM})\text{DIM} + u_C + u_{CQ}$$

Here Y is the response variable (EC, ln(SCC), ln(LDH), milk protein, or milk fat); Culture = Gram-positive versus Gram-negative mastitis as fixed effect; DIM = days in milk as fixed effect; β_{DIM} specify the overall linear effect of DIM, and $\gamma_{Culture.DIM}$ is used to specify the Culture × DIM interaction. Predicted means were calculated with corresponding 95% confidence intervals.

Comparison of SCC and LDH Activity to Predict Mastitis. To compare the effectiveness of SCC and LDH activity to predict Gram-positive or Gram-negative mastitis, each of these variables was standardised or re-scaled, for example, $x' = (x - \bar{x})/SD_x$, to compare variables across different scales, using the scale function in R version 3.2.5, (http://www.r-project.org).

The rescaled SCC or LDH activity data were assessed individually because of collinear association between them ($r_s \geq 0.8$), using the following nonlinear model (spline function in R):

$$\ln[\pi/(1-\pi)] = \beta_0 + \beta_1 x' + s(x') + u_C + u_{CQ,}$$

where x' is either the rescaled SCC or the rescaled LDH. Each is included as a fixed linear effect, together with a nonlinear spline term, $s(x')$, specified as a random effect in the model, and also included u_C and u_{CQ} as random Cow and Cow.Quarter effects. Rescaling allowed displaying both predictors with the corresponding fitted values on the same plot.

Correlation Test. To assess for correlation between predictors, pairwise Spearman's correlations were obtained between the 3 variables, namely EC, ln(SCC), and ln(LDH), independent of mastitis pathogen. Because of large variabilities in milk fat (particularly) and protein content between milking, we did not calculate the correlation of milk fat and protein with these 3 variables.

RESULTS

One cow had only three functional quarters, and several cows were sampled on several days as a result of the EC-threshold criterion (7 cows × 2 times, 5 cows × 3 times, 4 cows × 5 times, and 1 cow × 6 times). This resulted in 686 samples from 48 cows for the laboratory analysis and 343 samples for culture test. On average, milk samples before ejection were collected within 60 s of udder touch (Bruckmaier et al., 2004b). This limited the volume of milk collected, and therefore some measurements were missing in the analysis (insufficient volumes were 27 samples for SCC and 115 samples for fat and protein). Likewise, for milk sampled after ejection, we also found cases of insufficient volume (18 samples for SCC and 48 samples for protein and fat measurements) due to hostile behavior mostly by primiparous

cows or attributable to pain from clinical mastitis, or due to machine measurement error with limited samples. Additionally, after laboratory measurement of SCC and LDH activity, 3 quarters showed extreme deviation for SCC and LDH values between 2 sampling times and therefore were excluded from the analysis.

Bacteria Identification and Difference between Sampling Times

Out of 343 tested quarters of 48 cows, 157 (45.8%) were Gram-positive, 6 (1.8%) were Gram-negative (coliform), 6 (1.8%) were mixed, and 174 (50.7%) did not have bacterial growth (negative control). Out of the 157 Gram-positive pathogens, *Corynebacterium* spp. (44%, *n* = 69), *Strep. uberis* (17.2%, quarter samples = 27) and *Strep. dysgalactiae* (13.4%, quarter samples = 21), accounting for approximately 70% of all infections. Other mastitis-causing Gram-positive pathogens were coagulase-nagetive *Staphylococcus* (9.6%, quarter samples = 15), *Strep. agalactiae* (5.7%, quarter samples = 9), coagulase positive *Staphylococcus* (3.2%, quarter samples = 5), *Bacillus* spp. (2.6%, quarter samples = 4), *Enterococcus faecalis* (1.9%, quarter samples = 3), *Trueperella pyogenes* (1.3%, quarter samples = 2), *Aerococcus* spp. (0.6%, quarter sample = 1), environmental *Streptococcus* spp. (0.6%, quarter sample = 1). Milk before ejection had significantly greater EC ($P < 0.001$) and LDH activity ($P = 0.036$) but lower milk protein ($P < 0.001$) than after-ejection (Table 4. 1).

Assessment of Sampling Times by ROC

In the ROC evaluation (Table 4. 2), we obtained excellent (AUC > 0.9) mastitis prediction ability for both before- and after-ejection samples at different SCC thresholds. Samples before ejection had numerically higher or equal mastitis prediction ability compared with

Table 4. 1**.** Model-based means (95% CI in parenthesis) of electrical conductivity, SCC, lactate dehydrogenase activity, and milk protein and milk fat concentrations in milk samples before and after ejection, from linear mixed models[1]

Responses[2]	Times[3]		
	Before-ejection	After-ejection	*P* value
EC (mS)	5.08 (4.97, 5.19)	4.60 (4.51, 4.69)	<0.001***
SCC (× 1000 cells/ml)	536 (423, 679)	432 (351, 533)	0.18
LDH (U/L)	200.75 (171.56, 234.91)	160.36 (139.55, 184.28)	0.036*
Milk protein (%)	3.41 (3.33, 3.49)	3.59 (3.52, 3.67)	0.001**
Milk fat (%)	2.83 (2.61, 3.05)	2.86 (2.66, 3.05)	0.86

[1]Linear mixed models to calculate predicted means of 4 outcome variables (electrical conductivity, lactate dehydrogenase activity, protein content, and fat content in milk), with random effect estimates for each cow and cow-quarter (n = 515 quarters).
[2]EC = electrical conductivity; LDH = lactate dehydrogenase activity.
[3]Before ejection = comparison only before alveolar milk ejection; after ejection = comparison only after alveolar milk ejection.
*P < 0.05, **P < 0.01, ***P < 0.001.

after ejection, as specified by the AUC values (0.98 to 0.99 vs. 0.97 to 0.98, respectively; P > 0.1). At the optimum cutoff (maximum value of Youden's index) the differences between Se and Sp between before- versus after-ejection times were (-0.34% to 0.8%, and -0.9% to 8.4%, respectively), with an average 3.6% higher Sp among before-ejection samples.

Assessment of Sampling Times by ROC

In the ROC evaluation (Table 4. 2), we obtained excellent (AUC > 0.9) mastitis prediction ability for both before- and after-ejection samples at different SCC thresholds. Samples before ejection had numerically higher or equal mastitis prediction ability compared with after ejection, as specified by the AUC values (0.98 to 0.99 vs. 0.97 to 0.98, respectively; P >

Table 4. 2. Analysis of receiver operating characteristic (ROC) curves, sensitivity and specificity at optimum cut-off value for prediction of mastitis with different thresholds of SCC (gold standard for definition of mastitis) by multivariable generalised linear mixed model[1]

SCC (> × 1000 cells/ml)	Item[2]						
	Before-ejection			After-ejection			
	AUC (95% CI)	Se	Sp	AUC (95% CI)	Se	Sp	P -value
100	0.989 (0.979, 0.998)	0.929	1.00	0.970 (0.947, 0.992)	0.928	0.939	0.12
200	0.979 (0.963, 0.994)	0.954	0.932	0.982 (0.967-0.998)	0.968	0.941	0.74
300	0.984 (0.972-0.995)	0.892	0.989	0.969 (0.948-0.989)	0.926	0.944	0.22
400	0.986 (0.975-0.997)	0.926	0.981	0.976 (0.960-0.992)	0.928	0.948	0.32
500	0.983 (0.969-0.997)	0.930	0.964	0.968 (0.948-0.988)	0.956	0.88	0.23
750	0.986 (0.975-0.997)	0.962	0.942	0.978 (0.963-0.992)	0.954	0.949	0.37
1000	0.986 (0.974-0.999)	0.957	0.962	0.978 (0.963-0.993)	0.968	0.917	0.39

[1]Logistic generalised linear mixed models included 4 variables (electrical conductivity, lactate dehydrogenase activity, protein content, and fat content in milk), with the random effect estimates for each cow and cow-quarter. *n* = 512 quarters; Gram-positive mastitis = 232; Gram-negative mastitis = 6; mixed mastitis = 5; negative control = 269.

[2]Before ejection = comparison only before alveolar milk ejection; after ejection = comparison only after alveolar milk ejection; AUC = area under the curve; Se = sensitivity at Youden's index (cutoff point where the index is maximised); Sp = specificity at Youden's index.

0.1). At the optimum cutoff (maximum value of Youden's index) the differences between Se and Sp between before- versus after-ejection times were (-0.34% to 0.8%, -0.9% to 8.4%, respectively), with an average 3.6% higher Sp among before-ejection samples.

Effect of Sampling Times on EC Measurement

In assessment with backward elimination of the multivariable model including EC, LDH activity, milk protein and milk fat, only EC and LDH activity had significant interactions with sampling time. Hence the final model, including EC and LDH, shows that the EC in milk collected before milk ejection had greater power to predict mastitis than did milk collected after ejection. Among EC levels up to 5 mS, there was no substantial difference in the probability of mastitis between sampling times. However, above 5 mS, the difference in the probability of mastitis increased much more rapidly with increasing EC in the samples taken before milk ejection than it did in those obtained after milk ejection (Figure 4. 1).

Difference between Gram-positive and Gram-negative Mastitis

The differences between Gram-positive and Gram-negative mastitis for EC, SCC, LDH activity, milk protein, and milk fat, with or without separating the sampling times, are presented in Table 3. Irrespective of sampling time, and despite the relatively small sampling size for mastitis associated with Gram-negative pathogens, Gram-negative coliform mastitic milk had significantly greater LDH activity ($P < 0.01$) and higher protein levels ($P < 0.05$), and showed a trend ($P = 0.09$) for higher SCC, than did the Gram-positive mastitic milk. We discovered a significant effect of DIM on protein content ($P < 0.05$) as well as interaction of LDH activity with pathogen type.

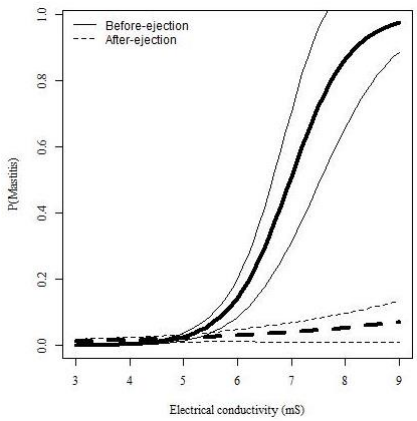


Figure 4. 1. Effect of sampling times before milk ejection (solid lines) and after milk ejection (dashed lines) on electrical conductivity (mS, mean ± SE) to predict mastitis; (*n* = 515 quarters). Quarters having > 530,000 cells/ml in the milk before ejection and > 440,000 cells/ml after ejection were considered as mastitic quarter (*n* = 104), and other quarters were negative control (*n* = 411). This logistic model included electrical conductivity, lactate dehydrogenase activity, and sampling times as fixed effects, and cow, or quarter nested within cow as random effects.

After separating the sampling times, Gram-negative mastitis showed significantly greater EC ($P < 0.01$) and LDH activity ($P < 0.05$) than did the Gram-positive mastitis in the samples taken before milk ejection. In the case of after-ejection sampling time, Gram-negative mastitic milk had significantly ($P < 0.01$) greater LDH activity than Gram-positive mastitic milk did. In addition, Gram-positive mastitic milk had a significantly lower protein content compared with Gram-negative ($P = 0.008$) mastic milk, although we found a significant interaction of DIM with pathogen type ($P < 0.001$).

Comparison of SCC and LDH Activity to Predict Mastitis

After re-scaling (standardising), both SCC and LDH activity showed positive associations with the probability of Gram-positive mastitis (Figure 4. 2) and Gram-negative mastitis (Figure 4. 3). In the case of Gram-positive mastitis, SCC ($P = 0.009$) and LDH activity ($P = 0.012$) had similar predicted probabilities of mastitis. However, LDH activity, expressed as standardised ln(LDH), showed a stronger positive association with Gram-negative coliform mastitis ($P = 0.027$) than did standardised ln(SCC) ($P = 0.044$), indicating better predictive ability of LDH.

Correlations

Overall, we observed a strong positive correlation between SCC and LDH activity (r_s = 0.89). The correlations of EC with LDH activity and SCC were similar (r_s for both = 0.61). All the r_s values were Spearman correlation coefficients, without accounting for quarter nested within cow, thus representing crude associations between pairs of parameters.

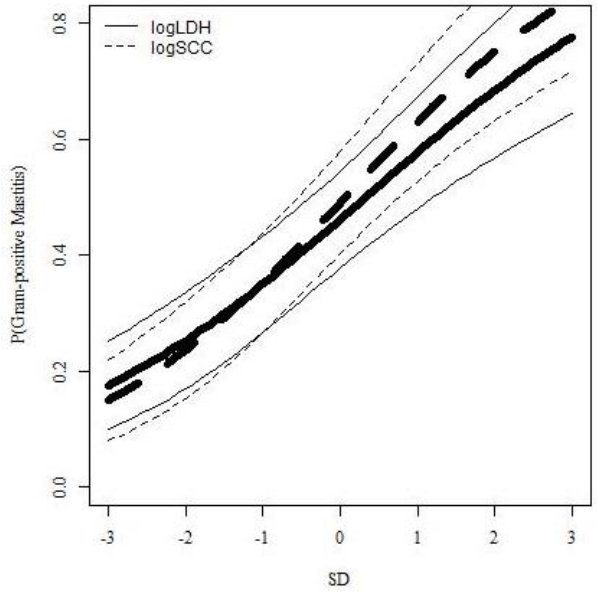
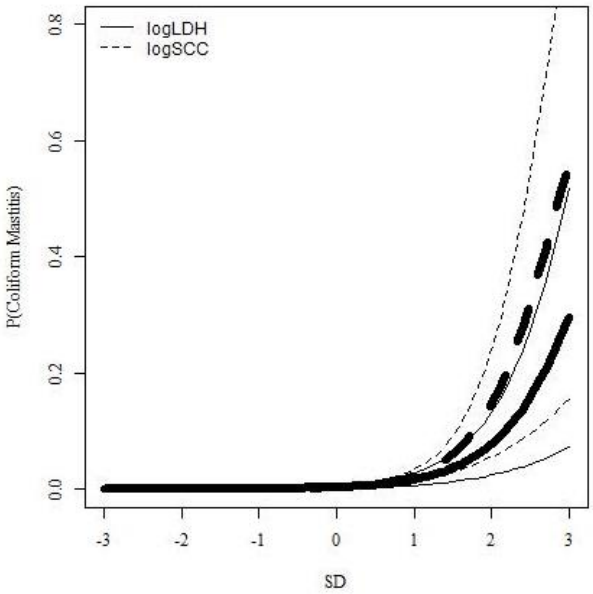


Figure 4. 2. Prediction of Gram-positive mastitis by SCC (dashed lines, estimated value ± SE) and by lactate dehydrogenase activity (solid lines, estimated value ± SE); n = 275 quarters. This logistic model inclu-ded rescaled SCC or LDH (express- ed as number of SD away from the mean) as fixed effects, with cow or quarter nested within cow as random effects. Because of positively ske-wed distribution, SCC and LDH data were log-transformed.

Figure 4. 3. Prediction of Gram-negative mastitis by SCC (dashed lines, estimated value ± SE) and lactate dehydrogenase activity (solid lines, estimated value ± SE); n = 506 quarters. This logistic model included rescaled SCC or LDH (ex-pressed as number of SD away from the mean) as fixed effect, with cow or quarter nested within cow as random effects. Because of positi- vely skewed distribution, SCC and LDH data were log-transformed.



Pathogen-specific Variation in SCC and LDH Responses

We found variations in average SCC (2,119, 5,388, 2,318, 1,435) × 1000 cells/ml and average LDH activity (215.42, 1619.87, 351.56, 140.13 U/L) responses between Gram-positive, Gram-negative, mixed mastitis, and control groups, respectively. Gram-positive *Aerococcus* spp., coagulase-positive *Staphylococcus*, *E. faecalis, Strep. dysgalactiae, Strep. uberis,* and *Trueperella pyogenes* had SCC responses and LDH activity similar to those Gram-negative coliforms (Figure 4. 4).

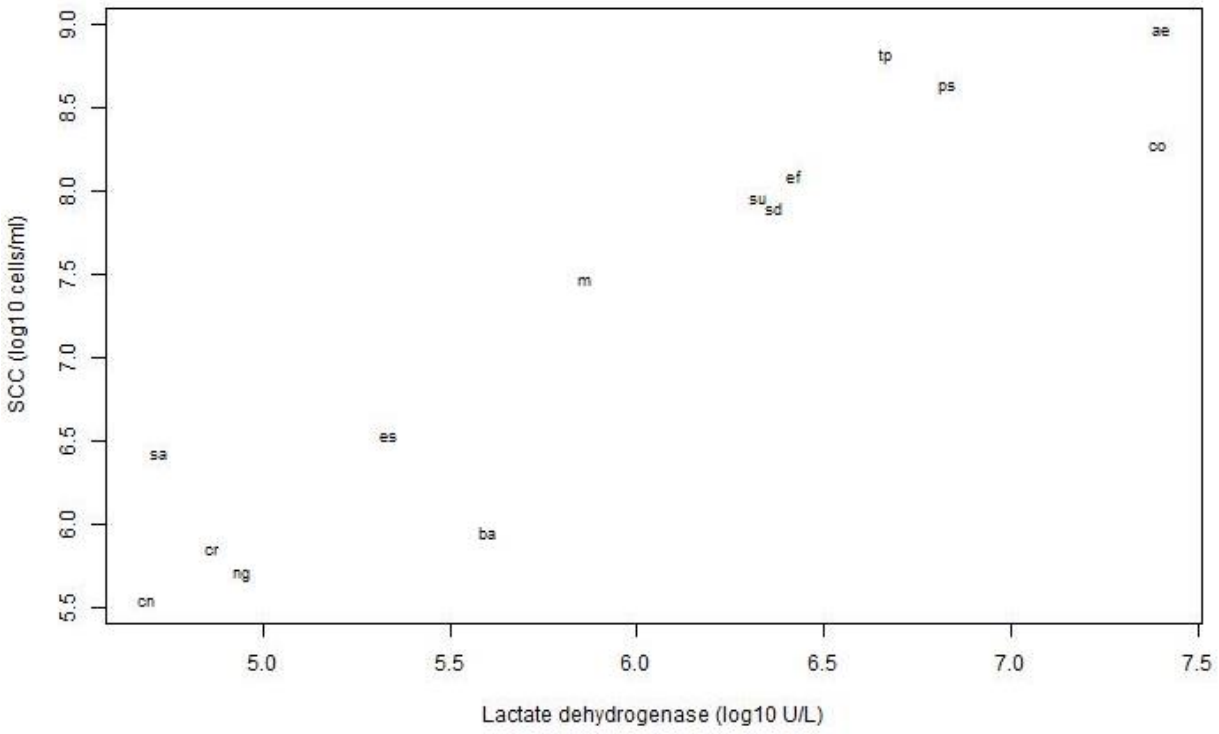


Figure 4. 4. Concentrations of SCC (log_{10}, cells/ml) and lactate dehydrogenase concentrations (LDH; log_{10}, U/L) in mastitis caused by different Gram-positive and Gram-negative bacteria (*n* = 512 quarters). Measured bacteria are as follows: ae = *Aerococcus spp.;* ba = *Bacillus spp.;* cn = Coagulase−*negative Staphylococcus*; co = Coliform bacteria; cr = *Corynebacterium spp.*; ef = *Enterococcus faecalis*; es = *Environmental Streptococcus*; m = mixed, ng = No growth; ps = Coagulase positive *Staphylococcus*; sa = *Streptococcus agalactiae*; sd = *Streptococcus dysgalactiae*; su = *Streptococcus uberis*; tp = *Trueperella pyogenes.*

Table 4. 3**.** Difference between Gram-positive and Gram-negative (coliform) mastitis for electrical conductivity, somatic cell count, lactate dehydrogenase activity, milk protein content, and milk fat content by linear mixed models[1]

Item[2]	Gram-positive[3]	Gram-negative[3]	*P*-value
	Electrical conductivity (mS)		
Both times	4.88 (4.71, 5.04)	5.18 (4.48, 5.88)	0.23
Before-ejection	5.24 (5.02, 5.46)	6.63 (5.70, 7.56)	0.003
After-ejection	4.64 (4.45, 4.84)	4.73 (3.97, 5.50)	0.66
	Somatic cell count (log_{10} cells/ml)		
Both times	6.44 (6.06, 6.83)	7.03 (6.13, 7.92)	0.09
Before-ejection	6.67 (6.23, 7.12)	7.55 (5.77, 9.33)	0.14
After-ejection	6.29 (5.90, 6.68)	7.01 (5.86, 8.16)	0.17
	Lactate dehydrogenase (log_{10} U/L)		
Both times	5.34 (5.09, 5.58)	6.19 (5.47, 6.91)	0.003
Before-ejection	5.52 (5.16, 5.88)	6.55 (4.95, 8.16)	0.04
After-ejection	5.23 (4.99, 5.47)	6.33 (5.46, 7.19)	0.008
	Protein %		
Both times	3.68 (3.45, 3.91)	3.93 (3.54, 4.32)	0.02
Before-ejection	3.64 (3.34, 3.94)	3.67 (3.08, 4.27)	0.12
After-ejection	3.75 (3.54, 3.95)	4.15 (3.75, 4.54)	0.008
	Fat %		
Both times	2.84 (2.42, 3.26)	2.55 (1.58, 3.53)	0.35
Before-ejection	2.70 (2.14, 3.25)	2.53 (0.63, 4.43)	0.71
After-ejection	2.85 (2.46, 3.24)	2.47 (1.52, 3.42)	0.30

[1]Linear mixed models with random effects for cow and cow-quarter. n = 238 quarters.
[2]Both times = comparison without separating sampling times; before ejection = comparison only before alveolar milk ejection; after-ejection = comparison only after alveolar milk ejection.
[3]Gram-positive *n* = 232; Gram-negative *n* = 6. Means show, with 95% CI in parentheses.
**P* < 0.05; ***P* < 0.01.

DISCUSSION

The main objective of this study was to compare milk sampled before and after ejection in their potential abilities as mastitis predictors. Our study has revealed that milk sampled before ejection is more informative for monitoring mastitis-related changes and therefore has higher mastitis prediction ability. Previous studies of healthy cows have reported that milk samples taken after ejection reduce the effectiveness of potential mastitis indicators such as SCC levels and LDH activity (Sarikaya and Bruckmaier, 2006; Lehmann et al., 2015). Such comparisons among cows with mastitis in this study support previous findings that sampled milk fractions influence potential mastitis indicators with significance (e.g., EC, LDH activity) or without significance (e.g., SCC) but also milk composition such as milk protein (Sarikaya and Bruckmaier, 2006).

Significantly higher EC in the before-ejection sample than after ejection might be associated with regulation of the milk osmotic pressure by higher ion concentrations (e.g., Na, Cl), which could be exacerbated by mastitis-related damage to the tight junctions (Nguyen and Neville, 1998; Ontsouka et al., 2003). Severe damage to tight junctions might lead to a higher EC, with passing of somatic cells at abnormal milk ranges, with greater prediction probability of before-ejection samples with > 530,000 cells/ml than after-ejection samples with > 440,000 cells/ml (Gordon et al., 1980). However, an SCC threshold much higher than 100,000 to 200,000 cells/ml would result in potential false-negative subclinical mastitis detection (dos Reis et al., 2011). It is worth noting that the predictors in our model (e.g., EC, LDH) might be affected by time elapsed from the start of infection and also by the degree of infection (Højsgaard and Friggens, 2010). Hence, an experimental longitudinal study, with in-depth observation of strict foremilk at different ranges of SCC, could assist in better prediction of subclinical mastitis. Overall, this is

important because, currently, higher EC, as an indicator for mastitis detection in AMS, is based on milk samples taken after ejection; milk ejection occurs during teat cleaning, meaning that the most informative data are lost (Kamphuis et al., 2008; Khatun et al., 2017). Thus the current system results in insufficient accuracy in the EC sensor to identify subclinical or clinical mastitis in cows (Hamann and Zecconi, 1998). The situation could potentially be improved by using milk taken before ejection (Bruckmaier et al., 2004b).

The nonsignificant differences in SCC ($P = 0.18$) in milk sampled before ejection compared with milk taken after ejection accords with the results reported in a previous study in healthy quarters (Ontsouka et al., 2003). In our study, the reason behind such nonsignificance might be related to our smaller sample size, with < 50% mastitic quarters, or to the delayed effect of mild ejection, as it took 60 s to collect before-ejection samples (within the recommended lag time of 50 to 100 s) instead of 40 s (recommended time required for strict foremilk before-ejection) due to different management situations (Bruckmaier and Hilger, 2001; Bruckmaier et al., 2004b). Additionally, 8 quarters were dipped before the sampling procedure, leading to missing data for before-ejection milk samples for the comparison.

The lower milk protein content in the milk of before-ejection samples might be due to mastitis-related damage to the tight junctions, with regulation of the milk osmotic pressure by higher levels of electrolytes than after-ejection samples (Nguyen and Neville, 1998; Ontsouka et al., 2003). Moreover, numerically lower ($P = 0.86$) fat content in milk before ejection might be due to lower specific gravity, as reported previously (Ontsouka et al., 2003).

We further evaluated our hypothesis using ROC assessment, which is a useful tool for assessing performance in predicting clinical mastitis (Khatun et al., 2018). In the ROC analysis, the mastitis diagnostic test in this study may be considered excellent (AUC > 0.9) compared with

those used in other studies (AUC ≤ 0.73; Norberg et al., 2004; Mollenhorst et al., 2010; Petzer et al., 2017). This might be due to our prediction model including LDH activity, milk protein, and milk fat in addition to EC, with the additional parameter increasing the AUC values. Moreover, the temperature differences in EC measurement in our study (e.g., room temperature) as opposed to those used in other studies (e.g., milking temperature, 38°C) might also responsible for such differences. However, the current approach of using multiple measurements produced results similar to those of previous study that used single measurements, comparing the benefits of before-ejection samples versus after-ejection samples (Bruckmaier et al., 2004b). Hence, despite statistical nonsignificance, the numerically higher AUC values in our study at different SCC thresholds, with, on average, 3.6% higher Sp of before-ejection samples, show that about 4 more mastitic cows (out of 100) will be correctly classified this way than by looking at the after-ejection samples.

Significantly higher EC only in before-ejection samples (not in after-ejection samples in the combined results of samples taken at both times) in cases of Gram-negative mastitis compared with Gram-positive mastitis further support the better efficiency of before-ejection samples for mastitis detection. As in previous studies, we found similar patterns of higher SCC response and LDH activity by Gram-negative coliform mastitis, likely associated with greater destruction of the tight junctions with cell disruption (Wellnitz et al., 2011, 2016; Hernández-Castellano et al., 2017). Differential LDH-based adaptive immune activation but similar SCC-based innate immune activation systems might explain the differences in the LDH activity (P = 0.003) and SCC (P = 0.09) responses between Gram-positive and Gram-negative mastitis, respectively (Hiss et al., 2007; Hernández-Castellano et al., 2017). Higher protein content in Gram-negative mastitis (3.93% vs. 3.68%, P = 0.02) might be due to effects of lower DIM (158 d vs. 186 d)

than among Gram-positive mastitis groups (Auldist et al., 2007). Overall, in this pathogen-specific analysis, the robustness of the outcomes warrants further investigation due to limited sample size (only 6), and should be investigated in larger number of samples from different parities with diversified pathogens.

Separate comparisons after re-scaling (because of different data scales) of SCC and LDH activity data were intended to better evaluate the relevance of types of mastitis (Gram-positive vs. Gram-negative) for rapid inline detection in AMS. Similar Gram-positive mastitis prediction probability of SCC and LDH activity but distinct Gram-negative mastitis prediction probability of LDH activity indicates that SCC is a valid marker to obtain alerts against Gram-positive mastitis, whereas LDH activity is particularly useful in detection of Gram-negative mastitis (Chagunda et al., 2006a; Sørensen et al., 2015; Hernández-Castellano et al., 2017). However, Gram-negative mastitis prediction probability needs to be further evaluated using a larger sample size.

We also noticed remarkable differences in SCC response and LDH activity by different Gram-positive bacteria, compared with those observed in Gram-negative coliforms. This is in agreement with using a combined SCC-LDH response to differentiate Gram-positive and Gram-negative mastitis (Hernández-Castellano et al., 2017). Another potential advantage of such an approach, using combined SCC and LDH, would be to detect chronic, latent mastitis status where there is scar tissue formation, with blockage of the blood-milk barrier that would prevent a massive SCC influx (Nickerson, 1993; Hébert et al., 2000). Further improvement of combined SCC-LDH analysis would be possible by incorporating other potential markers such as milk albumin or other protein to improve the mastitis pathogen differentiation capacity of the inline

sensors. This will be the focus for future studies with larger number of samples of different pathogens.

In this study, the predominant mastitis-causing pathogens (~44%) were *Corynebacterium* spp., and these might be associated with poor milking hygiene practice (lack of effective teat disinfection) in AMS before milking (Haltia et al., 2006). However *Corynebacterium* spp. are considered minor subclinical mastitis pathogen, representing contamination of milk with bacteria present in the teat canal, rather than real presence of an intramammary infection (Gonçalves et al., 2016).

CONCLUSIONS

We evaluated the sensitivity of strict foremilk (samples taken before milk ejection) and foremilk samples taken after milk ejection for quarter-level mastitis prediction. In summary, SCC, LDH activity, and milk protein levels were strongly associated with mastitis. Foremilk sampled before milk ejection was more sensitive for detection of mastitis than foremilk harvested after milk ejection, which is induced by udder preparation, including teat cleaning in AMS systems. Both LDH activity and milk protein contents were higher in quarters with Gram-negative coliform mastitis than in quarters with mastitis caused by Gram-positive bacteria. Overall, our results suggest that, in the future, sensors could be modified to monitor milk removed before teat cleaning, to improve the ability of the AMS to detect mastitis.

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Supplementary material

Milk from both sampling times was also assessed by producing a receiver operating characteristic (**ROC**) curve and calculating the area under the curve (**AUC**) to discriminate between disease-positive and disease-negative individuals.

Efficient mastitis detection provides opportunity to implement early and adequate treatment protocols and to avoid excessive use of antibiotics and thereby reduce the risk for antimicrobial resistance and associated human hazard (Hardefeldt et al., 2018), maintaining good animal health and welfare by reducing soreness, pain and discomfort; enhancing recovery rate; and improving economic return to the farmers (Milner et al., 1997; Lehmann et al., 2015).

Model-based means were calculated using ASReml-R using the method of Welham et al. (2004).

The generated area under the curve (**AUC**) value from the ROC curve is used to correctly discriminate between disease- positive and disease-negative individuals, classified as excellent (0.9–1), good (0.8–0.9), fair (0.7–0.8), poor (0.6–0.7), or fail (0.5–0.6; Swets, 1988).

The rescaled SCC or LDH activity data were assessed individually because of collinear association between them ($r_s \geq 0.8$), using the following logistic GLMM incorporating a spline function (Verbyla et al. 1999) to allow for a possible nonlinear response (on the log-odds scale) of each of these explanatory variables

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CHAPTER 5: Development of a new clinical mastitis detection method for automatic milking systems

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OVERVIEW OF CHAPTER 5

Although the sensitivity and specificity of single sensor such as electrical conductivity can be improved by measuring strict foremilk in AMS (Chapter 4), further improvement might be possible by accounting for multiple milking-related sensor data. Hence, the research reported in Chapter 5 was conducted to explore the mastitis detection ability of different milking-related inline sensor-derived information using retrospective data. Twelve different electronic measurements from AMS were analysed to develop a multivariable index based on best fitted model including measurements related to electrical conductivity, milk yield, milk flow rate and number of incomplete milkings. Three datasets from two pasture-based farms were used for the model development and assessments.

ABSTRACT

This study investigated the potential for accurate detection of clinical mastitis (**CM**) in an automatic milking systems (**AMS**) using electronic data from the support software. Data from cows were used to develop the model, which was then tested on two independent datasets, one with 311 cows (same farm but from a different year) and one with 568 cows (from a different farm). In addition, the model was used to test how well it could predict CM one to three days before actual clinical diagnosis. Logistic mixed models were used for the analysis. Twelve measurements were included in the initial model before a backwards elimination, which resulted in the following six measurements being included in the final model: quarter-level milk yield (MY, kg), electrical conductivity (EC, mS/cm), average milk flow rate (MF, kg/min), occurrence of incompletely-milked quarters in each milking session (IM, yes or no), MY per hour (MYH, kg/h), and EC per hour (ECH, mS/cm/h) between successive milking sessions. The other six measurements tested but not included in the final model were peak milk flow rate (kg/min), kick-offs (yes or no) in each milking session, lactation number, DIM (d), blood in milk (yes or no), and a calculated mastitis detection index used by DeLaval (DelPro software; DeLaval International AB, Tumba, Sweden). All measurements were assessed to determine their ability to detect CM, both as individual variables and combinations of the 12 above-mentioned variables. These were assessed by producing a receiver operating characteristic curve and calculating the area under the curve (**AUC**) for each model. Overall, nine measurements (e.g., EC, ECH, MY, MYH, MF, IM, peak flow rate, lactation number, and mastitis detection index) had significant mastitis detection ability as separate predictors. The best mastitis prediction was possible by incorporating six measurements (e.g., EC, ECH, MY, MYH, MF, IM) as well as the random cow

and quarter effects in the model, resulting in 90% sensitivity and 91% specificity with excellent AUC (0.96). Assessment of the model was found to produce robust results (AUC > 0.9) in different datasets and could detect CM with reductions in sensitivity and specificity with increasing days before actual diagnosis. This study demonstrated that improved mastitis status prediction can be achieved by using multiple measurements, and any new index based on that is expected to result in improved accuracy of mastitis alerts, thereby improving the detection ability and utility on farm.

Keywords: Dairy cow, clinical mastitis, automatic milking system, pasture-based.

INTRODUCTION

Bovine mastitis is an inflammation of the udder or mammary gland that is typically caused by invading bacteria belonging predominantly to *Enterobacteriaceae, Staphylococcaceae* or *Streptococcaceae* families (Bradley, 2002). Mastitis is commonly classified into subclinical, clinical or chronic forms, all of, which cause significant animal welfare concerns. The economic impact of clinical mastitis (**CM**) associated with production losses, treatment and culling rate ranged from $36 to $470/cow per year, with large differences between farms (Halasa et al., 2007; Huijps et al., 2008; Lam et al., 2013). Interest in and adoption of automatic (robotic) milking systems (**AMS**) have created the demand for reliable automatic detection of mastitis due to the reduction in inspection time required to identify mastitic cows that need veterinary intervention (Mollenhorst et al., 2012). Many commercial brands supplying AMS already incorporate a variety of milk monitoring or sensing equipment (e.g., electrical conductivity, milk yield, milk flow rate, incomplete milking, kick-off), and some researchers have been working to develop

algorithms that use and integrate data captured during the milking process to find the most accurate mastitis alert guideline (Hogeveen et al., 2010; Hovinen and Pyörälä, 2011; Rutten et al., 2013). Accuracy is determined by a high incidence of true-positive cases (high sensitivity, **Se)** and low incidence of false alerts (high specificity, **Sp**). Previous studies have shown that the use of only EC in different detection algorithms was unable to achieve the International Organization for Standardization (**ISO**, 2007) standard Se (> 70%) and Sp (> 99%) for CM detection (Khatun et al., 2017). In the past decade, many attempts have been made to improve the Se and Sp of CM detection using AMS data; however, they were not successful enough to detect at quarter level, and the search for an improved automated mastitis detection system still continues (Claycomb et al., 2009; Hogeveen et al., 2010; Penry et al., 2017). Moreover, in a pasture-based AMS, where cows are less visible to the farmers compared to indoor farming system, checking multiple alerts (either automatic or non-automatic) to improve Se and Sp for detection of mastitis requires an increase in workload (Steeneveld et al., 2010). Given that mastitis can be associated with multiple changes (Sordillo, 2005) in the cow's body and milk, it is possible that if we could achieve higher Se and Sp if we integrate additional measurements captured during milking (e.g., milk yield, milking frequency, milk flow rate, milking pattern). Exploiting multi-sensor data could lead to sustainable improvements in detection of mastitis (Brandt et al., 2010; Hogeveen et al., 2010; Steeneveld et al., 2010). Thus, the objective of this study was to develop a multiple measurement approach or index for inline AMS sensors to detect CM targeting > 80% Se and ≥ 99% Sp.

MATERIALS AND METHODS

Data Source

A retrospective longitudinal cohort study was conducted with data collected from two pasture-based robotic dairy farms. Farm 1 was located near Camden, New South Wales, Australia (34.0544°S, 150.6958°E, rainfall = 764 mm/yr) and belonged to the University of Sydney, and farm 2 was a commercial dairy farm located near Deloraine, Tasmania, Australia (41.5349°S, 146.6616°E, rainfall l= 1,016 mm/yr). Farm 1 had 85 ha of effective grazing land for about 350 Holstein-Friesian lactating cows with daily access to annual ryegrass (*Lolium multiflorum*) oversown on kikuyu (*Pennisetum clandestinum*) and oats (*Secale cereale*) in autumn, winter, and spring. Animals were supplemented with approximately 7 kg dry matter (**DM**) of grain-based commercial pelleted concentrate (18% protein) per cow in the post-milking area (in automated out-of-parlor feeders) after each milking session and with a partial mixed ration containing primarily brewer's grain, orange pulp and pasture silage to cover true pasture deficits. Cows were fitted with a neck-mounted electronic rumination and activity monitoring tag (SCR HR-LDn; SCR Engineers Ltd, Netanya, Israel). On Farm 2, cows were offered a combination of grazable pasture (*Lolium perenne*), partial mixed ration and grain-based commercial pelleted concentrate targeting daily DM intake 22.5 kg of DM/cow. The percentage of each feed in the daily allocation varied depending on the availability of pasture. Cows had access to grain-based commental pelleted concentrate (based on DIM) after milking in 20 automated out-of-parlor feeders (FSC400, DeLaval International AB, Tumba, Sweden) located in an area immediately postmilking. Both farms operated with voluntary cow traffic and 3-way grazing system (Lyons et

al., 2013b). The herds of both farms were predominately Holstein-Friesian with a year-round calving system in farm 1 and a split (2 batches) calving system in farm 2. In both farms, cows were milked through a robotic rotary system (DeLaval Automatic Milking Rotary, Tumba, Sweden; 24-unit platform, 5 robotic arms). All data were recorded and stored in the herd management software (DeLaval DelPro Software 5.1, DeLaval International AB).

Nine measurements (variables) relating to the individual milking event for each cow (out of 81 different measurements available in the software) were selected to identify the best CM predictors. These included milk yield (**MY**; kg/cow per milking), electrical conductivity (EC; mS/cm), incomplete milking (**IM**; yes or no), average milk flow rate (**MF;** kg/min), peak milk flow rate (**PF**; kg/min), kick-offs (yes or no), blood in milk (yes or no), lactation number and days in milk (**DIM**, d). In addition, the mastitis detection index (**MDi,** unitless) was also included within the variables to be tested. This is an index generated within DelPro software that incorporates EC, blood in milk, and milking interval per quarter to give an indication of likelihood of mastitis (unpublished metric). As MY (Ouweltjes, 1998) and EC (Fernando et al., 1981) are both affected by the milking interval, these two variables were divided by milking interval to estimate the MY per hour (**MYH**; kg/h) and EC per hour (**ECH;** mS/cm per hour). This resulted in a total of 12 variables to be included in the analysis.

Gold Standard for CM and Control

The quarters included in this study included both clinically infected and healthy quarters. In both farms the protocol used for definition of CM was a record of veterinary treatment and the day of treatment was considered as d 0. Normal farm practice was to monitor DelPro EC records at least

once daily to flag the suspected mastitis cases. Cows that were deemed by farm staff to have elevated EC in 1 or more quarters (> 7.5 mS/cm, without a strict threshold) were drafted before the next milking to allow visual inspection of the suspect quarter (for redness, heat and swelling) and its milk (for the presence of flakes, clots or lumps). The CM-positive cases were determined by trained farm staff's assessment of clinical cases after potentially affected animals had been identified by changes in EC. Thus, although unlikely, it is possible that some true cases of CM could have gone unnoticed (false negatives). In addition, the cows recorded as incompletely milked or with abnormal rumination or activity (e. g., SCR HR-LDn sensor) were separated by the farm staff and also checked for CM. The CM-positive cases were treated with antibiotics [3 doses of Special Formula 17900-Forte Suspension (Zoetis, Kirkland, QC, Canada) every 12 h], and milk was discarded during this period. On farm 2, in addition to looking at EC, quarters with poor milk letdown, 2 consecutive IM or kick-offs and MDi > 9 were also checked for signs of CM. The non-treated quarters of the CM-positive cows and 1,176 quarters of 294 milking cows without any record of CM during a 120-wk time window (chosen arbitrarily) were considered as the negative control.

Data Distribution

Three datasets were identified (2 datasets from farm 1 and one dataset from farm 2), with cows having CM in a single quarter or multiple quarters or no CM (negative controls) in any quarter. An initial dataset from farm 1 was used to develop the model. The model was then tested using a second dataset (collected in a different time period) from farm 1 (assessment 1). The dataset from farm 2 was used for further assessment of the model (assessment 2).

Data were selected from around 4 wk before CM treatment of the cow by extraction from DelPro. Due to the large dataset of healthy cows, 2,000 data records were selected randomly using the 'sample' function in R version 3.2.5 (http://www.r-project.org) to balance the data size of single-quarter and multiple-quarters CM datasets before analysis. In model selection, the proportion of single-quarter CM versus negative control versus multiple-quarter CM mastitis cow data was 1: 2: 3 (1,078: 2,000: 3,116). The number of single-quarter CM and multiple-quarter CM cases and other selection criteria of the 3 datasets is presented in Table 5. 1. Missing values ranged from 3-6% and any missing value or any single observation (predictor variable, 1.6-2.3%) more than 4 SD (based on expected extreme deviation of the normally distributed sample size used in the analysis) from its mean were not included in the analysis.

Statistical Analysis

Model Selection. The 12 different AMS variables extracted from the AMS were considered as predictor variables to test their association with CM. The data were analysed using ASReml-R (Butler et al., 2009) built under R version 3.2.5 (http://www.r-project.org). Three sets of logistic generalised linear mixed models (GLMMs) were constructed (univariable, multivariable, and multivariable interaction) to find the best predictors of CM, as a binary outcome. Initially, univariable logistic GLMM were fitted to each of the 12 variables as a fixed effect using the

Table 5. 1. Selection criteria of data sets

Item	Model selection and implementation Farm 1	Assessment 1 Farm 1	Assessment2 Farm 2
Cows without mastitis			
Records collected during study[1]	323	278	512
Records used in analysis[2]	294	268	504
Cows with mastitis in multi-quarters	40[3]	20[3]	10[4]
Quarters with mastitis in multi-quarters	210[5]	281[5]	64[5]
Cows with mastitis in single quarter[6]	24	23	54
Total cows	358	311	568
Total quarter milkings	24,776	24,464	25,008
Lactation number	1 to 9	1 to 9	1 to 7
DIM	191	157	94
Data extraction period	October 2014 to March 2016	April 2016 to September 2016	July 2016 to December 2016
Missing value (%)	3.4	6.0	3.01

[1]Total number of records from healthy cows during the study period.
[2] Total number of cow records used in the analysis selected by the 'sample' function in R (http://www.r-project.org/).
[3]Multiple mastitis records at the same quarter or different quarter (s) of the same cow at a certain interval in the same lactation.
[4] Single mastitis record in different quarters of the same cow at a certain interval in the same lactation.
[5] Total number of multiple mastitis records at the same quarter or different quarter (s) of the same cow at a certain interval in the same lactation.
[6]Single mastitis record in a single quarter of a cow in the same lactation.

following model:

$$\ln[\pi/(1-\pi)] = \text{Constant} + \text{Predictor} + \text{Cow} + \text{Cow.Quarter},$$

where $\pi = P(Y = 1)$ is the probability that the cow has CM in a particular quarter on a particular test session, predictor was one of the 12 variables listed above as a fixed effect, and cow and cow-quarter (quarter nested within a Cow) were random effects. As the distribution of MYH, ECH, MDi, MF, and PF were positively skewed, they were log (base *e*)-transformed before fitting the logistic regression model to reduce leverage of very large values.

Following this, variables identified in the univariable analyses as having indicative associations ($P < 0.2$) were included in an initial multivariable model along with the same random effects as in the univariable models, and a manual backwards elimination procedure was used to drop non-significant variables. Wald F and Wald chi-square tests were used for significance testing, and the final model included any variables with $P < 0.05$. The variable MDi was excluded from the multivariable model because it is a composite index including several variables (e.g., EC, blood in milk, milking interval) that are already in the model. The third stage of modeling included the evaluation of interactions between fixed effects. All possible 2-way interactions were evaluated between pairs of variables (categorical × categorical and categorical × quantitative). A backward elimination procedure was again used to eliminate non-significant interactions, and the cow- and quarter-specific information was used as random effects. The final multivariable model may be written as

$$\ln[\pi/(1-\pi)] = \beta_0 + \boldsymbol{\beta}'\mathbf{x} + u_C + u_{CQ},$$

where $\mathbf{x} = (x_1, x_2, \ldots, x_p)'$ is the set of fixed effect predictor values (including interaction terms) with associated regression coefficients $\boldsymbol{\beta}$, and u_C and u_{CQ} are the random cow and cow-quarter effects.

Model Implementation on Farm 1. After selecting and fitting the final model, the estimates of the fixed effects $\hat{\boldsymbol{\beta}}$ (excluding the estimated constant or intercept) and the random effect estimates for each cow ($\hat{u}_C$) and cow-quarter ($\hat{u}_{CQ}$) were obtained, and an index (1 index value including each observation used in model selection in farm 1) was created on the linear predictor **(LP;** unitless) scale as below:

$$\text{LP} = \hat{\boldsymbol{\beta}}'\mathbf{x} + \hat{u}_C + \hat{u}_{CQ} = \hat{\beta}_1 x_1 + \hat{\beta}_2 x_2 + \ldots + \hat{\beta}_p x_p + \hat{u}_C + \hat{u}_{CQ},$$

Increasing values of LP indicate greater probability of being CM, and different thresholds of LP can be applied. When compared against the known case-control status, Se and Sp values can be determined at each threshold LP value. Note that the estimated intercept $\hat{\beta}_0$ was not included in the LP value as its value would change according to the sampling fraction of control cow-quarters and its value does not influence Se and Sp calculations. Receiver operating characteristic curves **(ROC)** were used to visually present the Se and Sp using different threshold values.

ROC Curve. The ROC curve is a plot of Se (true positive rate; y-axis) versus 1 – Sp (false positive rate; x-axis) and is a graphical illustration of the diagnostic value of the test (Hanley and McNeil, 1982). The curve is traced out by applying varying thresholds to an index (LP, the linear predictor), and the area under the curve (**AUC**) was used as an overall measure of diagnostic test performance, classified as excellent (0.9-1), good (0.8-0.9), fair (0.7-0.8), poor (0.6-0.7) and fail (0.5-0.6) (http://gim.unmc.edu/dxtests/roc3.htm) with values under 0.5 worse than random classification. The construction of the ROC curves was performed using the AUC package (version 0.3.0) in R (version 3.2.4, https://www.r-project.org/). Among the ranges of LP values in the constructed ROC curve, the optimum cut-off value for the final multivariable model was

defined as a threshold point where the sum of Se and Sp was maximum; a value exceeding the cut-off is a CM indicator. Each predictor variable that was significant as a univariable model was also assessed independently by AUC values based on observed records of AMS values and LP values obtained from the univariable GLMM including the cow ($\hat{u}_C$) and quarter-specific ($\hat{u}_{CQ}$) information as random effects at the optimum cut-off value. A similar evaluation was performed for the final multivariable model.

Model Assessment 1 and 2. In this analysis, the fitted model from farm 1 was applied to a separate set of data recorded in different time periods on the same farm (assessment 1) as well as to a new dataset belonging to farm 2 (assessment 2). This was undertaken in 2 ways. First, an LP value was calculated for each observation in each data set using the fixed effect estimates from the model selection stage without any random effects included-for example, $\text{LP} = \hat{\boldsymbol{\beta}}'\mathbf{x} = \hat{\beta}_1 x_1 + \hat{\beta}_2 x_2 + \ldots + \hat{\beta}_p x_p$. Following that, the AUC value of the LP value and Se and Sp at the optimum cut-off were evaluated as described previously. Such a model without inclusion of random effects is appropriate in situations where no prior information is available for a cow or its four quarters. Second, when previous mastitis history (previous mastitis records within the same or previous 2 lactations) is available for the cow and quarter, this information in the form of estimates $\hat{u}_C$ and $\hat{u}_{CQ}$ can still be included in the LP calculation by initially fitting a model as follows:

$$\ln[\pi/(1-\pi)] = \hat{\boldsymbol{\beta}}'\mathbf{x} + \beta_0 + u_C + u_{CQ},$$

where $\hat{\boldsymbol{\beta}}'\mathbf{x}$ is specified as an offset using the estimates $\hat{\boldsymbol{\beta}}$ from model selection in farm 1 as fixed constants; $\mathbf{x} = (x_1, x_2, \ldots, x_p)'$ is the set of fixed effect predictor values in model assessment data sets (assessment 1 and 2). The estimates of the random effects $\hat{u}_C$ and $\hat{u}_{CQ}$ were

calculated for the model assessment datasets (second dataset of farm 1 and dataset of farm 2). Next, the LP was calculated for each observation, including the previous mastitis history of cow ($\hat{u}_C$) and quarter ($\hat{u}_{CQ}$) information, as in model implementation in farm 1 (e.g., $\text{LP} = \hat{\boldsymbol{\beta}}'\mathbf{x} + \hat{u}_C + \hat{u}_{CQ}$).

Model Assessment at Earlier Days Prior to CM Diagnosis. In this step, the final model was assessed using the same dataset as the development model selection in the first dataset of farm 1 and the dataset of farm 2 to determine the utility of the model predicting CM before actual diagnosis. Using the fitted model from farm 1, the test was evaluated assuming that CM was present only on the day of diagnosis (d 0), up to 1 d (prior d 1), up to 2 d (prior d 2) and up to 3 d (prior d 3) before diagnosis. Evaluations were also made to assess test Se on each specific prior day. During assessment at prior days, we used the same estimates $\hat{\boldsymbol{\beta}}$ from model selection in farm 1 following the same procedure as mentioned above in the assessment steps. R code was written to prepare the data set to set the CM status as positive for the specified prior day. The LP calculated for the models with and without the random effect were compared for AUC as well as Se at the optimum cut-off of d 0.

RESULTS

Model Selection and Implementation (Univariable Models)

The significance level of the 12 predictor variables to predict CM with the cow ($\hat{\sigma}^2_C$) and quarter ($\hat{\sigma}^2_{CQ}$) variance estimates is presented in Table 5. 2. In general out of the 12 predictor variables, 9

of these (EC, ECH, MY, MYH, MDi, IM, MF, PF, and lactation number) had associations ($P < 0.05$) with CM status in the univariable GLMM analyses, and 1 additional variable (DIM) had a weak ($P = 0.15$) association worth considering in the initial multivariable model. The other 2 (kick-offs and blood in milk) were nonsignificant ($P > 0.2$) in the univariable GLMM and therefore not included in the initial multivariable model. The CM prediction ability of each of the 9 univariable models ($P < 0.05$) based on visual measurement from AMS (observed record) were poor compared with GLMM with random effect at LP scale based on the AUC values (Table 5. 3). Among the univariable models, the best-performing univariable model was MYH based on observed record (AUC = 0.859) as well as at LP scale (AUC = 0.940), which takes into account prior history of CM in each cow and cow-quarter by inclusion of random effects.

Table 5. 2. Significance of the 12 predictor variables to predict clinical mastitis with the cow and quarter variance by univariable GLMM[1]

Variables[2]	*P*-value	Cow (σ^2)[3]	Quarter (σ^2)[3]
EC	< 0.001	0.27 ± 0.13	0.24 ± 0.14
[4]ECH	< 0.001	0.21 ± 0.14	0.68 ± 0.18
MY	< 0.001	0.34 ± 0.16	0.67 ± 0.19
[4]MYH	< 0.001	0.21 ± 0.15	0.71 ± 0.19
[4]MF	< 0.001	0.31 ± 0.16	0.71 ± 0.20
IM	< 0.001	0.23 ± 0.15	0.77 ± 0.20
[4]PF	< 0.001	0.38 ± 0.17	0.69 ± 0.19
[4]MDi	< 0.001	0.14 ± 0.13	0.61 ± 0.18
Lactation number	0.002	0.22 ± 0.15	0.67 ± 0.19
DIM	0.15	0.36 ± 0.17	0.64 ± 0.18
Blood in milk	0.43	0.32 ± 0.16	0.63 ± 0.18
Kick offs	0.39	0.32 ± 0.16	0.64 ± 0.18

[1]Logistic generalised linear mixed models included variables with estimated fixed effects.
[2]EC = electrical conductivity (mS/cm); ECH = electrical conductivity per hour (mS/cm per hour); MY = milk yield (kg); MYH = milk yield per hour (kg/h); MF = average milk flow rate (kg/min); IM = incomplete milking in each milking session (yes/no); PF = peak milk flow rate (kg/min); MDi = mastitis detection index.
[3]σ^2 = variance estimate ± SE.
[4]Because of positively skewed distribution data were log-transformed.
*$P < 0.05$; *** < 0.001.

Table 5. 3. Analysis of receiver operating characteristic curves, sensitivity and specificity at optimum cut-off value for prediction of clinical mastitis based on observed automatic milking systems records and by univariable GLMM[1] at linear predictor scale (n = 358 cows)

Variable[2]	Linear predictor scale[3]					Observed record[4]			
	Item[5]								
	AUC	Se	Sp	Cut-off	RC	AUC	Se	Sp	Cut-off
EC	0.879	0.841	0.778	> 7.95	1.08 ± 0.070	0.790	0.716	0.711	> 7.06
[6]ECH	0.900	0.915	0.798	> 1.26	-0.920± 0.143	0.725	0.871	0.551	< 0.429
MY	0.914	0.861	0.863	> -1.16	-0.928± 0.070	0.797	0.821	0.667	< 2.37
[6]MYH	0.940	0.915	0.880	> 2.59	-1.26 ± 0.070	0.859	0.776	0.865	< 0.097
[6]MF	0.935	0.900	0.861	> 1.78	-1.76 ± 0.104	0.829	0.746	0.824	< 0.755
IM	0.886	0.900	0.713	> 0.208	2.66 ± 0.151	0.708	0.478	0.938	Yes
[6]PF	0.907	0.866	0.824	> 0.453	-1.27±0.131	0.680	0.532	0.744	< 0.105
[6]MDi	0.850	0.696	0.904	> 2.82	2.29 ± 0.195	0.743	0.634	0.784	> 2.5
Lactation number	0.861	0.786	0.770	> 1.60		0.633	0.751	0.454	> 3
Lactation number-2					0.892 ± 0.380				
Lactation number-3					1.208 ± 0.405				
Lactation number-4					1.317 ± 0.377				
Lactation number-5					1.671 ± 0.392				
Lactation number-6					1.929 ± 0.452				
Lactation number-7					-4.894 ± 18.905				
Lactation number-8					0.781 ± 1.178				
Lactation number-9					1.709 ± 0.828				

[1]Logistic generalised linear mixed models included variables with estimated fixed effects.
[2]EC = electrical conductivity (mS/cm); ECH = Electrical conductivity per hour (mS/cm per hour); MY = milk yield (kg); MYH = milk yield per hour (kg/h); MF = average milk flow rate (kg/min); IM = incomplete milking in each milking session (yes/no); MDi = mastitis detection index.
[3]An index value including estimated fixed effects (excludes the estimated intercept) and the random effect estimates for each cow and cow-quarter.
[4]Receiver operating characteristic curve generated from visual record from automatic milking systems.
[5]AUC = area under the curve; Se = sensitivity; Sp = specificity; Cut-off = threshold point where sum of sensitivity and specificity is maximum, a value exceeding the cut-off is a mastitis indicator; RC = regression coefficient ± SE.
[6]Because of positively skewed distribution data were log-transformed before calculating linear predictor.

Model Selection and Implementation (Multivariable Models)

After backwards elimination, the final multivariable model included 6 variables (all $P < 0.001$). The 6 variables were EC, ECH, MY, MYH, MF, and IM. The nonsignificant variables were PF, lactation number, and DIM. Pairwise interactions were considered between variables (all $P > 0.05$). The estimated (regression coefficients, $\hat{\boldsymbol{\beta}} \pm \mathrm{SE}(\hat{\boldsymbol{\beta}})$, of the 6 predictor variables of the final model were EC (1.30 ± 0.095), ECH (-2.11 ± 0.341), MY (-0.875 ± 0.179), MYH (1.34 ± 0.333), MF (-1.75 ± 0.264), and IM (1.30 ± 0.187). These regression coefficients were used as fixed values in the model implementation (farm 1) and assessments (assessment 1 and assessment 2) to calculate the LP value, only cow and quarter random effects were estimated to reflect the history (previous mastitis information within the same or previous two lactations) of cow and quarter records. The estimated optimum LP cut-off of the final model based on the maximum sum of Se and Sp was > 8.24 for the model with random effects and > 7.84 for the model without random effects. These 2 cut-off values were used as reference values for all other assessments and compared with optimum cut-offs for other assessments (e.g., cutoffs to maximise Se + Sp for each specific model assessment). The CM prediction ability of the final model on the LP scale was excellent (AUC = 0.96 vs. 0.92) for the model with and without random effects, respectively. At the optimum cut-off, the calculated Se (90% vs. 84%) and Sp (91% vs. 88%) were better for the model with random effects than without random effects, respectively (Table 5. 4). Overall, the performances of the final multivariable model containing 6 predictors were better (in terms of both AUC and the maximum sum of Se and Sp) than any of the 9 univariable predictors. This was true regardless of whether random effects were included in the model.

Table 5. 4. Analysis of receiver operating characteristic curves, sensitivity and specificity at different cut-off values for prediction of clinical mastitis by multivariable GLMM[1] at linear predictor scale[2]

	With random effects[4]					Without random effects[4]					
Item[3]	MI[5]	Assessment 1[6]		Assessment 2[7]		MI[5]	Assessment 1[6]		Assessment 2[7]		
AUC	0.958	0.942		0.978		0.921	0.910		0.912		
Cut-off	> 8.24[8]	> 8.24[8]	> 7.80[9]	> 8.24[8]	> 6.52[9]	> 7. 84[10]	> 7. 84[10]	> 7.25[11]	> 8.24[8]	> 7. 84[10]	> 5.80[11]	> 8.24[8]
Se	0.900	0.850	0.897	0.667	0.933	0.836	0.762	0.864	0.650	0.533	0.8	0.433
Sp	0.906	0.900	0.872	0.996	0.972	0.884	0.899	0.846	0.928	0.994	0.927	0.996

[1]Logistic generalised linear mixed models included variables with estimated fixed effects (regression coefficient ± SE): electrical conductivity, 1.30 ± 0.095; log-transformed electrical conductivity per hour, -2.11 ± 0.341; milk yield, -0.875 ± 0.179; log-transformed milk yield per hour, 1.34 ± 0.333; log-transformed mean milk flow rate, -1.75 ± 0.264; and incomplete milking, 1.30 ± 0.187.

[2]An index value including estimated fixed effects (excludes the estimated constant or intercept) and the random effect estimates for each cow and cow-quarter.

[3]AUC = area under the curve; Se = sensitivity; Sp = specificity; Cut-off = threshold point where sum of sensitivity and specificity is maximum; a value exceeding the cut-off is a mastitis indicator.

[4]Random effect = cow- and quarter-specific information, including previous mastitis history.

[5]Model implementation using the first dataset of farm 1 (n = 358 cows).

[6]Assessment 1 using the second dataset of farm 1 (n = 311 cows).

[7]Assessment 2 using the dataset of farm 2 (n = 568 cows).

[8]Optimum cut-off as evaluated in farm 1 with random effect (model implementation).

[9]Farm- and data set-specific optimum cut-off with random effect.

[10]Optimum cut-off as evaluated in farm 1 without random effect (model implementation).

[11]Farm- and data set-specific optimum cut-off without random effect.

Model Assessment 1 and Model Assessment 2 (Multivariable Models)

Assessment 1 **(**using the second dataset of farm 1) and assessment 2 (using the dataset of farm 2) values of the final model were robust and had excellent AUC (> 0.9) value for the models with and without random effects. The variation in ROC curves for the model with and without random effects at the model implementation, assessment 1 and assessment 2 steps is presented in Figure 5. 1 and 5. 2, respectively. The optimum cut-off in assessment 1 (> 7.80 vs. > 7.25) and assessment 2 (> 6.52 vs. > 5.80) for the models with and without random effects differ from the optimum cut-offs (e. g. reference values) of model implementation (> 8.24 vs. > 7.84). Comparing the Se between model implementation versus assessment 1 and model implementation versus assessment 2 for the model with random effects only at the corresponding optimum cut-off, there was 5% higher Se in assessment 1 (cut-off: > 8.24 vs. > 7.80) and 27% higher Se in assessment 2 (cut-off: > 8.24 vs. > 6.52). Similarly, such Se comparison between model implementation versus assessment 1 and model implementation versus assessment 2 for the model without random effects only at the corresponding optimum cut-off, the Se was always higher (10% to 27%) in assessments (cut-off: > 7.25 in assessment 1, > 5.80 in assessment 2) compared with implementation (cut-off: > 7.84). In such comparisons, the Sp were 3 to 5% lower in assessment data sets compared with that obtained in the model implementation. When comparing the Se and Sp at the corresponding optimum cut-off of assessment 1 and 2, the values were higher for the model with random effects than the model without random effects as presented in Table 5.4. In addition, comparing the Se at the specific cut-off of > 8.24 for the model with and without random effects, we obtained 20% (assessment 1: 85% vs. 65%) to 23% (assessment 2: 66.7% vs. 43.3%) higher Se due to inclusion of random effects. The equivalent

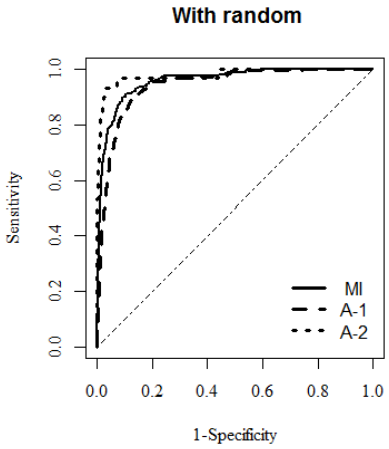

Figure 5. 1. Receiver operating characteristic curves of multivariable logistic generalised linear mixed models at implementation (MI) and assessments steps in Farm 1 (A-1: assessment-1) and Farm 2 (A-2: assessment-2). Random effects included cow, and quarter nested within cow. Models included variables with estimated fixed effects (regression coefficient ± SE) were: Electrical conductivity (1.30 ± 0.095), log-transformed electrical conductivity/h (-2.11 ± 0.341), milk yield (-0.875 ± 0.179), log-transformed milk yield /h (1.34 ± 0.333), log-transformed mean milk flow rate (-1.75 ± 0.264), and incomplete milking (1.30 ± 0.187).

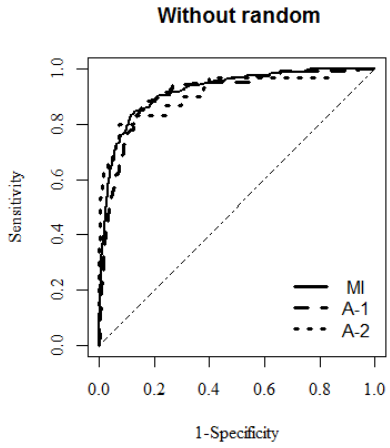

Figure 5. 2. Receiver operating characteristic curves of multivariable logistic generalised linear mixed models at implementation (MI) and assessments steps in Farm 1 (A-1: assessment-1) and Farm 2 (A-2: assessment-2). Models included variables with estimated fixed effects (regression coefficient ± SE) were: Electrical conductivity (1.30 ± 0.095), log-transformed electrical cond- uctivity /h (-2.11 ± 0.341), milk yield (-0.875 ± 0.179), log-transformed milk yield /h (1.34 ± 0.333), log-transformed mean milk flow rate (-1.75 ± 0.264), and incomplete milking (1.30 ± 0.187). These models do not include any cow, and quarter nested within cow as random effect.

comparison for Sp showed 2 to 6% higher Sp (in the models with or without random effects) at the corresponding optimum cut-off than the cut-off of > 8.24.

Model Assessments at Earlier Days Prior to CM Diagnosis (Multivariable Model)

Model assessment values for multiple consecutive prior days CM at the farm-specific optimum cut-off (farm 1: > 8.24 vs. > 7.84; farm 2: > 6.52 vs. > 5.80) for the model with and without random effects respectively are presented in Table 5. 5. In general, better AUC values with higher Se and Sp were found for the model with random effects than the model without random effects in both farms.

Table 5. 5. Comparison of the area under the curve, sensitivity, and specificity at optimum cut-off assuming clinical mastitis for different prior days by multivariable GLMM[1] at linear predictor scale[2]

	With random effects[4]				Without random effects[4]			
Time[3]	AUC	Se	Sp	Cut-off	AUC	Se	Sp	Cut-off
Farm 1[5]								
D 0	0.958	0.900	0.906	> 8.24[6]	0.921	0.836	0.884	> 7.84[7]
D 0 to prior d 1	0.947	0.858	0.890		0.886	0.708	0.887	
D 0 to prior d 2	0.936	0.834	0.870		0.852	0.613	0.890	
D0 to prior d 3	0.927	0.850	0.839		0.813	0.543	0.892	
Farm 2[8]								
D 0	0.978	0.933	0.972	> 6.52[9]	0.912	0.800	0.927	> 5.80[10]
D 0 to prior d 1	0.982	0.956	0.934		0.761	0.556	0.924	
D 0 to prior d 2	0.736	0.305	0.979		0.704	0.481	0.924	
D0 to prior d 3	0.711	0.249	0.978		0.669	0.424	0.925	

[1]Logistic generalised linear mixed models included variables with estimated fixed effects (regression coefficient ± SE): electrical conductivity, 1.30 ± 0.095; log-transformed electrical conductivity per hour, -2.11 ± 0.341; milk yield, -0.875 ± 0.179; log-transformed milk yield per hour, 1.34 ± 0.333; log-transformed mean milk flow rate, -1.75 ± 0.264; and incomplete milking, 1.30 ± 0.187.

[2]An index value including estimated fixed effects (excludes the estimated constant or intercept) and the random effect estimates for each cow and cow-quarter.

[3]D 0 = actual day of clinical mastitis diagnosis; prior day = prior single day of actual mastitis treatment; the corresponding number in column 1 indicates the number of prior days endorsed as pseudo-mastitis.

[4]Random effect = cow- and quarter-specific information, including previous mastitis history; AUC = area under the curve; Se = sensitivity; Sp = specificity; Cut-off = threshold point where sum of sensitivity and specificity is maximum; a value exceeding the cut-off is a mastitis indicator.

[5]Assessment for prior days using the first data set of farm 1 (n = 358 cows).

[6]Optimum cut-off as evaluated on d 0 with random effect in farm 1.

[7]Optimum cut-off as evaluated on d 0 without random effect in farm 2.

[8]Assessment for prior days using the data set of farm 2 (n = 568 cows).

[9]Optimum cut-off as evaluated on d 0 with random effect in farm 2.

[10]Optimum cut-off as evaluated on d 0 without random effect in farm 2.

In farm 1, the AUC values of the model decreased gradually (0.96 to 0.93 vs. 0.92 to 0.81) due to increasing number of prior days as CM in the model with and without random effects. For the model with random effects, the Se and Sp were always higher on d 0; Se decreased gradually from 90% to 85%, and Sp decreased from 91% to 83% with increasing number of prior days as CM in the model. For the model without random effects, Se also decreased from 83% to 54% with minor variation in Sp.

In farm 2, except d 0 to prior d 1 (model with random effect), the AUC and Se were always higher on d 0 compared with other times. There was minor variation in Sp in both models with and without random effects.

In the evaluation on each specific prior day at the same cut-off as mentioned above, Se decreased sharply in farm 1 (90% to 37% vs. 84% to 34%) and in farm 2 (93% to 13% vs. 80% to 29%) for the model with and without random effects, respectively. Similar to previous assessments, Se was higher for the model with random effects compared to the model without

random effects.

DISCUSSION

This study endeavored to predict CM by analysing and integrating multiple inline sensor data with robust assessment values in 2 pasture-based farms. We developed and tested a multivariable linear predictor index (LP) capable of detecting CM at quarter level, which to the best of our knowledge has better Se and Sp than any existing single inline sensor. We evaluated our models using ROC curves, which have also been used successfully for monitoring other diseases (e.g., hyperketonemia) and radiologic imaging diagnostics (Hanley and McNeil, 1982; van der Drift et al., 2012; Perkins et al., 2013).

The excellent AUC value of the final multivariable LP index with higher balance Se and Sp than any of the univariable LP index further supports the idea that better mastitis detection is possible by integrating multiple types of information and measurements rather than using single variable measurements (Brandt et al., 2010; Hogeveen et al., 2010; Steeneveld et al., 2010). If solely relying on AMS measurements, it is difficult to identify the CM cases early enough because milk fractions might influence sensor results (Sarikaya and Bruckmaier, 2006). Thus, in this study, we aimed to develop a multivariable index to maximise the Se and Sp by integrating multiple sources of sensor information that are available in all the different brands of AMS commercially available. As a baseline, we have compared with ISO standard Se (> 70%) and Sp (≥ 99%) recommended for automatic discarding of abnormal milk (Mein and Rasmussen, 2008; Sherlock et al., 2008). It should be noted that our multivariable model index can also achieve the ISO standard at a higher cut-off of the index than mentioned in the text, but we argue that the cost for missing the true positive cases might compensate for missing the true negative cases. This is why we aimed for the maximum sum of Se and Sp as the farm and data-specific optimum cut-off. The limitation at this optimum cut-off was that our index leaves 9% of cows as wrongly classified as having CM (e. g., up to 36 false alerts in a herd of about 400 cows, farm 1). However, this level of error may be acceptable for a pasture-based AMS compared to other more labor-demanding approaches as mentioned by Steeneveld et al. (2010). However, further optimisation might be possible by incorporating cows' immune information (DeLaval lactate dehydrogenase or others). Another encouraging outcome of our study was the 20-23% increase in Se at a fixed cut-off (> 8.24) due to inclusion of previous CM history of the cow (random effects) in the assessment datasets. This reflects the statistical prediction ability of biological relevance with repeated cases without knowing any causal factors (Abureema et al., 2014). However, to account for such random effects in on-farm situations, we need to ensure that there is enough information about

previous lactations, or previous days of the current lactation, before creating ROC curves; otherwise, Se may decline markedly. We noticed much lower Se in assessment 2 (66.7%) than in assessment 1 (85%) at the same cut-off (> 8.24) due to absence of cow-level and quarter-level mastitis history (Table 5. 1). However, such variation can also be due to different farm conditions with different sample size. Despite variation, such an approach of inclusion of information to account for random effects for milking-related information might be more useful when combined with other test procedures such as somatic cell count and lactate dehydrogenase (Chagunda et al., 2006; Kamphuis et al., 2008; Sørensen et al., 2016). Moreover, in such evaluation, we might consider cow- or quarter specific optimum cut-off as we noticed higher sum of Se and Sp at the farm- and data-specific cut-off both in assessment 1 (Sum = 1.769 vs. 1.75; cut-off = 7.8 vs. 8.24) and assessment 2 (sum = 1.905 vs. 1.663 cut-off: 6.52 vs. 8.24) with random effect or without random effect (assessment 1: sum = 1.71 vs. 1.661, cut-off = 7.25 vs. 7.84; assessment 2: sum = 1.727 vs. 1.527, cut-off = 5.8 vs. 7.84). The procedure did show ability to detect CM on days before actual diagnosis. However, the ability to do this reliably declined, with reductions in Se and Sp with increasing days before actual diagnosis showing the ability to monitor false-positive and false-negative cases.

Moreover, our collective study findings also support the observations from previous studies with single or limited information. For example, MYH, the best single inline measurement, has been previously found to be useful for detection of nonspecific health problems (Lukas et al., 2009). Moreover, a previous study reported by our research group found that MYH has a nonlinear association with the milking interval, with reduced MYH when milking interval is more than 16 h (Lyons et al., 2013a). As milking interval is not controlled in pasture-based AMS, MYH might not be a better CM predictor in such situations despite the high AUC (0.94) obtained in our study. Our study also suggests that EC alone is not powerful enough for CM prediction even using different statistical algorithms (Kamphuis et al., 2008; Khatun

et al., 2017). This might be explained by the impact of temperature, fat content or milk fraction on EC measurements (Nielen et al., 1992; Bruckmaier et al., 2004). The decreasing trend (< 0.429 mS/cm per hour, opposite to EC) of the ECH for the CM alert might be related to milking interval as mastitic cows are prone to have longer milking intervals, allowing longer harboring of the pathogens causing infection (Hogeveen et al., 2001; Hammer et al., 2012; Penry et al., 2017). Similar to a recent report by another group, our study also did not find strong CM prediction ability of PF, which is considered an important breeding parameter (Penry et al., 2017). The correlation of the parameters included in the final model ranged from -0.32 to 0.71, and PF was moderately correlated with MF (r = 0.76), but using the backward elimination procedure it was not included in the final multivariable model. Although many farmers use MDi for routinely checking for mastitis, MDi did not perform better than EC even though this index incorporates multiple measurements such as EC, MF, and blood in milk. The reasons behind the poor performance of MDi might be due to the effect of blood in milk or milk color as influenced by milk fat color and breed (Rasmussen and Bjerring 2005). Compared with MDi, a better AUC value of IM was achieved with higher Se and Sp at the optimum cut-off (LP scale), and this was supported by the use of direct visual observations in farm 2, where 50% of mastitis cases were detected twice based on an IM alert before MDi indicated an alert (personal communication with N. Dornauf, farm owner, Gala Farm, Tasmanis).

Another important aspect of our index is that it might be a cost effective and understandable approach for farmers (as the value is absolute) for automatic detection of cows at risk for CM with low false alerts, especially in a pasture-based system with minimal farmer-cow contact. In this way, it will reduce labor requirements and costs associated with visual inspection of cows. It is possible that even more accurate levels of CM prediction could be obtained if

immune and behavioral variables were incorporated; this will be the focus of further investigations.

CONCLUSIONS

We developed and evaluated a multivariable quarter-level CM prediction index in AMS. Overall, our study found that better CM prediction is possible by using multiple automatically recorded inline sensor data records rather than a single sensor data records. The best-fitting model used information on EC, ECH, MY, MYH, MF and IM. Incorporation of cow and quarter previous mastitis history (random effects) improved the performance of the test procedures. The present model is suited for estimation of the quarter-, cow- and herd-level mastitis alarm and expected to result in improved accuracy of mastitis alerts, thereby improving the detection ability and practicality on farm.

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Supplementary material

These were assessed by producing a receiver operating characteristic curve and calculating the area under the curve (**AUC**) to discriminate between disease-positive and disease-negative individuals.

Moreover, in a pasture-based AMS, contact between herd managers and their cows is less frequent compared to indoor farming system (Wildridge et al 2019), checking multiple alerts (either automatic or non-automatic) to improve Se and Sp for detection of mastitis requires an increase in workload (Steeneveld et al., 2010).

The data were retrieved for only selective cows (e.g. mastitis and control).

Gold Standard for CM and Control

The quarters included in this study included both clinically infected and healthy quarters. In both farms the protocol used for definition of CM was a record of veterinary treatment done by trained farm staff or by assigned veterinarian and the day of treatment was considered as d 0.

The data were analysed using ASReml-R (Butler et al., 2009) built under R version 3.2.5 (http://www.r-project.org) fitted using a penalised quasi-likelihood method (Breslow and Clayton, 1993).

The sharp decline in Se and Sp in false mastitis cases at earlier days prior to actual CM detection by farmers further validates the robustness of the approach.

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CHAPTER 6: Prediction of quarter level subclinical mastitis by combining in-line and on-animal sensor data

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OVERVIEW OF CHAPTER 6

In addition to milking-related changes (discussed until Chapter 5) mastitis is also responsible for behavioural changes as discussed in Chapter 2. Hence, Chapter 6 reports an investigation into mastitis detection ability of behavioural changes alone and in combination with electrical conductivity. A controlled experimental study, as well as retrospective data, were used in this study.

ABSTRACT

We investigated the potential for automatic detection of subclinical mastitis (**SCM**) in pasture-based automatic milking systems. The objective of the study was to determine the ability of electrical conductivity (**EC**), together with relative changes in daily activity (**activity**) and daily rumination (**rumination**) recorded using heat and rumination–long-distance tags, to predict quarter-level SCM. Activity (arbitrary unit/day) and rumination (min/day) data were determined across 21 days using heat and rumination–long-distance tags for 170 cows. Cows were allocated into the following three groups: SCM (n = 32, EC ≥ 7.5 milli Siemens/cm (mS/cm) in one or more quarters and a positive bacteriological culture in the corresponding quarter(s); true-negative (**TN**, n = 9, EC ≥ 7.5 mS/cm and a negative culture in all four quarters); and apparently healthy (n = 129, no culture test and EC < 7.5 mS/cm). Group mean differences in activity and rumination were compared using Welch's t-tests. Logistic mixed models were used to predict SCM by EC, activity and rumination changes before mastitis detection, including parity information between SCM and TN groups. Cow- and quarter-specific information were included as random effects, followed by model assessment by producing receiver operating-characteristic curve and area under the curve (AUC) value. In total, 287 quarters were used in the prediction model, including 143 quarters with a positive culture (Gram-positive; n = 131, Gram-negative; n = 6, mixed; n = 6) and 144 quarters with a negative culture. On average, SCM group had 4.65% greater (P < 0.01) activity and 9.89% greater (P < 0.001) rumination than did the TN group and 11.70% greater (P < 0.001) activity than did the apparently healthy group. A combined model with terms for EC, activity changes, rumination changes prior to detect SCM and parity had a better SCM prediction (AUC = 0.92) ability than did any of them separately (all AUC < 0.8). Hence, we

conclude that EC in combination with activity and rumination information can improve the accuracy of prediction of quarter-level SCM.

Keywords: Electrical conductivity, daily activity, daily rumination, automatic milking systems.

INTRODUCTION

Bovine mastitis is an inflammation of the mammary gland, typically caused by bacteria belonging to *Enterobacteriaceae*, *Staphylococcaceae* or *Streptococcaceae* families (Bradley, 2002; Pyörälä, 2003). Mastitis is an animal-welfare issue responsible for substantial economic loss due to milk loss (Bar et al., 2007; Schukken et al., 2009), treatment cost, increased culling or sometimes death (Hertl et al., 2011). Earlier mastitis detection helps in early treatment decision, thereby maintaining good animal health and welfare, and improving economic return to the farmers (Milner et al., 1997). Since 2000, the average dairy herd size has nearly doubled, creating opportunities for automatic milking systems (AMS; García and Fulkerson, 2005; Dairy Australia, 2018). In AMS, due to fewer opportunities for visual inspection of udders, there is an increasing demand for a reliable automatic tool for earlier identification and detection of mastitis (Mollenhorst et al., 2012). Mastitis-associated changes in milk, such as electrical conductivity (**EC**) and somatic cell count (**SCC**), have encouraged the use of sensor technology for automatic detection of mastitis (Koop et al., 2015; Sørensen et al., 2016; Khatun et al., 2018). Hence, the EC (determined primarily by sodium and chloride ion concentration) is the most commonly used mastitis-detection method in AMS, although results are variable (Kitchen, 1981; Kamphuis et al., 2010b; Khatun et al., 2017).

Apart from milk-related changes, behavioural changes are also observable during mastitis (Fogsgaard et al., 2012; Medrano-Galarza et al., 2012; Kester et al., 2015). Behavioural

changes such as activity and rumination can be automatically and continuously monitored by sensors located in collars or tags (Clark et al., 2015; Molfino et al., 2017). Such sensor information has been found to be useful in predicting clinical mastitis (Stangaferro et al., 2016). Hence, the objective of the present study was to assess the ability of EC, combined with activity and rumination changes in AMS, to predict subclinical mastitis (**SCM**). This approach of observing EC, activity and rumination in combination could be a cost-effective method to detect SCM earlier, particularly in pasture-based AMS with minimal farmer–cow contact.

MATERIALS AND METHODS

Animals

All procedures involving the use of the animals were approved by the University of Sydney Animal Ethics Committee (project number: 2017/1141). The study was conducted at the pasture-based dairy research farm of the University of Sydney (Corstorphine, Camden, NSW, Australia) for a period of 2 mo (Jun. 21 to Aug. 30, 2017). The herd consisted of 350 (predominantly Holstein–Friesian) lactating cows with a year-round calving system. The cows were milked through a robotic rotary system with a 24-unit platform and five robotic arms (DeLaval **AMR™**, Tumba, Sweden). The cows had daily access to pasture grass, and were supplemented with partial mixed ration and grain-based commercial pelleted concentrate (Khatun et al., 2018). All cows were fitted with a neck-mounted electronic heat and rumination–long-distance tag (HR–LD, SCR Dairy, Netanya, Israel), which contains an accelerometer to quantify activity and rumination movements (DataFlow, Netanya, Israel).

The raw data collected in 2-h periods were summarised for 24 h as daily activity (activity, arbitrary unit/day) and daily rumination (rumination, min/day).

Treatment Groups

Of the 350 lactating cows, 170 cows were enrolled in the study. The cows were divided into the following three groups: SCM (n = 32, EC ≥ 7.5 mS/cm) in one or more of the four quarters and a positive culture in the corresponding quarter(s)); true negative (**TN**, n = 9, EC ≥ 7.5 mS/cm and a negative culture in all four quarters); and apparently healthy (**AP**, n = 129, no culture test and EC < 7.5 mS/cm). We have chosen 7.5 mS/cm arbitrarily to find mastitis quarters with the SCC range of at least 425 000–531 000 cells/ml, according to Bruckmaier et al. (2004a). The mean (± SD) parity and days in milk (**DIM**; days) of the three groups were 3.85 ± 1.71 and 180 ± 115 (SCM), 4.18 ± 2.04 and 117 ± 113 (TN) and 1.71 ± 0.61 and 203 ± 135 (AP), respectively. As activity and rumination were associated with DIM and, in our study, there was a limited number of early lactation cows in the TN group, we investigated the AP group to get more statistical power for estimating the pattern of activity and rumination changes (Chaplin and Munksgaard, 2001; Bewley et al., 2010).

Gold Standard

Bacteriological culture was taken as the gold standard to assess the true infection status of the quarter with SCM (Sargeant et al., 2001). Any quarter identified as being either Gram-positive or Gram-negative, or mixed (e.g., with two or more than two bacterial genera isolated), were defined as SCM and quarters without any bacterial growth were defined as control in the prediction model between the SCM and TN groups. Hence, all cows in the

SCM group had either single- or multiple-quarter infection, whereas those in the TN group had no infection. On the basis of previous studies on the association between EC and SCC, AP group with EC of <7.5 mS/cm was expected to have <100 000 cells/ml (~3435–3880 cells/ml) in the present study (Nielen et al., 1993; Bruckmaier et al., 2004a).

Collection of Milk Samples

Aseptic quarter milk samples (10 ml) were collected from the individual quarter in SCM and TN groups for bacteriological culture. Prior to sampling, teats were dipped in iodine solution (Iodophor LF12, DeLaval, Melbourne, Vic. Australia) and cleaned with a 70% alcohol-soaked gauze (modified from Hogan et al., 1999). Because of the imposed EC-threshold criterion, several cows were sampled on several days (e.g., two samples, $n = 6$; three samples, $n = 4$; five samples, $n = 3$; six samples, $n = 1$). One cow had only three functional quarters. In total, there were samples from 283 quarters for culture tests.

Electrical Conductivity, Daily Activity and Daily Rumination

The EC data were recorded and stored by DeLaval DelPro Software 5.1 (DeLaval International AB, Tumba, Sweden) while milked through the robotic rotary system. The EC data of SCM and TN groups, from 21 days before SCM detection, were extracted from the software.

The activity and rumination data were recorded and stored by DataFlow software (SCR Dairy, Netanya, Israel) and extracted for all three groups (e.g., SCM, TN and AP). In the case of SCM and TN groups, the day of milk sampling was considered as Day 0 and, for the AP group, Day 0 was chosen arbitrarily so as to have 21 days of data during the 2-month study

period. Data of the AP group were analysed only to observe the activity and rumination changes, but were not included in the model prediction due to lack of culture tests. Apart from the imposed EC-threshold criterion, no other health disorder was recorded for any of the selected 169 cows. Data values that were identified with increased activity due to oestrus were deleted.

Statistical Analysis

The data were analysed using ASReml-R (Butler et al., 2009) built under R version 3.2.5 (http://www.r-project.org, accessed 10 May 2019) and the details of the model building are given below.

Daily activity and daily rumination change. The following formula were used to calculate change in activity (%) and change in rumination (%):

$$\%\ \text{Change in activity} = \frac{\left(\text{Activity on day } 0 - \text{Activity on day } -t\right) \times 100}{\text{Activity on day } -t}$$

$$\%\ \text{Change in rumination} = \frac{\left(\text{Rumination on day } 0 - \text{Rumination on day } -t\right) \times 100}{\text{Rumination on day } -t}$$

where Day $-t$ is t days before SCM detection, and differences were calculated for t = 1, 2, …, 21 days before SCM detection (Day 0). Welch's *t*-test was used to compare the activity and rumination changes among the SCM, TN and AP groups. Additionally, the ability (binary outcome) of quarter-level EC, and activity and rumination changes to predict SCM was tested in the SCM and TN groups by logistic generalised linear mixed model (GLMM), including cow and quarter nested within cow as random effects.

Prediction of SCM by single predictor. Three different predictors, namely EC, and activity and rumination changes, were tested separately to find the best predictor for SCM as a binary

outcome. Initially, three predictors were fitted individually with a univariable GLMM, using the following model:

$$\ln[\pi/(1-\pi)] = \text{constant} + \text{predictor} + \text{cow} + \text{cow.quarter}$$

where $\pi = P(Y = 1)$ is the probability that a particular quarter had SCM during the test session, predictor was one of the three variables listed above as a fixed effect, and cow and cow.quarter (quarter nested within a cow) were random effects. Wald *F* and Wald chi-square tests were used for significance testing.

Electrical conductivity up to 21 days before the detection of SCM was determined as a cumulative value of individual ECs before SCM detection, as a single predictor, i.e., as the sum of ECs from Day 0 to Day $-t$ (t = 1, 2, …, 21 days before the SCM detection). Similarly, % change in activity and % change in rumination (t = 1, 2,., 21 days before the SCM detection) were tested separately. The relative activity and rumination were determined for individual days for each cow, which added a constant prediction value to each quarter.

Receiver operating characteristic (**ROC**) curve. Each predictor variable was also assessed independently by creating a ROC curve and calculating the area under the curve (AUC) value. The generated AUC values from the ROC curves were classified as excellent (0.9–1.0), good (0.8–0.9), fair (0.7–0.8), poor (0.6–0.7) and fail (0.5–0.6), with values under 0.5 worse than random classification (Khatun et al., 2018). We evaluated the test performance at the optimum cut-off value, a threshold point where the sum of sensitivity (**Se**) and specificity (**Sp**) was the maximum; this is under the assumption of equal impact of a false positive and a false negative, although different weightings could be considered, a value exceeding the cut-off is taken as a SCM indicator. The construction of the ROC curves was performed using the AUC package (version 0.3.0) in R (http://www.r-project.org, accessed 10 May 2019).

Prediction of SCM by multiple predictors. The best single predictors in regard to changes across different days for EC, and activity and rumination changes, and additionally including

parity information, were fitted by multivariable GLMMs to obtain the best prediction model. The combined models were assessed by AUC values of the ROC curves (as described above) and also on the basis of the predicted Se and Sp.

RESULTS

Pattern of Daily Activity and Daily Rumination Change

On average, the SCM group had 4.65% greater ($P < 0.01$) activity and 9.89% greater ($P < 0.001$) rumination than did the TN group, and 11.70% greater ($P < 0.001$) activity and 1.11% greater ($P = 0.80$) rumination than did the AP group. The mean (± SE) changes in activity and rumination in each of the 21-day study period, along with the statistical significances among the groups, are presented in Table 6.1.

Pathogens

Culture tests showed that of the 287 quarters from 41 cows, 131 quarters were Gram-positive with *Aerococcus* sp., *Bacillus* sp., coagulase-negative *Staphylococcus*, coagulase-positive *Staphylococcus*, *Corynebacterium* sp., *Enterococcus faecalis*, environmental *Streptococcus* sp., *Staph. dysgalactiae*, *Strep. uberis*, and *Trueperella pyogenes*. Samples collected from only six quarters had growth of Gram-negative (coliform) bacteria. Six quarters showed growth of two or more than two bacterial genera (e.g., mixed infection) and 144 quarters were culture negative (control).

Table 6. 1. The mean ± standard error (SE) for differences in the % change in daily activity (activity, arbitrary unit/day) and daily rumination (rumination, min/day) for the period of 1–21 days before the day of mastitis detection (Day 0) for the subclinical mastitis (SCM), true-negative (TN) and apparently healthy (AP) groups

Days	Activity (Mean ± SE)			*P- value*		Rumination (Mean ± SE)			*P- value*	
	SCM	TN	AP	SCM vs. TN	SCM vs. AP	SCM	TN	AP	SCM vs. TN	SCM vs. AP
Day-1	13.07 ± 2.6	15.77 ± 3.8	2.10 ± 1.13	0.34	<0.001	6.99 ± 4.5	-17.16 ± 7.5	16.13 ± 6.77	0.02	0.02
Day-2	9.12 ± 2.1	-0.25 ± 3.6	-0.82 ± 1.08	0.08	<0.001	6.60 ± 3.0	9.99 ± 14.6	25.98 ± 6.00	0.96	0.003
Day-3	8.28 ± 2.1	11.61 ± 5.5	-2.13 ± 1.06	0.003	<0.001	4.86 ± 3.4	-8.01 ± 5.5	11.77 ± 3.39	0.39	0.02
Day-4	8.39 ± 2.2	4.24 ± 4.1	-2.66 ± 1.16	0.51	<0.001	17.30 ± 6.3	-13.08 ± 8.2	11.03 ± 6.03	0.07	0.79
Day-5	9.54 ± 2.0	8.80 ± 4.4	-0.74 ± 2.25	0.90	<0.001	15.28 ± 7.4	-7.43 ± 7.3	19.20 ± 4.52	0.15	0.005
Day-6	12.47 ± 2.6	7.97 ± 5.3	-2.89 ± 1.26	0.52	<0.001	12.84 ± 6.1	-19.20 ± 5.3	10.79 ± 7.73	0.01	0.65
Day-7	6.35 ± 2.2	7.09 ± 5.0	-1.20 ± 1.41	0.89	0.002	4.50 ± 4.1	-12.86 ± 7.7	26.16 ± 5.24	0.16	<0.001
Day-8	7.06 ± 2.2	5.28 ± 5.1	-2.35 ± 1.25	0.77	<0.001	0.01 ± 2.2	-10.89 ± 9.2	30.36 ± 3.95	0.16	<0.001
Day-9	8.97 ± 2.7	3.38 ± 5.2	-10.64 ± 0.95	0.47	<0.001	1.67 ± 3.1	-9.11 ± 8.3	8.75 ± 3.96	0.22	0.42
Day-10	10.82 ± 2.7	7.77 ± 5.0	-9.18 ± 0.98	0.76	<0.001	4.69 ± 4.3	7.77 ± 8.2	4.28 ± 3.53	0.12	0.51
Day-11	12.31 ± 2.5	4.02 ± 5.3	-3.56 ± 1.50	0.24	<0.001	1.40 ± 3.2	19.81 ± 17.8	-5.64 ± 3.87	0.58	0.01
Day-12	12.72 ± 2.7	5.96 ± 5.4	1.27 ± 1.15	0.42	<0.001	6.77 ± 3.7	11.95 ± 21.2	-7.68 ± 2.39	0.40	<0.001
Day-13	3.76 ± 2.3	3.28 ± 4.3	-5.22 ± 1.24	0.65	0.04	1.55 ± 5.3	-0.80 ± 11.1	-11.14 ± 2.34	0.97	0.08
Day-14	9.01 ± 3.3	1.95 ± 6.5	-0.89 ± 1.27	0.35	0.005	6.52 ± 6.1	-8.16 ± 10.2	-10.59 ± 2.46	0.34	0.002
Day-15	3.56 ± 2.6	5.80 ± 4.1	-2.69 ± 1.28	0.48	0.05	14.35 ± 5.6	-15.86 ± 4.7	6.51 ± 5.89	0.004	0.06
Day-16	7.18 ± 3.6	2.76 ± 5.9	0.68 ± 1.19	0.70	0.13	17.94 ± 6.7	-5.42 ± 10.8	6.90 ± 3.59	0.05	0.12
Day-17	11.31 ± 3.3	1.07 ± 7.1	-0.62 ± 1.25	0.24	<0.001	7.18 ± 4.6	4.67 ± 13.4	2.89 ± 2.99	0.41	0.50
Day-18	4.05 ± 2.6	0.33 ± 6.5	-5.12 ± 1.14	0.65	0.002	4.79 ± 3.9	-10.64 ± 8.5	8.79 ± 3.01	0.13	0.55
Day-19	7.21 ± 3.0	-2.16 ± 5.1	-7.08 ± 1.08	0.15	<0.001	13.25 ± 7.0	-20.84 ± 6.5	11.37 ± 3.60	0.02	0.86
Day-20	4.84 ± 3.8	0.67 ± 5.7	-3.90 ± 1.28	0.65	0.045	6.28 ± 4.8	1.23 ± 11.0	8.80 ± 3.07	0.34	0.89
Day-21	1.97 ± 2.8	3.09 ± 5.7	-4.96 ± 1.00	0.78	0.08	6.00 ± 6.9	2.94 ± 11.2	5.77 ± 2.95	0.76	0.61

Activity = Daily activity change on Day 0 (mastitis detection) from a prior single day (Days –1 to –21 in Column 1); Rumination = Daily rumination change on Day 0 (mastitis detection) from a prior single day (Days –1 to –21 in Column 1); SCM = a subclinical mastitic cow with electrical conductivity (EC) of ≥ 7.5 mS/cm in one or more of the four quarters and a positive culture in the corresponding quarter (s); TN = Cow with EC of ≥ 7.5 mS/cm in one or more of the four quarters, but culture negative in all four quarters; AP = an apparently healthy cow with EC of < 7.5 mS/cm and no culture test; *P*-values are based on Welch's *t*-tests.

Prediction of SCM by Single Predictor

All three predictors were associated with mastitis ($P \leq 0.01$) from 7 days before mastitis detection, with the exception of Day 2 before mastitis detection for rumination change (Table 6.2). From 8 to 21 days before mastitis detection, EC and change in rumination were significantly ($P < 0.05$) associated with mastitis, with the exception of change in rumination on Days 11 and 20 before mastitis detection. The change in activity was significantly ($P < 0.05$) associated with mastitis only on Days 10, 13 14 and 21 before mastitis detection.

Test performance based on AUC values ranged from fair (AUC: 0.7–0.8, 3 predictors) to poor (AUC: 0.6–0.7, 55 predictors) and fail (AUC: 0.5–0.6, 5 predictors). The best test performance was obtained by monitoring EC up to 3 days (Se = 0.444, Sp = 0.802) before the SCM detection. In regard to activity change, the best test performance was obtained from the 1st day (Se = 0.917, Sp = 0.310), the 4th day (Se = 0.588, Sp = 0.640) and the 13th day (Se = 0.568, Sp = 0.690) before the SCM detection. In regard to rumination change, the best test performance was from the 3rd day (Se = 0.621, Sp = 0.695), the 7th day (Se = 0.563, Sp = 0.772) and the 12th day (Se = 0.634, Sp = 0.759) before the SCM detection. These best-performing predictors by the univariable models were used in the subsequent multivariable model prediction.

Prediction of SCM by Multiple Predictors

In general, the test performances by multivariable models were better (good, AUC ≥ 0.8, to excellent, AUC ≥ 0.9) than any performances by the univariable models (fail, AUC ≤ 0.6, to fair, AUC ≤ 0.7) when comparing the AUC values (Table 6.3).

The best prediction model was found with EC up to 4 days before the SCM detection, activity change from the 4th day before the SCM detection, rumination change from the 3rd day before the SCM detection, and this resulted in an 'excellent' test-diagnostic value (AUC = 0.930) with more than 85% Se and Sp (Figure 6.1). By using the multivariable model, the Se and Sp were 22–41% and 11–27% greater respectively, than they were by using the best-performing univariable-model predictors, namely EC, activity and rumination.

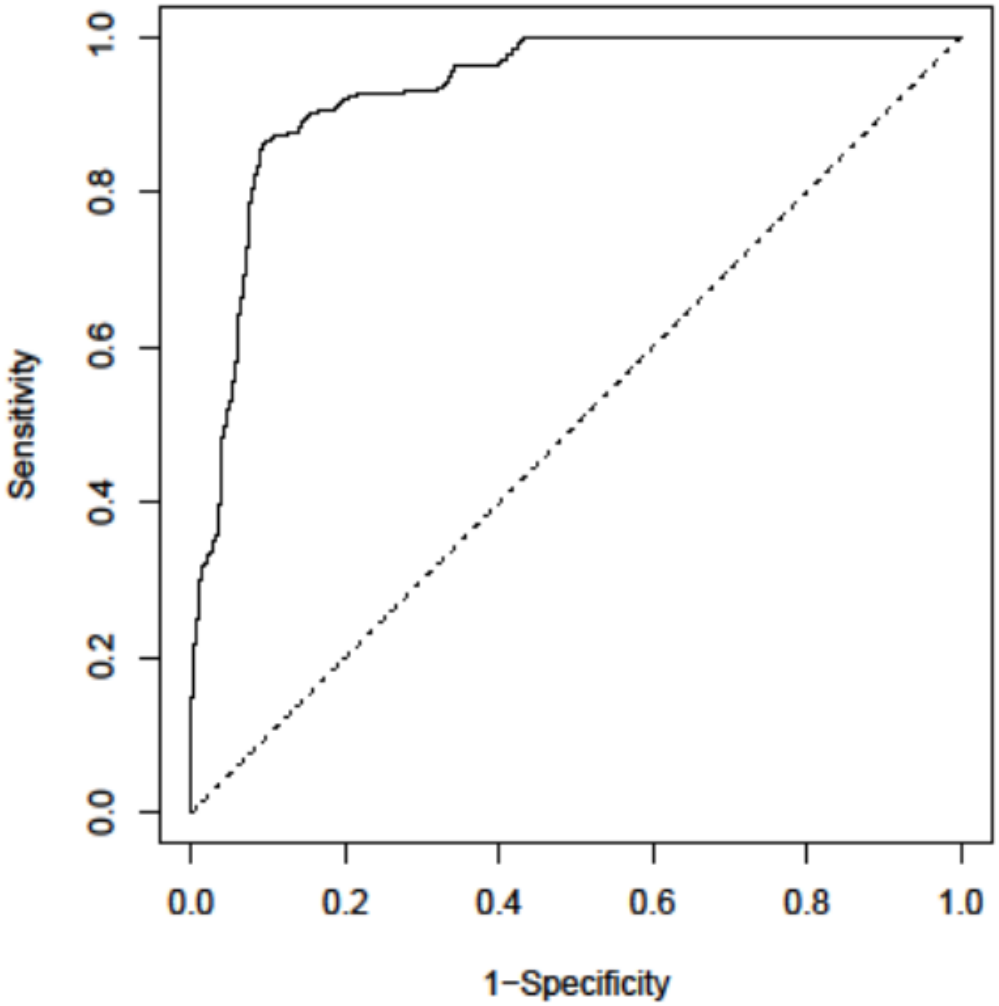

Figure 6.1. Receiver operating characteristic (ROC) curves of multivariable logistic generalised linear mixed models combining activity change from fourth day before, rumination change from third day before, and electrical conductivity record up to 4 days before subclinical mastitis detection (day 0). Random effects included cow- and quarter-specific information.

Table 6. 2. Analysis of receiver operating characteristic curve for prediction of mastitis based on monitoring changes in electrical conductivity (EC, mS/cm, recorded before mastitis detection), daily activity (activity, arbitrary unit/day) and daily rumination (rumination, min/day) for the period of 1–21 days before the day of mastitis detection (Day 0) by univariable logistic generalised linear mixed models in subclinical mastitis (SCM) and true-negative (TN) groups

Days	EC		Activity		Rumination	
	P-value	AUC	*P*-value	AUC	*P*-value	AUC
Day-1	<0.001	0.640	< 0.001	0.661	<0.001	0.657
Day-2	<0.001	0.641	0.01	0.622	0.247	0.611
Day-3	<0.001	0.644	0.002	0.636	<0.001	0.708
Day-4	<0.001	0.643	< 0.001	0.666	<0.001	0.682
Day-5	<0.001	0.642	0.001	0.638	<0.001	0.688
Day-6	<0.001	0.641	< 0.001	0.652	<0.001	0.654
Day-7	<0.001	0.642	0.003	0.614	<0.001	0.709
Day-8	<0.001	0.638	0.257	0.595	<0.001	0.651
Day-9	<0.001	0.637	0.141	0.616	0.005	0.648
Day-10	<0.001	0.634	0.03	0.623	0.002	0.644
Day-11	<0.001	0.633	0.188	0.610	0.896	0.609
Day-12	<0.001	0.633	0.471	0.620	<0.001	0.739
Day-13	<0.001	0.633	0.001	0.662	0.045	0.636
Day-14	<0.001	0.633	0.03	0.609	0.008	0.630
Day-15	<0.001	0.633	0.143	0.597	0.004	0.631
Day-16	<0.001	0.635	0.206	0.599	0.04	0.628
Day-17	<0.001	0.638	0.986	0.596	0.01	0.652
Day-18	<0.001	0.640	0.968	0.589	0.01	0.650
Day-19	<0.001	0.642	0.830	0.682	0.002	0.68
Day-20	<0.001	0.643	0.451	0.638	0.095	0.642
Day-21	<0.001	0.645	0.009	0.633	0.052	0.658

Activity = daily activity change on Day 0 (mastitis detection) from a prior single day (Days –1 to –21 in Column 1); Rumination = daily rumination change on Day 0 (mastitis detection) from a prior single day (Days –1 to –21 in Column 1); SCM = A subclinical mastitic cow with EC of ≥ 7.5 mS/cm in one or more of the four quarters and a positive culture in the corresponding quarter(s); TN = Cow with EC of ≥ 7.5 mS/cm in one or more of the four quarters, but culture negative in all four quarters; Days = Days in column 1 corresponding to EC indicate the cumulative number of prior days that recorded the EC included in the model; AUC = Area under the curve.

Table 6. 3. Analysis of receiver operating characteristic (ROC) curve for prediction of mastitis on the basis of monitoring changes in electrical conductivity (EC, mS/cm), daily activity (activity, arbitrary unit/day) and daily rumination (rumination, min/day) for the period of 1–21 days before the day of mastitis detection (Day 0), and parity, by multivariable logistic generalised linear mixed model in subclinical mastitis (SCM) and true negative (TN) groups.

EC day	AUC	Se	Sp	Cut-off	AUC	Se	Sp	Cut-off	AUC	Se	Sp	Cut-off	AUC	Se	Sp	Cut-off
	Activity-1, Rumination-3				Activity-1, Rumination-7				Activity-2, Rumination-3				Activity-3, Rumination-3			
Day 0	0.911	0.890	0.824	0.420	0.908	0.868	0.828	0.444	0.910	0.890	0.815	0.417	0.875	0.756	0.838	0.536
Day-1	0.928	0.882	0.894	0.444	0.927	0.876	0.894	0.477	0.927	0.884	0.888	0.466	0.915	0.895	0.834	0.425
Day-2	0.928	0.883	0.891	0.438	0.927	0.877	0.892	0.469	0.927	0.886	0.886	0.461	0.914	0.892	0.833	0.432
Day-3	0.928	0.880	0.894	0.457	0.928	0.878	0.891	0.461	0.928	0.886	0.885	0.451	0.915	0.877	0.845	0.459
Day-4	0.929	0.878	0.895	0.457	0.929	0.876	0.892	0.460	0.929	0.885	0.886	0.452	0.916	0.898	0.824	0.408
Day-5	0.929	0.876	0.895	0.449	0.929	0.874	0.891	0.444	0.929	0.883	0.887	0.446	0.916	0.885	0.835	0.430
	Activity-4, Rumination-3				Activity-4, Rumination-7				Activity-1, Rumination-12				Activity-4, Rumination-12			
Day 0	0.911	0.874	0.833	0.428	0.907	0.871	0.822	0.423	0.890	0.848	0.796	0.464	0.885	0.826	0.807	0.482
Day-1	0.929	0.863	0.903	0.531	0.928	0.871	0.891	0.439	0.819	0.877	0.879	0.481	0.919	0.859	0.887	0.521
Day-2	0.929	0.871	0.894	0.548	0.928	0.873	0.889	0.437	0.919	0.872	0.880	0.494	0.919	0.860	0.884	0.515
Day-3	0.929	0.864	0.900	0.517	0.929	0.861	0.902	0.536	0.919	0.873	0.881	0.495	0.919	0.859	0.885	0.519
Day-4	0.930	0.859	0.907	0.541	0.930	0.859	0.904	0.536	0.920	0.875	0.879	0.478	0.920	0.859	0.885	0.510
Day-5	0.930	0.857	0.908	0.533	0.930	0.857	0.905	0.529	0.920	0.871	0.882	0.482	0.919	0.855	0.887	0.510

Activity = daily activity change on Day 0 (mastitis detection) from a prior single day (Days –1 to –21 in Column 1); Rumination = daily rumination change on Day 0 (mastitis detection) from a prior single day (Days –1 to –21 in Column 1); SCM = a subclinical mastitic cow with EC of ≥ 7.5 mS/cm in one or more of the four quarters and a positive culture in the corresponding quarter (s); TN = Cow with EC of ≥ 7.5 mS/cm in one or more of the four quarters, but culture negative in all four quarters; EC day = corresponding number in column 1 indicate the cumulative number of prior days that recorded the EC included in the model; AUC = area under the curve; Se = sensitivity; Sp = specificity; Cut-off = a threshold point where sum of sensitivity and specificity is maximum, a value exceeding the cut-off is a mastitis indicator.

DISCUSSION

Our objective was to determine the predictive ability of EC, in combination with relative changes in activity and rumination, for automatic SCM detection in a pasture-based AMS. Combining all three predictors, as well as parity, in the model improved the SCM detection compared with using any of the individual predictors alone. Our best predictions were obtained from a model including up to 4 days of EC values before the SCM detection, activity change from the 4th day before the SCM detection and rumination change from the 3rd day before the SCM detection; this model had, on average, a 30% greater Se and a 17% greater Sp than did any of the best single predictors. These findings are in-line with the results of previous work, which has shown improved mastitis prediction with the incorporation of multiple data sources (Hogeveen et al., 2010; Khatun et al., 2018). In our study, incorporating the EC data with activity and rumination changes resulted in a difference of +18 percentage units (76% vs. 58%) in Se, compared with a study where EC information was not available (Stangaferro et al., 2016). Hence, EC is useful while predicting SCM, although EC alone can result in inconsistent results (Norberg et al., 2004; Khatun et al., 2017). Test performance was excellent (AUC > 0.9) when EC, activity and rumination were considered together, compared with the poorer (AUC < 0.8) test performance when they were considered individually, which supports the idea that incorporating activity and rumination can allow earlier mastitis detection (Stangaferro et al., 2016). The greater activity change in the SCM group than in the TN and AP groups contrasted with the lower activity of the clinical mastitis cows with severe damage to the udder reported by Stangaferro et al. (2016). This discrepancy could be related to different levels of severity of mastitis in each study (Schukken et al., 2011). In contrast, the similar activity change between the TN and SCM groups (except Day 3) might be due to lower DIM (117 vs. 180) in the TN group, as early

lactating cows spent more time eating to meet the nutritional needs of higher milk production (Chaplin and Munksgaard, 2001; Bewley et al., 2010). Despite the high levels of classification for our best model, there were up to 27 false alerts in the herd of 300 cows (9%), which does not fulfil the International Organization for Standardization (ISO, 2017) standard Sp (≥ 99%) recommended for automatic discarding of abnormal milk (Mein and Rasmussen 2008; Sherlock et al., 2008). In this regard, better clinical mastitis prediction is possible with ≥ 90% Se and Sp when milk yield, milk flow and incomplete milking are incorporated with EC (Khatun et al., 2018). We postulate that incorporating activity and rumination information with EC, milk yield, milk flow, incomplete milking and additional immune-related information may result in earlier and more accurate SCM detection in AMS. Our approach could be a cost-effective tool to detect affected cows in a pasture-based farm with minimal farmer–cow contact as the activity and rumination sensors are already incorporated in the AMS for heat detection.

CONCLUSIONS

We developed an approach for automatic detection of SCM in AMS by utilising EC, activity and rumination changes, on specific days before detection of SCM. Overall, our study found that activity and rumination changes add additional prediction strength to EC for SCM detection.

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Supplementary material

Treatment Groups

Of the 350 lactating cows, 170 cows were enrolled in the study based on the criteria of having EC, activity and rumination data for the three-week study period.

In all 169 cows, data values that were identified with increased activity due to oestrus during the three-week study period were deleted.

Each predictor variable was also assessed independently by creating a ROC curve and calculating the area under the curve (AUC) value by using the AUC package (Ballings and Poel, 2013). The generated AUC values from the ROC curves were classified as excellent (0.9–1.0), good (0.8–0.9), fair (0.7–0.8), poor (0.6–0.7) and fail (0.5–0.6), with values under 0.5 worse than random classification (Swets, 1988, http://gim.unmc.edu/dxtests/roc3.htm).

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CHAPTER 7: GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION

The primary purpose of this final chapter is to integrate the knowledge generated through each of the chapters, highlight those areas that require further research, and outline the key conclusions of these investigations. The overarching aim of this thesis was to improve the accuracy and timing of mastitis detection in AMS using inline sensor-derived data and other data-based available information. To accomplish this, it became apparent that this would be best achieved by combining milking-related and behavioural change-derived data collected through inline and on-cow sensors via various algorithms. Although integrating it all together was beyond the scope of this thesis, the success of these individual approaches can be assessed by evaluating to what extent they succeed in improving sensitivity (**Se**) and specificity (**Sp**) of mastitis detection in AMS. About six years of inline sensor-derived data (particularly electrical conductivity, milk yield, milk flow, number of incomplete milking or kick-off, daily activity and daily rumination time) of two pasture-based commercial dairy farms, together with original, controlled field experiments, were used in this study for improved Se and Sp of mastitis detection. It is also important to detect mastitis at the earliest possible time. Thus, about four-week' data prior to mastitis detection were explored to find the earliest time window for better mastitis detection by multiple sources of data.

Based on the extensive literature review (Chapter 2), it was confirmed that utilising multiple-inline sensor-derived information provides 'cutting-edge' guidance to improve mastitis detection performance (Hogeveen et al., 2010; Mollenhorst et al., 2010). Because all AMS farms are different in size, geographical location, physical and human resources and management aspects, there is a need for an easily interpretable system for routine application to detect mastitis with particular focus at quarter level (Kamphuis et al., 2008; Mollenhorst et al., 2010; Russell and Bewley, 2013). A contribution of this thesis was the development of an

"index" approach based on multiple sources of data (integrated by algorithms) to monitor mastitis automatically in AMS. The indices generated in this thesis were based on single inline sensor data (integrated by multiple algorithms, Chapter 3) or multiple inline sensors (combined by a single algorithm developed for a multivariable model, Chapter 5). Moreover, better suitability of the discarded foremilk samples to detect mastitis with the promising possibility of differentiating type of mastitis (Chapter 4) and mastitis prediction using behavioural change related data were also discussed (Chapter 6).

Chapter 3 demonstrated the advantage of the algorithms to improve mastitis alert based on single inline (e.g., EC) sensor-derived data (Khatun et al., 2017). For example, by means of algorithms, 16% to 21% greater Se was achieved at the same level of accuracy (80%), compared to a non-algorithm based single threshold. The major limiting factors of EC measuring inline sensors to reach the International Organization for Standardization (ISO, 2007) target of Se > 70% and Sp > 99% were the influences of milk temperature, milking interval, milk fractional variation (Fernando et al., 1982; Nielen et al., 1992; Ontsouka et al., 2003). This is why, despite exploration (EC as a stand-alone inline mastitis indicator) of the potential algorithms or options such as six different thresholds, > 250 algorithms-based indexes, and statistical process control, the achieved Se and Sp were not satisfactory. Currently, there is no consistent single EC threshold to be used by herd managers as a large number of false positive alerts are produced at lower thresholds or more missed mastitic cases (false negatives) at higher thresholds. Hence, in pasture-based AMS where cows spend most of the time in pasture land, it might be worth checking the cows while milking in the AMS to reduce the false positive or false negative cases.

The influence of milk fractions like milk sample before ejection was greater on EC measurement during Gram-negative *Escherichia coli* lipopolysaccharide experimentally induced mastitis (Bruckmaier et al., 2004b). Hence, how EC and other mastitis indicators

such as somatic cell count (**SCC**), lactate dehydrogenase (**LDH**) vary during naturally-occurring mastitis caused by both Gram-positive and Gram-negative pathogen needed to be investigated. In Chapter 4, strict foremilk before and after alveolar ejection collected before versus after udder stimulation to induce alveolar ejection, were compared to identify the better suitability of the milk fractions (differed in EC, somatic cell count, lactate dehydrogenase, milk protein and milk fat) to detect mastitis. Result from fitting logistic mixed models indicated that strict foremilk before alveolar ejection had better mastitis detection ability with ~ 4% greater Sp than the foremilk after ejection. Hence, based on the research findings reported in Chapter 4, a recommendation arising from this thesis is a modification of the current inline sensor measurement technique in AMS to capture strict foremilk rather discarding it during the cleaning process. This has the potential to increase accuracy of mastitis detection in AMS. Further investigation in Chapter 4 also revealed that strict foremilk could be used to differentiate Gram-positive mastitis from Gram-negative mastitis particularly by monitoring LDH concentration (sensor for LDH is already available in DeLaval's Herd Navigator^{TM}). The suitability of LDH for differentiating type of mastitis was also observed in a previous study (Hernández-Castellano et al., 2017). However, in both Hernández-Castellano et al. (2017) and in our studies there was a limited sample size to ascertain the robustness of the method.

As mastitis is associated with multiple changes in cows' physiology, Chapter 5 has focused on how multiple milking-related data could be used to improve mastitis detection performance in AMS. In this chapter, retrospective data (three data sets) from two pasture-based dairy farms were used to generate a multivariable "single index". The multiple variables incorporated into the index were EC, EC per hour, milk yield, milk yield per hour, mean milk flow rate, and number of incomplete milkings. Using this multiple data-based index, 6% greater Se and 13% greater Sp were achieved compared to the commonly used EC

measuring single sensor data. This index also exploited the usefulness of previous history of mastitis occurrence to identify mastitis at the quarter level. The further advantage of this index was that a single measure would be more useful to herd managers rather than them having to interpret multiple sources of data. In this study we use the AUC value generated from the ROC curve as an objective method to assess diagnostic test performance, an important consideration in the early stages of diagnostic test development (Halligan et al., 2015). One disadvantage of AUC of an ROC curve is that it ignores the trade-off between Se and Sp and a choice made on specific values of Se and Sp may be more appropriate for comparing between alternative diagnostic procedures (Halligan et al., 2015). To address this situation, we have used the Youden index, which takes into account specific values of Se and Sp, particularly the trade-off between them, in addition to the AUC value itself. The Se (90%) and Sp (91%) generated by this study have been calculated based on equal cost of false negatives and false positives, i.e. the point on the ROC curve when the Youden's index, J = Se + Sp – 1, is maximised (Ruopp et al., 2008). By altering the threshold, it may be possible to attain values closer to the ISO standard of a very high Sp of at least 99% and a moderate Se of 70% set for automatic discard of abnormal milk (Mein and Rasmussen, 2008; Sherlock et al., 2008). However, the cost for missing 30% of mastitic cows (false negatives) might be equally or more harmful than screening of these false positives. Hence, we might need to revise the current ISO recommendations and accept to increase the Se beyond 70%; this will come at the expense of more false positive alerts (lower Sp). The index generated in this study might be useful in such and to keep the AMS viable and more attractive. However, further improvement of such an index approach is possible by incorporating immunological data as concluded in Chapter 5. Until Chapter 5, the focus was only on using milking-related sensor data. However, being a multifactorial disease, mastitis is also responsible for animal behaviour changes. Such behavioural changes like daily activity and daily rumination time

had merit to predict mastitis according to a previous study (Stangaferro et al., 2016). In our investigation, we found that the SCM prediction ability of behavioural changes was greater when combined with milking-related data, for example EC data (Chapter 6). By combining EC with daily activity and daily rumination time, 24% to 42% (average 31%) greater Se and 11% to 27% greater Sp (~20%) were achieved than any of the individual sensors data. Thus, incorporation of behavioural changes along with other milking related data like milk yield, milk flow rate, incomplete milking (as incorporated in Chapter 5) as well as immunological change-related data like LDH, might allow us to generate a better SCM or clinical mastitis detection "index" with improved Se and Sp. Such an index to detect SCM or clinical mastitis will reduce the long-term production loss, prevent chronic mastitis and thereby reduce antimicrobial resistance. Currently, MDi, an index incorporating EC, blood in milk, milking interval (unpublished metrics) has been introduced by DeLaval but the performance is not satisfactory because of the influence of several factors on EC, blood in milk and milking interval. Developing an index incorporating diversified sources of information relating to milking and behavioural change will minimise the limitation of the influencing factors. Such an index might then be of practicable use to DeLaval or other AMS operators. This, however, will be the focus for future research, as compiling all individual findings into a fully integrated model or tool was outside the scope of the present research program. Moreover, there are also opportunities for installing additional remote sensing behaviour monitoring equipment such as infrared sensing technology, abnormal gait and posture by video recording that could be useful for better index development. Future research to develop such improved index will likely enhance the attractiveness of the AMS technology in the future.

CONCLUSIONS

The first specific objective of this thesis was the quantification of Se and Sp of single inline sensor (EC) data by algorithms. By using different algorithms, Se and Sp was improved to some extent but not at satisfactory level (ISO standard). Hence, we concluded that EC data alone cannot provide the required accuracy for viable and accurate solution of mastitis detection in AMS herds. Hence, further improvement was required and integration of multiple sources of data was hypothesised to be a possible solution.

The second specific objective was to demonstrate the suitability and comparative ability of strict foremilk compared to milk after ejection, to detect mastitis. In parallel this study also investigated the Gram-positive and Gram-negative mastitis differentiation capacity. In summary, foremilk before ejection was more sensitive than foremilk harvested after milk ejection, which is induced by udder preparation including teat cleaning in AMS systems. Overall, our results suggest that, in the future, sensors could be modified to monitor milk removed before teat cleaning to improve the ability of the system to detect mastitis in AMS. This is an original and innovative recommendation arising from this thesis.

The third objective was exploration of the mastitis detection ability of different sources of milking-related inline sensor data. Apart from EC, other useful milking related inline sensor data found in this study were milk yield, milk flow rate and number of incomplete milking when assembled into a multivariable index. The generated multivariable index was suited for estimation of the quarter-, cow- and herd-level mastitis alarm and resulted in much better Se and Sp than any of the single sensor data. However, further improvement might be possible by integration of immunological and behavioural changes related data.

The last objective was to investigate the mastitis prediction ability of using behavioural change-related data such as daily activity and daily rumination time captured by activity and rumination on-cow sensors. Overall, daily activity and daily rumination behavioural changes

add additional prediction strength to EC mastitis-related change with at least 13% higher Se and Sp.

Although integrating all the above individual findings into a new tool or detection system was outside the scope of this thesis, it is clear from this research that the potential of such tools or system (which would combine several sources of sensor-derived data with advanced statistical modelling as well as aspects of animal behaviour) to detect mastitis earlier and with increased accuracy would be substantially increased. This thesis, therefore, not only makes substantial and original contributions to research, but also paves the road for future developments and practical application of enhanced methods for automated mastitis detection. Whilst this thesis findings were based on pasture-based AMS, the generated knowledge could be also useful for AMS in general and also for conventional farms for improved mastitis detection. The overall findings will help dairy herd managers, researchers and consultants in their search for, and development of, early and more accurate mastitis diagnosis, with the associated benefits arising from improved animal welfare, reduced antibiotic use and losses in milk production, and improved economic return.

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