INVESTIGATING ALTERNATIVE METHODS TO DETECT BOVINE MASTITIS IN MILK

by

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DISSERTATION SUMMARY

The aim of this study was to investigate alternate measures for the diagnosis of bovine mastitis, which can either be done separately or coupled with the current use of somatic cell counts. Techniques that were assessed include measurement of pH, electrical conductivity (EC) and volatile organic compounds (VOCs) liberated by pathogens during metabolism in milk; the quantification of milk components (fats, whey proteins, lactose, caseins), and cell counts of *Staphylococcus aureus*. Various concentrations of *S. aureus* were used to assess the minimum bacterial inoculum level that could bring about detectable changes in the pH and EC values of milk. It was found that 10^{-2} diluted inoculum caused less changes in pH and EC compared to the undiluted bacterial inoculum size. On average, the pH of milk samples decreased from 6.45 to 5.31 after 32 hours of incubation at 37˚C. A corresponding EC value increased from 5.28 mS cm^{-1} to 6.68 mS cm^{-1} was observed due to the liberation of sodium and chloride ions during the incubation of inoculated milk after an incubation of 32 hours. The detected VOCs including compounds from hydrocarbon, ester, ketone, aldehyde and siloxane groups were observed from milk inoculated with common mastitis pathogens such as *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus agalactiae*, coagulase negative staphylococci (CNS) and *Escherichia coli*. Only 20% of a total of 50 inoculated samples released VOC's. Furthermore, the VOCs identified were not species-specific. However, by comparing the samples to a control (un-inoculated sample), the identified VOCs could be used as a rough monitoring tool to distinguish inoculated milk from un-inoculated milk. Near-infrared analysis (NIRA) was carried out using the Kernel partial least squares regression. However, the calibration models for milk composition and *S. aureus* were poor. We believe that this was affected by the technique used, measuring the NIR absorbance of milk samples in plastic Petri dishes. The absorptive abilities of polystyrene present in Petri dishes affected the NIRS scans. Secondly, insensitive wet chemistry methods, and the low sample number used in this study were concluded to be the major reasons for the poor predictive models that were obtained for the analysis of milk components and *S. aureus*. These analytic tools showed potential as diagnostic methods, however, further research must be conducted to solve these problems.

DECLARATION

I, Mduduzi Hendrick Shinga, declare that;

i. The research reported in this dissertation, except where otherwise indicated, is my original work.
ii. This dissertation has not been submitted for any degree or examination at any other university.
iii. This dissertation does not contain other persons' data, pictures, graphs or information, unless acknowledged as being sourced from other persons.
iv. This dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
    a. Their words have been re-written but the general information attributed to them has been referenced.
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v. Where I have produced a publication of which I am an author, co-author or editor, I have indicated in detail which part of the publication was written by myself alone and have fully referenced such publications.
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TABLE OF CONTENTS

Dissertation Summary **1**

Declaration **3**

ACKNOWLEDGEMENTS **4**

DISSERTATION INTRODUCTION **7**

REFERENCES 10

CHAPTER 1: **12**

Literature Review **12**

1. INTRODUCTION 12
2. TYPES OF MASTITIS 14
   2.1 Contagious Mastitis *15*
   2.2 Environmental Mastitis *17*
   2.3 Epidemiology of Contagious and Environmental Pathogens *19*
3. THE ECONOMIC IMPACT OF MASTITIS 20
4. PRINCIPAL MILK COMPONENTS 21
   4.1 Somatic Cells *21*
   4.2 Fats *22*
   4.3 Proteins *23*
   4.4 Lactose *24*
   4.5 Volatile Organic Compounds *24*
   4.6 Minerals *25*
5. NEAR-INFRARED SPECTROSCOPY 26
   5.1 NIRS Principles *27*
   5.2 NIRS Calibration *28*
   5.3 NIRS Validation *30*
   5.4 Previous NIRS Applications on Milk *30*
   5.5 Reference Methods *34*
6. DISCUSSION 36

REFERENCES 37

CHAPTER 2: **50**

Changes in pH and Electrical Conductivity (EC) of Milk Inoculated with *Staphylococcus aureus*, causal agent of bovine mastitis **50**

ABSTRACT 50

2.1 INTRODUCTION 51

2.2 MATERIALS AND METHODS 53

2.2.1 Culture Preparation and Milk Inoculation *53*

2.2.2 Monitoring of pH and EC *53*
2.3 RESULTS 54
2.3.1 Changes in pH and EC of milk with different S. aureus Inoculum Doses *54*
2.3.2 Changes in the pH and EC of Milk Inoculated with Bacillus spp. and a mixture of Bacillus spp. and S. aureus. *57*
2.4 DISCUSSION 60
REFERENCES 62

CHAPTER 3: 65

Gas chromatography-mass spectrophotometry Analysis of Volatile Organic Metabolites detected in Milk samples Inoculated with Mastitis Pathogens 65

ABSTRACT 65
3.1 INTRODUCTION 66
3.2 MATERIALS AND METHODS 67
3.2.1 Simulating mastitis *67*
3.2.2 GC-MS analysis *67*
3.2.3 Analysis of Data *68*
3.3 RESULTS 68
3.4 DISCUSSION 73
REFERENCES 75

CHAPTER 4: 77

Near-infrared analyses of the chemical composition of milk samples, and for the presence of *Staphylococcus aureus* 77

ABSTRACT 77
4.1 INTRODUCTION 78
4.2 MATERIALS AND METHODS 80
4.2.1 Milk Samples *80*
4.2.2 Spectral Acquisition *80*
4.2.3 Wet Chemistry *80*
5.3 RESULTS 84
4.4 DISCUSSION 89
REFERENCES 92

DISSERTATION Overview 95

INTRODUCTION 95
MAJOR FINDINGS AND THEIR IMPLICATIONS 96
CONCLUSION AND WAY FORWARD 98
REFERENCES 98

Appendix 1 100

DISSERTATION INTRODUCTION

Bovine mastitis is the most prevalent and costly disease affecting dairy industries across the world. Bovine mastitis results from the inflammation of the parenchyma in the mammary gland of dairy cattle following microbial infection (Blowey and Edmondson 2010; Mehmeti *et al.* 2016). Physical and chemical changes in milk, and pathological changes in the mammary glands are typical characteristics of bovine mastitis (Sharma, 2007). However, the severity of the disease is dependent on the causal pathogen and the breed, age, immunological health and lactation stage of the infected dairy cattle (Viguier *et al.* 2009). The severity of mastitis can be classified into two main forms: subclinical and clinical mastitis, with subclinical mastitis having major economic implications due to the lack of visible symptoms and therefore difficulty with detection (Viguier *et al.* 2009). Clinical mastitis is characterized by redness of the mammary gland and organoleptic changes in milk (Cvetnić *et al.* 2016). The incurred economic losses result mainly from decreased milk quality and quantity, discarded milk, veterinary and drug services, diagnostics and increased risks of culling (Hogeveen *et al.* 2011).

There are over 200 microorganisms that have been reported to cause mastitis, including algae, bacteria, chlamydia, mycoplasmas, fungi and viruses (Marques *et al.* 2006)**.** Of these different microorganisms, *Staphylococcus aureus*, coagulase-negative *Staphylococcus* (CNS), *Escherichia coli*, *Streptococcus dysgalactiae*, *Streptococcus agalactiae* and *Streptococcus uberis* are the most commonly isolated bacteria from mastitic milk from infected dairy animals and account for approximately 80% of all mastitis cases (Blowey and Edmondson 2010). Depending on the primary source and transmission of the causal pathogen, mastitis can be contagious (with the causal agents usually being *S. aureus*, *St. dysgalactiae* and *St. agalactiae*) or environmental (causal agents including *St. uberis*, and coliform bacteria such as *E. coli* and other *Enterococus* spp.) (Cvetnić *et al.* 2016; Gomes and Henriques, 2016).

In the light of increasing antibiotic resistance in the common mastitis pathogens, particularly *S. aureus* (Vanderhaeghen *et al.* 2010), effective diagnostic methods to detect bovine mastitis at early stages are essential for the management of the

disease before it becomes problematic. Current diagnostic methods rely primarily on an indirect measurement of the effect of pathogens on the host animal, resulting in enhanced somatic cell counts. Understanding the process by which mastitis develops is of paramount importance in the development of effective diagnostic techniques (Viguier *et al.* 2009).

Bacteria invade the udder through a dilated teat canal, and once inside, the bacteria produce toxins that stimulate leukocyte and milk secretory epithelial cells to produce chemo-attractants that attracts mostly polymorphonuclear neutrophils (PMNs) to the site of infection (Sharma *et al.* 2011). PMNs try to combat the invading bacteria by engulfing them and releasing oxidants and proteases. However, in the process, some epithelial cells are destroyed, resulting in reduced milk yields (Sharma *et al.* 2011). Putrefaction of milk results when PMNs activity also consumes fat globules and caseins in the milk (Opdebeeck, 1982). When PMNs have completed their task, they die off through apoptosis, and the dead PMNs remain in the milk, giving rise to the observed increased somatic cell count (SCC) (Zhao and Lacasse, 2008). If the infection persists and the mammary epithelium sustains continued damage, then the blood-milk barrier may be breached, causing blood to mix with milk (Sordillo and Streicher, 2002). An influx of sodium and chloride ions into the milk then results in an increase in the pH and EC of milk, and also an increase in bloodborne lipases and proteases, resulting in further degradation of caseins and fats in milk, and increased odors (Viguier *et al.* 2009).

The most common diagnostic techniques used to detect mastitis in most parts of the world, including South Africa, are based on determinations of the SCC, a milk clotting evaluation and enzymatic analyses of N-acetyl-b-D-glucosaminidase and lactate dehydrogenase (Pyörälä, 2003). These diagnostic techniques include the California mastitis test, Portacheck, fossomatic SCC and De Laval cell count (Viguier *et al.* 2009). Despite SCC based techniques being relatively inexpensive, they can be affected by various factors, which can make the SCC results difficult to interpret (Viguier *et al.* 2009). SCC is influenced by:

1. The severity of the mastitis: the nature and extent of the PMNs response is likely to be proportional to the scale of damage caused by bacterial infection (Dohoo and Meek, 1982);

2. The stage of lactation: a cow's immune system will produce more white blood cells when it is about to give birth to a calf, and thus there is an observed increase in PMN's, particularly neutrophils, at early and late lactation (Reichmuth, 1975);
3. Age: there is an increased prevalence of infection with age, and hence there is an observed increase in PMNs in older cows compared to younger cows (Sharma *et al.* 2011);
4. Breed of the cow: Singh (2002) showed that breed also affects the SCC because some breeds generate a higher SCC than other breeds, due to differences in immunological health, regardless of the absence or presence of infection;
5. Season: Microorganism populations tend to increase during summer to due favorable conditions for bacterial multiplication, and accordingly there are usually higher SCC scores in summer than in winter (Khate and Yadav, 2010).

Considering the limitations of SCC-based techniques, the primary aim of this study was to evaluate other diagnostic techniques based on the changes in pH, EC, fats, lactose, whey proteins, volatile organic metabolites (VOC's), caseins and the microbial population, in order to develop an integrated analytical tool that is simple to use and inexpensive, but which is more specific and sensitive, even at early infection stages, and which can be used for "on-site" measuring in any dairy. Therefore, the objectives of this study were as follows:

1. A literature review on bovine mastitis and the causal pathogens, in order to understand the basis of diagnostic methods; to review the current used SCC based diagnostic method and its limitations; and to review alternative diagnostic methods based on pH, EC, fats, proteins, lactose and volatile organic metabolites that might be used as alternatives or in conjunction with the SCC based diagnostic methods.
2. To monitor the changes in pH and EC of *S. aureus*-inoculated milk in order to evaluate the changes with incubation time.
3. To identify the VOC's associated with the most common bacteria causing mastitis, using a GC-MS instrument. The goal was to determine whether the VOC's could be used to differentiate between inoculated and un-inoculated milk, and to then assess whether the VOC's profiles were distinct enough to allow for species differentiation among the causal pathogens.

4. To develop diagnostic models for constituents of milk using near infra-red NIR analysis, correlated with “wet chemistry” methods for levels of fat, lactose, whey protein, caseins and bacterial counts. The goal was to develop accurate NIR models that could be used to estimate the commonly measured biochemical components, and the microbial population in milk inoculated with *Staphylococcus aureus,* while concurrently monitoring other parameters such as pH and EC.

This dissertation is composed of a literature review and three experimental chapters, each evaluating different parameters that can be used to detect bovine mastitis in dairy cattle. The dissertation is in the form of discrete research papers, with each chapter following a format endorsed by the University of KwaZulu-Natal. Each chapter is presented as a stand-alone research paper in order to facilitate publishing of material in this dissertation. As a result, there may be some repetition of references and introductory material among the chapters.

References

1. **Blowey, R. W. and Edmondson, P.** (2010). Mastitis Control in Dairy Herds, 2nd Ed. CABI, Wallingford, Oxfordshire, UK.
2. **Cvetnić, L., Samardžija, M., Habrun, B., Kompes, G., and Benić, M.** (2016). Microbiological monitoring of mastitis pathogens in the control of udder health in dairy cows. *Slovenian Veterinary Research* **53:** 130-140.
3. **Dohoo, I. R., and Meek, A. H.** (1982). Somatic cell counts in bovine milk. *The Canadian Veterinary Journal* **23:** 119.
4. **Gomes, F., and Henriques, M.** (2016). Control of bovine mastitis: old and recent therapeutic approaches. *Current Microbiology* **72:** 377-382.
5. **Hogeveen, H., Huijps, K., and Lam, T. J. G. M.** (2011). Economic aspects of mastitis: New developments. *New Zealand Veterinary Journal* **59:** 16-23.
6. **Khate, K., and Yadav, B. R.** (2010). Incidence of mastitis in Sahiwal cattle and Murrah buffaloes of a closed organized herd. *Indian Journal of Animal Sciences* **80:** 467-469.
7. **Mehmeti, I., Behluli, B., Mestani, M., Ademi, A., Nes, I. F., and Diep, D. B.** (2016). Antimicrobial resistance levels amongst staphylococci isolated from

clinical cases of bovine mastitis in Kosovo. *The Journal of Infection in Developing Countries* **10:** 1081-1087.

8. **Marques S., Silva, E., Carvalheira, J., and Thompson, G.** (2006) Short communication: In vitro antimicrobial susceptibility of *Prototheca wickerhamii* and *Prototheca zopfii* isolated from bovine mastitis. *Journal of Dairy Science* **89:** 4202-4204.
9. **Opdebeeck, J. P.** (1982). Mammary gland immunity. *Journal of the American Veterinary Medical Association* **181:** 1061-1065.
10. **Pyörälä, S.** (2003). Indicators of inflammation in the diagnosis of mastitis. *Veterinary Research* **34:** 565-578.
11. **Reichmuth J.** (1975). Somatic cell counting – Interpretation of results. In Proc. Of Sem. On Mastitis. Cont. IDF DOC. 85, pp 93 – 109.
12. **Sharma, N.** (2007). Alternative approach to control intramammary infection in dairy cows: A review. *Asian Journal of Animal and Veterinary Advances* **2:** 50-62.
13. **Sharma, N., Singh, N. K., and Bhadwal, M. S.** (2011). Relationship of somatic cell count and mastitis: An overview. *Asian-Australasian Journal of Animal Sciences* **24:** 429-438.
14. **Sordillo, L. M., and Streicher, K. L.** (2002). Mammary gland immunity and mastitis susceptibility. *Journal of Mammary Gland Biology and Neoplasia* **7:** 135-146.
15. **Vanderhaeghen, W., Cerpentier, T., Adriaensen, C., Vicca, J., Hermans, K., and Butaye, P.** (2010). Methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. *Veterinary Microbiology* **144:**166-171.
16. **Viguier, C., Arora, S., Gilmartin, N., Welbeck, K., and O'Kennedy, R.** (2009). Mastitis detection: current trends and future perspectives. *Trends in Biotechnology* **27:** 486-493.
17. **Zhao, X., and Lacasse, P.** (2008). Mammary tissue damage during bovine mastitis: causes and control. *Journal of Animal Science* **86:** 57-65.

CHAPTER 1:
LITERATURE REVIEW

1. Introduction

Mastitis (*mast*-breast and *itis*-inflammation) is a disease defined by the inflammation of the parenchyma of the mammary gland following bacterial infection (Wellenberg *et al.* 2002). Bovine mastitis is among the most important diseases affecting the world's dairy industry due to the heavy economic burden it creates for farmers (Hogeveen *et al.* 2011). Bacteriological, chemical and physical changes in milk and pathological changes in glandular tissues are some of the characteristics of mastitis (Sudhan and Sharma 2010). The symptoms of mastitis, however, primarily depend on the causal pathogen and properties of the host such as breed, age, immunological health and lactation stage (Blowey and Edmondson 2010).

There are more than 200 microorganisms that have been reported to cause mastitis; these include algae, bacteria, chlamydia, mycoplasmas, fungi and viruses (Watts 1988, Wellenberg *et al.* 2000, Malinowski *et al.* 2005). Bacterial pathogens are however, more prevalent than other causal microorganisms, accounting for 90-95% of all mastitis cases around the world (Viguier *et al.* 2010). Of the bacterial pathogens, *Staphylococcus aureus*, coagulase-negative staphylococci (CNS), *Escherichia coli*, *Streptococcus dysgalactiae*, *Streptococcus agalactiae* and *Streptococcus uberis* cause approximately 80% of mastitis cases (Barkema *et al.* 1998, Bradley 2002, Sudhan and Sharma 2010). Pathogens can be classified into either contagious pathogens, such as *S. aureus* and *St. agalactiae*, or environmental pathogens which is usually *E. coli* (Blowey and Edmondson 2010).

Once mastitic pathogens have invaded the interior of the mammary glands, they cause tissue injury due to toxins they produce (Sudhan and Sharma 2010) as it can be seen in Figure 1. Mastitis results in a substantial reduction in milk yields, a reduction in casein levels of up to 20%, and reduced lactose and fat content (Blowey and Edmondson 2010). Furthermore, infection leads to increased levels of somatic cells, lipase and plasmin, and sodium and chloride ions, which lead to the characteristic sour taste of mastitic milk (Blowey and Edmondson 2010).

Farmers need to identify mastitic milk, and the cows that produced it. Various methods for quantitative and qualitative analysis of milk components have been developed to diagnose mastitis (Norberg 2005). Somatic cell count (SCC) counters, protein assays, and spectral milk fat estimation and lactose determination have been used to diagnose mastitis (Norberg 2005). New methods have been developed as alternative diagnostic methods for the many advantages they offer (Barbano and Lynch 2006). These include speed, accuracy, sensitivity and the ability to be used on-site in commercial dairy production plants (Norberg *et al.* 2004). One such technique is near-infrared (NIR) spectrophotometry, which is an analytical technique with the ability to reliably and simultaneously monitor protein, lactose and fat contents of milk (Barbano and Lynch 2006).

This review evaluates the microbiology of mastitis, with discussions on the traditional diagnosis methods above, but primarily focusses on NIR analysis as a more practical diagnostic approach in a commercial dairy production environment.

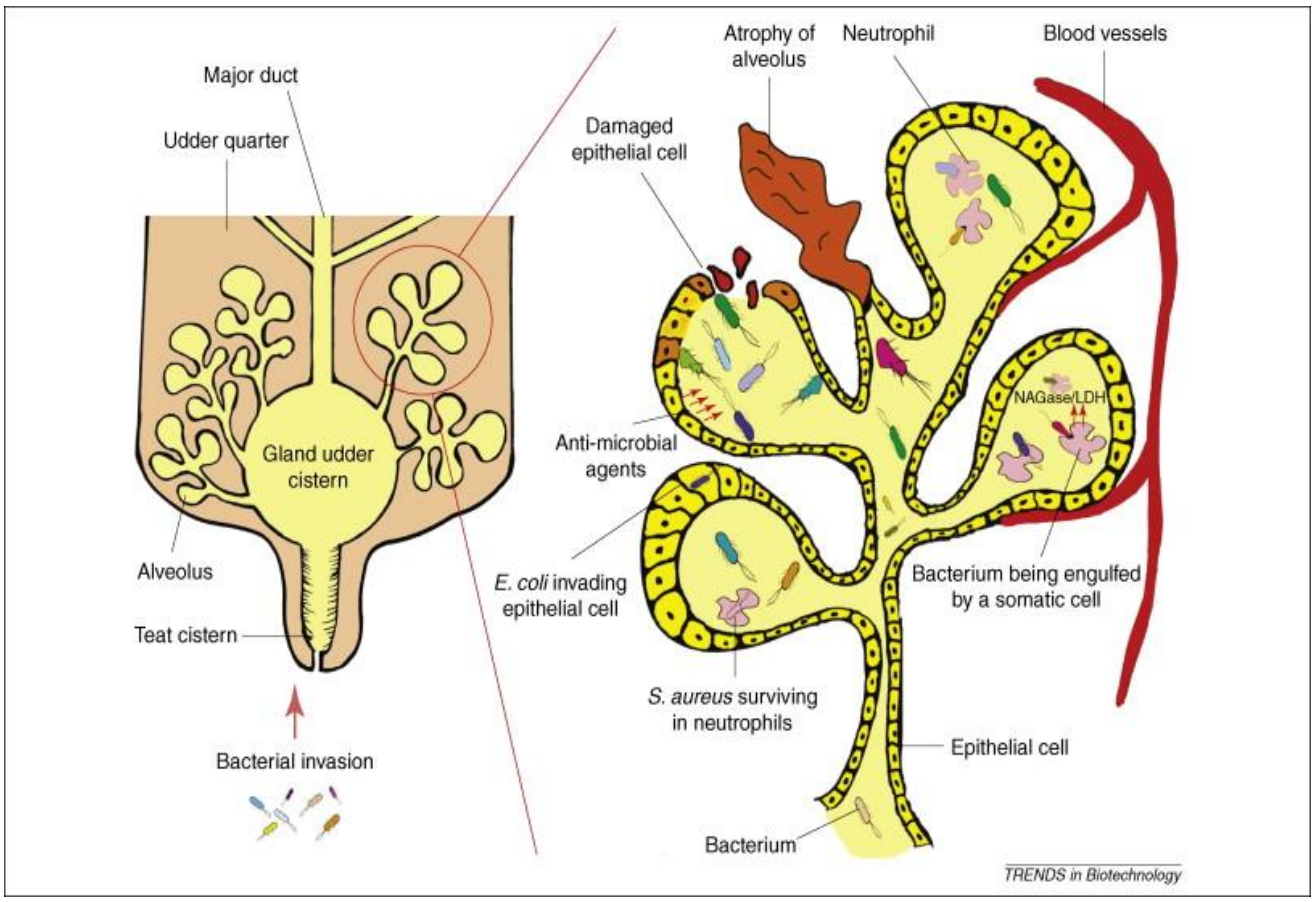

Figure 1: A simplified representation of mastitis in a mammary gland quarter upon infection (Viguier *et al.* 2010)**.**

2. Types of Mastitis

A healthy, non-infected cow usually has SCC value below $1.5x10^5$ cells ml^{-1}, bacterial infection can cause an SCC increase of above 10^6 ml^{-1}, whereas during mastitis, SCC values often reach above $2x10^6$ cells ml^{-1} (Leigh 1999; Bytyqi *et al.* 2010). Subclinical mastitis is the most common form of the disease. At this stage, there are no visible changes in milk appearance or the infected udder (Blowey and Edmondson 2010). It is characterized by milk compositional changes of lactose, electrolytes, casein and fat, changes in the SCC, the presence of bacteria in the milk and reduced milk yields (Ferrero *et al.* 2014). Such changes are also observed in clinical mastitis. However, clinical symptoms such as the formation of blood clots, hardness and swelling of the udder have also been reported (Oliver *et al.* 2004). Clinical mastitis can be further subdivided into *acute,* which is sudden in onset, and *chronic,* which persists for a long time (Blowey and Edmondson 2010).

Mastitis can be subdivided according to causal pathogens involved and their epidemiology, i.e., contagious and environmental mastitis types (Contreras and Rodríguez 2011). Understanding which pathogen is affiliated with a cow is important in knowing which control or future prevention measures to use (Blowey and Edmondson 2010). Mastitis incidence caused by different major pathogens in the UK in 1968, 1995 and 2007 are compared in Table 1. There was a decline in the overall incidence of clinical mastitis from 121 cases per 100 cows in year 1968, to 50 cases per 100 cows in year 1995 and 47 cases per 100 cows in year 2007. This observed decline was mainly because of control measures such as post-milking teat disinfection, dry cow therapy and culling of contagious mastitis infected cattle by UK dairy farmers (Blowey and Edmondson 2010). In South Africa, there was a reported 8.1 % prevalence of mastitis in 2002 which escalated to 15.4% by 2006 (Petzer et al., 2009a). Although Table 1 indicates a considerable increase of environmental cases between 1968 and 1995, this was due to a decrease in the number of contagious cases rather than to a rise in the actual number of environmental cases, and as research became more focused on environmental cases (Tenhagen *et al.* 2006, Blowey and Edmondson 2010).

Table 1: A mastitis survey results for the UK, showing the prevalence (%) of contagious and environmental pathogens in the years 1968, 1995 and 2007 (Blowey and Edmondson 2010).

	Year		
Pathogens type	**1968**	**1995**	**2007**
Coliforms (EP)[1]	5.4	26	19.6
Streptococcus agalactiae (CP)[2]	3	0	0
Staphylococcus aureus (CP)	37.5	15.4	3.3
Streptococcus dysgalactiae (CP)	20.1	10.8	1.5
Streptococcus uberis (EP)	17.7	32	23.5
Others	16.3	15.8	0
No. of cases per cow per year	121	50	47

[1]- EP indicates environmental pathogen and [2]-CP indicates contagious pathogen.

2.1 Contagious Mastitis

Contagious causal pathogens live and multiply on the animal's teat skin and udder and are well adapted to this environment (Watts 1988). Because of adaptation to surviving on the skin of the teat and udder, they can cause an infection that is long lasting (Blowey and Edmondson 2010). The infected mammary gland is the main source of contagious pathogens and transmission of contagious pathogens occurs primarily at milking time (Oliver *et al.* 2004). The most common contagious causal pathogens are *Staphylococcus aureus,* coagulase-negative staphylococci (CNS), *Streptococcus agalactiae and Streptococcus dysgalactiae* (Watts 1988, Blowey and Edmondson 2010).

S. aureus is a Gram-positive coccus that appears as creamy yellow colonies on blood agar plate and produces clear zones around its colonies, showing haemolytic ability. Most *S. aureus* strains agglutinate (form a clot) with rabbit serum, thus in a coagulase test, *S. aureus* tests positive (Kateete *et al.* 2010). The primary infection source of *S. aureus* is the teat and udder skin. After infection *S. aureus* is considered to be the most difficult mastitic pathogen to eradicate. It has been reported that cure rates of *S. aureus*-induced clinical and subclinical mastitis by cloxacillin may be as low as 25% and 40%, respectively. A reason for such poor response to antibiotic therapy in general, despite the known acquired resistance, is the lack of penetration by antibiotics into the sites of infection. This low penetration of antibiotics is due to *S. aureus* ability to live within white blood cells and epithelial cells. After damaging the

duct system, *S. aureus* is often shielded by fibrous scar tissue that results after tissue damage, which further complicates treatment by antibiotics. *S. aureus* infections can be avoided by strict hygiene during milking, and levels of infection can be reduced by dry cow therapy (Blowey and Edmondson 2010).

S. aureus infections damage epithelial cells, often resulting in a 15% reduction in milk production per infected cow. During mastitis caused by *S. aureus*, milk cultures from an infected cow generally do not show a high *S. aureus* microbial load. This is due to their ability to live within white blood cells, and the formation of abscesses during the early stages of infection. An SCC greater than 300,000 - 500,000 cells ml^{-1} in bulk milk is a good indication of a high prevalence of *S. aureus* infection in herds (National Mastitis Council, 2017a). Petzer *et al.* (2009a) reported a lower *S. aureus* prevalence (17.28 and 16.98% at dry and lactation periods) of mastitis cases in South Africa between the years 1996-2007 as shown in Table 2. However, *S. aureus* remains the principal pathogen causing mastitis, and causes the greatest costs due to the chronic and destructive nature of the mastitis cases it causes (Petzer *et al.* 2009a).

St. agalactiae is a Gram-positive, haemolytic coccus that usually colonizes the udder, although it can also be found on teat skin and in the teat canal (Blowey and Edmondson 2010). *St. agalactiae* causes subclinical mastitis and is one of the most infectious mastitis pathogens, which is easily transmitted from cow to cow during milking (Meiri-Bendek *et al.* 2002, Merl *et al.* 2003). *St. agalactiae* damages the cistern and the ductal system, producing irritants that causes inflammation of the mammary gland, followed by increased SCC (≥ 1,000,000 cells ml^{-1}) (National Mastitis Council, 2017b).

St. dysgalactiae is commonly found on teat skin, particularly when teat skin is damaged, although it can also be found on the tonsils (Blowey and Edmondson 2010). *St. dysgalactiae* is associated with both subclinical and clinical mastitis, occurring during lactation and the dry period (St Rose *et al.* 2003, Whist *et al.* 2007). *St. dysgalactiae* can live within secretory epithelial cells for extended periods of time without losing viability or damaging the secretory cells (Calvinho and Oliver 1998, Calvinho *et al.* 1998). However, when they ultimately damage the mammary secretory epithelial cells, there is a reduction in milk production in the infected

quarters (Almeida and Oliver 1995). Studies have reported an increase in SCC of greater than 500,000 cells ml^{-1} associated with *St. dysgalactiae* infections (Shephard *et al.* 2000, St Rose *et al.* 2003).

Table 2: South African survey perspective indicating percentage prevalence of major mastitis causal pathogens from year 1996 to 2009**.**

Pathogen	% Prevalence (Dry off) *n (3118)*	% Prevalence (Lactation) *n (112715)*	Reference
S. aureus	17.28	16.98	
CNS	61.71	60.96	
St. uberis	1.21	2.25	(Petzer *et al.* 2009a)
St. agalactiae	1.21	5.92	
E. faecalis	4.49	1.77	
St. dysgalactiae	2.51	2.27	
	n (648)	***n (648)***	
S. aureus	2.78	2.31	
CNS	21.14	16.98	(Petzer *et al.* 2009b)
St. uberis	0	0.15	
St. agalactiae	3.55	1.70	
E. faecalis	0.31	0.31	
St. dysgalactiae	1.54	0.46	
E. coli	0.15	0	

n= number of samples analysed in each study.

2.2 Environmental Mastitis

Environmental pathogens are found in the environment where animals live. The common pathogens causing environmental mastitis are *Streptococcus uberis* and *Escherichia coli* (Blowey and Edmondson 2010, Sudhan and Sharma 2010). The rate of transmission of environmental mastitis pathogens between dairy cattle is higher during the dry period than during the lactation period (National Mastitis Council, 2017b).

Coagulase-negative staphylococci (CNS) are a group of more than 30 Gram-positive cocci. These microorganisms are unable to form a clot during a coagulase test (Taponen *et al.* 2006). CNS can be haemolytic or non-haemolytic. The bacteria most associated with CNS mastitis cases are *S. epidermis*, *S. hyicus*, *S. intermedius* and *S. xylosus* (Sawant *et al.* 2009). These microorganisms are mostly found in the teat skin, teat end and teat canal of the udders (Blowey and Edmondson 2010). Reports indicate that CNS mastitis is generally mild compared to mastitis induced by the other major mastitis pathogens, and it is often subclinical (Koivula *et al.* 2007). A high incidence of 61.71% and 60.96% during the dry and lactation period, respectively, has been reported in South Africa between the years 1996-2007 as it can be seen in Table 2 (Petzer *et al.* 2009a). Petzer *et al.* (2009b) reported that in 648 quarter milk samples analysed in South Africa, CNS had the highest prevalence of 21.14% and 16.98% at the dry and calving periods, respectively (Table 2). Various studies reported a similar trend in CNS prevalence among mastitis cases of between 35-51% in regions such as Ontario, Finland and Germany (Pitkälä *et al.* 2004, Lim *et al.* 2007, Idriss *et al.* 2014). CNS infections are commonly associated with heifers (Rajala-Schultz *et al.* 2004, Taponen *et al.* 2006). Increased SCC levels in milk (between 200,000 and 600,000 cells ml^{-1}) and a slight decrease in milk yields are observed with CNS infection (Chaffer *et al.* 1999). Thus, if milk cultures from a cow having high levels of SCC shows pure CNS growth it is likely that the cow is mainly infected by CNS (Schukken *et al.* 2009). However, when sampling for CNS, it is essential to discard the first 4-6 milk squirts because the CNS's primary reservoir of infection is the teat canal (Blowey and Edmondson 2010).

In some countries, CNS mastitis (subclinical or mild clinical) is often left untreated or non-antimicrobial techniques such as milking out are used (Pyörälä and Taponen 2009). However, in cases where antimicrobial treatment is used, CNS has been reported to respond well to antibiotics, with cure rates ranging from 80 to 90% during lactation (Taponen *et al.* 2006, Pyörälä and Taponen 2009). Often, post-milking teat disinfection is important to reduce transmission of CNS infection and to prevent increased bulk milk levels of CNS (Blowey and Edmondson 2010). In cases where antibiotic therapy is suggested, the recommended period of administering antibiotic therapy for CNS mastitis is 2-3 days during lactation (Pyörälä and Taponen 2009).

St. uberis is a Gram-positive, non-haemolytic, esculin-hydrolyzing and catalase-negative coccus. Cases of mastitis caused by *St. uberis* have become more frequent in Canada and New Zealand in the last 15 years (Odierno *et al.* 2006; Riekerink *et al.* 2008). Increased SCC and reduced milk yields occur during *St. uberis* infection. However, the degree to which these changes occur varies from cow to cow (Green *et al.* 2004). *St. uberis* resides mostly in the cows' habitat, although housed cattle are at higher risks of *St. uberis* infection than those on pastures (Wyder *et al.* 2011). *St. uberis* causes about 35% of mastitis cases in the UK. Mastitis caused by *St. uberis* is difficult to control using antimicrobial therapy (Hillerton and Kliem 2002).

St. uberis damages the epithelial cells and the resulting mastitis is characterized by white clots in the milk. Infection can result in both subclinical and clinical mastitis (Odierno *et al.* 2006, Blowey and Edmondson 2010). *St. uberis* can respond poorly to phagocytosis by PMNs, due to the ability to resist recognition by antibodies (poor opsonization) (Blowey and Edmondson 2010). *St. uberis* can resists antimicrobial therapy through living within cells and mammary lymph nodes, which can lead to chronic and recurrent forms of mastitis (Varhimo *et al.* 2011).

E. coli are Gram-negative bacilli that grow as grey mucoid colonies on blood agar, with some strains showing haemolytic reactions (Blowey and Edmondson 2010). *E. coli* strains are commonly found in faeces, thus infection occurs most often in housed cows under poor conditions of hygiene (Blowey and Edmondson 2010). *E. coli* penetrates the teat canal, invades epithelial cells and causes acute clinical mastitis, characterized by a hard and swollen udder with a watery discharge (Döpfer *et al.* 2001, Blum *et al.* 2014). Severe cases of *E. coli* infections result in the production of endotoxins such as lipopolysaccharides, compounds that are involved in *E. coli* defence and toxicity (Blowey and Edmondson 2010). *E. coli* also produces invasins, which can cause severe shock reactions in infected animals and can cause an infected cow to die within hours (Kaipainen *et al.* 2002).

2.3 Epidemiology of Contagious and Environmental Pathogens

Contagious and environmental pathogens spread amongst the cows of a herd using different *modus operandi*. This affects the control measures that can be used to reduce or eradicate their populations (Blowey and Edmondson 2010). This is referred to as their epidemiology and the differences are summarized in Table 3.

Table 3: Major differences in the epidemiology of contagious and environmental pathogens (Adapted from Blowey and Edmondson, 2010).

	Contagious	**Environmental**
Reservoir of Infection	Teat and udder	Contaminated Environment
Transmission into udder	During milking	Between milking and the dry period
Types of mastitis	Mostly subclinical	Mostly clinical*
Control measures	Post-milking teat dipping	Environmental hygiene
	Dry cow therapy	Pre-dipping
	Milking hygiene	Dry period teat sealants
	Culling	

*(*St. uberis* can be subclinical)

3. The Economic Impact of Mastitis

Bovine mastitis is considered as one of the most prevalent and costly diseases of cows that affects the dairy industry, impacting on dairy farmers both directly and indirectly (Blowey and Edmondson, 2010). Calculating the costs of mastitis is crucial because this allows for increased awareness of economic losses that are often not directly observable, and for associated costs that are often underestimated (Sharifi *et al.* 2014).

Farmers need to understand mastitis as a disease but also as a cause of financial losses (Blowey and Edmondson 2010). Thus, dairy farmers need information on the costs of control measures relative to the losses resulting from not applying these control measures (Blowey and Edmondson 2010). Cost factors include discarded milk, diagnostics, culling, drug costs, veterinary services, decreased milk quality and quantity, and increased labour requirements (Halasa *et al.* 2007, Blowey and Edmondson 2010, Hogeveen *et al.* 2011). Information about the real cost of mastitis helps to evaluate the profitability of preventive measures with regards to a single cow, whole herds and the entire dairy sector (Heikkila *et al.* 2012). Mastitis costs are often calculated using budget calculations, simulation models and by using dynamic optimization models (Heikkila *et al.* 2012).

Production losses are a major component of the economic losses caused by mastitis, followed by culling, animal treatment and discarded milk costs (Østergaard

et al. 2005, Swinkels *et al.* 2005). Heikkila *et al.* (2012) concluded that to decrease costs associated with mastitis, more emphasis must be given to identifying and quantifying the hidden costs associated with this disease. Hidden costs include culling practices applied to chronically infected cows to prevent transmission of mastitis to healthy cows (Heikkilä *et al.* 2012). Currently, most dairy farmers go for replacements, which involve culling of young cows with mastitis. However, increases costs as cow replacement costs are high (Heikkilä *et al.* 2012).

4. Principal Milk Components

Principal milk components are components present in large quantities in milk, have high nutritional value, or are minor components that contribute in the taste of milk (Brandao *et al.* 2010). Knowledge about principal milk components such as somatic cells, fats, lactose, proteins and minerals is used to calculate the value of a consignment of milk, and for the consumer's information (Melfsen *et al.* 2012). However, these principal components can also serve as biomarkers for mastitis, the relationship of other milk components to SCC is graphically presented in Figure 2 (Hamann, 2002; Melfsen *et al.* 2012).

4.1 Somatic Cells

Somatic Cell Count (SCC) is used as a predictor of intramammary infection (IMI). In this method somatic cells are counted (Blowey and Edmondson 2010). Somatic cells are composed of around 75% white blood cells (neutrophils, macrophages, lymphocytes and erythrocytes) and 25% epithelial secretory cells (Bytyqi *et al.* 2010).

Polymorphonuclear neutrophils (PMN) have phagocytic ability, however, these cells can degrades fat globules and casein, leading to putrefaction of milk (Opdebeeck 1982). The primary function of lymphocytes is to recognize antigens through membrane receptors, after which they generate resistance responses for the elimination of bacteria (Bytyqi *et al.* 2010). During infection by foreign microorganism, macrophages reacts by moving towards the site of infection and producing proteases and reactive oxygen species to help kill invading bacteria (Mullan 1985). Epithelial cells produce inflammatory mediators, i.e., cytokine, chemokines, defence peptides and arachidonic acid metabolites, which help eradicate infection through ingestion and possible digestion of phagocytosed microbes (Sharma *et al.* 2011).

During infection, the major increase in SCC is due to the influx of neutrophils into the milk to fight infection, an influx that has been estimated to exceed 90% (Blowey and Edmondson 2010). In healthy mammary glands where the SCC is lower than $1x10^5$ cells ml^{-1}, infection can cause SCC counts to rise above $1x10^6$ cells ml^{-1} (Blowey and Edmondson 2010). During normal cases, at post-partum (after calving), the proportion of macrophages is highest at 68% and lowest in late lactation at 21% (Wellnitz and Bruckmaier 2012).

The bacterial infection level influences the numbers of somatic cells in milk. The degree and nature of the cellular response are likely to be proportional to the severity of the infection (Sharma *et al.* 2011). However, it has been reported that SCC increases with progressing lactation, regardless of whether the cow is suffering from mastitis or not (Viguier *et al.* 2010). An elevated SCC has been linked with an animal's innate immune response in preparation for calving and to enhance the mammary gland defence mechanism during the calving period (Dohoo and Meek 1982). At calving, SCC is usually higher than $1x10^7$ cells ml^{-1} and decreases to $1x10^5$ cells ml^{-1} at 7-10 days after calving (Jensen and Eberhart 1981). SCC increases with increasing age and this is mainly due to an increased prevalence of infection in older cows, and also varies between different dairy breeds (Sharma *et al.* 2011).

Currently, the common methods of measuring SCC are the California mastitis test (CMT), the fossomatic SCC (FSCC), and the De Laval cell counter (DCC) (Viguier *et al.* 2010). Other SCC based methods uses colorimetric and fluorometric assays that specialize in measuring enzymes such as NAGase and LDH, levels of which increase during mastitis (Paape *et al.* 2003). The disadvantages of SCC based techniques include: difficulty in interpreting high SCC counts; SCC varies so widely due to many external factors, so the link with mastitis is tenuous; SCC is an indirect measurement that does not directly reflect bacterial infections of teats of cows; and equipment which provides accurate SCC measurements are expensive, and not affordable for small-scale farmers (Viguier *et al.* 2010).

4.2 Fats

Lipids are primarily esters of fatty acids and or related molecular compounds. They are soluble in non-polar organic solvents and are nearly insoluble in water (Jensen 2002). Nearly all fat present in milk exists as oil-in-water emulsions referred to as fat

globules (diameter 1-10 µm) in the endoplasmic reticulum of epithelial cells (El-Loly 2011). The fat globules are secreted by epithelial cells and are enveloped by the apical plasma membrane of the secretory cell (Jensen 2002). After the envelopment process, the membrane is now referred to as the milk-fat-globule membrane (MFGM), which is generally 10-20nm in cross section (Fong *et al.* 2007). The primary functions of the MFGM are to act as an emulsifier, to protect against enzyme degradation and to prevent coalescence of the milk fat, to ensure an even distribution of fat globules throughout the aqueous phase (El-Loly 2011).

Fatty acids are the building blocks of lipids, comprised of aliphatic monocarboxylic acids (4-28 carbons) and can either be saturated, monounsaturated or polyunsaturated (Ruiz-Rodriguez *et al.* 2010). There are over 400 different types of fatty acids present in milk fat, making it the most complex of natural fats (Parodi 2004, Markiewicz-Kęszycka *et al.* 2013). Fatty acids composition in milk mainly depends on the diet being fed to the cow, and the breed of the cow (O'Brien 2009). Fatty acids are derived from body fat, dietary fat (most notably the long-chain fatty acids) and from acetate, a product of microbial fermentation in the rumen (Parodi 2004, Blowey and Edmondson 2010). Milk fat is approximately 98% triglycerides, 2% diacylglycerol, 0.5% cholesterol, 1% phospholipids and 0.1% free fatty acids, hydrocarbons, fat-soluble vitamins, flavour compounds and feed introduced compounds (Jensen 2002, Parodi 2004, O'Brien 2009). Mastitis results in a decrease in milk fats and an increase in free fatty acids (FFAs), during both sub-clinical and clinical mastitis (Lindmark-Månsson *et al.* 2006, Ogola *et al.* 2007, Hunt *et al.* 2013).

4.3 Proteins

Caseins comprises about 80% of the total milk proteins. They exist in the form of casein micelles containing 4 protein species (α_{s1}-, α_{s2}-, β- and Ƙ-caseins) (Dalgleish 1998). Caseins are bound together by calcium phosphate and hydrophobic interactions and they display very unusual features such as hydrophobicity and phosphorylated proteins (Ribadeau-Dumas and Grappin 1989). Caseins occur in large poly-disperse aggregates in aqueous solution at room temperature and neutral pH. The isoelectric pH of the caseins lies in the pH range of 4.5-5.5 (Ribadeau-Dumas and Grappin 1989). During mastitis, casein levels decrease due to increased proteolytic activity, which results from the influx of serum proteins such as plasmin into milk (Khan and Khan 2006).

Milk whey proteins include β-lactoglobulin and α-lactalbumin (50% and 12%, respectively). The remaining are serum proteins (serum albumin, immunoglobulins, lactoferrin) and enzymes such as lactoperoxidases, xanthines and oxidases (Ribadeau-Dumas and Grappin 1989). Studies reported an increase in total proteins due to the increased permeability of the mammary membranes to serum proteins such as serum albumin, immunoglobulin and transferrin, allowing them to move into the milk (Le Roux *et al.* 1995). The increased vascular permeability results from the ductal and secretory epithelium cell damage of the epithelia lining the interior of the teat and udder cistern (Myllys and Rautala 1995). However, casein content in milk decreases during mammary gland inflammation because of degradation by proteolytic enzymes such as plasmin due to a damaged blood-milk barrier (Ribadeau-Dumas and Grappin 1989).

4.4 Lactose

Glucose and galactose combine in the udder to form lactose, which serves as the osmotic determinant to achieve an optimum osmotic level (Blowey and Edmondson 2010). Lactose concentration fluctuates in milk as the concentration of other milk components such as fats, proteins, and sodium and chloride ions changes (Blowey and Edmondson 2010). During mastitis, lactose production is suppressed and its concentration is reduced to compensate for the increase in electrolyte influx from the blood that enters milk (Bruckmaier *et al.* 2004, Blowey and Edmondson 2010).

4.5 Volatile Organic Compounds

Volatile organic compounds (VOC's) can result from the breakdown of nutrients by extracellular enzymes of microorganisms present in milk during early stages of microbial growth. The VOC's are referred to as primary metabolites and include the hydrolysis of triglycerides by *S. aureus* lipases to free fatty acids (FFA's) (Smeltzer *et al.* 1992). Secondary metabolites such as branched FFA's, branched alcohols, and branched aldehydes and tertiary metabolites are all associated with cell lysis (Hettinga *et al.* 2008a). Primary and secondary metabolites can be detected after short incubation times (the lag and exponential phase of bacteria), while tertiary metabolites can be detected at the stationary or decline phase of the bacterium involved (Masson *et al.* 1966; de Vos Petersen *et al.* 2004).

4.6 Minerals

Milk minerals are derived directly from the blood, with most common minerals being sodium, potassium and chloride. Potassium is the most abundant mineral ion found in milk (Kitchen 1981). Most reports on subclinical / clinical mastitis studies in dairy animals have reported an increase in Na and Cl, and a decrease in K concentration (Kitchen 1981, Kasikci *et al.* 2012). During mastitis, the increased flow of Na and Cl to the teat and udder cistern causes the K concentration to decrease proportionately to maintain the osmotic pressure of the milk (Norberg *et al.* 2004). Due to the changes that occurs with these minerals, the concentration of these minerals can be used to monitor subclinical / clinical mastitis in dairy animals (Tale *et al.* 2007, Blowey and Edmondson 2010).

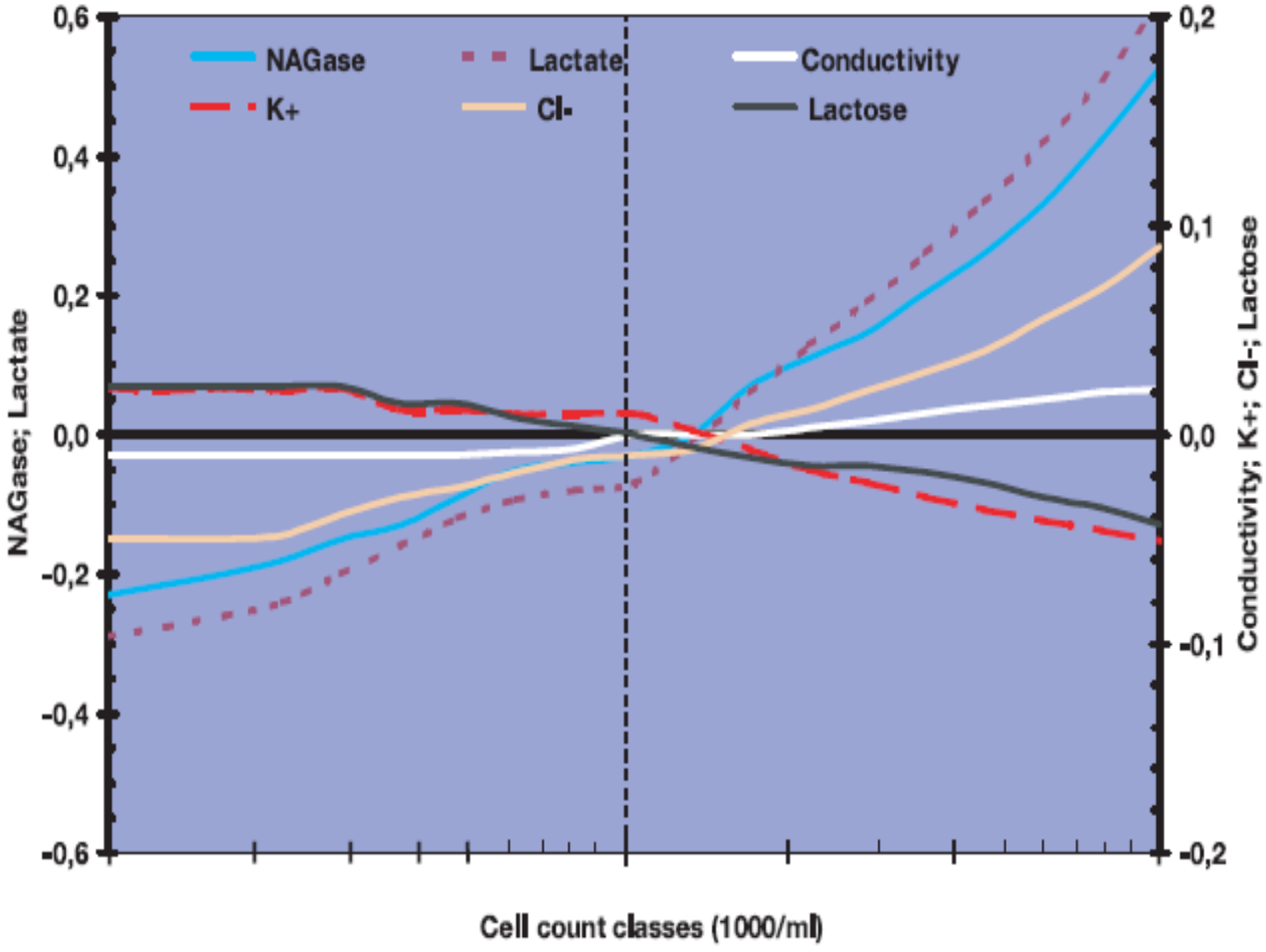

Figure 2: Mean deviation (in log 10) of milk properties from the overall means in relation to SCC. The zero-line represents a healthy quarter (Hamann, 2002).

5. Near-Infrared Spectroscopy

For the well-being of a dairy herd, continuous monitoring and early detection of disease is crucial, to allow for the early treatment of infected cows, and effective management of mastitis (Blowey and Edmondson 2010). Currently, mastitis detection is carried out using somatic cell counts (SCC) to a specific threshold of 200,000 cells ml^{-1}, a value that is often used as a threshold for mastitis (Melfsen *et al.* 2012). An ideal diagnostic method should be highly specific, non-complicated, allow early detection, be inexpensive and be compatible with “on-site” measuring (Viguier *et al.* 2009, Viguier *et al.* 2010).

In most cases, the analysis of basic milk constituents such as fats, proteins and lactose is done once every 3 to 4 weeks on commercial dairy farms (Aernouts *et al.* 2011, Melfsen *et al.* 2012). However, the current analytic methods have many disadvantages, including: they are time consuming, expensive, laborious, require complex pre-treatments, and require well trained personnel to operate the instruments (Balabin and Smirnov 2011). Therefore, there is a need for analytic tools capable of frequent sampling during the milking process, and concurrently ensuring the rapid and reliable quantification of milk components (Friggens *et al.* 2007). The ideal analytic tools should allow early detection of infections, thus providing a good opportunity to decide on strategic treatments (Melfsen *et al.* 2012).

Vibrational spectroscopic techniques such as mid-infrared (MIR) and near-infrared (NIR) spectroscopy provide faster, more accurate, more precise, non-destructive, and real-time analysis with minimal sample pre-treatment (Melfsen *et al.* 2012). The advantages of NIRS over MIRS are that NIRS is faster, is non-destructive, allows for simultaneous quantification of milk components and has the potential for in-line analysis (Brandao *et al.* 2010).

NIRS has been used to monitor fat, protein and lactose concentration in raw milk using reflectance, transmittance and transflectance modes (Brandao *et al*. 2010, Aernouts *et al.* 2011). NIRS is not limited to liquid milk samples but can also be applied to powdered milk and fermented milk products (Nicolai *et al.* 2007, Aernouts *et al.* 2011).

5.1 NIRS Principles

There are many NIRS instruments available. However, they are all based on the same principles (Aernouts *et al.* 2011), that a radiant energy source produces a light beam that passes through a grating device that allows a range of wavelengths to fall on the target material. The transmission or reflectance of the light spectrum covered is then measured (Givens and Deaville 1999). NIRS analysis is based on the absorption of light by material in the 780-2500 nm wavelength, which is the NIR region (Balabin and Smirnov 2011). Absorption of light by a substance at a specific wavelength within the NIR spectrum indicates the presence of a molecular bond (Shenk *et al.* 2001). In complex compounds, however, absorbance can also be obtained from wave overtones and combinations of fundamental vibrations of C-H, N-H, O-H and S-H bonds (Aernouts *et al.* 2011). Unfortunately, water that is present in biological samples dominates the absorption of the NIR spectrum since water strongly absorbs NIR radiation, which may complicate the analysis of biological samples (Shenk *et al.* 2001).

There are currently three scanning modes available for NIR analysis: reflectance, transmittance and transflectance, which differ in the process followed for absorbance of a substance that is taken (Reich 2005). In reflectance, the light beam is directed onto the surface of an opaque or solid sample and the amount of light reflected off it is measured (Workman *et al.* 2001). In transmittance, the instrument shins a light beam into the sample and amount of light absorbed by the semi-transparent sample is measured (Shenk *et al.* 2001). For transflectance, the instrument is set to the reflectance mode, but the light beam is directed through a transparent sample and reflected back to the sample using a white ceramic plate (Aernouts *et al.* 2011). In the transflectance mode, all radiation is collected, even radiation reflected within the sample, thus transflectance mode provides more information about the absorption of light (Workman *et al.* 2001).

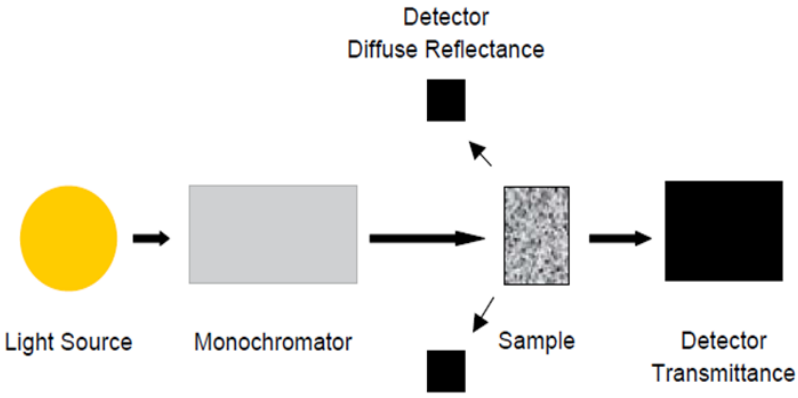

Figure 3: Basic configuration of a near infrared instrument (Reich, 2005).

As shown in Figure 3, during analysis, a wavelength from the wavelength discriminator is pointed onto the sample, the resultant reflected light is then collected by a photoelectric detector (Reich 2005). Once the light energy has been recorded in the photoelectric detector, the information is converted to log of a reciprocal (Reich 2005). From the Beer-Lambert law, the reflected light is inversely proportional to the light energy absorbed by a substance at a specific wavelength (Workman *et al.* 2001). The amount of light energy absorbed by the substance is proportional to the concentration of the substance at that wavelength value (Reich 2005). Hence, the concentration of the substance can be determined, if the relationship between the reflected light and concentration is known (Workman *et al.* 2001). This is where calibration during NIR analysis is crucial as its purpose is to determine the relationship between the two (Reich 2005).

5.2 NIRS Calibration

During analysis, establishing a relationship between log (1/R) and set of known concentrations of the component as determined by a reference method is called calibration (Reich 2005). The accuracy of an NIR instrument is dependent on sample-specific calibration, meaning that one must chose an accurate reference method for calibration (Williams *at el* 2001). Once the relationship between log (1/R) values and the reference method values has been determined, it is expressed as an approximation, and this involves a regression equation (Williams *et al.* 1984, Reich 2005).

In a regression equation, the regression constants are the y-intercept and the regression co-efficient, whereas a dependent variable is a reference method value

(Williams et al. 1984). During data treatment, log (1/R) values are mathematically combined at various wavelengths into independent variables, which are then used in the regression equation (Reich 2005).

During analysis, the data set for calibration is selected using statistical limits called Mahalanobis distances (H-statistic). These are the Global H (GH) and neighbouring H (NH) values (Shenk *et al.* 2001). These statistical limits aid in selecting a data set that is an accurate representation of the required population of samples (Shenk and Westerhaus 1991), they ensure that the samples selected to represent the data set are at a minimum, and that only samples making a significant input to the population are selected (Laporte and Paquin 1999). GH ensures the linearity of the relationship between reference method values and the NIR spectrum, while NH controls the closeness of samples representing the calibration set (Shenk and Westerhaus 1991). During a calibration experiment, the calibration method then tries to find the pattern/equation/model for predicting the reference values using the NIR spectral data (Workman *et al.* 2001).

The main problem encountered during calibration is the complexity of the nature of NIR spectra. Such complexity is characterized by overlapping, redundant and interfering peaks (Reich 2005). Methods used to minimize interference include partial least squares, multiple linear regression and principle component analysis, and can be used to create calibration equations (Boysworth and Booksh 2001). Methods that have been used for scatter correction are baseline correction, multiplicative scatter correction, standard normal variates, and first and second Savitzky-Golay derivatives (Aernouts *et al.* 2011).

Goodness of fit that indicates the predictive ability of models generated during calibration can be measured using the standard error of calibration (SEC) and the correlation co-efficient (r^2) (Velasco and Becker 1998). The SEC is a root mean square average of the errors about the fitted line, and it represents the mean difference from the prediction (Laporte and Paquin 1999). The r^2 lies between these, and it indicates the predictive ability of the fitted straight line relationship, relative to the variability of y-values of the calibration (Osborne *et al.* 1982, Velasco and Becker 1998). To inspect for deviations form linearity, outliers and other unusual features

such as skewness is important and these aspects can be inspected by plotting the data (Smith *et al.* 1998).

5.3 NIRS Validation

The purpose of a validation step is to measure the predictive ability of the model generated during the calibration step (Workman *et al.* 2001). During validation, samples not included in the calibration set are predicted using the calibration model. The predicted values are then compared to the reference values derived from "wet chemistry" techniques (Park *et al.* 2003). The standard error of cross-validation (SECV), standard error of prediction (SEP), correlation co-efficient (r^2) and ratio of prediction to deviation (RPD) are used to examine the accuracy of prediction (Workman *et al.* 2001). The SECV is used when the samples used for the validation process are from the same calibration set but are not used in the creation of the calibration model (Park *et al.* 2003). The SEP is used when the samples used are from an independent set and not affiliated with calibration set or model (Shenk *et al.* 1993). The ratio of prediction to deviation is the ratio of the standard deviation of the validation set to the SEP (Workman *et al.* 2001). RPD values ≥ 8.0 indicate excellent calibration models, values ≥ 3.0 can be used for analytic purposes, and values lower than 3.0 can only be used for rough screening (Melfsen *et al.* 2012).

5.4 Previous NIRS Applications on Milk

NIRS has been used previously to monitor most principal milk components, including monitoring for mastitis diagnosis *in vitro* and *in vivo* (Laporte and Paquin 1999, Melfsen *et al.* 2012, Porep *et al.* 2015). However, there are differences in the performances of calibration and validation across studies, and a number of factors affecting NIRS calibration may account for differences across studies (Melfsen *et al.* 2012). Possibly influences may include the wavelength range, sample presentation, scanning mode, differences in substrate temperature, sample set size and data pre-treatment (Tsenkova *et al.* 2000). This means that not all milk constituents are predicted with the same accuracy across the literature, and thus RPD, SEP and r^2 of validation varies considerably (Table 4) (Melfsen *et al.* 2012).

Laporte and Paquin (1999) determined milk fat, crude protein (CP), true protein (TP) and casein using transmission NIRS with a spectral range of 1100 – 2500 nm. The reference method used for fat determination was the Mojonnier method and Kjeldahl

method for CP, TP and casein determination. A NIRSystems model 6500 scanning spectrophotometer with a temperature module was used for the analysis of the major components of milk. NIR spectra (averaged for 32 completed scans) for each sample were obtained and the Mahalanobis distance was employed on 96 samples, which included both homogenized and unhomogenized raw milk samples. Using Global H and neighbouring H, 20% of the samples were set aside from the calibration set to be used as a validation set. The remaining 80% of the samples were used for the calibration experiment, and a modified partial least-squares regression was performed on the calibration set. For the optimization of calibration accuracy, scattering and mathematical treatments were applied, from which 512 calibration equations were generated. The standard normal variate (SNV) was used for scatter correction and the best calibration equation was selected, based on the highest r^2, and the lowest SEC and SECV. Validation set samples were then analysed with the calibration model that gave an SEP for each constituent. The performance of the validation model is summarized in Table 4. Prior to derivative mathematical treatment, the transmission of NIR was dominated by major bands at 1450 nm and 1940 nm, which correspond to the water present in milk samples. However, after the 2nd derivative and averaging of spectra, characteristic absorption bands corresponding to combinations of fundamental vibration and 2^{nd} overtones due to lipid C-H bonds were observed at 2320 nm and 2350 nm, respectively. Evidence of fundamental vibrations due to N-H bonds was observed at 2060 nm through to 2170 nm.

Melfsen *et al.* (2012) evaluated the accuracy of an NIRS device in predicting fat, proteins, lactose, urea and the SCC of raw milk at a milking parlour. The NIRS instrument used operated in a diffuse reflectance scanning mode in the wavelength region of 851 – 1649 nm, which is in the NIR spectral region. Pre-treatment methods were applied on the NIR spectra for noise reduction, baseline correction, resolution enhancement, mean centering and normalization. A total of 785 samples were analysed and 523 samples were randomly selected and used as a calibration set. The partial least squares method (PLS-1) was applied during the calibration experiment. Validation was carried out using 262 test sample set. Statistical values such as SEP, r^2 and RPD were used as validation criteria for each PLS model generated (Table 4). Milk fat content was characterized by absorbance at 851 and

1649 nm, which correspond to 2nd and 3rd overtone absorption by C-H bonds The authors concluded that NIR analysis of raw milk constituents cannot achieve the accuracy of recommendations required in the laboratory but that the prediction accuracy of constituents with regards to the reproducibility recommendations for in-line and at-line analytical devices can be achieved.

Determination of SCC in raw milk by NIRS is difficult because somatic cells cannot be defined through explicit chemical bonds (Melfsen *et al.* 2012). When calibrating an NIRS instrument with regards to SCC, the spectrum partly applies to milk compositional changes and partly to the scattering effects of somatic cells (Tsenkova *et al.* 2001). Hence SCC calibration and validation accuracy is relatively low in many studies of NIRS analysis due to these influences (Harmon 1994, Tsenkova *et al.* 2001, Melfsen *et al.* 2012).

Table 4: Different studies validation models based on the analysis of fats, proteins and lactose from milk samples.

Scanning mode and Spectral Range	Component (%)	Range of Validation Set	No. of samples in the Validation Set	SEP	R^2_{Val}	Bias	RPD	Reference
Transmittance	Fat	0.12 - 6.84	96	0.05	1.00	-0.01	-	(Laporte and Paquin 1999)
(1100 – 2500 nm)	Protein	1.90 - 4.97	96	0.09	0.95	-0.01	-	
	Lactose	-	-	-	-	-	-	
Transmittance	Fat	0.65 - 9.10	86	0.26	0.98	0.003	7.64	(Tsenkova *et al.* 2000)
(700 – 1100 nm)	Protein	2.60 - 3.62	86	0.13	0.45	-0.003	1.68	
	Lactose	4.08 - 4.74	86	0.08	0.72	-0.0002	1.9	
Diffuse Transmittance	Fat	0.94 - 6.19	72	0.25	0.95	-0.06	-	(Kawasaki *et al.* 2008)
	Protein	2.91 - 4.32	72	0.15	0.72	0	-	
(600 – 1050 nm)	Lactose	2.22 - 4.99	72	0.26	0.83	0	-	
Transflectance	Fat	2.63 - 5.47	47	0.06	0.99	0	11.6	(Saranwong and Kawano 2008)
(600 – 1000 nm)	Protein	2.86 - 3.81	43	0.08	0.91	0.01	3.3	
	Lactose	3.99 - 4.80	46	0.17	0.70	-0.01	1.2	
Transmittance	Fat	2.63 - 5.47	47	0.03	0.99	0	21.3	(Saranwong and Kawano 2008)
(400 – 1100 nm)	Protein	2.86 - 3.81	43	0.07	0.96	0.01	4.2	
	Lactose	3.99 - 4.80	46	0.09	0.89	0.03	2.4	
Reflectance	Fat	3.29 - 6.84	100	0.05	0.997	0.004	17.2	(Aernouts *et al.* 2011)
(1000 – 1700 nm)	Protein	2.80 - 4.68	100	0.10	0.96	-0.011	4.9	
	Lactose	3.48 - 5.09	100	0.28	0.31	-0.011	1.2	
Transmittance	Fat	3.29 - 6.84	100	0.04	0.997	-0.002	17.7	(Aernouts *et al.* 2011)
(1000 – 2500 nm)	Protein	2.80 - 4.68	100	0.13	0.93	-0.044	3.7	
	Lactose	3.48 - 5.09	100	0.16	0.76	-0.011	2.1	
Diffuse Reflectance	Fat	0.7 - 12.3	262	0.09	0.998	-0.0004	21.72	(Melfsen *et al.* 2012)
	Protein	2.4 - 4.0	262	0.05	0.98	0.0003	6.44	
(851 – 1649 nm)	Lactose	3.9 - 5.2	262	0.06	0.92	-0.0076	3.51	

Val= validation.

5.5 Reference Methods

Many different methods have been used for milk fat determination: gravimetrically (i.e. the Bligh and Dyer method) and densitometrically (Görs *et al.* 2009, El-Abassy *et al.* 2011). Most of these methods are automated such that they can be used on-site in dairy industries plants, to precisely and simultaneously determine milk constituents (El-Abassy *et al.* 2011). These methods have been used to monitor each individual cow's milk fat and to relate its milk fat composition to the cow's health status (Syring *et al.* 2012). The most traditional and commonly used methods available for milk fat analysis are the Rose-Gottlieb reference and the Gerber butyrometric method (Forcato *et al.* 2005). Standard method for milk fat determination as recognized by the Association of Official Analytical Chemists (AOAC) official methods of analysis is the Mojonnier ether extraction method (AOAC 1995). The principle underlying the Mojonnier ether extraction method is the separation of milk fats via liquid-liquid extraction using ether as solvent (Mills *et al.* 1983). Following evaporation of ether once the extract has been obtained, the dried fat is then weighed and expressed as a percentage fat by weight (AOAC 1995).

Milk lactose is estimated using an enzymatic assay. The principle behind the assay is the hydrolysis of lactose to D-galactose and D-glucose in the presence of β-galactosidase (AOAC 1995). Hydrolysis is then followed by mutarotation of α- and β-anomeric forms of D-galactose in the presence of galactose mutarotase, and finally oxidation of β-D-galactose anomers by oxidized nicotinamide-adenine-dinucleotide (NAD^+) to D-galactonic acid in the presence of β-galactose dehydrogenase (AOAC 1995). The reduced form of nicotinamide-adenine-dinucleotide (NADH) formed is measured at 340nm, which is stoichiometric with the amount of lactose in milk (Feldsine *et al.* 2002).

Milk protein is estimated by a Kjeldahl method. However, about 5-6% of the total N in milk is not associated with proteins (non-protein N, NPN), and the level varies among regions and seasons (Litwińczuk *et al.* 2011). Historically, TN (true nitrogen) content has been measured and used as a reference or basis for calibration of electronic milk analysis (AOAC 1995). The most common nitrogen fractions measured in milk composition studies are TN, NPN and non-casein-nitrogen (NCN). The NPN fraction includes urea, creatinine and amino acids (Barbano *et al.* 1992).

Bacteriological culturing has been used as the method of choice for the identification of mastitis pathogens. However, this approach has a number of disadvantages (Hettinga *et al*. 2008b), including being laborious, time consuming and technically demanding. Given that it takes several days to conduct culture tests, it is not possible to diagnose the causal organisms of mastitis from cultures in time to implement control measures effectively (Hettinga *et al.* 2008a).

Another approach is to analyse the volatile organic compounds (VOC's) released from milk during infection. This is based on the discovery that mastitis causing pathogens have different VOC's profiles due to the distinct metabolism each pathogen has (Hettinga et al. 2008b). Identification of VOC's using techniques such as gas chromatography and mass spectrophotometry (GC-MS) have proved to have a significant diagnostic value in medicine (Schulz and Dickschat 2007, Thorn *et al.* 2011, Wilson and Baietto 2011). There are factors affecting the clinical applicability of volatile biomarkers for pathogen identification, and several factors must be considered when using VOC's for diagnostic purposes (Bos *et al.* 2013). Firstly, the subtype of bacterial species/strain used must be noted because genomic variations between subtypes could result in differences in the efficacy of enzymes (Brooks 1976). Secondly, the growth medium used can influence the relative quantity and quality of VOC's produced because the growth medium is a source of building blocks for the VOC's produced (Scotter *et al.* 2005). Finally, incubation time intervals used for sampling and subsequent analysis also influence the profile and the amount of VOC's obtained (Scotter *et al.* 2005, Filipiak *et al.* 2012).

Minerals such as calcium, sodium, potassium, phosphorus, magnesium and chloride ions have been accurately measured using inductively coupled plasma atomic emission spectrometry (Feldsine *et al.* 2002). However, this technology is too expensive for routine use, and thus most studies have analysed minerals in milk using electrical conductivity (EC), which is cheap and easy to measure (Soyeurt *et al.* 2009). EC is the measure of the concentration of ions in solution and over the last decade electrical conductivity has been introduced as an indicator trait for mastitis hence a potential udder health measure (Norberg *et al.* 2004). Milk is enriched with compounds such as mineral salts such as calcium, chloride, magnesium, sodium and potassium which gives its conductive properties (Fahmid *et al.* 2016). Although EC is also influenced by a number of factors, during mastitis, there is an increase in

the concentration of Na^+ and Cl^-, hence EC increases with increasing microbial populations from a normal electrical conductivity of 4.0 – 5.5 mS cm^{-1} (Norberg *et al.* 2004; Janzekovic *et al.* 2009).

6. Discussion

Milk is a major component of the human diet in most cultures, for nutrients such as fats, lactose, minerals, proteins and vitamins (Aernouts *et al.* 2011). Knowledge about milk composition is important for establishing the value of milk samples, to inform the consumer and for quality control to monitor costly diseases such as mastitis (Núñez-Sánchez *et al.* 2016). Mastitis is the costliest disease in the dairy industry both nationally and globally and it is characterized by the inflammation of the cow's udder due to microbial infection (Sharifi *et al.* 2014). Therefore, the income of a dairy farmer, and the economic value of a batch of milk is defined by its composition, which defines its quality (Aernouts *et al.* 2011). Mastitis costs can be direct or indirect, and these costs range from veterinary treatments, increased labour demands, the costs of diagnostics, decreased production, the discarding of contaminated milk, and to decreased milk quality (Halasa *et al.* 2007).

Mastitis can progress with changes that are visible changes (clinical symptoms), during clinical mastitis, there are changes in colour and a presence of flakes in milk, swelling, hardening of tissue of the udder and general health of the animal can deteriorate (Janzekovic *et al.* 2009). However, subclinical mastitis occurs without noticeable changes in which case detection is dependent on bacteriological examination and or the examination of milk components such as fats, proteins, SCC, lactose and minerals (Schultz *et al.* 1977). Milk constituents have been considered as useful indicators of udder health, and hence can be used to detect mastitis (Bruckmaier *et al.* 2004).

Given that the traditional analytical methods are slow, laborious and costly, NIRS technology has become highly relevant to the dairy industry for its many advantages (Tsenkova *et al.* 2000). NIRS technology provides faster, accurate, non-destructive measurement for multiple traits simultaneously. Furthermore, real-time analysis with minimal sample pre-treatment is possible, which creates the potential for on-line analysis of milk (Melfsen *et al.* 2012).

NIRS is versatile enough to be used to measure constituents in food products of plant origin (Melfsen *et al.* 2012). Various studies have been published on the use of NIRS to estimate milk constituents. NIRS is based on indirect measurements, therefore calibration of “wet chemistry” analyses with NIR spectra is needed. This requires mathematical and statistical tools to interpret the data (Brandao *et al.* 2010). NIRS technology has largely been applied using bench-top equipment in laboratory conditions, with the advent of handheld NIRS units, NIRS is now industrially applied (Porep *et al.* 2015).

References

1. **Aernouts, B., Polshin, E., Lammertyn, J. and Saeys, W.** (2011). Visible and near-infrared spectroscopic analysis of raw milk for cow health monitoring: reflectance or transmittance? *Journal of Dairy Science* **94:** 5315-5329.
2. **Almeida, R. A. and Oliver, S. P.** (1995). Invasion of bovine mammary epithelial cells by *Streptococcus dysgalactiae*. *Journal of Dairy Science* **78:** 1310-1317.
3. **AOAC** (1995). Official Methods of Analysis of AOAC International. Arlington, Va.: AOAC Intl. pv (loose-leaf).
4. **Balabin, R. M. and Smirnov, S. V.** (2011). Melamine detection by mid- and near-infrared (MIR/NIR) spectroscopy: A quick and sensitive method for dairy products analysis including liquid milk, infant formula, and milk powder. *Talanta* **85:** 562-568.
5. **Barbano, D. and Lynch, J.** (2006). Major advances in testing of dairy products: Milk component and dairy product attribute testing. *Journal of Dairy Science* **89:** 1189-1194.
6. **Barbano, D., Lynch, J., Bauman, D., Hartnell, G., Hintz, R. and Nemeth, M.** (1992). Effect of a prolonged-release formulation of N-methionyl bovine somatotropin (sometribove) on milk composition. *Journal of Dairy Science* **75:** 1775-1793.
7. **Barkema, H., Schukken, Y., Lam, T., Beiboer, M., Wilmink, H., Benedictus, G. and Brand, A.** (1998). Incidence of clinical mastitis in dairy herds grouped in three categories by bulk milk somatic cell counts. *Journal of Dairy Science* **81:** 411-419.

8. **Blowey, R. W. and Edmondson, P.** (2010). Mastitis Control in Dairy Herds. Wallingford, Oxfordshire, UK; Cambridge, MA: CABI.
9. **Blum, S. E., Heller, E. D. and Leitner, G.** (2014). Long term effects of *Escherichia coli* mastitis. *The Veterinary Journal* **201:** 72-77.
10. **Bos, L. D., Sterk, P. J. and Schultz, M. J.** (2013). Volatile metabolites of pathogens: a systematic review. *PLoS Pathogens* **9:** e1003311.
11. **Boysworth, M. K. and Booksh, K. S.** (2001). Aspects of multivariate calibration applied to near-infrared spectroscopy. *Practical Spectroscopy Series* **27:** 209-240.
12. **Bradley, A.** (2002). Bovine mastitis: an evolving disease. *The Veterinary Journal* **164:** 116-128.
13. **Brandao, M., Carmo, A., Bell, M. and Anjos, V.** (2010). Characterization of milk by infrared spectroscopy. *Revista do Instituto de Laticinios Candido Tostes* **65:** 30-33.
14. **Brooks, J**. (1976). Detection of bacterial metabolites in spent culture media and body fluids by electron capture gas-liquid chromatography. *Advances in Chromatography* **15:** 1-31.
15. **Bruckmaier, R., Ontsouka, C. and Blum, J.** (2004). Fractionized milk composition in dairy cows with subclinical mastitis. *Veterinarni Medicina-UZPI* (Czech Republic) **49:** 283-290.
16. **Bytyqi, H., Zaugg, U., Sherifi, K., Hamidi, A., Gjonbalaj, M., Muji, S. and Mehmeti, H.** (2010). Influence of management and physiological factors on somatic cell count in raw cow milk in Kosova. *Veterinarski Arkiv* **80:** 173-183.
17. **Calvinho, L., Almeida, R. and Oliver, S.** (1998). Potential virulence factors of *Streptococcus dysgalactiae* associated with bovine mastitis. *Veterinary Microbiology* **61:** 93-110.
18. **Calvinho, L. and Oliver, S.** (1998). Invasion and persistence of *Streptococcus dysgalactiae* within bovine mammary epithelial cells. *Journal of Dairy Science* **81:** 678-686.
19. **Chaffer, M., Leitner, G., Winkler, M., Glickman, A., Krifucks, O., Ezra E. and Saran A.** (1999). Coagulase-negative *Staphylococci* and mammary gland infections in cows. *Journal of Veterinary Medicine* **46:** 707-712.

20. **Contreras, G. A. and Rodríguez, J. M.** (2011). Mastitis: comparative etiology and epidemiology. *Journal of Mammary Gland Biology and Neoplasia* **16:** 339-356.
21. **De Petersen, C. D. V., Beck, H. C., and Lauritsen, F. R.** (2004). On-line monitoring of important organoleptic methyl-branched aldehydes during batch fermentation of starter culture Staphylococcus xylosus reveal new insight into their production in a model fermentation. *Biotechnology and bioengineering* **85:** 298-305.
22. **Dalgleish, D.** (1998). Casein micelles as colloids: surface structures and stabilities. *Journal of Dairy Science* **81:** 3013-3018.
23. **Dohoo, I. R. and Meek, A.** (1982). Somatic cell counts in bovine milk. *The Canadian Veterinary Journal* **23:** 119-125.
24. **Döpfer, D., Nederbragt, H., Almeida, R. and Gaastra, W.** (2001). Studies about the mechanism of internalization by mammary epithelial cells of *Escherichia coli* isolated from persistent bovine mastitis. *Veterinary Microbiology* **80:** 285-296.
25. **El-Abassy, R., Eravuchira, P., Donfack, P., Von der Kammer, B. and Materny, A.** (2011). Fast determination of milk fat content using Raman spectroscopy. *Vibrational Spectroscopy* **56:** 3-8.
26. **El-Loly, M**. (2011). Composition, properties and nutritional aspects of milk fat globule membrane-a review. *Polish Journal of Food and Nutrition Sciences* **61:** 7-32.
27. **Fahmid, S., Hassan, E., Naeem, H., Barrech, S., Lodhi, S., & Latif, S.** (2016). Determination of mastitis by measuring milk electrical conductivity. *Int. J. Adv. Res. Biol. Sci*, **3:** 1-4.
28. **Feldsine, P., Abeyta, C. and Andrews, W. H.** (2002). AOAC International methods committee guidelines for validation of qualitative and quantitative food microbiological official methods of analysis. *Journal of AOAC International* **85:** 1187-1200.
29. **Filipiak, W., Sponring, A., Baur, M. M., Filipiak, A., Ager C., Wiesenhofer, H., Nagl M., Troppmair, J. and Amann, A.** (2012). Molecular analysis of volatile metabolites released specifically by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *BMC Microbiology* **12:** 133-129.

30. **Fong, B. Y., Norris, C. S. and MacGibbon, A. K.** (2007). Protein and lipid composition of bovine milk-fat-globule membrane. *International Dairy Journal* **17:** 275-288.
31. **Forcato, D., Carmine, M., Echeverria, G., Pecora, R. and Kivatinitz, S.** (2005). Milk fat content measurement by a simple UV spectrophotometric method: An alternative screening method. *Journal of Dairy Science* **88:** 478-481.
32. **Friggens, N., Ridder, C. and Løvendahl, P.** (2007). On the use of milk composition measures to predict the energy balance of dairy cows. *Journal of Dairy Science* **90:** 5453-5467.
33. **Givens, D. and Deaville, E.** (1999). The current and future role of near infrared reflectance spectroscopy in animal nutrition: a review. *Crop and Pasture Science* **50:** 1131-1145.
34. **Görs, S., Kucia M., Langhammer, M., Junghans, P. and Metges, C.** (2009). Technical note: milk composition in mice—methodological aspects and effects of mouse strain and lactation day. *Journal of Dairy Science* **92:** 632-637.
35. **Green, M., Green, L., Schukken, Y., Bradley, A., Peeler, E., Barkema, H., De Haas, Y., Collis, V. and Medley, G.** (2004). Somatic cell count distributions during lactation predict clinical mastitis. *Journal of Dairy Science* **87:** 1256-1264.
36. **Halasa, T., Huijps, K., Østerås, O. and Hogeveen, H.** (2007). Economic effects of bovine mastitis and mastitis management: A review. *Veterinary Quarterly* **29:** 18-31.
37. **Hamann, J.** (2002). Milk quality and udder health in relation to modern milking. Recent developments and perspective in bovine medicine. Proceedings of the XXII World Buiatrics Congress, Hannover. Germany.
38. **Hamann, J. and Krömker, V.** (1997). Potential of specific milk composition variables for cow health management. *Livestock Production Science* **48:** 201-208.
39. **Harmon, R.** (1994). Physiology of mastitis and factors affecting somatic cell counts. *Journal of Dairy Science* **77:** 2103-2112.
40. **Heikkilä, A. M., Nousiainen, J. and Pyörälä, S.** (2012). Costs of clinical mastitis with special reference to premature culling. *Journal of Dairy Science* **95:** 139-150.
41. **Hettinga, K. A., Van Valenberg, H., Lam, T. and Van Hooijdonk, A.** (2008a). Detection of mastitis pathogens by analysis of volatile bacterial metabolites. *Journal of Dairy Science* **91:** 3834-3839.

42. **Hettinga, K. A., Van Valenberg, H. and Van Hooijdonk, A.** (2008b). Quality control of raw cows' milk by headspace analysis. *International Dairy Journal* **18:** 506-513.
43. **Hillerton, J. E. and Kliem, K. E.** (2002). Effective treatment of *Streptococcus uberis* clinical mastitis to minimize the use of antibiotics. *Journal of Dairy Science* **85:** 1009-1014.
44. **Hogeveen, H., Huijps, K. and Lam, T. J.** (2011). Economic aspects of mastitis: New developments. *New Zealand Veterinary Journal* **59:** 16-23.
45. **Hunt, K. M., Williams, J. E., Shafii, B., Hunt, M. K., Behre, R., Ting, R., McGuire, M. K. and McGuire, M. A.** (2013). Mastitis is associated with increased free fatty acids, somatic cell count, and interleukin-8 concentrations in human milk. *Breastfeeding Medicine* **8:** 105-110.
46. **Idriss, S. E., Foltys, V., Tancin, V., Kirchnerova, K., Tancinova, D. and Zaujec, K.** (2014). Mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Nitra, Slovakia. *Slovak Journal of Animal Science* **47:** 33-38.
47. **Janzekovic, M., Brus, M., Mursec, B., Vinis, P., Stajnko, D., and Cus, F.** (2009). Mastitis detection based on electric conductivity of milk. *Journal of Achievements in Materials and Manufacturing Engineering*, **34**, 39-46.
48. **Jensen, D. and Eberhart, R.** (1981). Total and differential cell counts in secretions of the non-lactating bovine mammary gland. *American Journal of Veterinary Research* **42:** 743-747.
49. **Jensen, R. G.** (2002). The composition of bovine milk lipids: January 1995 to December 2000. *Journal of Dairy Science* **85:** 295-350.
50. **Kaipainen, T., Pohjanvirta, T., Shpigel, N., Shwimmer, A., Pyörälä, S. and Pelkonen, S.** (2002). Virulence factors of *Escherichia coli* isolated from bovine clinical mastitis. *Veterinary Microbiology* **85:** 37-46.
51. **Kasikci, G., Çetin, Ö., Bingol, E. B. and Gunduz, M. C.** (2012). Relations between EC, somatic cell count, California mastitis test and some quality parameters in the diagnosis of subclinical mastitis in dairy cows. *Turkish Journal of Veterinary and Animal Sciences* **36:** 49-55.
52. **Kateete, D. P., Kimani, C. N., Katabazi, F. A., Okeng, A., Okee, M. S, Nanteza, A., Joloba, M. L. and Najjuka, F. C.** (2010). Identification of *Staphylococcus*

aureus: DNase and mannitol salt agar improve the efficiency of the tube coagulase test. *Annals of Clinical Microbiology and Antimicrobials* **9:** 23-30.

53. **Kawasaki, M., Kawamura, S., Tsukahara, M., Morita, S., Komiya, M. and Natsuga, M.** (2008). Near-infrared spectroscopic sensing system for on-line milk quality assessment in a milking robot. *Computers and Electronics in Agriculture* **63:** 22-27.
54. **Khan, M. and Khan, A.** (2006). Basic facts of mastitis in dairy animals: a review. *Pakistan Veterinary Journal* **26:** 204-208.
55. **Kitchen, B. J**. (1981). Bovine mastitis: milk compositional changes and related diagnostic tests. *Journal of Dairy Research* **48:** 167-188.
56. **Koivula, M., Pitkälä, A., Pyörälä, S. and Mäntysaari, E. A.** (2007). Distribution of bacteria and seasonal and regional effects in a new database for mastitis pathogens in Finland. *Acta Agriculturae Scandinavica Section A* **57:** 89-96.
57. **Laporte, M. F. and Paquin, P.** (1999). Near-infrared analysis of fat, protein, and casein in cow's milk. *Journal of Agricultural and Food Chemistry* **47:** 2600-2605.
58. **Le Roux, Y., Colin, O. and Laurent, F.** (1995). Proteolysis in samples of quarter milk with varying somatic cell counts. 1. Comparison of some indicators of endogenous proteolysis in milk. *Journal of Dairy Science* **78:** 1289-1297.
59. **Leigh, J.** (1999). *Streptococcus uberis*: a permanent barrier to the control of bovine mastitis? *The Veterinary Journal* **157:** 225-238.
60. **Lim, G., Leslie, K., Kelton, D., Duffield,T., Timms, L. and Dingwell, R.** (2007). Adherence and efficacy of an external teat sealant to prevent new intramammary infections in the dry period. *Journal of Dairy Science* **90:** 1289-1300.
61. **Lindmark-Månsson, H., Bränning, C., Aldén, G. and Paulsson, M.** (2006). Relationship between somatic cell count, individual leukocyte populations and milk components in bovine udder quarter milk. *International Dairy Journal* **16:** 717-727.
62. **Litwińczuk, Z., Król, J., Brodziak, A. and Barłowska, J.** (2011). Changes of protein content and its fractions in bovine milk from different breeds subject to somatic cell count. *Journal of Dairy Science* **94:** 684-691.
63. **Masson, P.L., Heremans, J.F., Prignot, J.J., and Wauters, G**. (1966). Immunohistochemical localization and bacteriostatic properties of an iron-binding protein from bronchial mucus. *Thorax* **21:**538–544

64. **Malinowski, E., Lassa, H., Kłlossowska, A., Smulski, S., Markiewicz, H. and Kaczmarowski, M.** (2005). Etiological agents of dairy cows' mastitis in western part of Poland. *Polish Journal of Veterinary Sciences* **9:** 191-194.
65. **Markiewicz-Kęszycka, M., Czyżak-Runowska, G., Lipińska, P. and Wójtowski, J.** (2013). Fatty acid profile of milk-A review. *Bulletin of the Veterinary Institute in Pulawy* **57:** 135-139.
66. **Meiri-Bendek, I., Lipkin, E., Friedmann, A., Leitner, G., Saran, A., Friedman, S. and Kashi, Y.** (2002). A PCR-based method for the detection of *Streptococcus agalactiae* in milk. *Journal of Dairy Science* **85:** 1717-1723.
67. **Melfsen, A., Hartung, E. and Haeussermann, A.** (2012). Accuracy of in-line milk composition analysis with diffuse reflectance near-infrared spectroscopy. *Journal of Dairy Science* **95:** 6465-6476.
68. **Merl, K., Abdulmawjood, A., Lämmler, C. and Zschöck, M.** (2003). Determination of epidemiological relationships of *Streptococcus agalactiae* isolated from bovine mastitis. *FEMS Microbiology Letters* **226:** 87-92.
69. **Mills, B. L., Van de Voort, F. and Usborne, W.** (1983). Mojonnier method as reference for infrared determination of fat in meat products. *Journal-Association of Official Analytical Chemists* **66:** 1048-1050.
70. **Mullan, F.** (1985). Seasons of survival: reflections of a physician with cancer. *New England Journal of Medicine* **313:** 270-273.
71. **Myllys, V. and Rautala, H.** (1995). Characterization of clinical mastitis in primiparous heifers. *Journal of Dairy Science* **78:** 538-545.
72. **Nicolai, B. M., Beullens, K., Bobelyn, E., Peirs, A., Saeys, W., Theron, K. I. and Lammertyn, J.** (2007). Non-destructive measurement of fruit and vegetable quality by means of NIR spectroscopy: A review. *Postharvest Biology and Technology* **46:** 99-118.
73. **National Mastitis Council, 2017a.** Contagious Mastitis, retrieved November 27 2017, from https://nmconline.org/contmast.html.
74. **National Mastitis Council, 2017b.** Environmental Mastitis, retrieved November 27 2017, from https://nmconline.org/environmental.html.
75. **Norberg, E.** (2005). Electrical conductivity of milk as a phenotypic and genetic indicator of bovine mastitis: A review. *Livestock Production Science* **96:** 129-139.

76. **Norberg, E., Hogeveen, H., Korsgaard, I. R., Friggens, N. C., Sloth, K. H. M. N. and Løvendahl, P.** (2004). Electrical conductivity of milk: ability to predict mastitis status. *Journal of Dairy Science* **87:** 1099-1107.
77. **Núñez-Sánchez, N., Martínez-Marín, A., Polvillo, O., Fernández-Cabanás, V., Carrizosa, J., Urrutia, B. and Serradilla, J.** (2016). Near Infrared Spectroscopy (NIRS) for the determination of the milk fat fatty acid profile of goats. *Food Chemistry* **190:** 244-252.
78. **O'Brien, R. D**. (2009). Fats and Oils: Formulating and Processing for Applications. 6000 Broken Sound Parkway NW, Suite 300, Boca Raton, Florida, USA: CRC Press, Taylor and Francis Group.
79. **Odierno, L., Calvinho, L., Traverssa, P., Lasagno, M., Bogni, C. and Reinoso, E.** (2006). Conventional identification of *Streptococcus uberis* isolated from bovine mastitis in Argentinean dairy herds. *Journal of Dairy Science* **89:** 3886-3890.
80. **Ogola, H., Shitandi, A. and Nanua, J.** (2007). Effect of mastitis on raw milk compositional quality. *Journal of Veterinary Science* **8:** 237-242.
81. **Oliver, S., Gillespie, B., Headrick, S., Moorehead, H., Lunn, P., Dowlen, H., Johnson, D., Lamar, K., Chester, S. and Moseley, W.** (2004). Efficacy of extended ceftiofur intramammary therapy for treatment of subclinical mastitis in lactating dairy cows. *Journal of Dairy Science* **87:** 2393-2400.
82. **Opdebeeck, J.** (1982). Mammary gland immunity. *Journal of the American Veterinary Medical Association* **181:** 1061-1065.
83. **Osborne, B. G., Douglas, S., Fearn, T. and Willis, K. H.** (1982). The development of universal calibrations for measurement of protein and moisture in UK home-grown wheat by near-infrared reflectance analysis. *Journal of the Science of Food and Agriculture* **33:** 736-740.
84. **Østergaard, S., Chagunda, M., Friggens, N., Bennedsgaard, T. W. and Klaas, I. C.** (2005). A stochastic model simulating pathogen-specific mastitis control in a dairy herd. *Journal of Dairy Science* **88:** 4243-4257.
85. **Paape, M. J., Bannerman, D. D., Zhao, X. and Lee, J.W.** (2003). The bovine neutrophil: Structure and function in blood and milk. *Veterinary Research* **34:** 597-627.
86. **Park, B., Abbott, J. A., Lee, K. J., Choi, C. H., and Choi, K. H.** (2003). Near-infrared diffuse reflectance for quantitative and qualitative measurement of

soluble solids and firmness of Delicious and Gala apples. *Transactions of the ASAE*, **46**, 1721.

87. **Parodi, P. W.** (2004). Milk fat in human nutrition. *Australian Journal of Dairy Technology* **59:** 3-59.
88. **Petzer, I. M., Karzis, J., Watermeyer, J. C., Van der Schans, T. J. and Van Reenen, R.** (2009a). Trends in udder health and emerging mastitogenic pathogens in South African dairy herds. *Journal of the South African Veterinary Association* **80:** 17-22.
89. **Petzer, I. M., Lourens, D. C., Van der Schans, T. J., Watermeyer, J. C., Van Reenen, R., Rautenbach, G. and Thompson, P.** (2009b). Intramammary infection rate during the dry period in cows that received blanket dry cow therapy: efficacy of 6 different dry-cow intra-mammary antimicrobial products. J*ournal of the South African Veterinary Association* **80:** 23-30.
90. **Pitkälä, A., Haveri, M., Pyörälä, S., Myllys, V. and Honkanen-Buzalski, T.** (2004). Bovine mastitis in Finland 2001—prevalence, distribution of bacteria, and antimicrobial resistance. *Journal of Dairy Science* **87:** 2433-2441.
91. **Porep, J. U., Kammerer, D. R. and Carle, R.** (2015). On-line application of near infrared (NIR) spectroscopy in food production. *Trends in Food Science and Technology* **46:** 211-230.
92. **Pyörälä, S. and Taponen, S.** (2009). Coagulase-negative staphylococci—emerging mastitis pathogens. *Veterinary Microbiology* **134:** 3-8.
93. **Rabiee, A. and Lean, I.** (2013). The effect of internal teat sealant products (Teatseal and Orbeseal) on intramammary infection, clinical mastitis, and somatic cell counts in lactating dairy cows: a meta-analysis. *Journal of Dairy Science* **96:** 6915-6931.
94. **Rajala-Schultz, P., Smith, K., Hogan, J. and Love, B.** (2004). Antimicrobial susceptibility of mastitis pathogens from first lactation and older cows. *Veterinary Microbiology* **102:** 33-42.
95. **Reich, G.** (2005). Near-infrared spectroscopy and imaging: basic principles and pharmaceutical applications. *Advanced Drug Delivery Reviews* **57:** 1109-1143.
96. **Ribadeau-Dumas, B. and Grappin, R.** (1989). Milk protein analysis. *Le Lait* **69:** 357-416.

97. **Riekerink, R. O., Barkema, H., Kelton, D. and Scholl, D.** (2008). Incidence rate of clinical mastitis on Canadian dairy farms. *Journal of Dairy Science* **91:** 1366-1377.

98. **Ruiz-Rodriguez, A., Reglero, G. and Ibañez, E.** (2010). Recent trends in the advanced analysis of bioactive fatty acids. *Journal of Pharmaceutical and Biomedical Analysis* **51:** 305-326.

99. **Saranwong, S. and Kawano, S.** (2008). System design for non-destructive near infrared analyses of chemical components and total aerobic bacteria count of raw milk. *Journal of Near Infrared Spectroscopy* **16:** 389–398.

100. **Sawant, A., Gillespie, B. and Oliver, S.** (2009). Antimicrobial susceptibility of coagulase-negative *Staphylococcus* species isolated from bovine milk. *Veterinary Microbiology* **134:** 73-81.

101. **Schukken, Y. H., González, R. N., Tikofsky, L. L., Schulte, H. F., Santisteban, C. G., Welcome, F. L., Bennett, G. J., Zurakowski, M. J. and Zadoks, R. N.** (2009). CNS mastitis: Nothing to worry about? *Veterinary Microbiology* **134:** 9-14.

102. **Schultz, L. H.** (1977). Somatic cells in milk-physiological aspects and relationship to amount and composition of milk. *Journal of food protection*, **40**, 125-131.

103. **Schulz, S. and Dickschat, J. S.** (2007). Bacterial volatiles: the smell of small organisms. *Natural Product Reports* **24:** 814-842.

104. **Scotter, J. M., Langford, V. S., Wilson, P. F., McEwan, M. J. and Chambers, S. T.** (2005). Real-time detection of common microbial volatile organic compounds from medically important fungi by Selected Ion Flow Tube-Mass Spectrometry (SIFT-MS). *Journal of Microbiological Methods* **63:** 127-134.

105. **Sharifi, H., Adeli Sardooei M., Bodagh Abadi M. and Babaei H.** (2014). Economic impact of mastitis in dairy cows: case study of Tehran Province, Iran. *Iranian Journal of Veterinary Surgery* **9:** 39-44.

106. **Sharma, N., Singh, N. and Bhadwal, M.** (2011). Relationship of somatic cell count and mastitis: An overview. *Asian-Australasian Journal of Animal Sciences* **24:** 429-438.

107. **Shenk, J. and Westerhaus, M.** (1991). Population definition, sample selection, and calibration procedures for near infrared reflectance spectroscopy. *Crop Science* **31:** 469-474.

108. **Shenk, J. S., Fales, S. L. and Westerhaus, M. O.** (1993). Using near infrared reflectance product library files to improve prediction accuracy and reduce calibration costs. *Crop Science* **33:** 578-581.

109. **Shenk, J. S., Workman, J. J. and Westerhaus, M. O.** (2001). Application of NIR spectroscopy to agricultural products. *Practical Spectroscopy Series* **27**: 419-474.

110. **Shephard, R., Malmo, J. and Pfeiffer, D.** (2000). A clinical trial to evaluate the effectiveness of antibiotic treatment of lactating cows with high somatic cell counts in their milk. *Australian Veterinary Journal* **78:** 763-768.

111. **Smith, K., Simpson, R. and Armstrong, R.** (1998). Using near infrared reflectance spectroscopy to estimate the nutritive value of senescing annual ryegrass (*Lolium rigidum*): a comparison of calibration methods. *Animal Production Science* **38:** 45-54.

112. **Soyeurt, H., Bruwier, D., Romnee, J.M., Gengler, N., Bertozzi, C., Veselko, D. and Dardenne, P.** (2009). Potential estimation of major mineral contents in cow milk using mid-infrared spectrometry. *Journal of Dairy Science* **92:** 2444-2454.

113. **St Rose, S. G., Swinkels, J. M., Kremer, W. D., Kruitwagen, C. L. and Zadoks, R. N.** (2003). Effect of penethamate hydriodide treatment on bacteriological cure, somatic cell count and milk production of cows and quarters with chronic subclinical *Streptococcus uberis* or *Streptococcus dysgalactiae* infection. *Journal of Dairy Research* **70:** 387-394.

114. **Sudhan, N. and Sharma, N.** (2010). Mastitis—an important production disease of dairy animals. *SMVS'Dairy Year Book, Ghaziabad* 72-88.

115. **Suojala, L., Kaartinen, L. and Pyörälä, S.** (2013). Treatment for bovine *Escherichia coli* mastitis–an evidence-based approach. *Journal of Veterinary Pharmacology and Therapeutics* **36:** 521-531.

116. **Swinkels, J., Hogeveen, H. and Zadoks, R.** (2005). A partial budget model to estimate economic benefits of lactational treatment of subclinical *Staphylococcus aureus mastitis*. *Journal of Dairy Science* **88:** 4273-4287.

117. **Syring, C., Boss, R., Reist , M., Bodmer, M., Hummerjohann, J., Gehrig, P. and Graber, H.** (2012). Bovine mastitis: The diagnostic properties of a PCR-based assay to monitor the *Staphylococcus aureus* genotype B status of a herd, using bulk tank milk. *Journal of Dairy Science* **95:** 3674-3682.

118. **Tale, S., Kolte, A., Waghmare, S. and Handa, A.** (2007). Alteration in electrolyte and biochemical profile of milk during subclinical mastitis in cows. *Royal Veterinary Journal of India* **3:** 140-141.

119. **Taponen, S., Simojoki, H., Haveri, M., Larsen, H. D. and Pyörälä, S.** (2006). Clinical characteristics and persistence of bovine mastitis caused by different species of coagulase-negative staphylococci identified with API or AFLP. *Veterinary Microbiology* **115:** 199-207.

120. **Tenhagen, B. A., Köster, G., Wallmann, J. and Heuwieser, W.** (2006). Prevalence of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Brandenburg, Germany. *Journal of Dairy Science* **89:** 2542-2551.

121. **Thorn, R. M. S., Reynolds, D. M. and Greenman, J.** (2011). Multivariate analysis of bacterial volatile compound profiles for discrimination between selected species and strains in vitro. *Journal of Microbiological Methods* **84:** 258-264.

122. **Tsenkova, R., Atanassova, S., Itoh, K., Ozaki, Y. and Toyoda, K.** (2000). Near infrared spectroscopy for biomonitoring: cow milk composition measurement in a spectral region from 1,100 to 2,400 nanometers. *Journal of Animal Science* **78:** 515-522.

123. **Tsenkova, R., Atanassova, S., Kawano, S. and Toyoda, K.** (2001). Somatic cell count determination in cow's milk by near-infrared spectroscopy: a new diagnostic tool. *Journal of Animal Science* **79:** 2550-2557.

124. **Varhimo, E., Varmanen, P., Fallarero, A., Skogman, M., Pyörälä, S., Livanainen, A., Sukura, A., Vuorela, P. and Savijoki, K.** (2011). Alpha-and β-casein components of host milk induce biofilm formation in the mastitis bacterium *Streptococcus uberis*. *Veterinary Microbiology* **149:** 381-389.

125. **Velasco, L. and Becker, H. C.** (1998). Estimating the fatty acid composition of the oil in intact-seed rapeseed (*Brassica napus L.*) by near-infrared reflectance spectroscopy. *Euphytica* **101:** 221-230.

126. **Viguier, C., Arora, S., Gilmartin, N., Welbeck, K. and O'Kennedy, R.** (2010). Mastitis detection: current trends and future perspective. *Trends in Biotechnology* **27:** 486-493.

127. **Watts, J. L.** (1988). Etiological agents of bovine mastitis. *Veterinary Microbiology* **16:** 41-66.

128. **Wellenberg, G., Van der Poel, W., Van der Vorst, T., Van Valkengoed, P., Schukken, Y., Wagenaar, F. and Van Oirschot, J.** (2000). Bovine herpesvirus 4 in bovine clinical mastitis. *Veterinary Record* **147:** 222-225.

129. **Wellenberg, G., Van Der Poel, W. and Van Oirschot, J.** (2002). Viral infections and bovine mastitis: a review. *Veterinary Microbiology* **88:** 27-45.

130. **Wellnitz, O. and Bruckmaier, R. M.** (2012). The innate immune response of the bovine mammary gland to bacterial infection. *The Veterinary Journal* **192:** 148-152.

131. **Whist, A., Østerås, O. and Sølverød, L.** (2007). *Streptococcus dysgalactiae* isolates at calving and lactation performance within the same lactation. *Journal of Dairy Science* **90:** 766-778.

132. **Williams, P. C**. 2001. *Implementation of near infrared technology*. In New infrared technology in the agricultural and food industries (ed. Williams, P. C. and Norris, K. H.), pp. 145–171.

133. **Williams, P. C., Preston, K. R., Norris, K. H. and Starkey, P. M.** (1984). Determination of amino acids in wheat and barley by near-infrared reflectance spectroscopy**.** *Journal of Food Science* **49:** 17–20**.**

134. **Wilson, A. D. and Baietto, M.** (2011). Advances in electronic-nose technologies developed for biomedical applications. *Sensors* **11:** 1105-1176.

135. **Workman, J., Creasy, K. E., Doherty, S., Bond, L., Koch, M., Ullman, A. and Veltkamp, D. J.** (2001). Process analytical chemistry. *Analytical Chemistry* **73:** 2705-2718.

136. **Wyder, A. B., Boss, R., Naskova, J., Kaufmann, T., Steiner, A. and Graber, H.** (2011). *Streptococcus* spp. and related bacteria: Their identification and their pathogenic potential for chronic mastitis–A molecular approach. *Research in Veterinary Science* **91:** 349-357.

CHAPTER 2:
CHANGES IN PH AND ELECTRICAL CONDUCTIVITY (EC) OF MILK INOCULATED WITH *STAPHYLOCOCCUS AUREUS,* CAUSAL AGENT OF BOVINE MASTITIS

Abstract

The aims of this study was to investigate the extent to which pH and electrical conductivity (EC) of milk changes when exposed to variable concentrations of *Staphylococcus aureus* and also how the pH and EC is influenced upon the addition of a common commensal microorganism such as *Bacillus* spp. since mastitis generally results from infection by a mixture of microorganisms. For the axenic culture of *S. aureus*, sterile commercial milk was inoculated with three different concentrations of *S. aureus:* undiluted, 10^{-1} diluted and 10^{-2} diluted. The inoculated milk was incubated at 37°C, pH and EC values were measured at intervals of 0, 2, 4, 8, 16 and 32 hours. For the co-culture experiment, *Bacillus* spp was added to a culture of *S. aureus* after which 0.1 ml of milk was transferred onto a sterile TSA plates and incubated at 37°C for 24 hours. For the axenic culture of *S. aureus*, the pH decreased with incubation time from an average value of 6.45 to 5.31 after 32 hours of incubation. The decrease in pH was matched by an increase in EC from 5.28 mS cm^{-1} to 6.68 mS cm^{-1} after 32 hours of incubation. In the second experiment there was an observed increase in pH by the axenic *Bacillus* isolates to pH values of 7.30 and 7.41 and EC values of up to 8.22 mS cm^{-1} and 6.35 mS cm^{-1} for isolates 1 and 2 respectively after 32 hours of incubation and *S. aureus* had a pH of 5.36 and an EC value of 6.49 mS cm^{-1} after 32 hours. A decrease in pH is indicative of the degradation of lactose to lactic acid by *S. aureus* and the increase by Bacillus isolates was due to amino acid degradation which resulted in alkaline pH. An increase in EC has been attributed to the release of sodium and chloride ions as well as lactose. It was concluded that the measurement of pH and EC could be used to estimate the levels of *S. aureus* contamination of milk, reflecting mastitis present.

2.1 Introduction

Bovine mastitis presents a major burden for dairy farmers worldwide. This disease results from the inflammation of the mammary inside the udder, with *Staphylococcus aureus* being the most common causal pathogen (Viguier *et al.* 2009). The severity of bovine mastitis can be classified into subclinical, clinical and chronic forms, and the severity is influenced by a number of factors including causal pathogen, breed of cow, lactation stage, and immunological health of the cow etc. (Tangorra *et al.* 2010). In mastitis diagnostics, currently the common method used is the somatic cell count (SCC) method, which relies on the principle of there being elevated somatic cells in milk during infection. However, this method has proved to have major shortfalls, including the cost of the equipment, slow delivery of results from centralized laboratories and that SCC is an indirect, unreliable measure of mastic infection of a cow's udder. Therefore there is a need for more reliable, user-friendly, cost effective and rapid methods of detection and diagnosis of mastitis (Viguier *et al.* 2009).

Electrical conductivity is defined as a measure of the degree to which a specific material conducts an electrical current, and it is measured in Siemens per meter, normal milk has a standard range EC of 4.0 – 5.5 mS cm^{-1} (Norberg, 2005; Fahmid *et al.* 2016). In milk, an increase in sodium and chloride ion concentrations in infected milk typically causes an increase in EC (Kitchen, 1981). These ionic changes are caused primarily by changes in the pH and fat concentration in milk (Korhonen and Kaartinen, 1995; Tangorra *et al.* 2010), the active transport systems present in sensory cells of the mammary glands of the udder thereby is responsible for pumping the Na^+ and Cl^- into the extra cellular fluid and potassium ions into the cells (Fahmid *et al.* 2016). However, the change in EC is not only influenced by mastitis, but also by non-mastitis related factors, namely, the breed, age of the dairy cow and the stage of its lactation, all of which are also known to influence the EC of milk (Ford *et al.* 1998). The use of EC as a method of detecting mastitis in dairy animals present several potential advantages, namely, EC can be automatically measured in milk, and the EC measurements can be easily converted into a computer readable signal, making this method easily applicable

to an online automatic monitoring of udder health, and can be installed on milking machines (Tangorra *et al.* 2010). Hand-held EC meters are relatively cheap, which would make the testing for mastitis feasible for small scale farmers, using this approach and a recent study on the sensitivity and specificity of the use of EC meters has reported values of 70% and 98% respectively (Steenveld *et al.* 2010).

EC has been used as a diagnostic method to detect subclinical mastitis in dairy cattle but the results have not been consistent between studies (Stenager *et al.* 1991; Hamann and Krömker 1997). In studies on the axenic culture of *S. aureus* Fujikawa and Morozumi (2006) found an increase in EC in inoculated milk compared to an un-inoculated control. Other studies have also found that EC values were higher in both subclinical and clinical mastitic milk than in milk from a healthy cow (Nielen *et al.* 1992*;* Norberg 2005). Furthermore, although the milk from a cow suffering from mastitis may not always show elevated EC levels, there can be a large difference in EC measurements between milk from infected and uninfected quarters (Tangorra *et al.* 2010).

In this study, we monitored the EC and pH of milk inoculated with *S. aureus*, for a period of 32 hours, to observe the changes in both of these parameters, in order to assess the sensitivity of EC and pH in diagnosing bovine mastitis.

We observed that *Bacillus* spp. were usually present in the commercial milk we were using, and realized that *Bacillus* spp. is normally present in milking parlours. Given that *Bacillus* spp. could influence the changes in pH and EC that resulted from the addition of *S. aureus* to milk, we conducted a further experiment that was aimed at evaluating the changes in the EC and pH of milk during axenic culturing of either *S. aureus* or *Bacillus* spp., or a mixed culture of both species*.*

2.2 Materials and Methods

2.2.1 Culture Preparation and Milk Inoculation

A field isolate of *Staphylococcus aureus* was inoculated on tryptone soy agar (TSA) and incubated at 37°C for 18 hours, after which, a loopful of culture was used to inoculate 50 ml tryptone soy broth (Sigma Aldrich) in a 100 ml flask and incubated for 24 hours at 37°C on an orbital flask shaker while stirring at 100 rpm. Serial dilutions of 10^{0}, 10^{-1} and 10^{-2} of the original *S. aureus* liquid culture ($1x10^{8}$ CFU ml^{-1}) was prepared using quarter strength Ringer's solution amounting to $1x10^{8}$ CFU ml^{-1}, $1x10^{7}$ CFU ml^{-1} and $1x10^{6}$ CFU ml^{-1} . An amount of 1 ml of the resultant suspensions was used to inoculate 39 ml of autoclaved milk sample in a 50 ml Erlenmeyer flasks (commercial milk brand: Clover; Supermarket: Pick' n Pay). The culture was incubated at 37°C while stirring at 100 rpm. The concentration of each isolate was quantified using a viable plate count approach to determine the initial concentration used for the inoculation of the sterile milk.

Another experiment was set-up to determine the effect of the addition of *Bacillus* spp., a common bacterial genus often found in milking parlours, to milk inoculated with *S. aureus* in terms of the pH and EC of milk incubated for 32 hours at 37°C while stirring at 100 rpm. Two isolates of *Bacillus* spp. were isolated from milk used in this study. A milk container was opened aseptically and 0.1 ml of milk was transferred onto sterile 90 mm TSA plates, which were incubated at 37°C for 24 hours.

2.2.2 Monitoring of pH and EC

Autoclaved milk was inoculated with 10^{0}, 10^{-1} and 10^{-2} suspension of *S. aureus* liquid culture in triplicates. Each dilution was sampled by removing 4 ml of each sample in duplicates at incubation times of 0, 2, 4, 8, 16 and 32 hours of incubation, for the determination of their pH and EC values.

To investigate how the isolated *Bacillus* isolates affected the changes in pH and EC, relative to the impact of *S. aureus*, another experiment was conducted. 100 ml flasks inoculated with the two *Bacillus* isolates and *S. aureus* or a mixture of a *Bacillus* isolate and *S. aureus* were prepared and the changes in pH and EC were monitored at 0, 2, 4, 8, 16 and 32 hours.

One flask per inoculum was used to monitor the effect of sampling for instance any interruptions caused by flask removal and volume reduction in the duplicate set hence measurements in these flasks were only done at the onset and the end of incubation. A control was prepared by the addition of 1 ml of sterile TSB in 39 ml of autoclaved milk.

2.3 Results

2.3.1 Changes in pH and EC of milk with different *S. aureus* Inoculum Doses

Figure 4a indicates the change in pH across the investigated inoculum doses of *S. aureus* (10^0, 10^{-1} and 10^{-2}) and the un-inoculated control. The pH of the milk of the un-inoculated control flask remained almost constant through incubation for 32 hours, only decreasing by 0.01 pH units, from 6.36 to 6.35.

Inoculation of milk with *S. aureus* consistently resulted in a decrease of the pH of the milk over the three concentration, and over 32 hours of incubation. The higher the concentration of *S. aureus*, the faster the pH decrease (for dilutions of 10^0, 10^{-1} and 10^{-2} dilutions). At 8 hours the difference were large (pH 5.83, 6.09 and 6.30, respectively). At 32 hours the differences in pH had diminished (pH 5.25, 5.32, 5.37, respectively), as the populations of bacteria evened out. The decrease of pH was almost linear for the dilution of 10^{-2} over the period measured.

In Figure 4b the EC of the milk of the untreated control was stable over the 32 hours, increasing slightly from 5.33 to 5.38 mS cm^{-1}. In contrast, the EC values of the milk of the inoculated samples all increased, the increases being relative to dose of bacteria used (for dilutions of 10^0, 10^{-1} and 10^{-2} dilutions) and the length of incubation. At 8

hours, the EC values were 5.94, 5.52 and 5.30, respectively. These increased to 6.49, 6.12 and 5.64 at 16 hours, and 6.98, 6.60 and 6.45 at 32 hours, respectively.

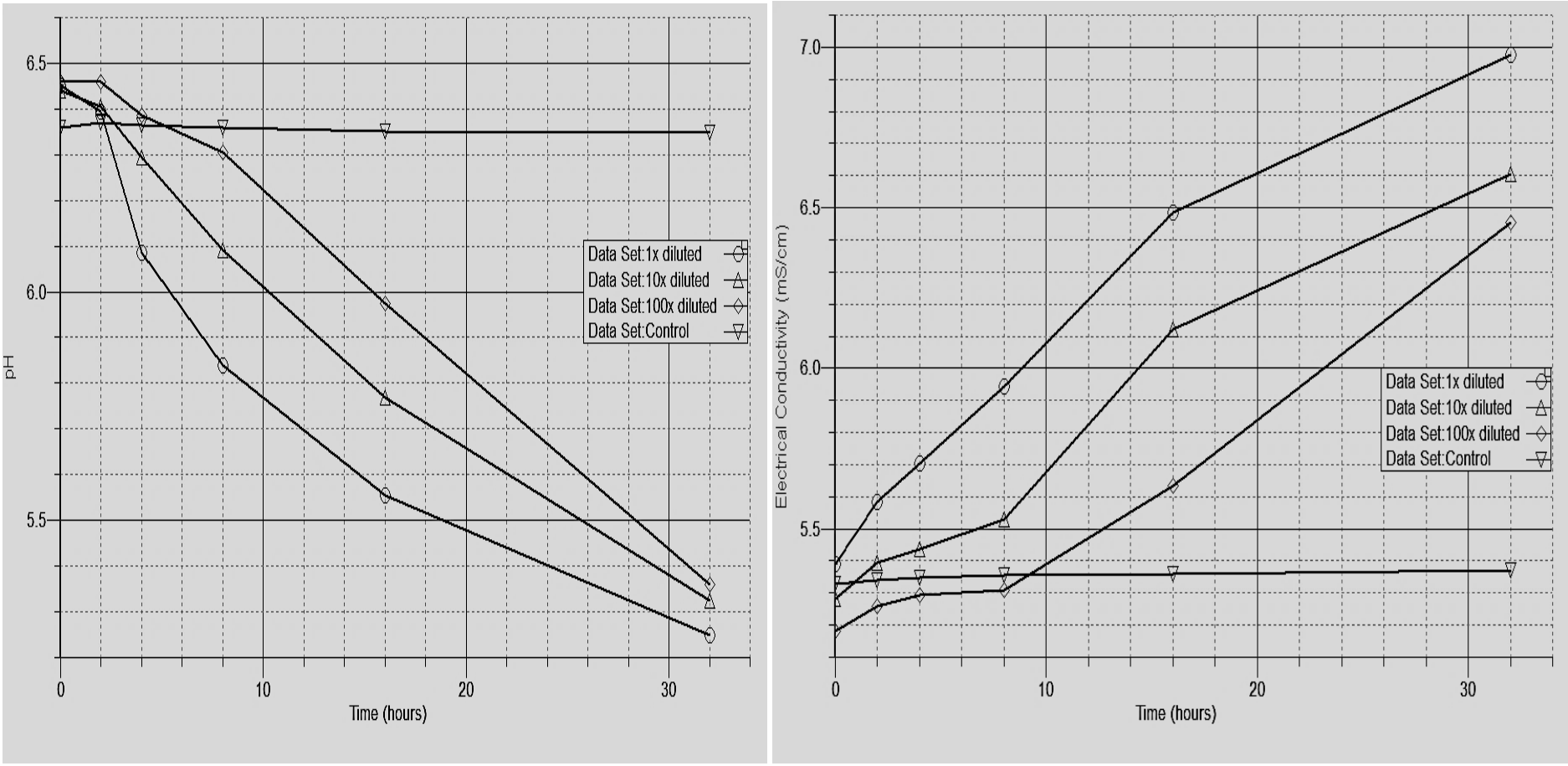

Figure 4: Changes in pH (a) and EC (b) of milk inoculated with three different doses of *S. aureus* at 37°C from 0 – 32 hours. 1x = 10^0 dilution of *S. aureus*, 10 x =10^{-1} dilution of *S. aureus*, 100x = 10^{-2} dilution of *S. aureus*.

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2.3.2 Changes in the pH and EC of Milk Inoculated with *Bacillus* spp. and a mixture of *Bacillus* spp. and *S. aureus*.

Figure 5a displays how the pH changed during the incubation of milk samples inoculated with *Bacillus* Isolate 1 (B1), *Bacillus* Isolate 2 (B2) and *S. aureus* (SA). The pH of the control sample remained almost constant throughout the course of incubation, changing from 6.39 after 0 hours to 6.37 after 32 hours of incubation at 37°C. Both B1 and B2 caused an increase of the pH, to values of 7.30 and 7.41, respectively, whilst SA decreased the pH to a value of 5.36.

Figure 5b shows the changes in EC as a result of the same treatments. At the start of incubation (0 hours), the milk samples had similar EC values. B1, B2 and SA had EC readings of 5.49, 5.33 and 5.19 mS cm^{-1} respectively, relative to a control sample at 5.50 mS cm^{-1}. After 32 hours of incubation, the test flasks of B1, B2, SA and the control had EC values of 8.22, 6.35, 6.49 and 5.48 mS cm^{-1}, respectively.

In Figure 6 are the graphical representation of EC and pH changes of milk samples treated with mixed culture of B1, B2 and SA. Figure 6a indicates a similar trend with regards to pH change during incubation, for the milk samples inoculated with mixtures of *S. aureus* and the *Bacillus* isolates. All the milk samples treated with suspensions of *S. aureus* and *Bacillus* spp showed a decrease in pH (from about 6.40 to less than 5.0). The milk samples treated with undiluted inoculum (1x SA, B1 and B2) showed a rapid drop in pH, but the drop in pH started to level off at 8 hours, and these samples had the highest pH at 32 hours at just above a pH of 5.20. The milk sample with only *Bacillus* spp. (B1 and B2) inoculum had an initial pH of 6.45 that decreased to a pH of 4.28, the lowest final pH.

Figure 6b represents the change in EC of the same milk samples. In the milk samples treated with the highest dilution of inoculum (10^{-2}), there was a gradual increase in EC, whereas in the milk samples treated with undiluted inoculum there was a rapid increase in EC but this started to level off at 13 hours, and these samples had the lowest EC at 32 hours. The milk sample with only *Bacillus* spp. inoculum had an initial EC of 5.03 that increased to 8.45 mS cm^{-1}, the highest final EC. The results with pH measurements were parallel but inverse to the EC results (decreasing pH versus increasing EC) for all treatments.

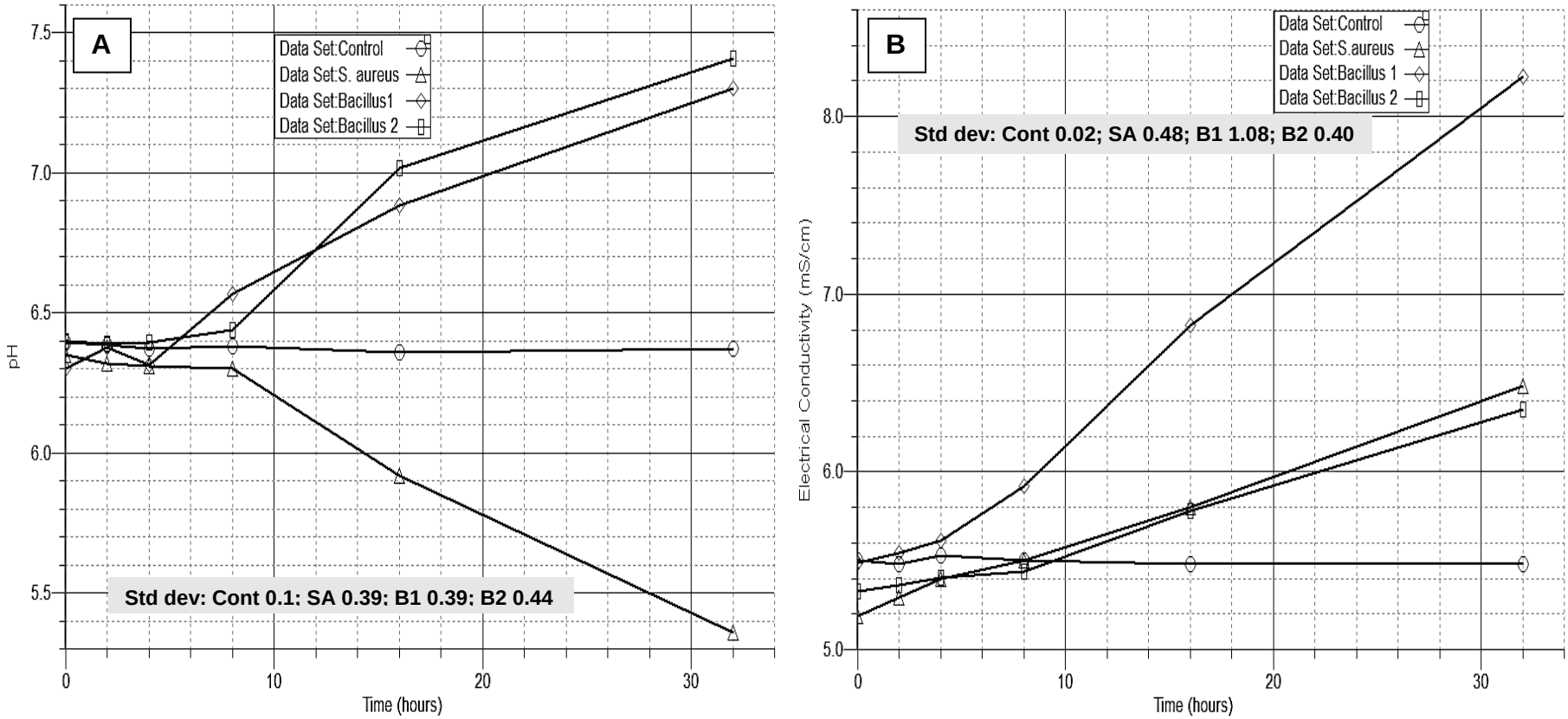
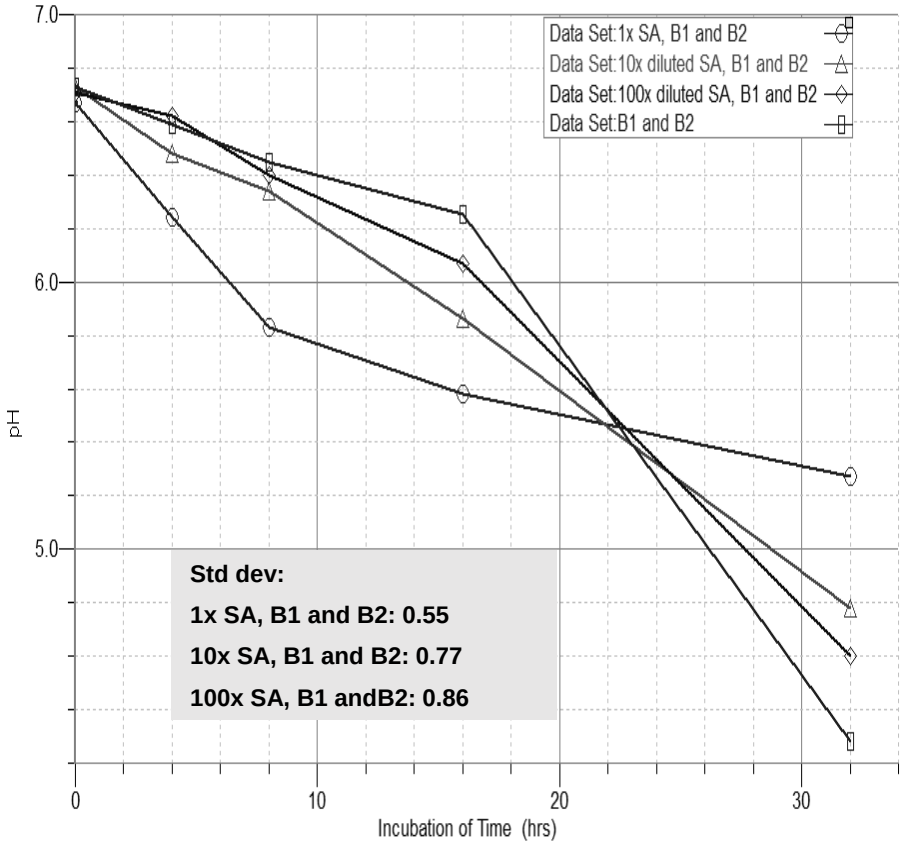


Figure 5: A graphical representation of pH (a) and EC (b) changes of pasteurized milk samples inoculated with either *S. aureus* or *Bacillus* spp. isolates. Cont = uninoculated control; SA = *Staphylococcus aureus*; B1= *Bacillus* spp. Isolate 1; B2= *Bacillus* spp. Isolate 2; std dev = standard deviation.



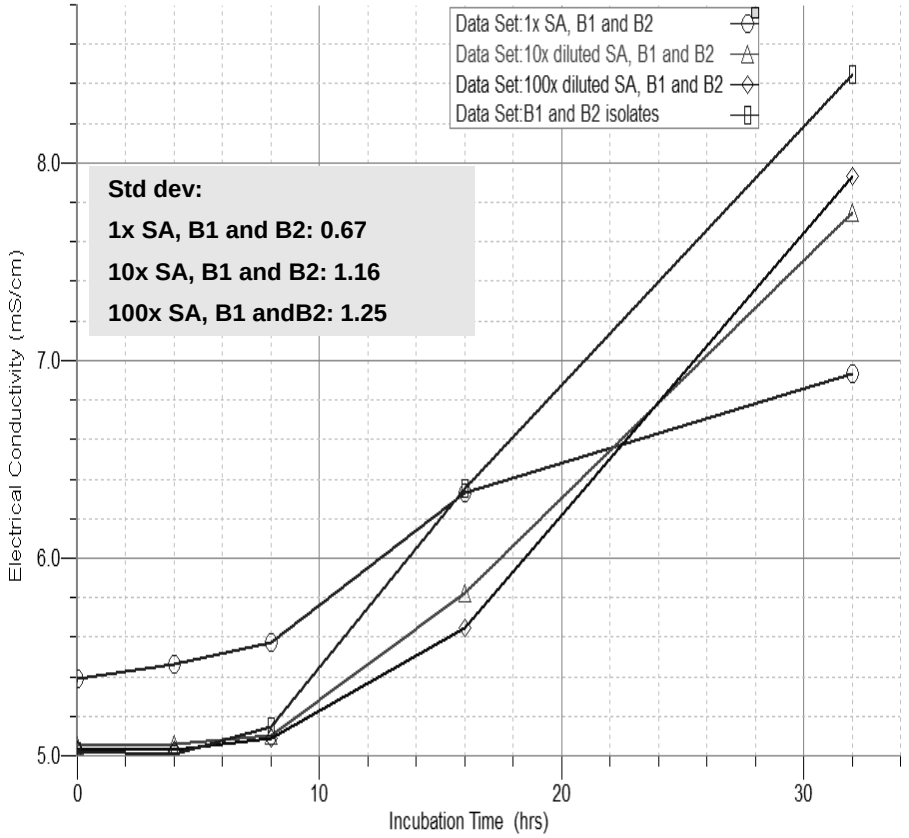


Figure 6: A representation of changes in pH (a) and EC (b) of pasteurized milk samples with mixed culture of *S. aureus* and *Bacillus* spp. isolates. The samples were read in duplicates and the values averaged to create these graphs. In this case, a flask containing only *Bacillus* spp. Isolate 1 and 2 served as a control. SA = *Staphylococcus aureus*; B1= *Bacillus* spp. Isolate 1; B2= *Bacillus* spp. Isolate 2; std dev = standard deviation.

2.4 Discussion

Healthy milk has a pH range between 6.5 and 6.7 and an EC of between 4.0 and 5.5 mS cm^{-1} at 25^0C (Norberg, 2005; Batavani *et al.* 2007). In this study a decrease in pH was observed from a value of 6.36 to 5.25, 5.32 and 5.37 for 10^0, 10^{-1} and 10^{-2} *S. aureus* inoculum levels respectively (Figure 4a). A decrease in pH of milk with *S. aureus* has been reported before and it is in accordance with other studies (Mucchetti *et al.* 1994; Fujikawa and Morozumi, 2006), Fujikawa and Morozumi (2006) observed a lower pH value with milk inoculated with *S. aureus* compared to an un-inoculated control and Janstova *et al.* (2013) also reported a pH value of 4.47 after 102 hours at 25^0C. A decrease in pH has been attributed to the ability of *S. aureus* to ferment lactose into glucose then into lactic acid hence creating acidic conditions (Moon and Parulekar 1993).

As it can be seen in Figure 4b, all the investigated inoculum dosages (10^0, 10^{-1} and 10^{-2}) of *S. aureus* had initial EC values below 5.5 mS cm^{-1} which increased to EC values above 6.90 mS cm^{-1} at 32 hours. The un-inoculated control flask remained between 5.33 to 5.38 mS cm^{-1} throughout the 32-hour incubation period, which is within the normal range of healthy milk and it is reported that when the EC of milk is below 5.5 mS cm^{-1} and when the difference between quarters is less than 0.6 mS cm^{-1} the corresponding quarter or the cow can be considered healthy (Janzekovic *et al.* 2009). Norberg *et al.* (2005), reported estimate mean EC values for healthy, sub-clinically and clinically infected cows at 5.3, 5.75 and 6.73 mS cm^{-1} respectively.

Other similar studies have found that mastitic milk had a higher pH than healthy milk (Wielgosz-Groth and Groth, 2003; Batavani *et al.* 2007). These observed discrepancies may have been due to different experimental set-up in which case the milk used may have had more than just one mastitis causal pathogen (Ogola *et al.* 2007). In this study, two *Bacillus* spp. isolates changed the pH to alkaline conditions rather than acidic conditions (Figure 5a), Moon and Parulekar, (1993) reported that such microorganisms can switch from consuming lactose to amino acids thereby in an increase in the pH of milk, however, the electrical conductivity in Figure 5b showed an increased nonetheless

due to the increase of ions in the culture. Figure 6a shows a decrease in pH, which can be attributed to the fact that a co-culture was used instead and hence the resultant pH decreased, and the resultant increase in EC shown in Figure 6b.

An increase in EC as a result of mastitis has been attributed largely to the influx of sodium and chloride ions from blood into milk because of the increased permeability of damaged epithelial membranes (Viguier *et al.* 2009). Ionic changes are not only caused by mastitis related factors but also external factors that include temperature, fat content, breed, age and the lactation stage of a cow. These confounding factors that reduce the diagnostic value of EC to detect mastitis reliably (Oshima, 1978). In order to maximize the diagnostic value of the EC of milk, two approaches have been tested, absolute and differential EC measurements (Tangorra *et al.* 2010). The absolute EC approach involves measuring milk samples' EC values and comparing them to a set threshold value. If the measured EC values are above this threshold, then the milk samples are considered to be from mastitic cows. With the differential EC approach, the assumption is that when the EC of one quarter of a single cow is greater or equal to 16% or more than the minimum EC of another quarter, then the cow probably has mastitis (Nielen *et al.* 1992). Differential EC has proven to be more sensitive and specific, and thus it is a more reliable approach when using EC technology (Nielen *et al.* 1992; Hamann and Zecconi, 1998). Steenveld *et al.* 2010 reported an EC sensitivity and specificity 70% and 98% respectively.

From this study, we can conclude that markers such as the incubation time at which certain values of pH and EC are obtained relative to the values of a control (un-inoculated sample) could be used to develop a diagnostic tool for the preliminary detection of mastitis. In this study, 16 hours of incubation yielded pH and EC values that were very different to the un-inoculated control. However, for use in the field one should take into consideration other factors affecting milk pH and EC, and whether the pathogens inducing the changes in pH and EC are mixed or a single dominant pathogen since the design of this experiment was focused primarily on *S. aureus* which is the most common causal agent of mastitis and also the use of commercial milk was

to ensure consistency as commercial milk contains a standardized milk components. To improve the accuracy of this study, we recommend that in future, a differential measurement of pH and EC of the quarters be used for the analysis rather than absolute measurements.

References

1. **Batavani, R., Asri, S. and Naebzadeh, H.** (2007). The effect of subclinical mastitis on milk composition in dairy cows. *Iranian Journal of Veterinary Research* **8:** 205-211.
2. **Ford, D., Easton, D., Stratton, M., Narod, S., Goldgar, D., Devilee, P., Bishop, D., Weber, B., Lenoir, G. and Chang-Claude, J.** (1998). Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. *The American Journal of Human Genetics* **62:** 676-689.
3. **Fujikawa, H. and Morozumi, S.** (2006). Modeling *Staphylococcus aureus* growth and enterotoxin production in milk. *Food Microbiology* **23:** 260-267.
4. **Hamann, J. and Krömker, V.** (1997). Potential of specific milk composition variables for cow health management. *Livestock Production Science* **48:** 201-208.
5. **Hamann, J. and Zecconi, A.** (1998). Evaluation of the electrical conductivity of milk as a mastitis indicator. Bulletin of the International Dairy Federation. **334:** 5-22.
6. **Janštová, B., Necidová, L., Janštová, B. and Vorlová, L**. (2013). *Staphylococcus aureus* growth and enterotoxin production in different types of milk. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis*, **60**, 103-108.
7. **Janzekovic, M., Brus, M., Mursec, B., Vinis, P., Stajnko, D. and Cus, F.** (2009). Mastitis detection based on electric conductivity of milk. *Journal of Achievements in Materials and Manufacturing Engineering*, **34:** 39-46.
8. **Kitchen, B.J.** (1981). Bovine mastitis: milk compositional changes and related diagnostic tests. *Journal of Dairy Research* **48:** 167-188.
9. **Korhonen, H.J. and Kaartinen, L.** (1995). Changes in the composition of milk induced by mastitis. In: The Bovine Udder and Mastitis. Editors, Sandholm, M., Honkanen-Buzalski, T., Kaartinen L., and Pyörala, S. Gummerus Kirjapaino, Oy, Jyvaskyla, Finland.

10. **Mucchetti, G., Gatti, M. and Neviani, E.** (1994). Electrical conductivity changes in milk caused by acidification: determining factors. *Journal of Dairy Science* **77:** 940-944.
11. **Moon, S. H. and Parulekar, S. J.** (1993). Some observations on protease production in continuous suspension cultures of Bacillus firmus. *Biotechnology and bioengineering*, **41:** 43-54.
12. **Nielen, M., Deluyker, H., Schukken, Y. and Brand, A.** (1992). Electrical conductivity of milk: measurement, modifiers, and meta-analysis of mastitis detection performance. *Journal of Dairy Science* **75:** 606-614.
13. **Norberg, E.** (2005). Electrical conductivity of milk as a phenotypic and genetic indicator of bovine mastitis: A review. *Livestock Production Science* **96:** 129-139.
14. **Ogola, H., Shitandi, A. and Nanua, J.** (2007). Effect of mastitis on raw milk compositional quality. *Journal of Veterinary Science* **8:** 237-242.
15. **Oshima, M.** (1978). Empirical formula for correcting electrical conductivity values of milk in relation to temperature. *Japanese Journal of Zootechnical Science*. **49:**180–8.
16. **Stenager, E., Knudsen, L. and Jensen, K.** (1991). Acute and chronic pain syndromes in multiple sclerosis. *Acta Neurologica Scandinavica* **84:** 197-200.
17. **Steeneveld, W., van der Gaag, L. C., Ouweltjes, W., Mollenhorst, H. and Hogeveen, H.** (2010). Discriminating between true-positive and false-positive clinical mastitis alerts from automatic milking systems. *Journal of dairy science*, **93**, 2559-2568.
18. **Tangorra, F.M., Zaninelli, M., Costa, A., Agazzi, A. and Savoini, G.** (2010). Milk electrical conductivity and mastitis status in dairy goats: Results from a pilot study. *Small Ruminant Research* **90:** 109-113.
19. **Viguier, C., Arora, S., Gilmartin, N., Welbeck, K. and O'Kennedy, R**. (2009). Mastitis detection: current trends and future perspectives. *Trends in Biotechnology* **27:** 486-493.
20. **Wielgosz-Groth, Z. and Groth, I.** (2003). Effect of the udder health on the composition and quality of quarter milk from black-and white cows. *Electronic*

Journal of Polish Agricultural Universities Animal Husbandry **6**. http://www.ejpau.media.pl/volume6/issue2/animal/abs-01.html

CHAPTER 3:
GAS CHROMATOGRAPHY-MASS SPECTROPHOTOMETRY ANALYSIS OF VOLATILE ORGANIC METABOLITES DETECTED IN MILK SAMPLES INOCULATED WITH MASTITIS PATHOGENS

Abstract

Conventional methods of pathogen identification in milk are time consuming, laborious and often lack accuracy. In the current study, gas chromatography-mass spectrophotometry was tested as a means of indirect identification of pathogens by the analysis of species-specific volatile organic compounds (VOCs). Milk samples were inoculated with the most common mastitis pathogens in South Africa: *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus agalactiae, Escherichia coli* and coagulase negative staphylococci. The primary aim of this study was to detect VOCs produced by these common pathogens in milk, in order to discover whether the VOC profiles could be used to differentiate between inoculated and un-inoculated milk samples, and to find out whether the profiles of the VOCs obtained were species-specific. Sterile commercial milk was inoculated with each of the pathogens, incubated at 37˚C for 24 hours before GC-MS analysis was performed. Subsequently profiling of the detected VOCs was done. A total of 58 VOCs were identified, but only 15 VOCs were commonly produced by the investigated pathogens. The detected VOCs were: hydrocarbons, esters, ketones, aldehydes and siloxanes. Profiling of the detected VOCs indicated that VOCs analysis could be used to distinguish between inoculated and un-inoculated milk samples. However, the detected VOCs were similar between species, so the VOC profiles could not be used for the rapid identification of the causal organisms of mastitis.

3.1 Introduction

There are many microorganisms that can cause mastitis. However, *Staphylococcus aureus*, coagulase-negative staphylococcus (CNS), *Streptococcus uberis*, *Streptococcus dysgalactiae* and *Escherichia coli* cause approximately 80% of mastitis cases (Hettinga *et al.* 2008). Bacteriological culturing has been the choice of identification of these pathogens. However, it has been noted that this technique has many important shortfalls that render it ineffective for microbial detection in milk samples. Typical disadvantages include it being laborious and time consuming, the high risk of contamination during sampling of milk, frequent low bacterial loads in milk, and the occurrence of false negatives (Yamagishi *et al.* 2007). However, considering that microorganisms have species-specific enzymes that facilitate their metabolism, that result in the production of specific volatile metabolite profiles, profiling the released volatile organic compounds (VOCs) during infection might allow for indirect identification of mastitis pathogens (Eriksson *et al.* 2005; Hettinga *et al.* 2008).

VOCs result from the breakdown of nutrients by extracellular enzymes during early stages of bacterial growth (Hettinga *et al.* 2008). These are referred to as primary metabolites, and a typical example includes the hydrolysis of triglycerides (milk fat) by *S. aureus* lipases to free fatty acids (Smeltzer *et al.* 1992). Secondary metabolites include branched free fatty acids, branched alcohols and aldehydes, and tertiary metabolites associated with cell lysis, include as acetoin (Zechman *et al.* 1986). VOCs have been observed to facilitate communication between the microorganisms and their environment, and to act as antimicrobial agents during growth (Stoppacher *et al.* 2010). Primary and secondary metabolites can be detected after short incubation times (lag and exponential phase) while tertiary metabolites can be detected during the stationary or decline phases, thus at longer incubation times (Petersen *et al.* 2004).

The detection and identification of VOCs using techniques such as gas chromatography and mass spectrophotometry (GC-MS) have a significant diagnostic value in medicine.

This technology has been used as a standard for the analysis of VOCs, which includes separation, detection and identification of VOCs *in vivo* and *in vitro* (Bos *et al.* 2013).

The first aim of this study was to identify VOCs associated with each of the most common mastitis causal pathogens using a GC-MS instrument. Secondly, we aimed to see if we could use the data to differentiate between inoculated and un-inoculated milk, and lastly, to determine if the profile of the VOCs produced by each of the investigated pathogens was distinct enough to allow for species differentiation among the pathogens used in this study.

3.2 Materials and Methods

3.2.1 Simulating mastitis

Field strains of *Staphylococcus aureus,* coagulase negative Staphylococcus (CNS), *Streptococcus uberis*, *Streptococcus dysgalactiae* and *Escherichia coli* were subcultured from blood agar plates onto tryptone soy agar (Sigma Aldrich) and incubated for 18°C at 37°C. Thereafter, a loopful of each pathogen was used to inoculate tryptone soy broth (Sigma Aldrich) in McCartney bottles, followed by incubation at 37°C for 24 hours. 1 ml of each culture was then used to inoculate 9 ml sterile fresh commercial milk samples in McCartney bottles, in replicates of 10. An uninoculated milk sample was the designated control (10 replicates).

3.2.2 GC-MS analysis

Inoculated milk samples were incubated at 37°C for 24 hours, and 1 ml samples were sent for GC-MS analysis of VOCs to the Chemistry Department, School of Chemistry and Physics, University of KwaZulu-Natal (Pietermaritzburg Campus). Analysis was performed using a Shimadzu QP2010 instrument (Shimadzu South Africa, (Pty) Ltd). Samples were collectively pre-heated at 60°C for 1 minute, then an extraction of VOCs from the head-space of the containment vials was performed for 15 minutes with constant stirring, at 45°C. A desorption process was then carried out at 250°C for 5 minutes. An APOLAR BPX-5 column was used for the separation of VOCs. The oven

was set at 70°C (2 minutes) to 140°C at 6°C /minute with a 2-minute holding period, then to 220°C at 6°C /minute with a 5-minute holding time. Mass-to-charge ratio (m/z) was set to 33-300 with helium as a carrier gas with a flow rate of 35 cm second^{-1}.

3.2.3 Analysis of Data

The VOCs from each pathogen were identified by the Chemistry Department, School of Chemistry and Physics, University of KwaZulu-Natal, Pietermaritzburg campus. Thereafter, they were analyzed using Microsoft Excel to sort out their concentration within each sample of treated milk compared to the control samples.

3.3 Results

After the GC-MS analysis of inoculated and inoculated milk, Figures 7, 8 and 9 were produced using the data generated. In the Figures, the pathogens were labeled SA (*Staphylococcus aureus*), EC (*Escherichia coli*), CNS (coagulase negative *Staphylococcus*), SUB (*Streptococcus uberis*), SAG (*Streptococcus agalactiae*), and CONT (un-inoculated control sample).

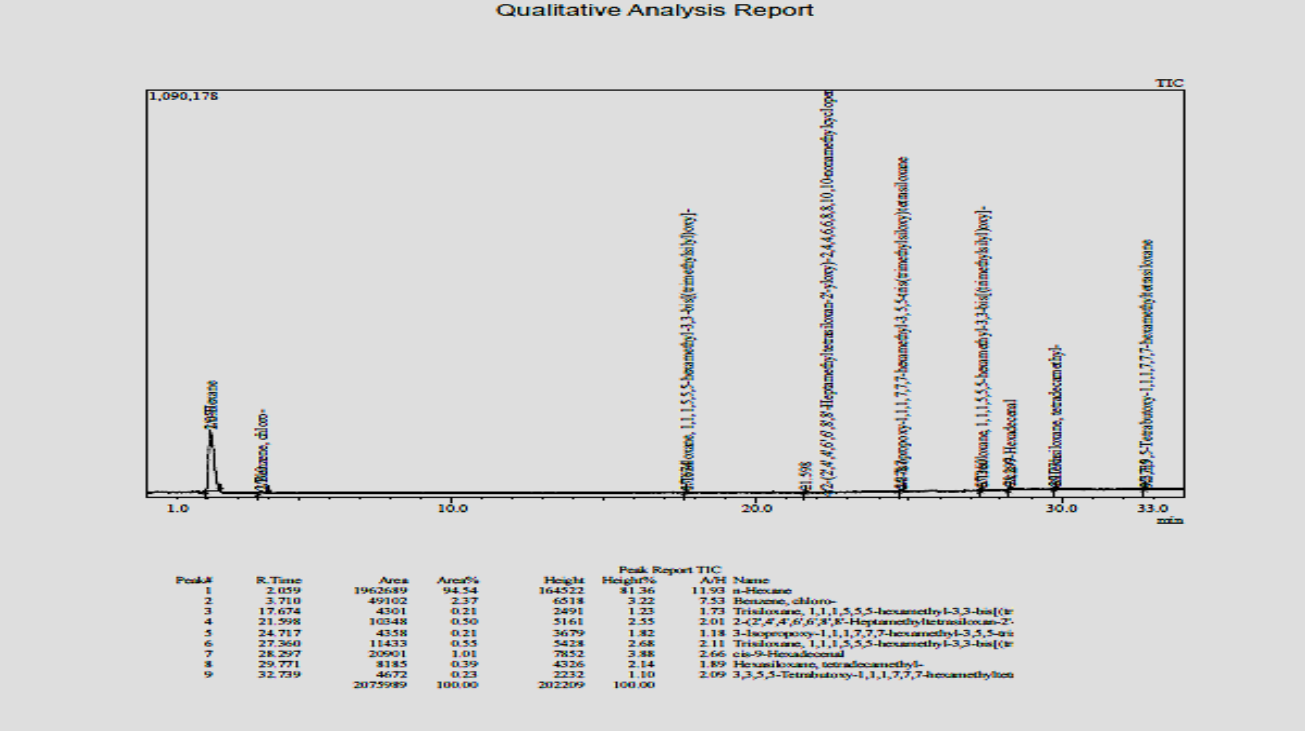


Figure 7: GC-MS analysis report of *S. aureus* inoculated milk sample.

Shown in Figure 7 is an example of a qualitative report corresponding to one of the samples that was inoculated with *S. aureus*. It can be seen that there were 9 different VOCs detected by the GC-MS instrument in this sample. Differences in the amount of VOCs produced from one of the 10 replicates of the *S. aureus* inoculated samples can also be seen based on the area percentages.

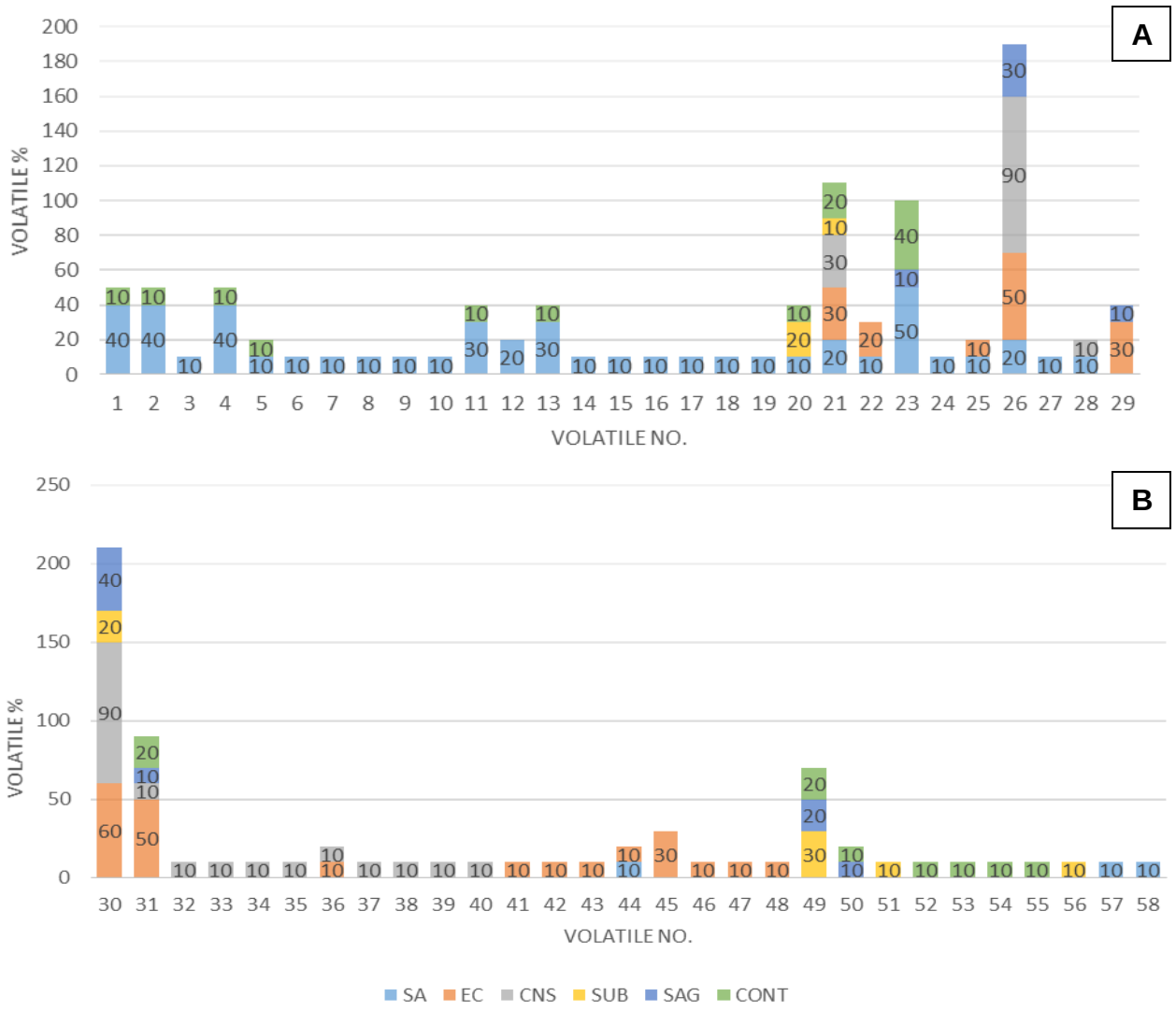


Figure 8: Qualitative and quantitative representation of VOC's detected from inoculated milk samples, including un-inoculated control samples (CONT). The VOCs were numbered 1 – 29 (**A**) then 30-58 (**B**). The inoculated samples were incubated at 37°C for 24 hours. **SA** (*Staphylococcus aureus*), **EC** (*Escherichia coli*), **CNS** (coagulase negative *Staphylococcus*), **SUB** (*Streptococcus uberis*), **SAG** (*Streptococcus agalactiae*), **CONT** (un-inoculated control sample).

From the results shown in Figure 8 (A-B), it can be noticed that in general, un-inoculated control sample had far less or a zero level of the detected volatile metabolites compared to milk samples inoculated with one of the mastitis causal pathogens. Volatiles L-alanine ethylamide-S, **(21)**, carbon dioxide **(23)**, alpha-amyrin

acetate **(26)**, acetic acid **(29)** and acetate **(31)** were the most shared volatile metabolites among the inoculated milk samples.

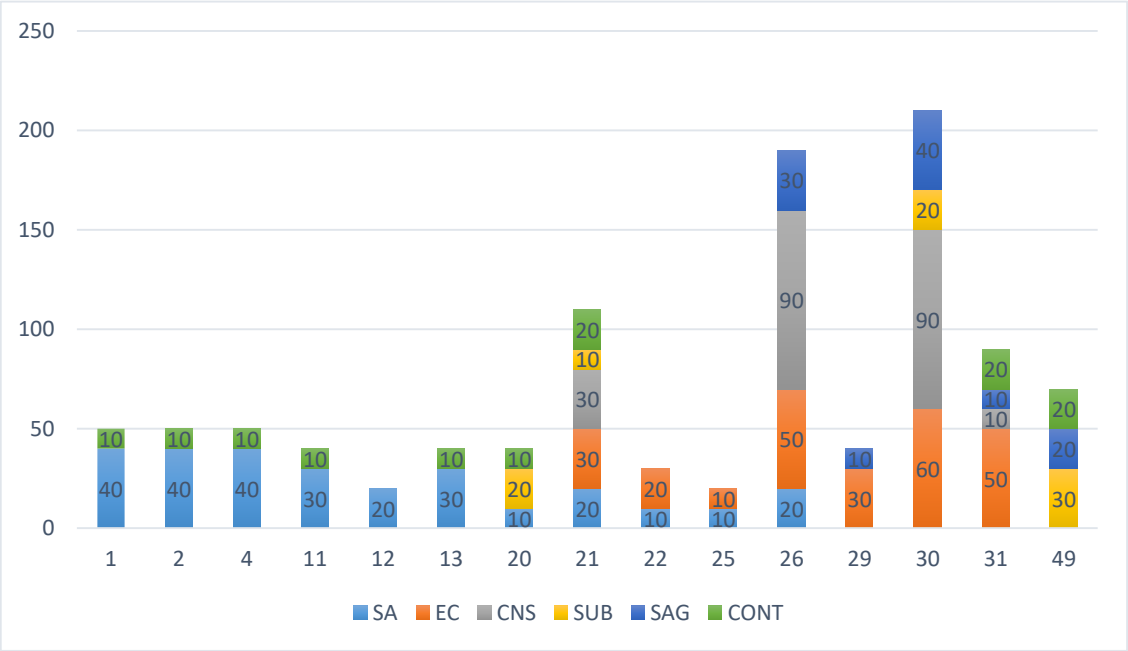
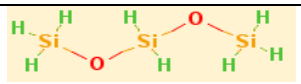
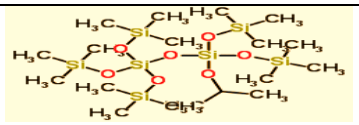
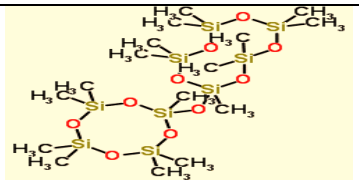
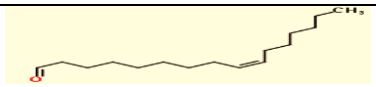
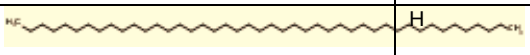
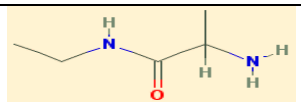
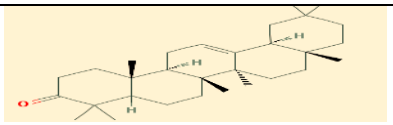
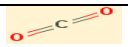
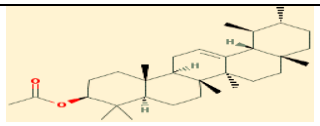
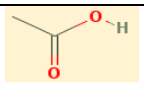
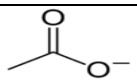
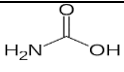


Figure 9: Volatile organic metabolites detected in milk at a prevalence of 20% or more. **SA** (*Staphylococcus aureus*), **EC** (*Escherichia coli*), **CNS** (coagulase negative *Staphylococcus*), **SUB** (*Streptococcus uberis*), **SAG** (*Streptococcus agalactiae*), **CONT** (un-inoculated control sample).

The prevalence of each volatile organic metabolite distribution among the milk samples inoculated with mastitis causal pathogens is shown in Figure 9. From this plot it can be noted that VOCs such as hexane **(1)**, benzene **(2)**, trisiloxane **(4)** and cis-9-hexadecanal **(13)** were associated with *S. aureus,* while L-alanine ethylamide-S **(21)** and alpha amyrin acetate **(26)** were associated with coagulase negative staphylococcus (CNS). Acetate **(31)** and acetic acid **(29)** were commonly associated with *E. coli.* The volatile metabolites tetratetracontane **(20)** and carbamic acid **(49)** are mostly distributed among *St. uberis* replicates, whilst *St. agalactiae* shared volatile metabolites alpha-amyrin acetate **(26)**, acetic acid **(29)**, acetate **(31)** and carbamic acid **(49)** with most of the investigated pathogens.

Table 5: The characteristics each of the VOCs that were more prevalent in milk inoculated with common mastitis causal pathogens.

Compound Name	Chemical formula	Atomic mass (g/mol)	Structure	Type
Hexane **(1)**	C_6H_{14}	86.178		H
Benzene **(2)**	C_6H_6	78.114		H
Trisiloxane **(4)**	$H_8O_2Si_3$	124.317		S
3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane (**11**)	$C_{18}H_{52}O_7Si_7$	577.200		S
(2-[(2,4,4,6,6,8,8-heptamethyl-1,3,5,7,2,4,6,8-tetraoxatetrasilocan-2-yl)oxy]-2,4,4,6,6,8,8,10,10-nonamethyl-1,3,5,7,9,2,4,6,8,10-pentaoxapentasilecane) **(12)**	$C_{16}H_{48}O_{10}Si_9$	653.316		S
cis-9-Hexadecenal **(13)**	$C_{16}H_{30}O$	238.409		Al
Tetratetracontane **(20)**	$C_{44}H_{90}$	619.185		H
l-Alanine ethylamide, (S)- (**21**)	$C_5H_{12}N_2O$	116.164		N-containing
Olean-12-en-3-one (**22**)	$C_{30}H_{48}O$	424.713		K
Carbon dioxide **(23)**	CO_2	44.009		-
α-amyrin acetate (**26**)	$C_{32}H_{52}O_2$	468.766		E
Acetic acid **(29)**	$C_2H_4O_2$	60.052		A
Acetate **(31)**	$C_2H_3O^-$	59.04		E
1S,6R,9S)-5,5,9,10-Tetramethyltricyclo[7.3.0.0(1,6)]dodec-10(11)-ene **(45)**	**N/A**	**N/A**	**N/A**	**-**
Carbamic acid **(49)**	CH_3NO_2	61.04		A

A-Organic acid; **N**-nitrogen; **E**-ester; **K**- ketone; **S**-Siloxanes; **H**- hydrocarbon; **Al**-aldehyde

3.4 Discussion

A systematic review of 31 literature/articles on volatile organic metabolites production by clinical microorganisms including *Staphylococcus aureus* and *Escherichia coli* reported that VOCs produced can be classified into hydrocarbons, alcohols, organic acids, aldehydes, ketone, esters, and sulfur-containing compounds (Bos *et al.* 2013).

Hydrocarbons are believed to be produced from the degradation of fatty acids, mainly through the methylerythritol phosphate and β-oxidation pathways (Schulz and Dickschat, 2007). Examples of commonly detected hydrocarbons include: isoprene, 1-undecene and 1, 3-butadiene (Ney and Boland, 1987). The most common alcohols produced are ethanol, methanol, propanol and butanol, the latter (methanol, propanol and butanol) are most commonly produced by *E. coli*, although not exclusively (Bos *et al.* 2013). 1-alcohols are known to be produced by acetyl coenzyme A through either β or α-oxidation of fatty acid derivatives (Kubo *et al.* 1995; Schulz and Dickschat, 2007).

Acetic acid is the most studied and prevalent organic acid metabolite produced by almost all pathogens, whereas isovaleric acid has been reported to be associated exclusively with *S. aureus* (Hettinga *et al.* 2009; Bos *et al.* 2013). Acetaldehyde and benzaldehyde have been reported to have antimicrobial properties (Larsen and Knöchel, 1997). Other aldehydes such as methylpropanal, 3-methylbutanal and 2-methyl-butanal are known to be intermediate metabolites for the formation of esters and ketones from amino acids (Schulz and Dickschat, 2007). Of these acetaldehydes, 2-methyl-butanal is mostly produced by *S. aureus* while methylpropanal is commonly produced by most pathogens (Bos *et al.* 2013). Ketones with a methyl group are produced when the derivatives of fatty acids are decarboxylated. The most common ketones are acetone, 2-nonanone, 2-dodecanone, 2-pentanone, 2-heptanone and acetoin, with virtually all pathogens producing these ketones (Xiao and Xu, 2007; Bos *et al.* 2013). Esters are produced during the esterification of acetic acid and fatty acids. Most pathogens can produce these volatile organic compounds (Bos *et al.* 2013). S-

containing metabolites such as hydrogen sulfide and methyl mercaptan are among the most common and highly toxic VOCs produced. They are reported to be involved when inflammation is induced (Yoshimura *et al.* 2000). S-containing metabolites have been observed to be generated by all mastitis causal pathogens. However, *E. coli* has most often been associated with hydrogen sulfide (Yoshimura *et al.* 2000).

In this study, various VOCs were observed. Amongst them were metabolites belonging to groups such as the hydrocarbons, organic acids, aldehydes, esters, nitrogen-containing compounds, ketones and siloxanes (having the Si–O–Si linkage) (Table 5). Various studies have detected VOCs generated by mastitic bacteria that belong to most of these groups (Kubo *et al.* 1995; Schulz and Dickschat, 2007; Xiao and Xu, 2007; Hettinga *et al.* 2009). Figure 9 shows that there were hydrocarbons were produced by *S. aureus*. However, hydrocarbons are produced by most pathogens as are not useful as a diagnostic VOC (Bos *et al.* 2013). The VOCs identified in this study, and their prevalence, did not provide a distinct VOC profile for each pathogen because these VOCs are produced by all of the pathogens (Bos *et al.* 2013).

To our best knowledge, the siloxanes identified in this study have not been reported by other authors in studies on VOCs profiling. The siloxane VOCs are numbered (**4, 11** and **12**) in Table 5. Authors Pandey *et al*. (2014) and Saif *et al*. (2008) reported that siloxanes polymers have antimicrobial activities towards *S. aureus* and *E. coli*. Their detection in inoculated milk probably reflects them being released by the pathogens to enhance their competitiveness for space and nutrients.

The different VOC profiles found in this study compared to previous studies may have been influenced by a number of factors that affect all studies on VOCs profiling. These factors include: the use of novel strains of the investigated pathogens; the growth medium used; and differences in sampling times. An example of these differences was demonstrated by Hettinga *et al*. (2009), where a probabilistic neural network was used to determine the optimum incubation time at which most VOCs in milk samples were produced, which was after 8 hours incubation at 37°C.

A total of 58 volatile organic metabolites were detected in both the inoculated (released by pathogens) and un-inoculated milk samples. The results showed a clear distinction between inoculated and un-inoculated milk. However, analysis of the sampled VOCs showed that the detected volatiles were not differential or species-specific. For future experiments on VOC's released from inoculated milk, we recommend assays with several time intervals during incubation and the inclusion of additional mastitis causal pathogens.

References

1. **Bos L. D. J., Sterk, P. J. and Schultz, M. J.,** 2013. Volatile metabolites of pathogens: a systematic review**.** *PLOS Pathogens* **9:** 1-8.
2. **Eriksson, Å. Waller, K. P., Svennersten-Sjaunja, K., Haugen, J., Lundby, F. and Lind, O.** (2005). Detection of mastitic milk using a gas-sensor array system (electronic nose). *International Dairy Journal* **15:** 1193-1201.
3. **Hettinga, K., Van Valenberg, H., Lam, T. and Van Hooijdonk, A.** (2008). Detection of mastitis pathogens by analysis of volatile bacterial metabolites. *Journal of Dairy Science* **91:** 3834-3839.
4. **Hettinga K. A., van Valenberg, H. J. F., Lam, T. J. G. M. and. van Hooijdonk, A. C. M.,** 2009. The influence of incubation on the formation of volatile bacterial metabolites in mastitis milk. *Journal of Dairy Science* **92:** 4901–4905
5. **Kubo, I., Muroi, H. and Kubo, A**. (1995). Structural functions of antimicrobial long-chain alcohols and phenols. *Bioorganic and Medicinal Chemistry* **3:** 873-880.
6. **Larsen, A. and Knöchel, S**. (1997). Antimicrobial activity of food-related *Penicillium* sp. against pathogenic bacteria in laboratory media and a cheese model system. *Journal of Applied Microbiology* **83:** 111-119.
7. **Ney, P. and Boland, W.** (1987). Biosynthesis of 1-alkenes in higher plants. *European Journal of Biochemistry* **162:** 203-211.
8. **Pandey, S., Satpathy, G. and Gupta, R. K.** (2014). Evaluation of nutritional, phytochemical, antioxidant and antibacterial activity of exotic fruit *Limonia acidissima*. *Journal of Pharmacognosy and Phytochemistry* ***3*****:** 81–88.

9. **Petersen, C. D. V., Beck, H. C. and Lauritsen, F. R**. (2004). On-line monitoring of important organoleptic methyl-branched aldehydes during batch fermentation of starter culture *Staphylococcus xylosus* reveal new insight into their production in a model fermentation. *Biotechnology and Bioengineering* **85:** 298-305.
10. **Saif, M. J., Anwar, J. and Munawar, M. A**. (2008). A novel application of quaternary ammonium compounds as antibacterial hybrid coating on glass surfaces. *Langmuir* **25:** 377-379.
11. **Schulz, S. and Dickschat, J. S.** (2007). Bacterial volatiles: the smell of small organisms. *Natural Product Reports* **24:** 814-842.
12. **Smeltzer, M. S., Hart, M. E. and Landolo, J. J.** (1992). Quantitative spectrophotometric assay for staphylococcal lipase. *Applied and Environmental Microbiology* **58:** 2815-2819.
13. **Stoppacher, N., Kluger, B., Zeilinger, S., Krska, R. and Schuhmacher, R**. (2010). Identification and profiling of volatile metabolites of the biocontrol fungus *Trichoderma atroviride* by HS-SPME-GC-MS. *Journal of Microbiological Methods* **81:** 187-193.
14. **Xiao, Z. and Xu, P.** (2007). Acetoin metabolism in bacteria. *Critical Reviews in Microbiology* **33:** 127-140.
15. **Yamagishi, N., Jinkawa, Y., Omoe, K., Makino, S. and Oboshi, K.** (2007). Sensitive test for screening for *Staphylococcus aureus* in bovine mastitis by broth cultivation and PCR. *Veterinary Record: Journal of the British Veterinary Association* **161:** 381-383.
16. **Yoshimura, M., Nakano, Y., Yamashita, Y., Oho, T., Saito, T. and Koga, T.** (2000). Formation of Methyl mercaptan from L-methionine by *Porphyromonas gingivalis*. *Infection and Immunity* **68:** 6912-6916.
17. **Zechman, J. M., Aldinger, S. and Labows, J. N**. (1986). Characterization of pathogenic bacteria by automated headspace concentration-gas chromatography. *Journal of Chromatography B: Biomedical Sciences and Applications* **377:** 49-57.

CHAPTER 4:
NEAR-INFRARED ANALYSES OF THE CHEMICAL COMPOSITION OF MILK SAMPLES, AND FOR THE PRESENCE OF *STAPHYLOCOCCUS AUREUS*

Abstract

Near-infrared spectroscopy can be used to monitor milk components directly affected by mastitis infection. These components include milk constituents such as fats, sugars (lactose) and proteins (casein and whey proteins), and the bacterial cells that cause infection. The aim of this study was to generate an NIRS tool to detect changes in the composition of milk, and for the presence of *S. aureus,* in order to quickly and effectively identify mastitic milk. A total of 100 raw milk samples were collected and wet chemistry methods were used to calibrate an NIR spectrophotometer for milk fats, whey proteins, caseins, lactose and *S. aureus*. Cold ethanol precipitation of fats was followed by the spectrophotometric determination of fats at 205 nm. Lactose was determined by an enzymatic method based on the hydrolysis of lactose to its monosaccharides, while proteins were assayed using the Bicinchoninic acid method. A profile of bacterial cell counts of *S. aureus* was created by determining the bacterial cell counts at intervals of 0, 3, 6, 9, 12, 15, 18, 21 and 24 hours, after inoculation of pasteurised milk. The NIR spectral data obtained was subjected to MOVAV, MSC, SNV, and 1st and 2nd Savitzky-Golay derivatives pre-processing, after which a Kernel partial least squares regression was applied to produce predictive models. The raw milk spectrum obtained was elevated but flat at a long wavelength range (after 1500 nm) instead of the expected topographic shape. This may have been due to the absorptive properties of polystyrene present in the Petri dishes used for the scanning of milk, which resulted in poor calibration models for fats, lactose, whey proteins and caseins. This was specifically characterized by low correlation coefficients and high root mean square errors of calibration and prediction values for all the measured parameters.

4.1 Introduction

Milk is a universal nutrient source comprising of the main components of the human diet, including fats, sugars, minerals, proteins and vitamins (Aernouts *et al.* 2011, Núñez-Sánchez *et al.* 2016). Knowledge of the concentrations of the principal milk constituents is not only important for liquid raw milk but for various milk products that exist in the dairy industry such cheese, butter, yoghurt, ice cream and milk powder (Laporte and Paquin, 1999). The income of a dairy farmer and the economic value of the milk is defined by its composition (i.e., primarily the fat and protein content), which is related to its quality (Aernouts *et al.* 2011).

Animal selection and management schemes in dairy farms can be improved by daily measurement of milk composition for each individual cow (Aernouts *et al.* 2011). In this way, abnormalities in individual cow health status and nutritional changes can be monitored using changes in the concentrations of milk components (Tsenkova *et al.* 2000; Melfsen *et al.* 2012). The balance of energy supply and concentration of protein in the feed is obtained from the knowledge about the protein and urea contents of milk. Thus the energy balance and any metabolic imbalances in an individual cow can be identified by measuring milk constituents such as milk fatty acids, milk total fat and milk protein (McParland *et al.* 2011, Toni *et al.* 2011). Somatic cell count (SCC), minerals, total bacterial count and lactose in raw milk have been considered as common indicators of udder disease, and can be used to monitor udder health (Bruckmaier *et al.* 2004).

Economic losses have compelled dairy farmers to become more efficient and to specialize over the last decade to increase milk production per cow through the use of genetic selection, feed quality improvement and management on the farm (Aernouts *et al.* 2011). A pre-condition for increased profitability of dairy farming is to ensure an increase in the lactation (both duration and yield) and the lifetime production per cow (Aernouts *et al.* 2011). However, highly productive cows are more prone to the

development of diseases, and therefore, early diagnostic techniques that enable effective treatment or prevention strategies are urgently needed (Aernouts *et al.* 2011). Effective diagnostic techniques will help to meet the demands of monitoring the diet and health status of individual cows, so as to decrease economic losses and to optimize animal welfare in dairy industries (Melfsen *et al.* 2012). In most cases, the analyses of basic milk constituents such fats, proteins, lactose and presence of pathogens is done once every 3 to 4 weeks using “wet chemistry” methods on milk samples (Melfsen *et al.* 2012). Disadvantages associated with analyses such as spectrophotometry and bacterial culturing include: high costs, a need for skilled labour and sophisticated equipment, complex pre-treatments, and the delay between sampling and getting results (Balabin and Smirnov, 2011). Therefore there is a need to develop management tools capable of frequent sampling of milk during milking to provide real-time analysis of milk components, ideally on a per-cow basis. This would allow for the early detection of systemic and local infections, allowing for the timeous strategic treatment of infected cows (Friggens *et al.* 2007).

Vibrational spectroscopic techniques such mid-infrared (MIR) and near-infrared (NIR) provides faster, more accurate, non-destructive, and real-time analysis, with minimal sample pre-treatment when compared to wet chemistry methods (Balabin and Smirnov, 2011). NIRS provides for faster, non-destructive measuring, simultaneous measuring, and the potential for online analysis, when compared to MIRS. NIRS has been applied to a wide range of analytic applications, from medical and biochemical studies to petroleum industries (Balabin and Smirnov, 2011). NIRS has been used to measure fat, protein and lactose concentrations in raw milk using reflectance, transmittance and transflectance modes in both liquid and powdered milk samples (Jankovská, 2003; Navrátilová, *et al.* 2006).

The aim of this study was to develop and validate NIRS calibrations models for the most commonly measured chemical and bacteriological parameters of milk, compared with commonly used wet chemistry methods. This would create a tool for the NIRS-based measurement of the main chemical components and the population of *S. aureus* in milk.

4.2 Materials and Methods

4.2.1 Milk Samples

Initially raw milk samples were obtained from Denleigh Dairy Farm, located in Karkloof, Howick, KwaZulu-Natal Province, South Africa. A total of 100 samples were taken, each sample corresponding to one quarter per cow. The cattle were manually milked, and milk was collected in sterile 20 ml McCartney bottles. Samples were stored at -20°C until analysis and no preservatives were added. Commercial milk, brand Clover, was used in subsequent experiments. It was purchased from Pick 'n Pay supermarket, Hayfields Pietermaritzburg, KZN, South Africa.

4.2.2 Spectral Acquisition

Small plastic Petri dishes (55 mm x 16 mm) were used as a sample cell for the NIRS measurements. Prior to scanning each sample, the samples were submerged and warmed to 40°C using a water bath. Scans were done in triplicates for each sample, using the reflection scanning mode at a wavelength range of 400-2500 nm, in the NIR region. The NIRS instrument used in this study was a FOSS NIRSystem model 6500 II (USA).

4.2.3 Wet Chemistry

4.2.3.1 Fat Assay

The experiment was carried out according to Forcato *et al.* (2005) with a few modifications. Standardized milk samples (5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 mg of fat per 1000 µL) were prepared in 2 ml micro-centrifuge tubes by mixing appropriate volumes of commercial fat free milk (200 mg fat per 100 ml / 0.2% fat), whole milk (3400 mg fat per 100 ml / 3.4% fat) and milk cream (37,000 mg fat per 100 ml / 37% fat). Commercial milk products (i.e., fat free, whole milk and milk cream) were purchased from Pick 'n Pay, Pietermaritzburg, South Africa and these products are from

Clover Manufacturers. Standard samples were prepared in triplicates and no preservatives were used. Samples were stored at -20ºC to prevent or minimize sample deterioration prior to fat measurement.

Standard or test milk samples were warmed up to 40ºC in a water bath, to ensure milk homogeneity, as milk often separates to layers (with the majority of milk fat at the top layer) at room temperature. Then 60 µL of each of the standard or test samples was mixed with 1500 µL cold absolute ethanol in 2 ml micro-centrifuge tubes (the ethanol was cooled at -20 ºC freezer for approximately 20 minutes prior to this step). The resultant solutions were stored immediately at -20 ºC for 1 hour, to facilitate the precipitation of proteins and peptides, that would otherwise interfere with the UV measurement of milk fat (Forcato *et al.* 2005).The solutions were centrifuged at 10,406 g for 15 minutes (using a Vacutec Heat Force Neofuge 13 bench-top centrifuge) at room temperature. The supernatant from each sample was transferred to a separate micro-centrifuge tube and the absorbance values for each solution were recorded at 205 nm using 2 µL of solution on a Nanodrop 2000 (Thermo Scientific) set to the UV/VIS mode.

4.2.3.2 Lactose Assay

The lactose assay was carried out using 1 ml of commercial milk, according to manufacturer's instructions provided with a kit used to estimate lactose in milk (Lactose and D-galactose kit, K-LACGAR, Megazyme). Briefly, the sample of 1 ml in duplicate was first clarified to remove a significant amount of fats and proteins, then the resultant sample was used for the assay.

Carrez I solution was prepared by dissolving 3.6 g of potassium hexacyanoferrate (II) ($K_4[Fe(CN_6).3H_2O$) (Sigma Aldrich) in 100 ml distilled water*. Carrez II solution* was prepared by dissolving 7.20 g of zinc sulphate ($ZnSO_4.7H_2O$) (Sigma Aldrich) in 100 ml of distilled water.

1 ml of the pre-treated milk sample was pipetted into 60 ml of distilled water in a 100 ml volumetric flask, mixed and incubated at 50˚C for 15 minutes while stirring. 2 ml of *Carrez I solution* was added and mixed, then 2 ml of *Carrez II solution* was added and mixed, after which 4 ml of 100 mM sodium hydroxide (NaOH) solution was added and

the solution mixed. The samples were made up to a volume of 100 ml using distilled water, then mixed and filtered and 0.2 ml of the filtrate used in the assay.

The principle behind the assay is first the hydrolysis of lactose to D-galactose and D-glucose, in the presence of β-galactosidase, followed by mutarotation of α- and β-anomeric forms of D-galactose in the presence of galactose mutarotase, and finally the oxidation of β-D-galactose anomers by oxidized nicotinamide adenine dinucleotide (NAD^+) to D-galactonic acid in the presence of β-galactose dehydrogenase. The reduced nicotinamide adenine dinucleotide (NADH) that is formed is measured at 340 nm, which is stoichiometric with the amount of lactose.

The spectrophotometric analysis was carried out according to the manufacturer's instructions. Briefly, one cuvette was labelled as a "blank" (B) and the other as the "test sample" (TS), then 0.2 ml aliquot of the test sample was transferred into the TS cuvette and 0.20 ml of β-galactosidase solution was added to both the B and TS cuvettes and the resultant solutions mixed, and incubated at approximately 25^0C for 10 minutes. Then 2.00 ml and 2.20 ml of distilled water was added to the TS and B cuvettes, respectively, after which 0.20 ml of a pH 8.6 buffer containing ethylenediaminetetraacetic acid (EDTA) was added to both cuvettes (EDTA chelates divalent metal ions that would otherwise inhibit β-galactose dehydrogenase). Thereafter, 0.10 ml of a solution containing NAD^+ was added to both cuvettes, the cuvettes were stirred, and incubated for 3 minutes. Then and the absorbance (A_1) of each solutions in the two cuvettes was measured at 340nm using a NanoDrop 2000C (Thermo Scientific). These steps ensured hydrolysis of lactose to galactose and glucose. For the mutarotation and oxidation steps 0.02 ml of a solution containing both galactose mutarotase and β-galactose dehydrogenase was added into both cuvettes. After 6 minutes, when the absorbance values stabilized, which marks the end of oxidation of β-D-galactose, the absorbance values (A_2) at 340nm were taken using a NanoDrop 2000C (Thermo Scientific).

4.2.3.3 Protein (whey proteins and casein) Assay

This methodology was adapted from Hogarth *et al*. (2004) with some modifications. In duplicates, 5 ml of commercial milk were transferred into Falcon conical centrifuge tubes (ThermoFisher Scientific) and centrifuged at 5000g for 10 minutes at 4^0C using an Allegra X-30 R refrigerated benchtop centrifuge (Beckman Coulter). This was done primarily in order to remove milk fats and any material that has pelleted in the bottom of the tube. Casein was then removed from the resultant supernatant by the dropwise addition of 1 M hydrochloric acid until the pH of solution was 4.6 (the iso-electric point of casein). Precipitated casein was removed by centrifugation at 5000g for 10 minutes at 4^0C, dried for 24 hours at 55^0C and then weighed using an analytical balance.

1 ml of the remaining supernatant was dialysed (to remove salts) against a 1X phosphate buffered saline (PBS) at room temperature for 2 hours, followed by dialysis against a new buffer, a 10x diluted PBS, for a further 2 hours, then lastly a dialysis overnight at 4^0C using a 100x diluted PBS buffer. Whey proteins from the dialysed solution were then estimated using a Pierce Bicinchoninic acid (BCA) assay kit (ThermoFisher Scientific), with bovine serum albumin (BSA) as a standard.

4.2.3.4 Microbial Population Assay

This method was adapted from Saranwong and Kawano (2008). One litre of sterile commercial milk (Clover brand) was inoculated with 1 ml of an axenic culture of *Staphylococcus aureus* and then distributed into 45 McCartney bottles at 11 ml each, these samples were grouped into 9 groups, with each group having 5 replicates.. These groups were subjected to incubation at 37^0C for periods of 0, 3, 6, 9, 12, 15, 18, 21 and 24 hours. Microbial populations of *S. aureus* were estimated by determining the CFU ml^{-1} at the above intervals using tryptone soy agar through a spread plate technique after incubation of agar plates at 37^0C for the above mentioned time periods. The remaining 10 ml of each sample was used to obtain NIR spectra in triplicates per sample.

4.2.3.5 Chemometric analysis

Chemometric analysis was performed on the spectral and wet chemistry data to develop predictive models that could be used for each milk constituent and the total *S. aureus* bacterial count in milk, and to determine the precision and accuracy of the generated models. From the data of wet chemistry methods, a column-wise mean was generated for each of the three scans to give one composite scan for each sample in a comma-separated value (csv) file. This was done for the four milk constituents, namely, fats, lactose, whey proteins and casein. A similar approach was used for total microbial count of *S. aureus*. The wet chemistry samples were randomly divided to a calibration set containing 65% of samples and a validation set with the remaining 35%. Pre-processing of spectra included moving averaging (MOVAV), multiple scatter correction (MSC), standard normal variate (SNV), and 1st and 2nd Savitzky-Golay derivatives, in order to provide normalized and smooth spectral data prior to regression. After pre-processing, the calibration was performed by subjecting the data to a kernel partial least squares (PLS) regression algorithm to produce a model able to predict the investigated variables.

5.3 Results

Table 6 is a representation of the data sets that were used for generating calibration models and a set of samples that were used to validate the predictive models, as well as the corresponding statistical values for both data sets.

Table 6: The statistical values of milk constituents and *Staphylococcus aureus* population counts in milk samples used for calibration and validation.

	Calibration					Validation				
Milk components	**n_c**	**Range**	**ave**	**r^2_c**	**RMSEC**	**n_v**	**range**	**Ave**	**r^2_v**	**RMSEP**
Fats (%)	65	0.018 – 12.61	3.23	0.75	14.20	35	0.699 – 13.054	4.54	0.51	15.72
Lactose (%)	65	0 – 6.58	3.98	0.90	2.05	35	0 – 4.933	0.69	-	8.76
Whey Proteins (%)	65	0.641 – 1.74	0.99	0.45	1.77	35	0.574 – 1.361	1.00	0.14	2.17
Caseins (%)	65	0.114 – 5.472	3.13	0.71	5.90	35	1.260 – 5.382	3.31	0.50	9.10
BP (logCFU ml^{-1})	45	6.52 - 11.78	8.75	-	-	45	0.652 – 1.178	0.88	-	-

BP: Bacterial population of *Staphylococcus aureus*, **n_c**: number of samples in the calibration data set, **ave**: average, **r^2_c**: correlation co-efficient of the calibration data set, **RMSEC**: Root mean square error of calibration, **n_v**: number of samples in the validation set, **r^2_v**: correlation co-efficient of the validation data set, **RMSEP**: Root mean square error of prediction, - means negative values for correlation co-efficient and RMSEC/SEP.

Figure 10 (a), represents a typical spectrum of milk obtained in this study. From the spectra, there are characteristic, notable peaks at approximately 1000 nm, 1200 nm, 1400–1500 nm and 1700 nm. After the spectral range of 1700–2500 nm, there seems to be a fairly constant absorbance without distinct peaks. Figure 10 (b) represents a 2nd Savitzky-Golay derivative of the obtained raw spectra with clear absorbance at approximately the same wavelengths as observed in the raw spectra. However, after the 1700–2500 nm spectral range, there are distinct peaks visible at wavelengths of 1750, 1850, 2150, 2300, 2350, and 2450 nm.

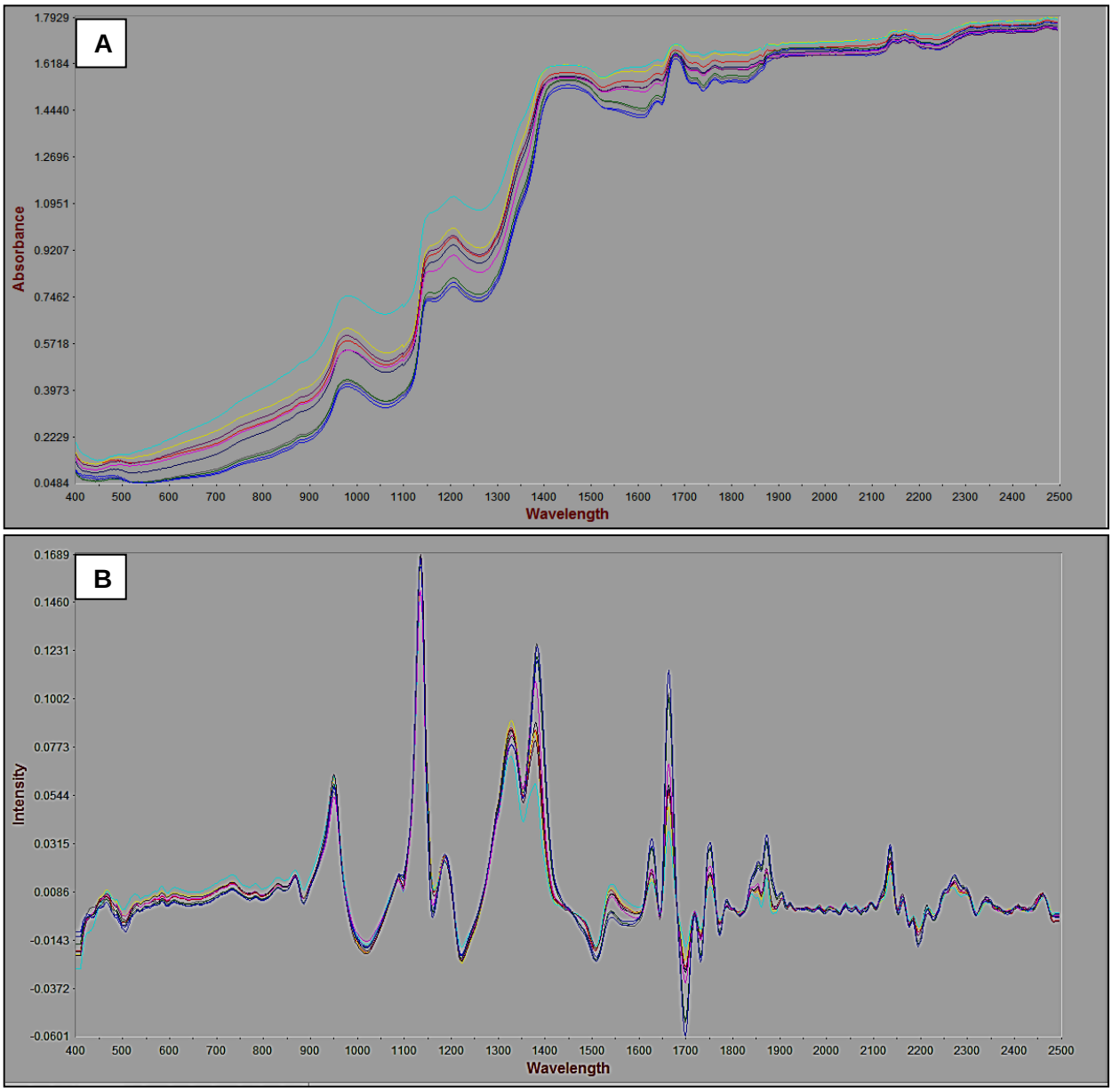


Figure 10: Spectral representation of raw milk (a) versus the Savitzky-Golay derivative (b).

Figure 11 shows scatter plots relating the predicted NIRS values using the calibration models versus the wet chemistry values obtained from the milk samples. There was a poor correlation between the NIRS predicted values versus wet chemistry values for lactose (a), whey proteins (b), casein (c), and fats (d).

There was also a poor correlation between predicted NIRS values versus the microbiological estimates of cell counts for *S. aureus*, as shown in Figure 11 (e).

The reliability of the scatter plot was based on the closeness of the data points to the line of best fit, which is mathematically interpreted as the correlation coefficient.

In the Appendix Figure 1 shows the Petri dish characteristic spectra against a Petri dish with milk spectra and Figures 2-5 illustrate characteristic spectra (raw and differentiated) of milk based on investigated milk components.

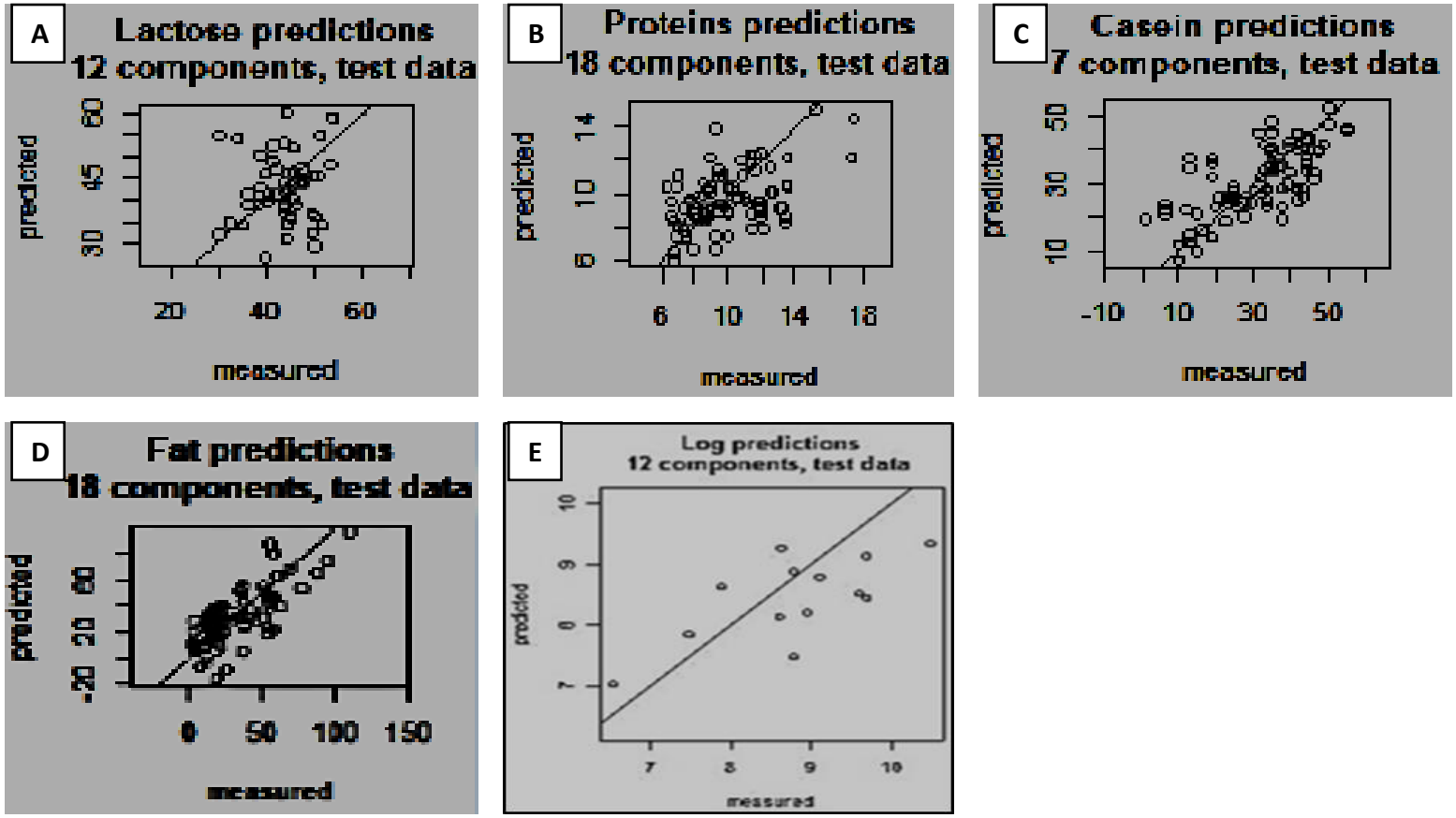


Figure 11: Scatter plots of the NIRS predicted vs the wet chemistry measured values of the investigated milk components: lactose (a), whey proteins (b), casein (c), and fats (d) and (e) population of *S. aureus* in milk.

4.4 Discussion

From the basic statistics presented in Table 6, the mean values of the milk constituents that were used for calibration in this study were similar to the values reported in the literature. Tsenkova *et al*. (2001) reported values of 3.724%, 3.106%, and 4.458% for fats, total proteins and lactose, respectively from Holstein cows. Aernouts *et al*. (2011) reported mean values of Flemish herds for fats, proteins and lactose of 4.61%, 4.127% and 4.58%, respectively. In both these studies, a Milkoscan NIRS device was used for the determination of fats, lactose and total proteins. The estimated means obtained in this study were 3.228%, 4.127% and 3.983% for fats, proteins (whey protein plus casein) and lactose, respectively.

However, there were anomalies observed with the corresponding means of the validation data set that was used, particularly with the lactose and *S. aureus* population count. A reliable predictive model is characterized by a high correlation coefficient for validation (r^2_v), as well as low RMSEC and RMSEP values (Alvarez-Guerra *et al*. 2010). From this study, as shown in Table 4.1, there were low r^2_v values of ≤ 0.51 and high RMSEP values for the milk constituents investigated and the bacterial populations of *S. aureus* obtained from the algorithm used. The obtained r^2 values for validation are indicative of unreliable predictive models for the determination of the investigated parameters. The fact that there were also poor correlation coefficients and RMSEC present in the calibration set suggest that there were factors affecting accuracy of measurement during the acquisition of spectra. Two primary factors were identified: (1) the use of Petri dishes as measuring chambers. Unfortunately, the plastic petri dishes used contained polystyrene which absorbs light at a critical range of the spectrum; (2) the milk samples were warmed to 37^0C instead of using ambient room temperature, which would have affected the NIR spectra gathered.

It is also probable that the wet chemistry methods used were also not most accurate methods. The standard methods that are most approved for NIRS calibration of milk include the Mojonnier methods for fats, the Kjeldahl method for determination of

proteins, and polarimetric and photometric methods for lactose determination (Burns, *et al.* 2007).

The NIR spectra of milk samples obtained in this study using a FOSS NIRSystem model 6500 II (USA) was comparable to milk spectra reported in the literature (Tsenkova *et al.* 2000), especially in the mid wavelength range. There were strong absorbance peaks at approximately 950–1000 nm, 1150–1250 nm, 1450–1500 and 1700nm observed in the raw reflectance spectra, as shown in Figure 10 (a). However, the spectra captured in the wavelength range after 1700 nm was different from raw milk spectra reported in the literature (Tsenkova *et al.* 2001). Specifically, the milk spectra obtained in this study had a fairly constant absorption in the longer wavelength range (1700–2500 nm), whereas the characteristic spectra of milk reported in the literature has a low level of absorbance in this region except at 1940 nm, where an O-H stretch vibration occurs, corresponding to water found in milk (Tsenkova *et al.* 2000; Aernouts *et al.* 2011). Due to the dominant presence of water in milk, studies have reported overtone and combination broad bands of the O-H bonds of water to fall between 900–1000 nm; 1400–1500 nm; and 1900–2000 nm, and consequently there are no O-H bands from 700–900 nm; 1500–1900 nm; and 2000–2500 nm, which allows for the detection of other milk constituents (Burns, *et al.* 2007). In this study, the characteristic absorbance pattern observed for milk in the longer wavelength region may have been distorted due to the polystyrene present in the plastic Petri dishes that were used to hold the milk samples during scanning. Polystyrene absorbs intensely in this region as the overtone and combination bands of $(C\text{-}H)_n$ bonds constituting polystyrene have been observed to absorb light strongly at 1143.7 nm, 1680.2 nm, 2167.2 nm, and 2306.8 nm, which is in the NIR region (Burns, *et al.* 2007). There are peaks in the raw spectra and the 2^{nd} derivative in Figure 10 that can be approximately assigned to these wavelength values. Thus the polystyrene in the Petri dishes would have absorbed light in the informative regions normally used detection of milk's primary constituents. The absorption peaks of polystyrene, 1143.7 nm, 1680.2, 2167.2, and 2306.8 nm are in the 1000–2500 nm wavelength range, which Tsenkova *at al*. (1999) reported as being the most informative region of the NIR spectrum for the analysis of milk components. This is consistent with

other studies, which have reported more absorbance in the longer wavelength region than in the short wavelength region for milk fats, proteins and lactose. Laporte and Paquin (1999) and Aernouts *et al*. (2011) reported 3rd overtone C-H stretch vibrations of triglycerides making up fats to appear at 930 nm; 1st overtone C-H stretching of fat molecules at 1690 nm; and a combination of fundamental vibration and 2nd overtones due to C-H bonds at 2300 nm and 2350 nm. For proteins, the prominent absorbance bands are present in the range 1640–1670 nm, corresponding to the 1st overtone amide B group; and at 2060 nm and 2170 nm corresponding to the vibration due to N-H bonds (Czarnik-Matusewicz *et al.* 1999; Aernouts *et al.* 2011). Stuart and David (1997) reported an absorbance range corresponding to lactose in the region of 1480–1500 nm, with 1490 nm specifically corresponding to the 1st overtone O-H stretch vibration of lactose. The fact that polystyrene absorbance bands are also present in this longer wavelength informative region was identified as the primary reason that predictive models obtained in this study were so poor. Clearly, the absorbance of the sample testing containers/cells is extremely important for NIR analyses, and cannot interfere with the absorbance spectra of the target compounds.

Figure 11 (e) shows the correlation between NIRS values and log CFU ml^{-1} of the predictive model obtained for the monitoring of *S. aureus* population in milk. From the scatter plot, there was a poor correlation, hence a poor predictive mode. This is also shown by the negative $r^2_{c/v}$ and RMSEC/SEP values, as shown in Table 4.1. Saranwong and Kawano (2008) investigated the capability of an NIRS instrument to quantify cells of bacteria such as *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Lactococcus* sp. present in milk. They reported that the spectral range of 700–900 nm corresponded to the light scattering effects of bacterial colonies in milk, and that the region of 900–1100nm was useful for the detection of the chemicals generated during the bacterial degradation of proteins and lactose, producing compounds such as urea and lactic acid. The poor correlation between the NIRS predicted values and log CFU ml^{-1} actual values obtained for *S. aureus* calibration may also be attributed to the masking effect of polystyrene, and secondly, to the use of a relatively small number of samples (n=45). Thirdly, there was a problem with the microbiological method used, namely, using the

population growth of SA on TSA, a solid medium, as a model for the growth of SA in milk, a liquid medium.

To improve this study one would need approximately 300 milk samples for both the calibration of milk components and for the cell counts of the *S. aureus* population in milk. Furthermore, the NIRS scanning instrument should have a temperature module to ensure that the sample and the instrument temperatures are identical and that the temperature remains constant throughout the scanning session. A quartz or glass cuvette with no detectable absorptive properties within the NIR region should be used during scanning, to ensure that there is no interference in the NIR informative regions where fats, proteins, lactose and bacterial cells absorb light.

References

1. **Aernouts, B., Polshin, E., Lammertyn, J. and Saeys, W.** (2011). Visible and near-infrared spectroscopic analysis of raw milk for cow health monitoring: reflectance or transmittance? *Journal of Dairy Science* **94**: 5315-5329.
2. **Balabin, R. M. and Smirnov, S. V.** (2011). Variable selection in near-infrared spectroscopy: benchmarking of feature selection methods on biodiesel data. *Analytica Chimica Acta* **692:** 63-72.
3. **Bruckmaier, R., Ontsouka, C. and Blum, J.** (2004). Fractionized milk composition in dairy cows with subclinical mastitis. *Veterinarni Medicina-UZPI* (Czech Republic) **49:** 283-290.
4. **Czarnik-Matusewicz, B., Murayama, K., Tsenkova, R. and Ozaki, Y.** (1999). Analysis of near-infrared spectra of complicated biological fluids by two-dimensional correlation spectroscopy: protein and fat concentration-dependent spectral changes of milk. *Applied Spectroscopy*, **53,** 1582-1594.
5. **Burns, D. A., and Ciurczak, E. W.** (Eds.). (2007). Handbook of Near-Infrared Analysis. CRC Press, New York, Basel, Hong Kong.
6. **Forcato, D., Carmine, M., Echeverria, G., Pecora, R. and Kivatinitz, S.** (2005). Milk fat content measurement by a simple UV spectrophotometric method: An alternative screening method. *Journal of Dairy Science* **88:** 478-481.

7. **Friggens, N., Ridder, C. and Løvendahl, P.** (2007). On the use of milk composition measures to predict the energy balance of dairy cows. *Journal of Dairy Science* **90:** 5453-5467.
8. **Hogarth, C. J., Fitzpatrick, J. L., Nolan, A. M., Young, F. J., Pitt, A. and Eckersall, P. D.** (2004). Differential protein composition of bovine whey: a comparison of whey from healthy animals and from those with clinical mastitis. *Proteomics* **4:** 2094-2100.
9. **Jankovská, R., and Sustová, K.** (2003). Analysis of cow milk by near-infrared spectroscopy. *Czech Journal of Food Sciences*, **21:** 123-128.
10. **Laporte, M .F. and Paquin, P.** (1999). Near-infrared analysis of fat, protein, and casein in cow's milk. *Journal of Agricultural and Food Chemistry* **47:** 2600-2605.
11. **McParland, S., Banos, G., Wall, E., Coffey, M. P., Soyeurt, H., Veerkamp, R. F., and Berry, D. P.** (2011). The use of mid-infrared spectrometry to predict body energy status of Holstein cows. *Journal of Dairy Science*, **94:** 3651-3661.
12. **Melfsen, A., Hartung, E. and Haeussermann, A.** (2012). Accuracy of in-line milk composition analysis with diffuse reflectance near-infrared spectroscopy. *Journal of Dairy Science* **95:** 6465-6476.
13. **Navrátilová, P., Hadra, L., Dračková, M., Janštová, B., Vorlová, L. and Pavlata, L**. (2006). Use of FT-NIR spectroscopy for bovine colostrum analysis. *Acta Veterinaria Brno* **75:** 57-63.
14. **Núñez-Sánchez, N., Martínez-Marín, A. L., Polvillo, O., Fernández-Cabanás, V. M., Carrizosa, J., Urrutia, B., and Serradilla, J. M.** (2016). Near Infrared Spectroscopy (NIRS) for the determination of the milk fat fatty acid profile of goats. *Food Chemistry*, **190:** 244-252.
15. **Saranwong, S. and Kawano, S.** (2008). System design for non-destructive near infrared analyses of chemical components and total aerobic bacteria count of raw milk. *Journal of Near Infrared Spectroscopy* **16:** 389-398.
16. **Stuart, B. H. and Ando D. J.** (1997). Biological applications of infrared spectroscopy. John Wiley & Sons, pp. 150-154.

17. **Toni, F., Vincenti, L., Grigoletto, L., Ricci, A. and Schukken, Y.** (2011). Early lactation ratio of fat and protein percentage in milk is associated with health, milk production, and survival. *Journal of Dairy Science* **94:** 1772-1783.
18. **Tsenkova, R., Atanassova, S., Itoh, K., Ozaki, Y. and Toyoda, K.** (2000). Near infrared spectroscopy for biomonitoring: cow milk composition measurement in a spectral region from 1,100 to 2,400 nanometers. *Journal of Animal Science* **78:** 515-522.

DISSERTATION OVERVIEW

Introduction

Bovine mastitis is the most common and costly disease affecting dairy industries across the globe. Mastitis results from the inflammation of the parenchyma of the mammary gland after microbial infection (Wellenberg *et al.* 2002). There is a wide range of microorganisms reported to cause bovine mastitis, including algae, viruses, chlamydia, mycoplasmas, fungi and bacteria (Watts 1988; Wellenberg *et al.* 2000; Malinowski *et al.* 2005). However, bacterial infections are the most common, accounting for approximately 90-95 % of all mastitis cases, and *Staphylococcus aureus* is amongst the most common bacterial pathogens (Sudhan and Sharma 2010; Viguier *et al.* 2010).

The symptoms of bovine mastitis are not always observed in mastitis cases. For example, during subclinical mastitis, symptoms are not visible to the naked eye and can therefore be difficult to detect (Viguier *et al.* 2009). The clinical form of mastitis where the milk has visible changes such as blood clots, a strong odour, and a watery appearance, which is accompanied by redness of the mammary gland, is more easily observed (Cvetnić *et al.* 2016). Following infection, there are characteristic chemical, physical and pathological changes in milk, and in the glandular tissue of the udder, that have been used for diagnosing mastitis (Norberg, 2005).

The most commonly used diagnostic technique to detect mastitis is based on a count of somatic cells in milk. During a mastitic event, there is an increase in the somatic cell count (SCC), which is an immune response to invading pathogenic cells in the udder of the cow. Thus the determination of SCC is an indirect measure of mastitis (Pyörälä, 2003). The use of SCC has some limitations.These include a low sensitivity; difficulties in interpreting results because a high SCC can also be the result of other infections or stresses affecting a cow, or a residual effect despite the absence of pathogens; and a significant delay existing between the farmer taking milk samples, and the farmer

receiving the results, which reduces the opportunity to treat infected cows timeously (Viguier *et al.* 2009).

With the disadvantages of the SCC based method of detection in mind, the aim of this study was to develop alternative mastitis detection techniques based on the changes in in milk of pH, EC, levels of fats, lactose, whey proteins, volatile organic metabolites (VOC's) and caseins, and quantifying microbial populations. The objectives of this study were firstly, to monitor the pH and EC (*in vitro*) of milk inoculated with *S. aureus,* aiming to find a threshold in both pH and EC values that could be used diagnostically to differentiate between healthy and mastitic milk. Secondly, it was to identify volatile organic compounds (VOCs) in contaminated milk that are specifically associated with pathogens that cause mastitis. This was in the hope that we could develop a diagnostic tool to differentiate between mastitic milk and healthy milk, and to identification the key pathogens based on specific VOC "fingerprints". And thirdly, it was to develop predictive models for the estimation of fat, lactose, whey protein and casein levels, and *S. aureus* populations in milk using NIRS.

Major Findings and their Implications

Milk samples inoculated with three different concentrations (10^{0}, 10^{-1}, and 10^{-2}) of *S. aureus* and incubated at 37°C was monitored for pH and EC changes at time intervals of 0, 2, 4, 8, 16 and 32 hours. The pH and EC of uninoculated milk was remarkably stable over 32 hours. However, for all the inoculated milk samples the pH decreased from 6.45 to 5.31 after 32 hours of incubation at 37°C. There was a corresponding increase in EC from 5.28 mS cm^{-1} at 0 hours to 6.68 mS cm^{-1}. There was a clear distinction in terms of both the pH and EC between the control sample and all inoculated samples in the region of 8-16 hours after incubation at 37°C. Another experiment was conducted to evaluate how *S. aureus* changes the pH and EC of milk if it is in a mixed culture with naturally occurring *Bacillus* spp. Despite the presence of two strains of *Bacillus* sp., the pH decrease and EC increase was fairly consistent with the results for milk inoculated with *S. aureus* only These results mean that mastitis caused

by *S. aureus* can be monitored using pH and EC values. However, given that mastitis can be caused by a variety of number of microorganism and the fact that pH and electrical changes in milk can be influenced by many other factors both internally and environmentally, more research needs to be done to see if these results would also hold for other pathogens, and how the environment and the cow itself affect these results. However, pH and EC are quick, cheap and fast to measure, so this approach could be valuable to farmers, both commercial and small scale.

Another finding was that the VOCs released from milk samples inoculated with the most common mastitis causal pathogens such as *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus agalactiae, Escherichia coli* and coagulase negative staphylococci, can be detected using GC-MS, even at low concentrations. The detected VOCs were not species specific so it is unlikely that VOCs will provide a chemical "fingerprint" unique to each pathogen. However, VOCs do provide a useful tool for farmers to quickly distinguish between infected and uninfected milk. Whilst a GC-MS machine is too complex and expensive for most dairies to operate, an "electronic nose" such as mass spectrometer could be developed to do the same job to detect specific VOCs relatively cheaply.

A major goal of the study was to develop predictive models based on NIR spectra correlated with wet chemistry measurements, to be used for the estimation of fats, lactose, protein levels, and cell counts of *S. aureus* in milk. However, plastic Petri dishes were used as the vessels to contain the milk samples during NIRS scanning. Unfortunately, the $(C\text{-}H)_n$ bonds of polystyrene, which was present in the plastic Petri dishes that were used, absorb light in the region 1000-2500 nm, which is the light region where the most reliable constituent bands of milk are present for the capturing of NIR spectra. As a result, the NIRS predictive models for milk fats, lactose and proteins, and for cell counts of *S. aureus* in milk, were not satisfactory. The study needs to be repeated using quartz cuvettes for capturing of NIR spectral absorption, and with a larger number of milk samples.

Conclusion and Way Forward

Globally, the standard test for mastitis is to count somatic cells in milk (SCC). However, there are a number of problems with SCC alone. SCC results can be both false negative (usually with the early stages of mastitis) and false positive (usually late stage, post-mastitis), and the test requires sophisticated equipment usually housed and operated in a centralized laboratory, a logistic situation that creates delays in the testing of milk samples. This study evaluated alternative technologies for the detection of mastitic milk. The techniques tested showed potential as relatively quick, simple and quick ways to detect mastitis in cows at an early stage of mastitis.

There is a need to expand the testing of pH and EC in milk to include other pathogens. The detection of VOC's using GC-MS was good for discriminating between clean and contaminated milk. This approach could be adopted in the dairy industry using electronic noses. Finally, NIRS has great potential to be used for the rapid, non-destructive and relatively cheap assessment of milk samples. The study undertaken needs to be repeated using quartz cuvettes instead of Petri dishes during milk scanning, increasing milk sample numbers to more than 300 samples, using different algorithms, and using better wet chemistry assays. Once reliable predictive models have been developed for the major constituents of milk, NIRS could be investigated as a tool to qualitatively and quantitatively detect mastitic pathogens alone or in mixtures.

References

1. **Cvetnić, L., Samardžija, M., Habrun, B., Kompes, G. and Benić, M.** (2016). Microbiological monitoring of mastitis pathogens in the control of udder health in dairy cows. *Slovenian Veterinary Research* **53:** 131-140.
2. **Malinowski, E., Lassa, H., Kłlossowska, A., Smulski, S., Markiewicz, H. and Kaczmarowski, M.** (2005). Etiological agents of dairy cows' mastitis in western part of Poland. *Polish Journal of Veterinary Sciences* **9:** 191-194.
3. **Norberg, E.** (2005). Electrical conductivity of milk as a phenotypic and genetic indicator of bovine mastitis: A review. *Livestock Production Science* **96:** 129-139.

4. **Pyörälä, S.** (2003). Indicators of inflammation in the diagnosis of mastitis. *Veterinary Research* **34:** 565-578.
5. **Sudhan, N. and Sharma, N.** (2010). Mastitis—an important production disease of dairy animals. *SMVS'Dairy Year Book, Ghaziabad* pp 72-88.
6. **Viguier, C., Arora, S., Gilmartin, N., Welbeck, K. and O'Kennedy, R.** (2009). Mastitis detection: current trends and future perspectives. *Trends in Biotechnology* **27:** 486-493.
7. **Viguier, C., Arora, S., Gilmartin, N., Welbeck, K. and O'Kennedy, R.** (2010). Mastitis detection: current trends and future perspective. *Trends in Biotechnology* **27:** 486-493.
8. **Watts, J. L.** (1988). Etiological agents of bovine mastitis. *Veterinary Microbiology* **16:** 41-66.
9. **Wellenberg, G., Van der Poel, W., Van der Vorst, T., Van Valkengoed, P., Schukken, Y., Wagenaar, F. and Van Oirschot, J.** (2000). Bovine herpesvirus 4 in bovine clinical mastitis. *Veterinary Record* **147:** 222-225.
10. **Wellenberg, G. J., Van Der Poel, W. H. M. and Van Oirschot, J. T**. (2002). Viral infections and bovine mastitis: a review. *Veterinary Microbiology* **88:** 27-45.

APPENDIX 1

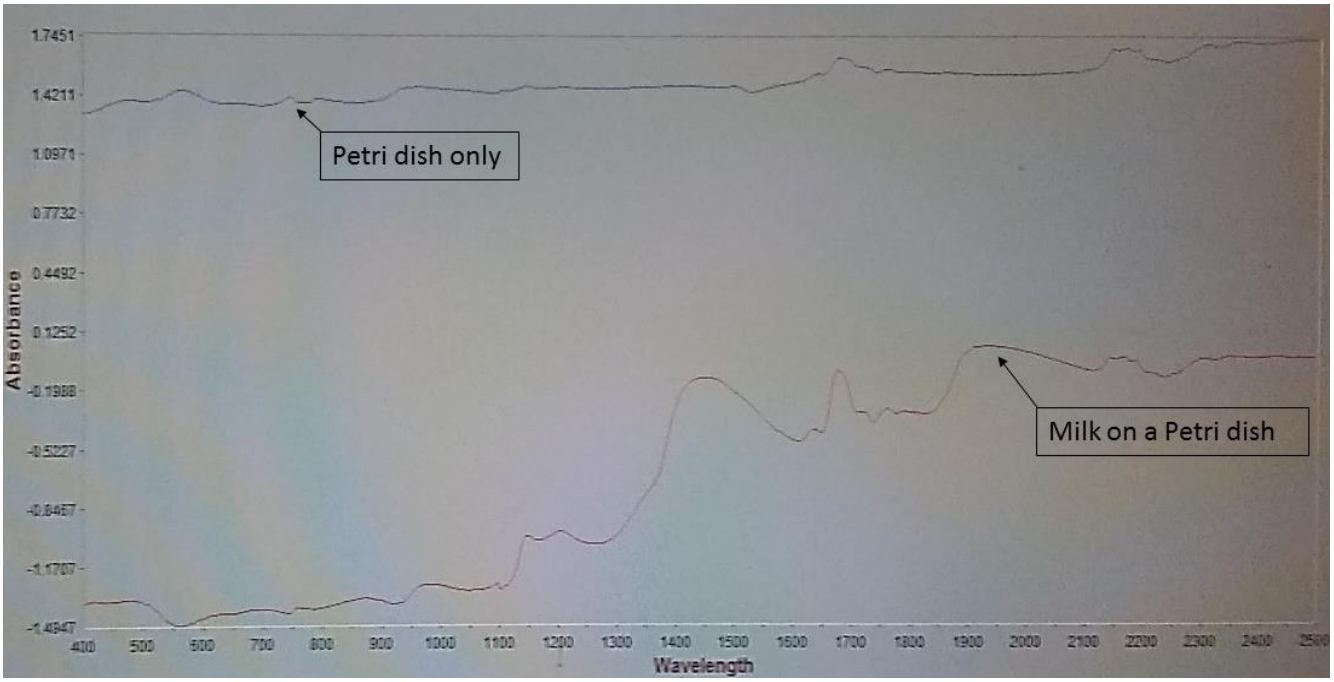


Figure 1: A spectra of a Petri dish used during spectral acquisition and a spectra of milk housed in a Petri dish. This demonstrates the absorptive abilities of polystyrene present in the Petri dish, which interfered with the most informative NIR region of milk where the investigated milk constituents are known to absorb light.

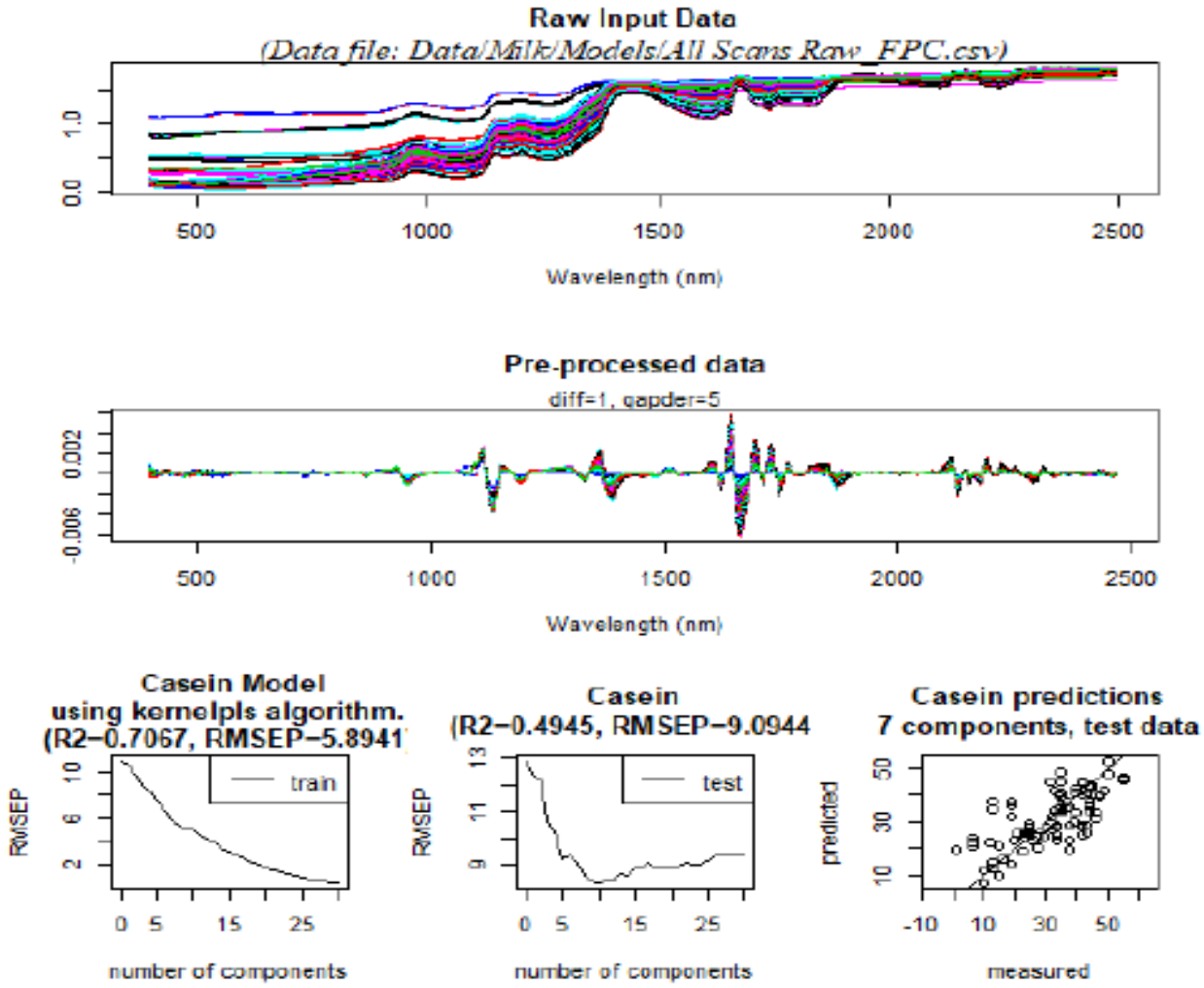


Figure 2: Characteristic spectra of milk (raw and differentiated) and the casein associated scatter plots showing the statistical values during calibration and validation.

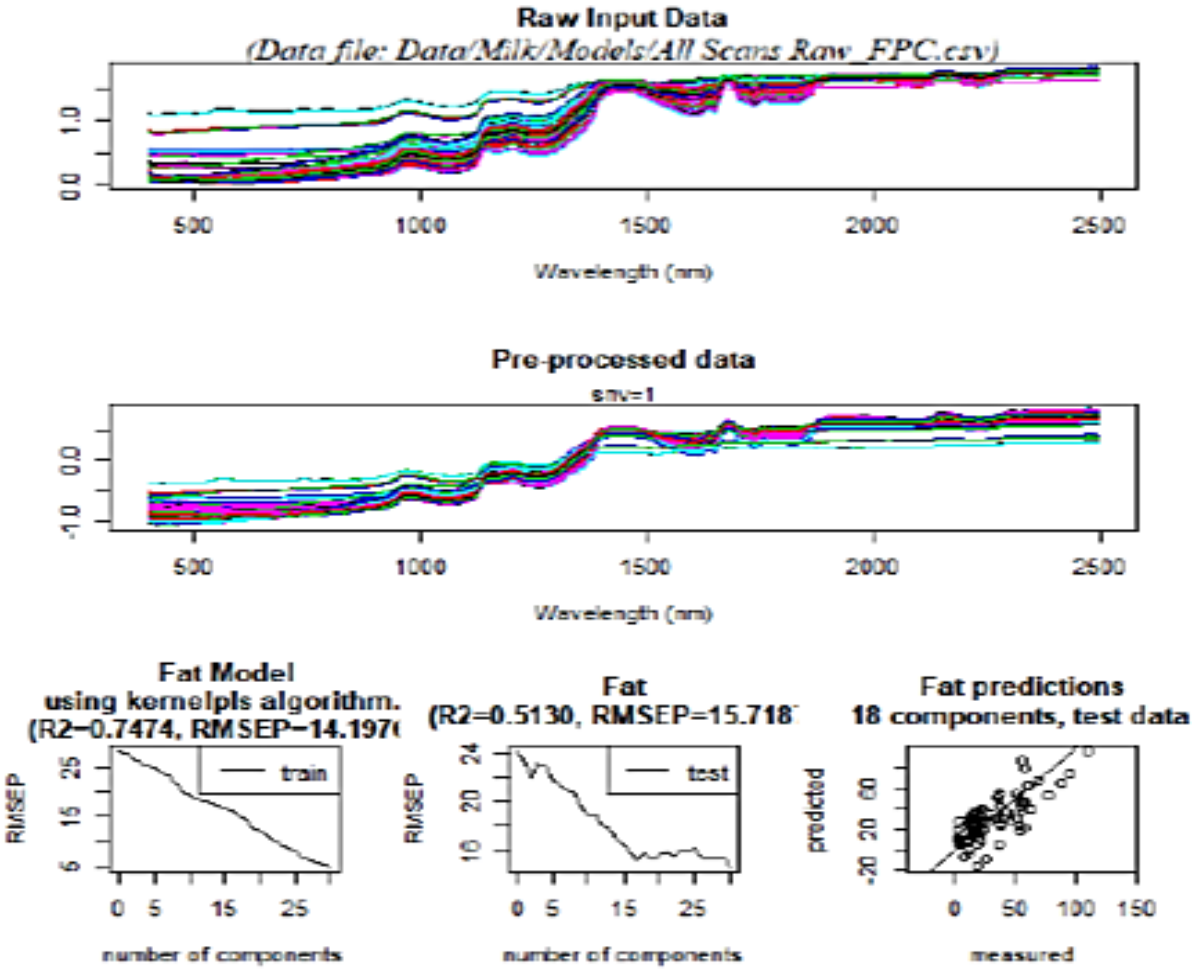


Figure 3: Characteristic spectra of milk (raw and differentiated) and the milk fat associated scatter plots showing the statistical values during calibration and validation.

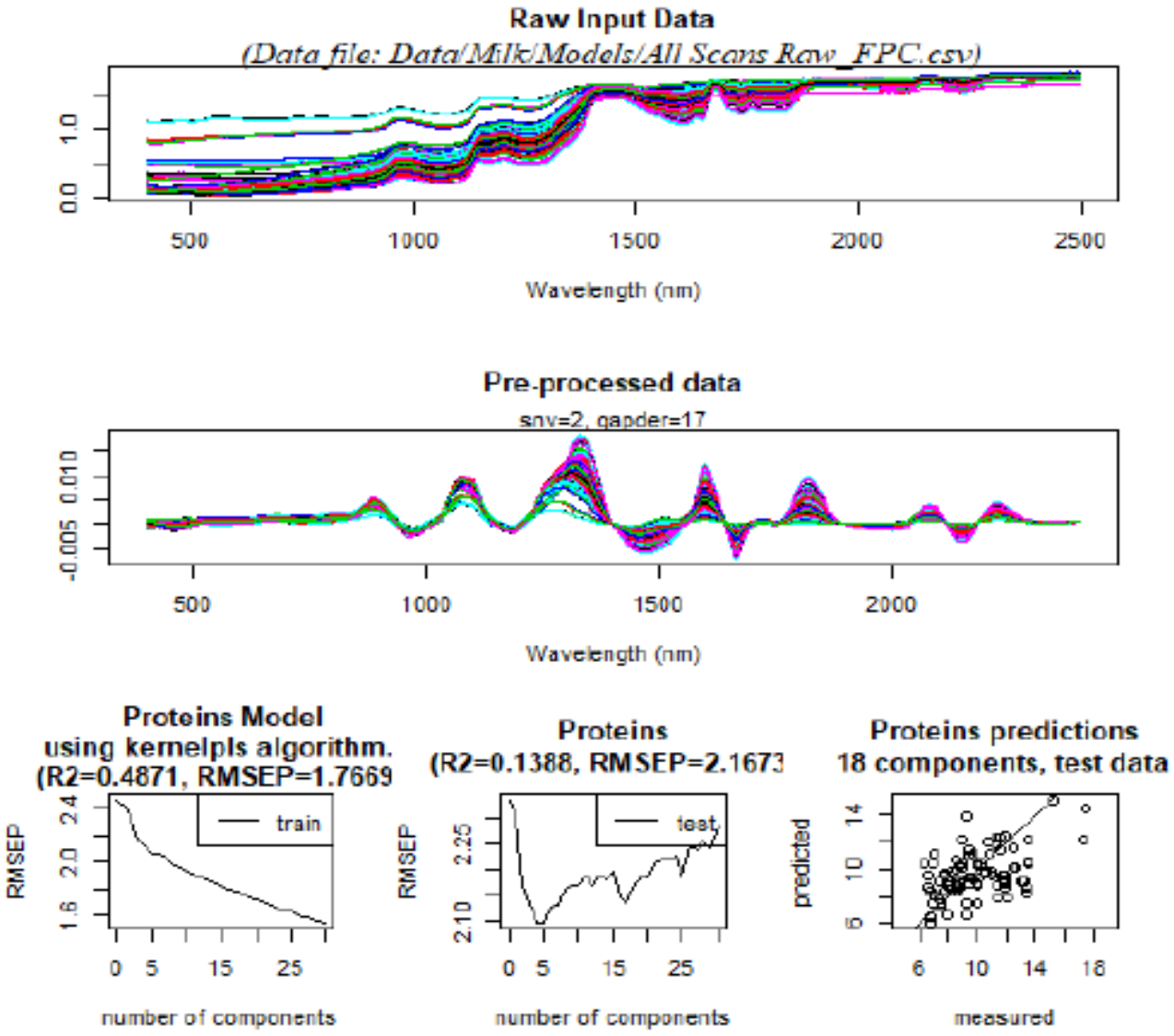


Figure 4: Characteristic spectra of milk (raw and differentiated) and the milk whey proteins associated scatter plots showing the statistical values during calibration and validation.

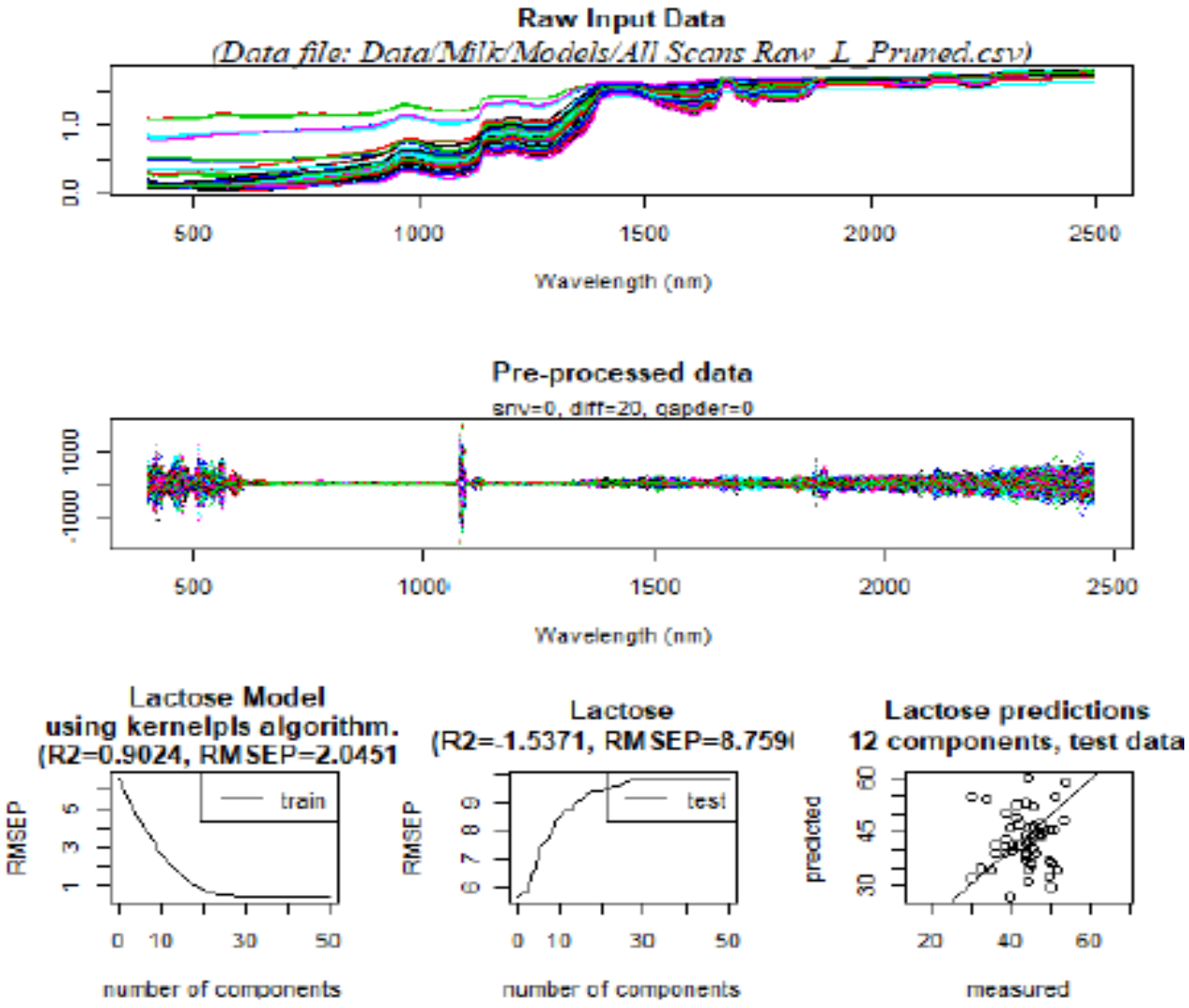


Figure 5: Characteristic spectra of milk (raw and differentiated) and the milk lactose associated scatter plots showing the statistical values during calibration and validation.