The physiology of the keratin plug formation in the teat canal of dairy cattle and its interaction with current and novel methods for prevention of intramammary infections

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Abstract

The incidence of intramammary infections (IMI) in dairy cows in the early dry period is the highest of the lactation cycle when methods to prevent IMI are not applied. This high incidence is comparable only with that observed near calving. At the end of lactation, the teat is sealed by a plug formed mainly by keratinised cells, detritus and proteinaceous material. Research suggests that the keratin plug acts as a physicochemical barrier throughout the dry period that impedes the entrance of bacteria. However, the physiological mechanism of keratin plug formation is still uncertain. The main objectives of this thesis were to characterise the physiological functions of the teat canal (TC) during the early dry period and assess how they associate with the presence of IMI. A further objective was to evaluate the modes of action of a current mastitis preventative containing bismuth subnitrate and a novel formulation of micronized keratin that is under investigation as a teat seal for preventing IMI during the early dry period.

To address these objectives a novel biopsy method was developed to allow investigation of the physiological characteristics of the epithelial tissues of the TC. A transcriptomic analysis of the TC epithelium after drying off showed that epithelial cells decreased expression of mitotic and immune-response related genes.

A *Streptococcus uberis* strain was used in a challenge study aiming to examine mechanisms of colonization in the TC and the response of the epithelial tissue to progressing infection. This *Streptococcus uberis* challenge did not result in colonization of the TC nor in IMI with *S. uberis*. Nevertheless, a reduction in the thickness of the *stratum granulosum* and the keratin layer of the TC epithelium was observed. This coincided with an increase in TC colonization by non-pathogenic bacteria and a decline

in the concentration of certain cytokines after drying off. These changes observed in the TC epithelium support previous reports showing increased incidence of IMI by non-pathogenic bacteria during the early dry period.

Antimicrobial effects and neutrophil cell responses were evaluated *in vitro* in two studies to test previously hypothesised action mechanisms for bismuth subnitrate and a novel keratin-based internal teat sealant (ITS) formulation. Bismuth subnitrate showed an inhibitory effect on bacterial growth, contrary to the current description of ITS as non-pharmacological, inert physical barriers. No activation of a cellular response was observed for keratin or bismuth formulations *in vitro*. Bismuth subnitrate and keratin were also tested *in vivo* for their effect on the formation of the keratin plug. The hypothesis of this study was that these treatments induce expression of mitogenic genes that induce a faster sealing of the teat canal. There was no modification of gene expression after treating cows with bismuth subnitrate or the novel keratin-based ITS formulation during the formation of the natural keratin plug, and no modification of the closure status of the teat canal lumen, suggesting that neither of the two treatments induced an improved sealing of the teat canal after drying off through increased keratin production.

These findings contribute to the knowledge of keratin plug formation and physiological characteristics of the TC during involution. They align with and partially explain some of the literature-reported events observed during the early dry period. The knowledge gained provides support for future product development aimed to increase protection of the mammary gland during the dry period.

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Preface

I have undertaken this thesis in the form of publishable experimental chapters using a format of thesis by publication. The current status and publication outlet including publication in conference proceedings are described in the following list.

Chapter 1: General Introduction

Chapter 2: Literature review

A New Method for Obtaining Biopsies from the Teat Canal of Dairy Cows

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Chapter 3: A novel biopsy technique in dairy cows and its suitability to obtain samples of the teat canal for mRNA expression analysis

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Chapter 4: Gene expression of the teat canal epithelium during the keratin plug formation and early lactation

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Chapter 5: Teat end response to *S. uberis* challenge in the early dry period

Notcovich S., Williamson N., Yapura M.J., Heuer C., Schukken Y.

Chapter 6: Effect of bismuth subnitrate on *in vitro* growth of major mastitis pathogens

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Chapter 7: Cellular response of neutrophils to bismuth subnitrate and micronized keratin products in vitro

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Chapter 8: Effects of bismuth and keratin-based teat sealant formulations on the epithelia of the teat canal at dry off

S. Notcovich, N.B. Williamson, J. Yapura, Y.H Schukken, C. Heuer

Chapter 9: General discussion

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List of abbreviations

AIC: Akaike information criterion

BIS: Bismuth

BA: blood agar

CFU: Colony forming units. Number of viable colonies

CNS: Coagulase negative *Staphylococcus*

DCT: Dry cow therapy

FBS: Foetal bovine serum

FDR: False discovery rate

FFA: Free fatty acids

FFPE: formalin-fixed paraffin-embedded

H_2O_2: Hydrogen peroxide

IFN-γ: Interferon gamma

IHC: Immunohistochemistry

IHF: Immunofluorescence

IL-6: Interleukin 6

IL-8: Interleukin 8

IL-10: Interleukin 10

K: Keratin

IMI: Intramammary infections

ITS: Internal teat sealant

LPO: Lactoperoxidase

LPS: Lipopolysaccharides

MHC: Major histocompatibility complex

MPO: Myeloperoxidase

MSB: Mean square between

MSW: Mean square within

NC: Negative control

NGS: Next generation sequencing

NO- NO2: Nitric oxide

PBS: Phosphate buffer solution

PC: Positive control

PMN: Polymorphonuclears

QC: Quality control

RCC: Resource script files

RIN: RNA integrity number

RMT: Rapid mastitis test

RNA-seq: RNA sequencing

ROS: reactive oxygen species

RSNO: S-Nitrosothiols

SCC: Somatic cell counts

SCN: thiocyanate

SDCT: Selective dry cow therapy

SEM: Scanning electron microscopy

TC: Teat canal

TNF-α: Tumoral necrosis factor alpha

TS: Teat sinus

TSA: Trypticase soy agar

TSB: Trypticase soy broth

UHT: Ultra high temperature

XO: Xantine oxidase

1 General introduction

Mastitis is inflammation of the mammary gland. This inflammation is frequently related to bacterial infection but can also be caused by viruses, traumatic injuries, and other pro-inflammatory factors. Due to the intensification of dairy systems, mastitis has been one of the main concerns for dairy farmers over the last century. Mastitis of dairy cows is a costly disease affecting the dairy industry around the world (Cha et al., 2011). The estimated cost per case of clinical mastitis may vary between $95.31 to $211 US dollars, including the costs of treatment, milk loss, animal welfare costs, pregnancy loss and potential increased culling rates of cows due to mastitis (Cha et al., 2011). Intramammary infections (IMI) are characterised by colonization of the mammary gland by bacteria. These infections can potentially cause clinical mastitis. The incidence of IMI and mastitis in cows varies throughout the lactation cycle. The phases with the highest risk of infection are the early dry, late dry, and post calving periods (Bradley and Green, 2004).

The teat canal (TC) is one of the first physical barriers of the mammary gland. This barrier is formed by the epidermis of the TC which consists of four well-defined strata which are the same strata that form skin: *stratum basale*, *stratum spinosum*, *stratum granulosum* and *stratum corneum* (keratin lining) (Comalli et al., 1984, Smolenski, 2018). Although the external skin and epithelium of the TC exert a protective barrier against environmental impacts, and have the same embryological origin, TC epithelial tissue is thicker than its counterpart of the external teat skin (Paulrud, 2005). After the end of lactation, during the early dry period, the TC epithelial tissue forms a plug which is mainly composed of keratin that “seals” the TC until parturition (Capuco et al., 1990). However, mechanisms of formation of the

keratin plug and the role of the TC in preventing IMI during drying off and the early lactating periods are not known in detail.

Two methods of reducing IMI and subsequent clinical mastitis in the high-risk periods are by intramammary infusion of antibiotics as dry cow therapy (DCT) or the use of internal teat sealants (ITS). Dry cow therapy is the application of a long acting antibiotic into each quarter of a cow after her last milking before drying off (Smith et al., 1967, Ziv et al., 1981). It has been used successfully for over 50 years to prevent and treat IMI over the dry period. However, the indiscriminate use of antimicrobials in production animals, including blanket treatment of non-infected quarters has raised public concerns (Swann et. al., 1969, Østerås et al., 1999, Huxley et al., 2002, Vanhoudt et al., 2018). Alternative, methods such as the use of ITS and selective dry cow therapy are being considered in an attempt to reduce antimicrobial usage in dairy cows (Østerås et al., 1999, Huxley et al., 2002, Vanhoudt et al., 2018). In response to the above-mentioned public concerns, the New Zealand Veterinary Association indicated that their aspirational goal is that “by 2030, New Zealand will not need antibiotics for the maintenance of animal health and wellness" (NZVA statement on dry cow therapy, https://www.nzva.org.nz/page/drycowtherapy). This statement indicates that soon, clear evidence of an existing bacterial infection or a quantifiable high risk of imminent infection will be required in order to justify antibiotic treatments. Such evidence includes an accurate diagnosis of the bacteria causing the infection and antibiotic sensitivity testing of the identified bacteria. The application of blanket DCT in a dairy herd does not comply with either of these conditions.

Internal teat sealants applied at the last milking before drying off are claimed to occlude the TC and prevent bacteria from entering the udder (Meaney, 1977,

Woolford et al., 1998). Currently used ITS are based on bismuth subnitrate, a salt of the heavy metal, bismuth. Bismuth, in different salt formulations, has been in long-term use in human health for different purposes such as antacid and anti-ulcerative products and included in dental products (Athanikar, 1998, Sun et al., 2003). When introduced for widespread use in dairy cattle in the late 1990s, ITS presented new and promising means for preventing mastitis during the dry period and in early lactation (Woolford et al., 1998, Huxley et al., 2002). Bismuth subnitrate effectively assists in the prevention of IMI during these high-risk periods. For example, there are reports describing a decrease in IMI of 6.3%, and a reduction in IMI in cows of 31% at 1 to 3 days in milk (DIM) when ITS were used in combination with DCT at drying off, (Godden et al., 2003). Others, reported a significant reduction in IMI occurring in cows treated with ITS (n= 103/928) *vs* cows treated with DCT (n= 145/940) $p<0.01$ Huxley et al. (2002). However, the mechanisms of action and interactions between the TC and bismuth-based products in preventing IMI remain poorly understood.

Given the current trend in terms of antibiotic use and considering the scarce knowledge in the area of TC physiology, developing products that prevent the occurrence of IMI during the dry period is challenging. This thesis aims to improve our scientific understanding of the physiology of the TC during the dry period and its involvement in the occurrence and prevention of IMI and clinical mastitis. In order to achieve this objective, the work describes the molecular mechanisms of keratin plug formation in the TC after drying off; it investigates mechanisms by which bacteria enter the mammary gland during the dry period and explores innate TC defence mechanisms against bacterial invasion. In addition, it investigates the impact of

current prophylactic and therapeutic interventions for mastitis on TC physiology. The main objectives of each chapter in this thesis are as follows:

1. General introduction: To define the main area of research by identifying limitations of current approaches to mastitis prevention, especially during the dry period.
2. Literature Review: To outline and critically evaluate the existing relevant literature in this field.
3. Chapter 3: A novel biopsy technique in dairy cows and its suitability to obtain samples of the teat canal for mRNA expression analysis: To develop a method to obtain serial biopsy samples from the teat canal and use it as tool to analyse the gene expression of the teat canal in the early dry and lactating periods to gain an enhanced understanding of its physiology.
4. Chapter 4: Gene expression of the teat canal epithelium during the keratin plug formation and early lactation: Next-generation sequencing (NGS) was used to profile the transcriptome of the teat canal epithelium during lactation and the early stages of the dry period (pilot experiment), and Nanostring nCounter Technology to validate and extend the results obtained in the first experiment (longitudinal study). Both studies were used to characterise the physiology of the teat canal epithelium during the keratin plug formation.
5. Chapter 5: Teat canal response to *Streptococcus uberis* challenge in the early dry period: To study the morphological and physiological changes in the teat canal and teat sinus after being challenged with a known strain of *S. uberis* at different stages in the early dry period.

6. Chapter 6: Effect of bismuth subnitrate on *in vitro* growth of major mastitis pathogens. To evaluate the effect of a component of internal teat sealant products on bacterial growth *in vitro*.
7. Chapter 7: Cellular response of neutrophils to bismuth subnitrate and micronized keratin products *in vitro*. To evaluate *in vitro* the effect of two putative teat sealant components on the immune response.
8. Chapter 8: Effects of bismuth and keratin-based teat sealant formulations on the epithelia of the teat canal at dry off. The objective of this study was to evaluate the effect of administration of two internal teat sealant formulations on the closure of the teat canal and keratin plug formation.
9. Chapter 9: General Discussion and conclusions. To integrate, compare, and contrast the scientific findings of this thesis with those reported in the literature in order to provide relevant conclusions, limitations, and recommendations to inform future research efforts in the area.

2 Literature review

2.1 Mammary glands

Mammary glands are the source of milk, which provides nutrients for mammals during their early life. The development of the mammary gland, the onset of lactation, and milk synthesis are highly related to reproductive function. Milk production is associated with the final stages of a successful reproductive cycle, which culminates in parturition of an offspring and its nourishment.

The lactation cycle is defined as the period in which the mammary gland is functional and includes a stage of involution at the end of the cycle, also known as the dry period in commercially milked animals (Akers and Capuco, 2002). In cows, the udder is situated below the caudal part of the abdomen forming one single mass, divided by connective tissue into four independent glands (quarters). The right and left sides of the udder are clearly divided by a median suspensory ligament that can be recognised from the outside as a median or intramammary groove (Figure 2.1, Left). The front and rear quarters are barely distinguishable one to the other by the naked eye. Still a functional distinction between the quarters is present, milk will not move from one quarter to another (Figure 2.1, Right).

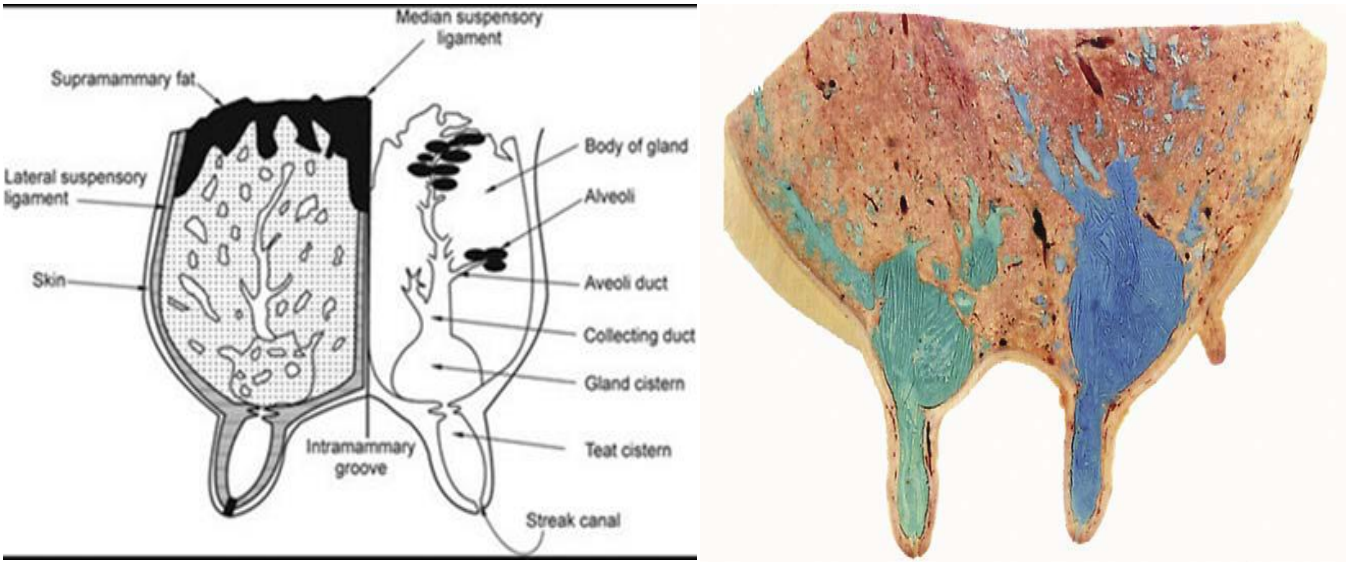


Figure 2.1 Left: Dairy cow udder diagram, caudal view. Right: lateral view of a sectioned cow udder - (green) front quarter, (blue) rear quarter (modified from Dyce et al. (2009).

The parenchyma of each mammary gland consists of tubule-alveolar structures comprising secretory units (alveoli) grouped into lobuli and a series of interlobular ducts connecting them and forming lobes, all inserted in supramammary adipose tissue called “supramammary fat” or “fat pad” (Figure 2.2, Left). The alveoli comprise mammary epithelial cells, which are the milk production units. They are situated on a basal membrane surrounded by a layer of myoepithelial cells (Helmboldt et al., 1953, Alkafafy et al., 2012). Milk ducts connect the alveoli and they are covered by non-secretory cuboidal epithelial cells (Helmboldt et al., 1953). Progressing towards the teat, the ducts increase in diameter to become lactiferous ducts (Figure 2.2). A dilation in the lumen of the lactiferous ducts at the base of the teat is the lactiferous sinus or gland cistern (Figure 2.2). The lumen of the gland cistern connects with a teat sinus or teat cistern and the two spaces are partially separated by a fold of mucosa surrounded by a ring of veins (Figure 2.2). This udder conformation can be seen only in the functional period of the mammary gland during lactation. After milk harvesting stops,

the mammary gland undergoes involution and most of these structures disappear. The teats, once fully developed after pregnancy and parturition, remain mainly the same for the rest of a cow's life. However, the teat sinuses or cisterns become smaller and shorter in the dry period.

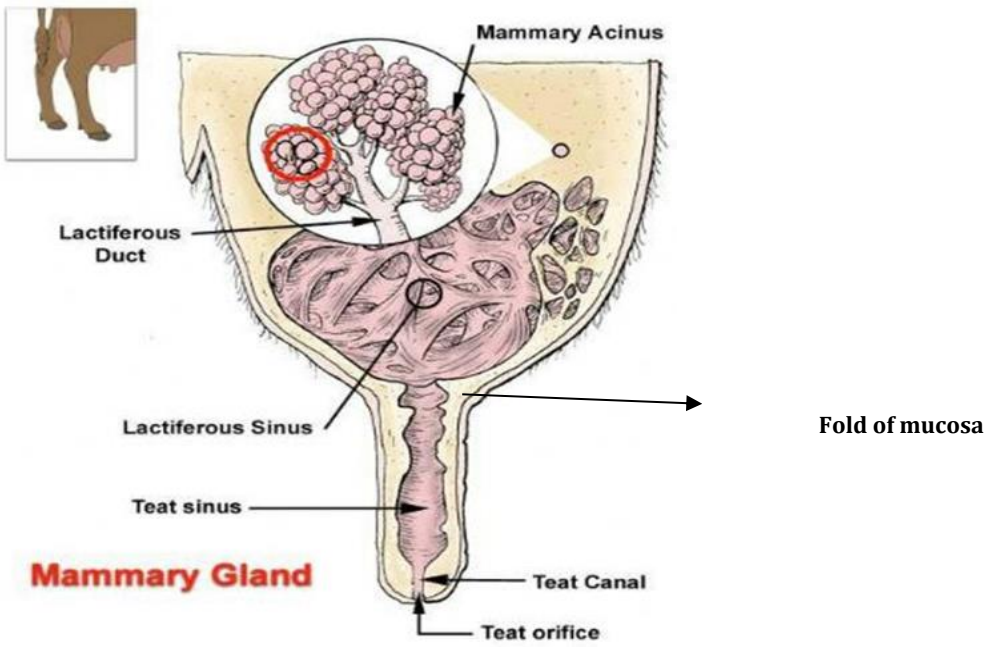


Figure 2.2 Mammary gland and teat structure Modified from (https://www.slideshare.net/waleedtareen2/anatomy-affection-of-udder-teat)

2.2 The teats

The teats are cylindrical structures that have evolved to provide feed for calves. Each mammal has differently shaped teats adapted to the mouth contour of their new-borns. In cows, the teat is between five and eight cm long, and one to 1.5 cm wide (Figure 2.2). Smooth multi-directional muscle, collagen fibres, elastic tissue, thick-walled veins, blood and lymphatic vessels form the teat barrels. These structures are

held together by fibrous tissue and are covered by skin (Paulrud, 2005). At the teat apex, the external teat skin invaginates approximately 10 mm to form the teat canal (TC). The average length of the TC ranges from three to 18 mm (Giesecke et al., 1972). The teat walls are generally about six mm thickness in the upper portion and one cm thickness at the lower end (around the TC). The internal wall is the teat sinus, lined by mucosa.

The teat sinus (TS) or teat cistern is a saccule situated in the teat barrel that can hold 30-40 mL of milk between milkings when cows are lactating. It is limited dorsally by a fold of mucosa and the gland cistern and ventrally by the Fürstenberg's rosette, which defines the limit between the sinus and the TC. The lining membrane of the sinus has several sacks or pouches, with an epithelium consisting of one or two layers of columnar or cubical cells. The underlying connective tissue is loose and contains elastic tissue (Johnston, 1938, Helmboldt et al., 1953, Adams et al., 1961). The teat sinus mucosa, and Fürstenberg's rosette are involved in the early immune response to invading microbial agents (Nickerson and Pankey, 1983, Rinaldi et al., 2010). Neutrophils and dendritic cells have been found in the stroma of the teat sinus showing the readiness of the tissue to respond to harmful bacteria (Asti et al., 2011, Smolenski, 2018). The TS opens into the TC where the external teat orifice is surrounded by a sphincter.

The sphincter of the TC is formed by smooth muscles situated at the distal end of the teat. The contraction of the muscles forming the sphincter occlude the TC and impede the escape of milk from the teat between suckling or milking times (Lefcourt, 1982b). Bacteria and environmental substances are prevented from contacting the milk in this way. However, the sphincter itself has no antibacterial activity. Van der

Merwe and others postulated that the name "sphincter" for the smooth muscular layer in the TC was incorrect (Van Der Merwe, 1985). Using serial histological cuts, it was demonstrated that the muscular layer of the TC is spiral-shaped, and the closing forces are mainly exerted by the connective/elastic tissue that surrounds the teat orifice (Van Der Merwe, 1985) (Figure 2.3). However, Van Der Merwe's observations are contrary to those of others who found rhythmic contractions of the muscle after milking and muscular response to alpha and beta agonists that suggested the teat orifice does function as an active sphincter (Bernabe and Peeters, 1980).

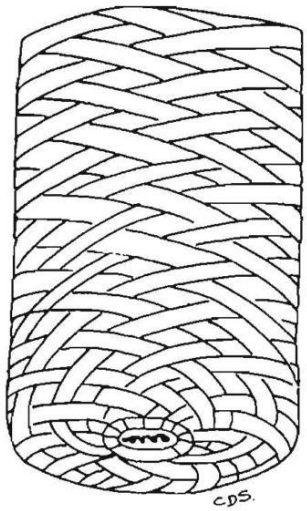


Figure 2.3 A schematic representation of the lamellated netlike arrangement of the elastic fibres and smooth muscle fibres in the bovine teat canal (Van Der Merwe, 1985)

The teat or streak canal extends from the squamocolumnar junction with the TS proximally (Fürstenberg's rosette) to the external teat orifice distally. The TC has a conical shape, with a diameter at the tip (external orifice) of 0.4 mm, middle of 0.5 mm and proximally around the Fürstenberg's rosette of 0.8 mm (Little and Plastridge,

1946, Giesecke et al., 1972, Ngatia et al., 1991). Similar to the stratified cornified epithelium that covers the exterior teat, the epidermis of the TC consists of the four well-defined strata: *stratum basale, stratum spinosus, stratum granulosum and stratum corneum* (Figure 2.4). It contains mainly keratinocytes, along with a minor population of dendritic and stem cells (Nickerson and Pankey, 1983, Paulrud, 2005, Smolenski, 2018). The *stratum granulosum* of the TC is thicker than in normal skin. The TC epithelium has folds situated in a coil shape in accord with the shape of the muscular layer of the teat, which extend from proximal to distal (Giesecke et al., 1972, Vesterinen et al., 2015). These folds are called "rete ridges" by some authors and they increase the elasticity of this TC epithelial tissue (Smolenski, 2018). Some folds of stromal tissue interface with the *stratum granulosum* of the epidermis. These structures receive the name of "Marksäulchen" (Figure 2.4). They contain neutrophils, which has been interpreted as them having a surveillance function in the TC epidermis (Smolenski, 2018). The openness of the TC and the thickness of the keratin layer (*corneum stratum*) influences the vulnerability of the mammary gland to IMI (Murphy, 1944, Davidov et al., 2011).

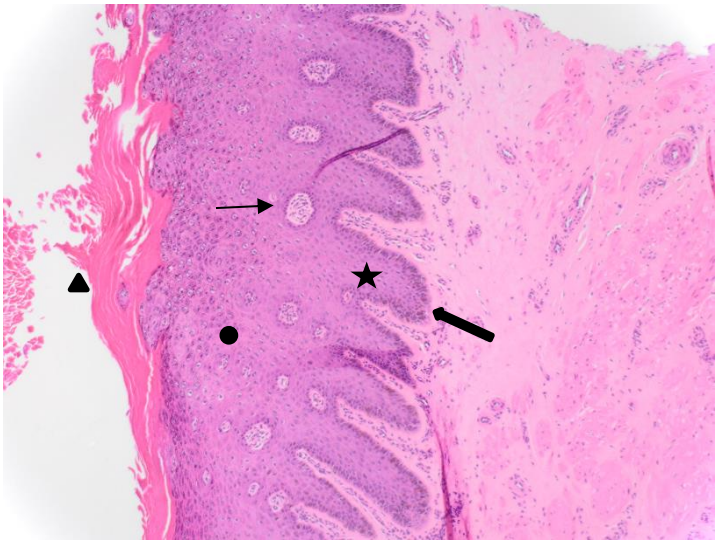


Figure 2.4 Microscopic image of the epithelial layer of the teat canal in a cow (10x). Thick arrow: Basal membrane. Star: *stratum spinosus*. Circle: *stratum granulosum*, Triangle: *stratum corneum*. Thin arrow: "Marksäulchen". Haematoxilin-Eosin stain. Microscopic image produced by S. Notcovich

2.3 Mammary gland and teat end physiology during the dry period

Termination of lactation is associated with partial loss of the mammary cell population and de-differentiation (Wilde et al., 1997). Evidence of decreased synthesis of lactose, casein, alpha-lactalbumin and increased synthesis of transferrin are reported (Wilde et al., 1997, Zarzyńska and Motyl, 2005). However, little is known about the changes occurring in the TC during drying off.

In many studies, the presence of a "keratin plug" is stated as sealing the teat lumen during the dry period (Woolford et al., 1998, Dingwell et al., 2004, Paulrud and Rasmussen, 2004). However, questions such as whether the keratin plug is formed by an increased synthesis of keratin or by passive accumulation due to the lack of flushing of *stratum corneum* from the canal by milk remain unanswered. Keratin plug formation is a dynamic process that occurs as a result of the balance between the synthesis and

removal of the keratin present in the TC. Studies of the changes occurring in the TC have been challenging up to now, due to the lack of a technique which enables the study of its physiology *in vivo* (Paulrud and Rasmussen, 2004).

A significant increase in the TC lumen diameter and a decrease in the mitotic index of the epithelial tissue are observed in the first 16 days after drying off (Comalli et al., 1984). An increased number of dendritic and macrophage cells are found in the Fürstenberg's rosette and teat sinus 14 days after drying off (Smolenski, 2018).

2.4 Intramammary infections during the dry period

The incidence rates of IMI observed in the dry period are higher than in mid-lactation (Figure 2.5) (Dingwell, 2002, Bradley and Green, 2004). The absence of flushing by milk might allow increased multiplication of bacteria in the TC and the mammary tissues. Most mastitis cases in this period are caused by environmental bacteria, like *Escherichia coli* and *Streptococcus uberis* (Bradley and Green, 2001, Bryan et al., 2011).

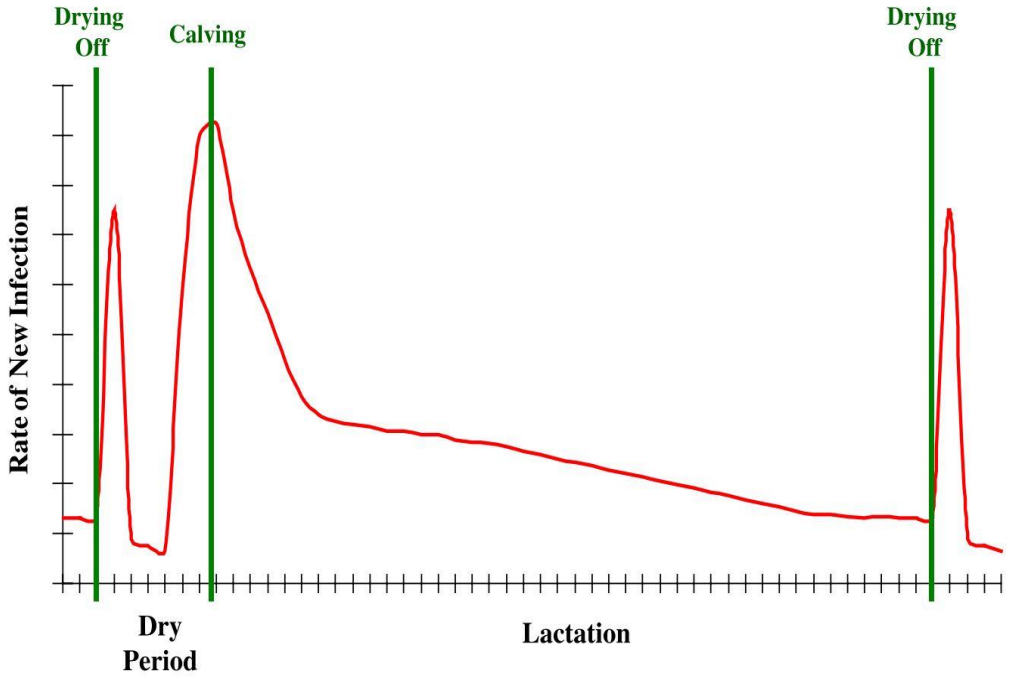


Figure 2.5 A schematic illustration of the incidence of new intramammary infections during the lactation cycle. A peak in new infection rate occurs immediately after drying off (Bradley and Green, 2004).

Many authors have stressed that a delay in the closure of the TC after the last milking is a major factor increasing the incidence of IMI. One study reported that approximately 50% and 5% of teats had an incomplete keratin plug present after seven and 50 days of the dry period, respectively (Williamson et al., 1995). Similarly, in another study, 50 and 23% of teat ends were still open after one and six weeks of the dry period (Dingwell et al., 2003). "Open teats" is the name given to teat ends which still allow the expulsion of some secretion when gently stripped by hand (Williamson et al., 1995). Heifers with open teats before calving are more prone to IMI than heifers that do not have open teats before calving (Krömker and Friedrich, 2009).

The assessment method to differentiate "open" from "closed" teats is somewhat subjective. The condition of "openness" of the teats might also arise due to the amount of milk remaining in the udder and leaking through the TC, softening the keratin plug and allowing residual secretions through the TC to be in contact with the environment. The keratin at the exterior tip of the TC, instead of being air dried because of the lack of flushing, may remain wet and provide conditions to allow bacteria to grow through the canal and colonise the rest of the gland (Dingwell et al., 2004).

Mechanisms by which bacteria enter the TC and hence the mammary gland are not definitively known. It was hypothesised that during milking, the liner pinches the tip of the teats, propelling keratin and some bacteria into the teat sinus (Forbes and Gehm, 2012). Others observed that when the internal pressure in the udder is high it may be sufficient to open the teat orifice, allowing milk to escape and form a film of milk through the TC in which bacteria can multiply (Neave et al., 1950). Another hypothesis suggested that a fluctuating vacuum during milking might briefly cause a

lower pressure in the teat sinus than at the apex of the teat, allowing bacteria to traverse the canal from exterior to interior (Forbes, 1968).

Different strains of *Staphylococcus aureus* and micrococci can colonize the TC (Forbes, 1968, 1970). In addition, close contact between cows is an important method by which skin bacteria are transferred between animals (Forbes, 1970). During the dry period, however, there are no liners in contact with teats, no apparent horizontal transmission and no reverse vacuum, so the question remains "how does a high rate of infection occur in this early dry period?"

Some clinical mastitis cases observed during the early dry period might be produced by bacterial colonies that live in the mammary gland in a latent phase while shielded in biofilms. Biofilms are hydrated matrices of polysaccharide and protein, forming a slimy layer surrounding the bacterial colonies (Stewart and Costerton 2001). The sudden changes in tissue structure and in milk constituents that occur after drying off may cause these bacteria to detach from the biofilm and induce infection (Yarwood et al., 2004, Melchior et al., 2006). The removal of nutrients from the environment where bacteria live might induce cell detachment from biofilms (Boles and Horswill, 2008). In dry cows, the reduced synthesis of lactose in the mammary gland could induce that change (Melchior et al., 2006). Other hypotheses of colonization are that environmental bacteria can invade and multiply in the thin film of milk remaining in the TC after drying off and the changes in milk composition from lactating to drying off secretion could be a key factor that enables the multiplication of some bacteria (Melchior et al., 2006). For example, the increased lactoferrin content of the milk during the early dry period can enhance the adherence to mammary

epithelial cells of *Streptococcus uberis,* which hold a lactoferrin binder protein, facilitating multiplication (Fang et al., 2000).

2.5 The teats as protective systems and structures against intramammary infections

Intramammary infections most commonly originate from the penetration of infective bacteria through the TC, followed by adhesion, migration and proliferation. The defence mechanisms are classified as innate and acquired immunity. Innate immune defence is a non-specific immunity because there is no need of previous exposures to infecting agents to launch the innate immune response. Innate immune defence includes the physical protective barriers of a cow (*stratum corneum* of skin, hair, mucus, etc.) whose functions are to prevent bacterial invasion. If these barriers are breached, innate immunity responds to phagocytose and destroy the foreign material. These mechanisms are particularly important to protect the udder against initial exposure to bacteria. Acquired immunity is related to the immune system of the cow. It requires repeated exposure to a mastitis-causing agent and it involves the synthesis of specific immunoglobulins. The success of this response is highly influenced by the general health of the cow (Sordillo, 2005).

The innate immune defence mechanism is an important part of the defence of the teats as most of the potential infections are stopped here by polymorphonuclear leucocytes (PMN) and other local mechanisms and structures that are described below.

2.5.1 Shape and size of the teats

The length, width and shape of the teat and the TC are associated with the cow's susceptibility to mastitis (Klein et al., 2005, Guarín et al., 2017). The TC length is directly associated with the amount of keratin content in the TC (Paulrud and Rasmussen, 2004) and consequently, longer teat canals might be at lower risk of IMI. On the other hand, wider TC are associated with more susceptible quarters to IMI (McDonald, 1975). Cows with higher production and greater ease of milking have shorter and wider TC and are more susceptible to mastitis than others (Kuhne, 1959). Older cows are more susceptible to mastitis during lactation and this can be due to a change in TC size and shape over time (Poutrel, 1982). The length and diameter of the canal varies with age and this can affect the resistance to mastitis (McDonald, 1955).

2.5.2 Keratin

A keratin layer covers the epithelium of the TC on its luminal surface. Keratin proteins are structural fibrous proteins that are key components of hair, wool, feathers, horns, hooves and nails as well as skin. Keratinocytes originate in the basal layer of the epidermis, through division of a population of stem cells. The daughter cells then divide several times as transit amplifying cells, which lose proliferative capacity and differentiate into post-mitotic, keratin rich cells as they migrate upward to the spinous layer of the epidermis. As these cells continue their transit through the granular layer, they lose their nucleus and form the uppermost, cornified layers of the epithelium (Helmboldt et al., 1953). Turnover of the epidermis allows the teat to maintain its barrier function and repair injuries. It was estimated that the keratin layer of the TC can be restored within one day of being manually removed (Capuco et al., 1990). However, this is far below the estimations made by Murphy and Stuart (1954),

who estimated that the turnover of the *stratum corneum* of the TC was two to four weeks. The regeneration of the keratin stratum in one day does not explain the increased susceptibility to infection of cows during the early dry period when cows seem to be more susceptible to mastitis for the first 14 to 50 days after drying off (Bradley and Green, 2004, Davidov et al., 2011). If the keratin layer of the TC was completely restored so quickly, the incidence of mastitis in this period would likely have been lower. Turnover of the corneum stratum of the TC epithelium might not be directly related to mastitis incidence. However, sealing of the TC lumen by desquamating keratinised tissue could be a key point in preventing bacterial invasion.

The layer of keratin in the TC is between 30 and 300 μm with the highest proportion of cows ranging between 200-300 μm (Uppal et al., 1994, Davidov et al., 2011). The keratin layer functions as a physical obstruction to bacteria and it traps them, reducing their ability to ascend to the teat sinus and mammary gland. The thicker the layer of keratin covering the TC, the lower the leukocyte migration is into the mammary gland, suggesting that it exerts an effective protective function in the TC (Davidov et al., 2011). The keratin lining is desquamated and flushed out during each milking and bacteria are removed from the TC with this lining (Capuco et al., 1994). During the dry period, the lack of flushing effect of milk allows the keratin to accumulate forming a protective plug. However, the keratin plug formation process takes from 16 to 50 days to seal the teats completely, leaving the mammary gland less protected during that time (Comalli et al., 1984, Williamson et al., 1995). Fatty acids and proteins of the keratin layer of the TC might have bactericidal and bacteriostatic characteristics (Hogan et al., 1986, Hogan et al., 1987, Bitman et al., 1988). Xanthine oxidase and the presence of S100 proteins found in the keratin lining have a

bacteriostatic effect *in vitro* (Collins et al., 1988, Smolenski et al., 2015). However, certain bacteria can survive and grow in keratin (Chandler et al., 1969, Forbes, 1970) and the bacteriostatic effect of the xanthine oxidase or S100 is not adequate to stop the growth of mastitis pathogens.

There is no agreement on whether the presence of keratin or its removal is the most effective defence mechanism against IMI. Keratin appears to prevent the entry of bacteria into the udder and the deliberate removal of keratin increases udder infection rates (Murphy, 1959, Hillerton and Lacy-Hulbert, 1995). Susceptibility to mastitis in cows which had thinner layers of keratin covering the lumen of the TC was higher than in cows that had thicker layers of keratin (Nickerson, 2011). In contrast, others found that removal of keratin decreased the risk of infection (Capuco et al., 1994, Lacy-Hulbert et al., 1996, Gleeson et al., 2003). If keratin with bacteria attached is jetted into the teat sinus by milking machine forces or by direct physical transport into the teat cistern via intramammary infusion, these mechanisms might be a means of IMI. Also, bacteria living in the keratin layer of the TC may multiply and ascend along the TC using milk within the canal as vehicle (Forbes, 1968, Chandler et al., 1969, Capuco et al., 1994).

2.5.3 Smooth muscle

The TC remains open for approximately two hours after milking and the risk of bacteria infecting the udder during this period is high (McDonald, 1975). The rhythmic contractions of the teat muscles increase after milking and constrict the orifice back to its closed position. This contraction of the teat sphincter has been shown to be peristaltic from the proximal to the distal end and it has been suggested that peristalsis of the smooth muscle of the canal might eject bacteria between milkings (Lefcourt,

1982a). In some cows, the poor tone of the muscle allows the milk to escape from the udder through the teat. This phenomenon of "leaky teats" in cows, which leak milk for long periods after drying off or before calving, increases their susceptibility to IMI (Dingwell, 2002, Krömker and Friedrich, 2009). Hypocalcaemia after parturition may also produce loss of tone in the teat sphincter and increase the chance of cows to be infected by mastitis bacteria (Curtis et al., 1983).

2.5.4 Fürstenberg's rosette

The dorsal termination of the TC is the Fürstenberg's rosette. The Fürstenberg's rosette is a folded epithelial structure at which the stratified squamous epithelium of the TC transitions into two layers of cuboidal cells towards the sinus (Figure 2.6). The stroma under the Fürstenberg's rosette epithelium has a population of leukocytes, which is thought to leave the teat wall and enter the lumen during mastitis (Asti et al., 2011). Knowledge about the function of the Fürstenberg's rosette in protecting the mammary gland is scarce with few research works revealing details of its physiology. Some of the cells within the Fürstenberg's rosette epithelium are antigen presenter cells (MHC class II) (Nickerson and Pankey, 1983, Smolenski, 2018). Plasma cells, PMN leukocytes and mononuclear phagocytes appear to invade or reside in the stroma and migrate to the teat lumen during mastitis (Nickerson and Pankey, 1983). However, more studies on the dynamics of the immune response of the TC are required to explain how these observed responses occur.

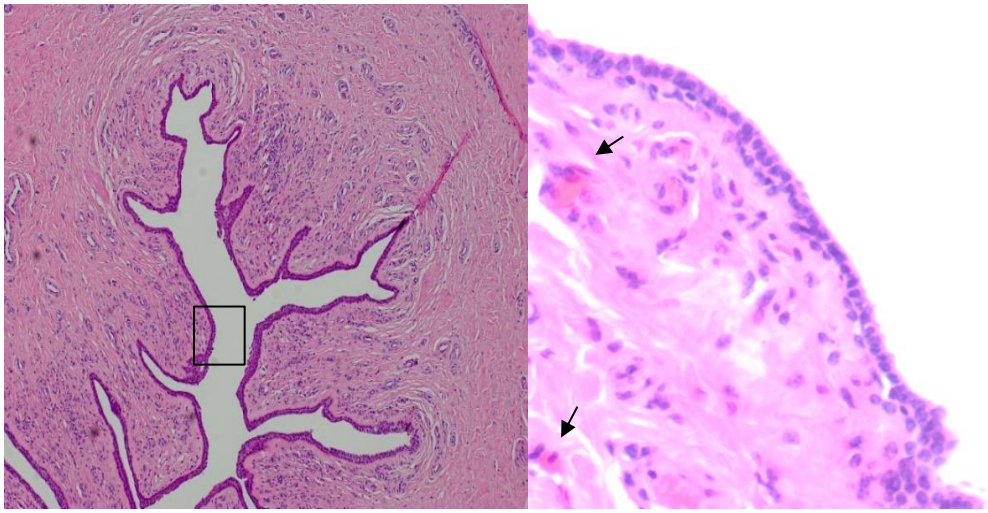


Figure 2.6 Histological structure of the Fürstenberg's rosette of a cow, thirteen days after drying off (Left- 4x). Square indicates the area of higher magnification shown on the right. Epithelial tissue consisting of two layers of cuboidal/cylindrical cells and high number of PMN leucocytes (arrows) in the stroma immediately under the epithelial tissue (Right-40x) Haematoxilin-Eosin. Micrograph produced by S. Notcovich.

2.5.5 Teat ends

Normal teat ends appear as smooth and/or slightly concave around the teat orifice. Callosity of the teat duct orifice initially appears externally during lactation as white rings around the external orifice. These white rings are the result of a normal physiological process of adaptation during the initiation of lactation and happen in more than 90% of machine milked as well as manually milked cows (Neijenhuis et al., 2000). Skin covering the teat is smooth and hosts a microflora of bacteria that do not represent a mastitis risk for the host cows. Most pathogens have only a limited chance of survival on the *stratum corneum* of healthy teats. Several studies showed that small lesions and erosions of teat skin significantly increased the rate of new IMI by *S. aureus* and other pathogens (Francis, 1984, Neijenhuis et al., 2001). The increased roughness and eversion of the teat end is known as hyperkeratosis. Cows with hyperkeratosis

induced by a malfunction of the milking machine are more prone to IMI than cows with normal teat ends (Neijenhuis et al., 2000, Bhutto et al., 2010). Muddy conditions, cold weather, dung and dirt can affect the skin condition of the teats producing chaps and small erosions, which can harbour bacteria and predispose the cows to clinical mastitis and high somatic cell counts (Hamann et al., 1988).

The TC has a surveillance function, being the first internal "point of contact" between entering bacteria and the epithelial tissue of the mammary gland (Rainard and Riollet, 2006, Rinaldi et al., 2010). Pathogens meet the immune system once past the physical barrier of the teat ends, streak canal and its keratin lining. In most tissues, the immune system overcomes bacteria. However, in the mammary gland, several factors, like the stage of lactation, age and health status of the cow can compromise the effectiveness of the immune response.

2.6 Cellular and soluble non-specific immunity

The functions of the innate immune defences are to phagocytise and destroy foreign material. During mastitis, PMN leucocytes are the first immune cells to enter the mammary tissue (Aitken et al., 2011). They migrate from the blood to the lumen of the mammary gland in response to cytokines and other chemotactic signals. This PMN migration is the first active step in udder immunity against bacteria (Sordillo et al., 1997). Macrophages, neutrophils and natural killer cells react to limit bacterial infections through phagocytosis (Sordillo, 2005). Much of the tissue damage that occurs during inflammation is caused by the release of lysosomal enzymes and other hydrolytic components from phagocytes (Harmon, 1994, Zhao and Lacasse, 2008). Concurrent with phagocytosis of pathogens, there is a major burst of oxidative metabolism, especially in neutrophils, that is associated with increased oxygen consumption and increased production of hydrogen peroxide. This results in the production of several microbicide oxidizing agents in the phagolysosomes. These oxidizing agents oxidise lipids in bacterial membranes causing lysis and can even oxidize or cross-link proteins, terminating their function (Sordillo and Streicher, 2002).

2.6.1 Neutrophils

Neutrophils are called polymorphonuclear due to the shape of their characteristic lobulated nucleus. This multilobulated nucleus allows them to migrate between endothelial cells with ease, arriving to sites of infection fast and efficiently (Paape et al., 2003). Neutrophils have been observed migrating through mammary epithelial tissue and crossing desmosome unions (Nickerson and Heald, 1981, Asti et

al., 2011). Neutrophils, plasma and dendritic cells and macrophages infiltrate the TS and the Fürstenberg's rosette (Nickerson and Pankey, 1983, Nickerson and Pankey, 1984). Neutrophils and macrophages of the mammary gland engulf entering bacteria. During mammary involution, neutrophils and macrophages phagocytose mammary debris and apoptotic cells (Sordillo et al., 1987, Paape et al., 2002). Most of the neutrophils found in these tissues are localised beneath the epidermal bilayer, suggesting a surveillance function. Macrophages migrate into the gland a few days after the first neutrophil reaction and present antigens to T lymphocytes to initiate both humoral and cellular immune responses. In the mammary gland, less than 2% of total milk somatic cells are epithelial cells. Neutrophils predominate during early stages of inflammation or involution and may account for greater than 90% of total milk somatic cells.

Myeloperoxidase (MPO) is one of the killing agents that neutrophils release that stops bacteria from infecting the mammary gland (Ledbetter et al., 2001). Myeloperoxidase is stored in azurophilic or primary granules within the cytoplasm of neutrophils and they are the first granules to appear during neutrophil maturation in the bone marrow. They are large electron-dense granules containing peroxidase. Peroxidase can kill bacteria directly by oxidising their cell membranes. Lysozyme is another potent bactericidal agent contained in lower amounts in the cytoplasmic secondary granules in neutrophils (Ledbetter et al., 2001). Bovine neutrophils also contain granules rich in lactoferrin, beta defensins and alkaline phosphatase which display bactericidal activity against many Gram-positive, Gram-negative, anaerobic bacteria, fungi and various viruses (Zecconi et al., 2002). Although the abundance of neutrophils is not high in normal TC tissue, the abundance of antigen presenter cells

(macrophages, plasma and dendritic cells) in the Fürstenberg's rosette show that this tissue has the ability to initiate an immune response to bacteria entering the udder (Smolenski, 2018). However, questions that are still not answered are how and how often is the Fürstenberg's rosette able to stop mastitis infection by actively responding to microorganisms.

2.6.2 Humoral immune response in the teat canal

Little is known about the local immune response and the importance of the TC immune response to first contact with bacteria. Within hours after infusion of lipopolysaccharide (LPS) from *E. coli* there was a change in the expression of some of the immune related genes in the Fürstenberg's rosette and in the teat sinus (Rinaldi et al., 2010). According to this study, an increased synthesis of lactoferrin occurred after teats were challenged with *E. coli*, *S. aureus* and *S. uberis* bacterial strains (Rinaldi et al., 2010, Smolenski, 2018). Lactoferrin is also known as a natural antimicrobial that is synthesised in the mammary gland (Harmon et al., 1976). However, all substances isolated as part of the soluble defence mechanisms and chemical components of the streak canal showed little or no effect when tested experimentally, even though they may have a role to play in the overall response of the teat to invading agents (Hogan et al., 1987, Trujillo et al., 1998, Lind et al., 2015). Xanthine oxidase, fatty acids and protein S100 are some examples of chemical components of the TC that have antimicrobial activities as are elaborated below.

2.6.3 Xanthine oxidase

Xanthine oxidase (XO) is a superoxide-producing enzyme, with low specificity. Xanthine oxidase is present in milk and can generate reactive oxygen. It also decomposes S-Nitrosothiols (RSNO) into nitric oxide (NO), which reacts with a

superoxide anion to form peroxynitrite under aerobic conditions (Trujillo et al., 1998). Xanthine oxidase is effective at inhibiting *S. uberis* growth in interaction with lactoperoxidase and thiocyanate (SCN) (Marshall et al., 1986). The action mechanism of XO is to substrate hypoxanthine and provide an essential component for anti-streptococcal activity mediated by lactoperoxidase. Xanthine oxidase is synthesised in the mammary tissue as well as in the TC, but the TS does not produce XO (Collins et al., 1988).

2.6.4 Lactoperoxidase

Lactoperoxidase (LPO) is commonly synthesised in the mammary gland of ruminants as well as humans. By its interaction with XO it converts nitrites to produce nitric dioxide (NO_2) a potent radical with antibacterial activity, using SCN as an electron donor (Silanikove et al., 2005). The three-dimensional protein structure of LPO is similar to that of MPO (Kussendrager and Van Hooijdonk, 2000). The action mechanism of LPO consists of a long chain of chemical reactions which also include XO and SCN as mentioned before. The oxidation of sulfhydryl groups of microbial enzymes and other proteins is essential to the antimicrobial action of the LPO function (Kussendrager and Van Hooijdonk, 2000).

2.6.5 Free fatty acids

The action of free fatty acids (FFA) may result from the inhibition of enzyme activity, impairment of nutrient uptake, generation of peroxidation and auto-oxidation degradation products, or direct lysis of bacterial cells. The prime target of FFA action is the cell membrane, where FFAs disrupt the electron transport chain and oxidative phosphorylation (Desbois and Smith, 2010). However, their detailed mode of antibacterial action is still poorly understood. The most abundant fatty acids in the TC

are polyene C18:2 and C18:3. Both showed bactericidal activities to *S. aureus, Staphylococcus hyicus*, *Streptococcus agalactiae*, and *Corynebacterium bovis in vitro* (Hogan et al., 1987). The most bacteriostatic saturated fatty acids were C12 and C14 that acted through interfering with cellular energy production.

2.6.6 S100

S100A7, also called psoriasin, has been found highly expressed in the streak canal epithelium of bovine mammary glands (Tetens et al., 2010). S100A7 has antimicrobial effect against *E. coli* and is been postulated as a strong candidate in development of an alternative to the use antimicrobial products in many other studies (Rinaldi et al., 2010, Smolenski et al., 2015). However, S100A7 is also highly expressed in any vastly proliferative tissue as well as in skin undergoing wound healing and as a marker of dendritic cells (Richters et al., 2016). Hence, it is possible that the function of S100 in the TC is not solely to target mastitis causing agents but also as a protein that assists the renovation of the normal epithelium of the TC.

2.7 Current mastitis preventive methods and future directions

Methods to prevent mastitis during the dry period include the use of antimicrobials prophylactically on all quarters of all cows and lately the use of ITS based-on bismuth subnitrate and paraffin. Nowadays, the use of selective DCT is recommended and encouraged (Vanhoudt et al., 2018).

2.7.1 Dry cow therapy

Dry cow therapy is the infusion of a long acting antimicrobial into the udder on the last day of lactation in order to cure and/or prevent mastitis infections during the

dry period. The earliest studies on DCT were performed in the fifties and sixties and the results showed high efficacy in curing *S. agalactiae* and *S. uberis* infections whilst preventing most new infections (Pearson JK, 1951, Smith et al., 1967, Wilkinson, 1969). However, the efficacy against *S. aureus* was lower. The idea of treating the whole herd with antimicrobial substances gained momentum during the seventies to the nineties when the dairy industry flourished globally. Despite promotion of the use of antimicrobial substances in the entire herd by the pharmaceutical industry and veterinarians, the idea of treating cows which are not infected, and the indiscriminate use of antibiotics, has now become a public concern. Without significant changes to udder health, selective dry cow therapy (SDCT) and ITS use are now viewed as more desirable options, since they reduce the use of antimicrobials and *prima facie* reduce the opportunities for bacteria to develop resistance to antibiotics (Vanhoudt et al., 2018, Dufour et al., 2019).

2.7.2 Internal teat sealants

Bismuth formulations used as ITS are classified as "medical devices", non-pharmacological barrier. There is currently a claim that there is no antimicrobial effect from these products (Williamson et al., 1995, Codex, 2016). However, the sole effect of creating a physical barrier against bacteria traversing the TC might not be sufficient to provide the efficacy that has been observed for the bismuth-based products (30% to 90% reduced incidence of new IMI during the dry period when compared to the positive control (Berry and Hillerton, 2002, Huxley et al., 2002). Other products creating physical barriers in the TC have failed to show the efficacy that bismuth-subnitrate based product have (Paape et al., 1988, Serna-Cock and Pabón-Rodríguez, 2016). There are studies showing inhibitory effects on bacterial growth and on biofilm

formation of bismuth-based products used for treating chronic diseases in humans (Folsom et al., 2011, Vega-Jiménez et al., 2012). However, an inhibitory effect of bismuth on mastitis causing agents during the dry period has not been investigated in literature reports.

The use of bismuth in teat sealants is currently being reviewed as its application has been demonstrated to have detrimental effects in the cheese industry in the past, bringing major economic losses to dairy processors (Lay et al., 2007). Bismuth subnitrate is a heavy metal and as such, it has toxicity involved with its use. Bismuth subnitrate is considered the least toxic salt from the bismuth derived group (Slikkerveer and de Wolff, 1989), but an outbreak of bismuth toxicity was reported in France and Australia between 1972 and 1980 related to bismuth subnitrate use (Emile 1981). An alternative product to the current teat sealants is desirable to reduce the potential risk of overuse of bismuth subnitrate in the future.

2.8 RNA sequencing as a tool for mammary gland studies

RNA sequencing (RNA-seq) analyses the transcriptome of a cellular sample. This can provide information about changes occurring in the cells. RNA sequencing can tell us which genes are expressed in a cell, what their level of expression is and whether they are activated or inactivated. This allows scientists to understand the biology of a cell or tissue and assess changes that may indicate disease. Some of the most popular techniques that use RNA-seq are transcriptional profiling, next generation sequencing (NGS), SNP identification, RNA editing and differential gene expression analysis.

With the development of the *Bos taurus* genome assembly records, bovine milk and mammary gland studies have changed. There is now an increasing interest in studying the transcriptomes of the bovine mammary gland through the lactation cycle (Bionaz and Loor, 2007, Asselstine et. al., 2019). These studies focus on fundamental stages of the mammary gland lactation cycle such as the drying off, the onset of lactation or mastitis events. Nearly 20 years ago, complex cDNA and RNA microarrays were designed to shed some light on the physiology of the bovine mammary gland (Suchyta et al., 2003, Swanson et al., 2009). More recently, NGS and now Nanostring complement the information obtained by the originally created microarrays and qPCR research works using these more advanced technologies (Crookenden et al., 2016, Asselstine et al., 2019). The long-term objective of having an in-depth knowledge of mammary gland physiology is to characterise events occurring in the tissue and consequently to make better decisions on how to prevent or treat IMI or to provide better management techniques for the lactation cycle in dairy cows.

Technologies such as NGS allow researchers to have a complete "picture" of the genes expressed in a tissue at the time of sampling. The use of transcriptomics analysis by NGS implies broadening the scale of studies as well as the information obtained. On the other hand, when using q-PCR or Nanostring, a researcher must choose which genes they will target. The advantage of using Nanostring technologies over qPCR is that it excludes some of the complexity of qPCR studies, providing shorter and simpler protocols, more consistent results that reduce the variations previously observed between real time PCR studies in the past (Malkov et al., 2009, Veldman-Jones et al., 2015).

2.9 Hypotheses

The leading hypothesis of this thesis is that active mechanisms occur in the epithelial tissue of the TC and TS during the early dry period that result in keratin plug formation. It is also proposed that bismuth-based teat sealants might have other than only a physical barrier function, e.g. antimicrobial activity, and that additional mechanisms could contribute to its efficacy. The exploration of these hypotheses will provide valuable data to assist in developing new treatment alternatives to prevent mastitis during the dry period.

3 A novel biopsy technique in dairy cows and its suitability to obtain samples of the teat canal for mRNA expression analysis

A technique to examine physiological changes of teat canal (TC) epithelial tissue during keratin plug formation has not previously been available. This chapter reports the development of a novel biopsy technique, established to obtain repeated samples of the TC tissue. The technique described in Experiment 1 of this Chapter allowed this research to continue and was used to achieve different objectives throughout the studies reported in this thesis:

1) To analyse the gene expression of the TC epithelial tissue (Experiment 2, Chapter 3).

2) To observe changes occurring in the TC before and after drying off and after treatment with internal teat sealants (Chapters 4 and 8).

The technique has been effective in retrieving tissue that is suitable for RNA extraction and transcriptomic analysis. It has allowed the generation of new information about the physiological state of the TC.

A novel biopsy technique in dairy cows and its suitability to obtain samples of the teat canal for mRNA expression analysis

3.1 ABSTRACT

At the end of lactation, epithelial tissue in the teat canal (TC) forms a keratin plug that seals the teat opening. How the keratin plug forms is unknown. The teat may be actively sealed by an increase in keratin synthesis and tissue proliferation at the end of lactation or by passive accumulation of detritus and desquamated tissue after the flushing effect of milking ceases. It has been shown that there is a reduced mitotic index of the basal cells in the *stratum corneum* of the TC at the end of lactation, but more studies are required to examine the function of the epithelial cells to understand the processes involved. A technique to examine physiological changes of the TC epithelial tissue throughout the keratin plug formation process was not available. Hence, the aims of the two studies were to 1) develop an effective biopsy technique to obtain serial tissue samples from the TC epithelium; 2) detect any changes in mRNA expression of the TC epithelium after sampling, and if so, assess the impact of the sampling technique on the TC by mRNA sequencing. In Study 1 (*ex vivo*), five biopsy techniques (cytobrush, automatic biopsy needle, tumour extractor, curette, and punch biopsy) were evaluated on all udder quarters of seven recently slaughtered lactating dairy cows. In Study 2 (*in vivo*), nine late lactating dairy cows were randomly assigned to be sampled on Day 0 of the dry period with one of two selected techniques (curette and tumour extractor) applied to two quarters of each cow (n=9 per technique) with the objective of testing the suitability of the samples obtained, and the effect of the

sampling techniques on indicators of pain and animal welfare. The remaining two quarters per cow served as non-sampled control teats (n=18). In a second sampling on Day 11 (D11), biopsy samples were obtained with a curette from the same anatomical area as the first sample to assess the impact of the first sampling on the mRNA expression of the TC tissue at second sampling. Five biopsy samples from teats that served as controls on Day 0 (D11-S), and four samples from teats previously sampled with a curette on Day 0 (D11-RS) were obtained. The tumour extractor technique was not evaluated on D11 based on the results obtained in Study 1 and on Day 0 of Study 2. RNA extracted from the samples was measured and the three RNA samples with the highest yield from each group were selected for analysis. Transcriptomes of D11-S n=3; D11-RS n=3 were analysed with Illumina RNA-sequencing and annotation of differentially expressed genes was performed using the Database for Annotation, Visualisation and Integrated Discovery (DAVID). Mixed linear models of measurements obtained from the histological photomicrographs showed that the curette was the most suitable technique resulting in repeatable high-quality biopsy samples. Results of RNA-sequencing revealed 1167 upregulated and 300 downregulated genes in the comparison between D11-S and D11-RS samples. Gene ontology (GO) enrichment analysis showed that the biological functions activated in the repairing tissue (D11-S vs. D11-RS) were mainly cell adhesion, oxidation-reduction processes, inflammatory response, immune response, proteolysis, angiogenesis, intracellular signal transduction, and positive regulation of cell migration. Gene Ontology molecular functions analysis showed higher binding and growth activities in D11-RS than in D11-S samples. Transcriptomic analysis was a suitable method to obtain information on changes occurring in the physiology of the TC and this may

assist in the development of new protective treatments or new management techniques to prevent mastitis in the early dry period.

3.2 INTRODUCTION

The early dry period is a stage of high risk for intramammary infection (IMI) in the lactation cycle in dairy cows (Dingwell, 2002, Bradley and Green, 2004). The teat canal (TC) is the first physical barrier that bacteria must overcome in order to infect the mammary gland (Forbes, 1968, Hillerton and Lacy-Hulbert, 1995). The epithelium covering the TC is a modified portion of the teat skin that invaginates over the distal 10-15 mm of the canal. The TC epithelium is thicker than normal skin, which is possibly related to the renewal rate of the TC epithelium being increased by the constant erosion induced by milking (Chandler et al., 1969, Paulrud, 2005).

During the first part of the dry period, the TC becomes sealed by a plug formed by lipids and a proteinaceous substance (Bright et al., 1990). Histological and physiological features of the TC are typically examined by immunohistochemistry (IHC) and immunohistofluorescence (IHF) (Nickerson and Pankey, 1983, Nickerson and Pankey, 1984, Smolenski, 2018). These techniques allow researchers to view static images from slaughtered cows to visualise processes occurring in the mammary gland or TC after bacterial invasion or during different stages of lactation or involution (Persson and Åström, 1989, Molenaar et al., 2000, Smolenski, 2018). However, the colonization of the mammary gland by mastitis-causing agents, involution and keratin plug formation are far from static. Epithelial cell proliferation, bacterial invasion and immune responses are dynamic and progressive events; hence, repeated sampling and

measurements are required to examine these changes (Paulrud and Rasmussen, 2004). Repeated sampling of the keratin mass formed in the TC lumen has been described (Bitman et al., 1988, Bright et al., 1990, Capuco et al., 1990). However, the mass of desquamated keratin extracted might be due to sampling trauma and not represent the true physiological state of the TC epithelium. Analysis of the physiological changes occurring in the TC throughout the lactation cycle would provide further knowledge on keratin plug formation in a more comprehensive and dynamic manner.

Elucidating the physiology of the TC and keratin plug formation is required for the development of novel preventive treatments and improvements of dry period management techniques. Therefore, the objectives of the studies presented herein are (1) to develop an effective biopsy technique to obtain serial tissue samples from the TC epithelium; (2) to analyse the impact of the sampling technique on animal wellbeing; and (3) to analyse the impact of the sampling technique on the gene expression of the TC epithelium.

3.3 MATERIALS AND METHODS

3.3.1 Study 1, ex vivo

Seven udders from recently slaughtered lactating dairy cows were suspended from a frame in a close-to-natural position. Each of the 28 quarters was randomly assigned to one of five sampling techniques using the random function of Microsoft Excel 2010 (Microsoft, USA). The sampling techniques were: a) cytobrush (Thermo Fisher Scientific NZ, North Shore City, New Zealand), b) “Achieve” automatic biopsy

needle (Care Fusion, Vernon Hills, Illinois, USA), c) tumour extractor (Shoof International Ltd. Cambridge, New Zealand), d) curette (Curette 160 mm, Moses pattern, [206689], Shoof International, 2017, Cambridge, New Zealand), and e) punch biopsy (Health link, Jacksonville, Florida, USA).

Cytobrush sampling method: while holding the teat, the cytobrush was inserted into the TC to a depth of approximately 10 mm. The teat was pressed by the thumb and index finger against the cytobrush while this was rotated in the TC four times.

Automatic biopsy needle sampling method: the instrument was inserted through the teat orifice with the plunger in the sampling position. The trigger was pushed, aiming to include the TC epithelium in the sample. The cylindrical TC specimen was pulled gently from the needle using forceps.

Tumour extractor sampling: the funnel-shaped end of the tool was inserted into the teat via the teat orifice, and the plunger was then withdrawn. With the non-dominant hand, the back end of the instrument was located over the TC. Pulling with the hand holding the instrument, a flap of epithelium inside the TC was pinched, cut and removed.

Curette sampling technique: the curette was inserted into the TC with the sharp side of the curette facing the area to be sampled. The teat was held with one hand and the curette was pulled out through the TC whilst applying pressure over it with the hand holding the teat.

Punch biopsy: the punch biopsy instrument was held vertically 1 mm lateral to the TC orifice and rotated upward using a twirling motion. Once the instrument

penetrated 8-10 mm deep, it was removed. The cylindrical specimens obtained were removed gently from the punch with forceps or a needle.

Tissue samples obtained using all sampling techniques except the cytobrush, were immediately submerged in 4% formalin for 24 hours after retrieval and were prepared for histological observation using a Haematoxylin and Eosin stain. The cytobrush samples were smeared onto pre-labelled glass slides and air-dried before fixation in 4% formalin for five minutes.

The techniques were scored (1 to 3) according to their ease of use, amount of tissue retrieved and wound evident in the teat after sampling. Ease: 1= difficult to handle, 2= practicable, 3= easy to handle. Amount: 1= low amount of tissue (cannot divide the sample in two), 2= enough tissue (sample can be divided into two equal parts), 3= can be divided into more than two parts. Wound: 1= big wound (>3mm), 2=medium wound (1.5mm>3mm), 3= Small wound (1mm or less). Sampling techniques were also classified as "Suitable" or "Unsuitable" according to the tissue structure observed in the photomicrographs and the variation between similar samples (repeatability of the technique). A technique was defined as suitable, if all the tissue samples obtained presented all the four layers of epithelial tissue (*stratum basale, stratum spinosus, stratum granulosum and stratum corneum*) Table 3.1.

3.3.2 Study 2, in vivo

Based on the results obtained in Study 1, a second study was performed *in vivo* at Massey University Dairy Number 4. The study was approved by MUAEC (Massey University Animals Ethics Committee, Palmerston North, New Zealand), protocol number 16/126. A general health assessment was performed on 14 cows ten days

prior to the beginning of the study. Nine lactating dairy cows of variable ages, (5 to 8 years old), 194 (±8.3) days in milk (DIM) and with four closed teat ends (absence of signs of hyperkeratosis in the teat ends) and no cracks or chaps in the teat skin were selected. One quarter of each cow was randomly assigned to one of two teat sampling techniques (curette and tumour extractor) because both techniques showed the best potential in the results of study 1. The remaining two quarters served as non-sampled control teats.

3.3.3 Curette and tumour extractor sampling - Day 0

On the last day of the lactation (Day 0) cows participating in the study were held in a head bail and a sedative dose of 0.8 ml of Xylazine 2% was administered intravenously into the caudal vein. Once the cows showed signs of sedation (slower movements), the hind legs were loosely tied with a rope to the sides of the cattle crush as a precaution to avoid injuries to the operator and cows during local anaesthetic infusion and sampling. After teat end inspection, a tourniquet was placed at the base of the teat using an elastic bandage in order to prevent secretion from entering the teat cistern and dilution of the local anaesthetic, to prevent the anaesthetic from diffusing into the mammary tissue, and to reduce bleeding from the site of sampling. Milk from the teat cistern was stripped to remove any accumulation. The teat orifice was thoroughly cleaned using cotton balls moistened with methylated spirits and local anaesthetic solution was infused into the teat cistern through the streak canal using a mastitis treatment syringe pre-filled with five millilitres of 2% lidocaine (Nopaine, Phoenix Pharm Distributors Ltd. Auckland, New Zealand). The local anaesthetic solution was stripped from the cistern after approximately 30 seconds. The teat ends were cleansed again with a cotton swab moistened with methylated spirits and

sampling was conducted as described above with individually sealed sterile curettes and tumour extractors. The tissue samples of the TC epithelium were placed in 1mL of an aqueous, non-toxic tissue and cell storage buffer that stabilizes and protects cellular RNA in intact, unfrozen tissue (RNA later, Invitrogen, Thermofisher Scientific, Baltics) until processed for RNA extraction.

The behavioural response of cows and inflammation at the sampling site during and after sampling were assessed on Days 0, 1, 4, 7 and 11 using a scoring scale from 3 to 0 (3= no discomfort or inflammation observed, 2= mild signs of discomfort, no inflammation, 1= showing signs of discomfort, red- inflamed teat, 0= Very uncomfortable-inflamed, oedema in the teat).

3.3.4 Curette sampling - Day 11

On Day 11, biopsy samples were obtained using the curette technique from teats that served as a control on Day 0 (D11-S, n=5) and from teats which were previously sampled on Day 0 of the dry period (D11-RS, n= 4). Samples D11-RS were obtained from the same area that was sampled on Day 0 (cranial area of the TC). The tumour extractor technique was not evaluated on Day 11 due to the poor results obtained in Day 0 of Study 2. Samples were immersed in 1 mL of "RNA later" for stabilisation.

3.3.5 Sample preparation and analysis

Total RNA was extracted from the curette tissue samples using a mirVana miRNA Isolation Kit (Life Technologies, Carlsbad, California, USA). The RNA integrity was assessed in a Bio analyser (Agilent Technologies, Inc.) and used to prepare the sequencing library. The RNA extracted from the samples was measured using Qubit

(Qubit® 2.0 Fluorometer, Q32866 Invitrogen, Life technologies, USA). Samples having RNA of suitable quality (n=3 D11-S and n= 3 D11-RS) were sent to New Zealand Genomics Limited (University of Otago, Dunedin, New Zealand) for sequencing. The submitted RNA was made into libraries using Illumina TruSeq Stranded mRNA kit. Samples were analysed with Illumina based on the quality and yield of the RNA obtained (Illumina, San Diego, CA, USA). The libraries were sequenced on 3 lanes of HiSeq 2x125bp PE v4 (Illumina, San Diego, CA, USA) and the gene expression of the TC epithelium in the early dry period (D11-S) was compared with the gene expression of the tissue after sampling with the curette (D11-RS). Annotation of differentially expressed genes was performed using the Database for Annotation, Visualisation and Integrated Discovery (DAVID) https://david.ncifcrf.gov/ (Sherman and Lempicki, 2009). Functional profiles for the differentially expressed genes were derived for each of the gene ontology (GO) categories: cellular component, molecular function, and biological process. Evaluation of inflammation and healing after sampling

On day 13 of the study, the cows were slaughtered, and the udders retrieved for histological inspection and evaluation of the healing. The photomicrographs of the histological slides were scored from ++++ to + for the inflammatory signs observed: ++++ = very inflamed determined by the presence of a high number of neutrophils and corpora amylacea (Table 3.2and Figure 3.2-C) +++ = moderate inflammation, ++ = slight inflammation and signs of re-epithelization + = no inflammation observed. Nine teats sampled with tumour extractor, five with a curette and 14 non-sampled control teats were evaluated. D11-RS teats (n= 9 and 1 that was sampled but did not retrieve any tissue) were not analysed on Day 13 (Table 3.2) because of the high degree of

inflammation present in all the photomicrographs (higher than ++++) due to the proximity of sampling and observation time points (2 days).

3.3.6 Statistical analyses

The scores from Study 1 (ease of use of the instrument, amount of tissue retrieved and wound) were analysed using the (nlme) function in R (R Core Team, 2015, R Foundation for Statistical Computing, Vienna, Austria) with the biopsy technique as fixed effect and cow (udder) as a random effect. In Study 2, the behavioural score (sum of discomfort, inflammation-oedema and wound) during and after sampling (days 0, 4, 7 and 11) and the inflammation score of the healing process from the micrographs on Day 13 were analysed by least-squares ANOVA (PROC MIXED, SAS 9.4 for Windows, SAS, 2019) after homoscedasticity of the residuals was confirmed. Cow was used in the model as a random effect instead of using a repeated measure analysis due to the low variability between the scores obtained. Instrument, quarter location and day were fixed effects for which least square means were calculated and compared. For the bioinformatics and statistical analyses of the transcriptomics data, the raw reads were filtered for adaptor sequences and low quality base pairs (phred<10) using BBduck (version 36.86) from the BBtools package (Bushnell B (2016)) Bbmap short read aligner University of California, Berkeley, California (URL http://sourceforge. net/projects/bbmap). The quality control (QC) filtered reads were aligned to the *Bos taurus* genome (ENSEMBL release 90) using HISAT2 (version 2.1.0) short read aligner using the default settings, except –rna-strandness set to incorporate strand information into the mapping. The short read alignment files were used to generate per gene read counts with htseq-count (version 0.9.1) using the 'union' mode (Anders et al., 2015) based on the ENSEMBL gene

models. To reduce the likelihood of obtaining $p<0.05$ by chance, a Benjamini-Hochberg procedure was carried out with the data obtained in the GO biological process annotations. Statistical significance was set at $p<0.05$ and false discovery rate (FDR) <0.1.

3.4 RESULTS

3.4.1 Study 1, ex-vivo

The five sampling techniques tested in this study were scored for their ease of use, amount of tissue retrieved, and the wound observed in the teat after sampling. There was no significant difference in these criteria between the three highest-scoring techniques: Achieve biopsy needle, curette and tumour extractor. Cytobrush and punch were significantly less suitable based on the comparison with the highest scored technique (Achieve biopsy needle Mean= 2.93±0.12) Table 3.1.

Table 3.1 Mean and standard deviation (SD) of the scores (1-2-3) obtained for each instrument in Study 1 (ex-vivo): Achieve biopsy needle, curette, tumour extractor, cytobrush and punch. Ease: 1= difficult to handle, 2= practicable, 3= easy to handle. Amount: 1= low amount of tissue (cannot divide the sample in two), 2= enough tissue (sample can be divided into two equal parts), 3= can be divided into more than two parts. Wound: 1= big wound (>3mm), 2=medium wound (1.5mm>3mm), 3= Small wound (1mm or less). Asterisks mean: * = p<0.05, *** = p<0.001

Instrument	Ease	Amount	Wound	N° of samples	Mean score of ease, amount and wound	SD	Suitability
Achieve biopsy needle	3.00	2.80	3.00	5	2.93	0.12	Unsuitable
Curette	3.00	2.17	2.83	6	2.67	0.58	Suitable
Tumour extractor	2.67	2.67	2.50	6	2.61	0.48	Suitable
Cytobrush	3.00	1.00	3.00	6	2.33*	1.15	Unsuitable
Punch	1.60	1.00	2.20	5	1.60***	0.81	Unsuitable

The techniques were also scored as “Suitable” or “Unsuitable” by observing the photomicrographs of the tissue samples obtained from the histological slides (Table 3.1;Figure 3.1). The cytobrush biopsy technique was not suitable in any sample (Figure 3.1-A). Only a low number of cells were identified as keratinocytes with no appreciable tissue structure. Histological slides taken with the automatic Achieve biopsy needle, retrieved only muscular rather than epithelial tissue making it unsuitable for further research (Figure 3.1-B). Samples obtained with the tumour extractor had an appropriate size (amount of tissue), they were well-structured biopsy samples, but were poorly repeatable based on a high variability between the amounts of tissue obtained in each sampling time point (Figure 3.1-C). The samples acquired by curette were well preserved in structure and repeatable (Figure 3.1-D). Some of the biopsies taken with the punch had muscular tissue with absent or low numbers of epithelial cells (Figure 3.1- E). Although the samples taken with the tumour extractor were not

consistent in terms of size between samples, the tumour extractor and the curette technique were chosen for further research *in vivo* due to practicality, and suitability of the samples obtained.

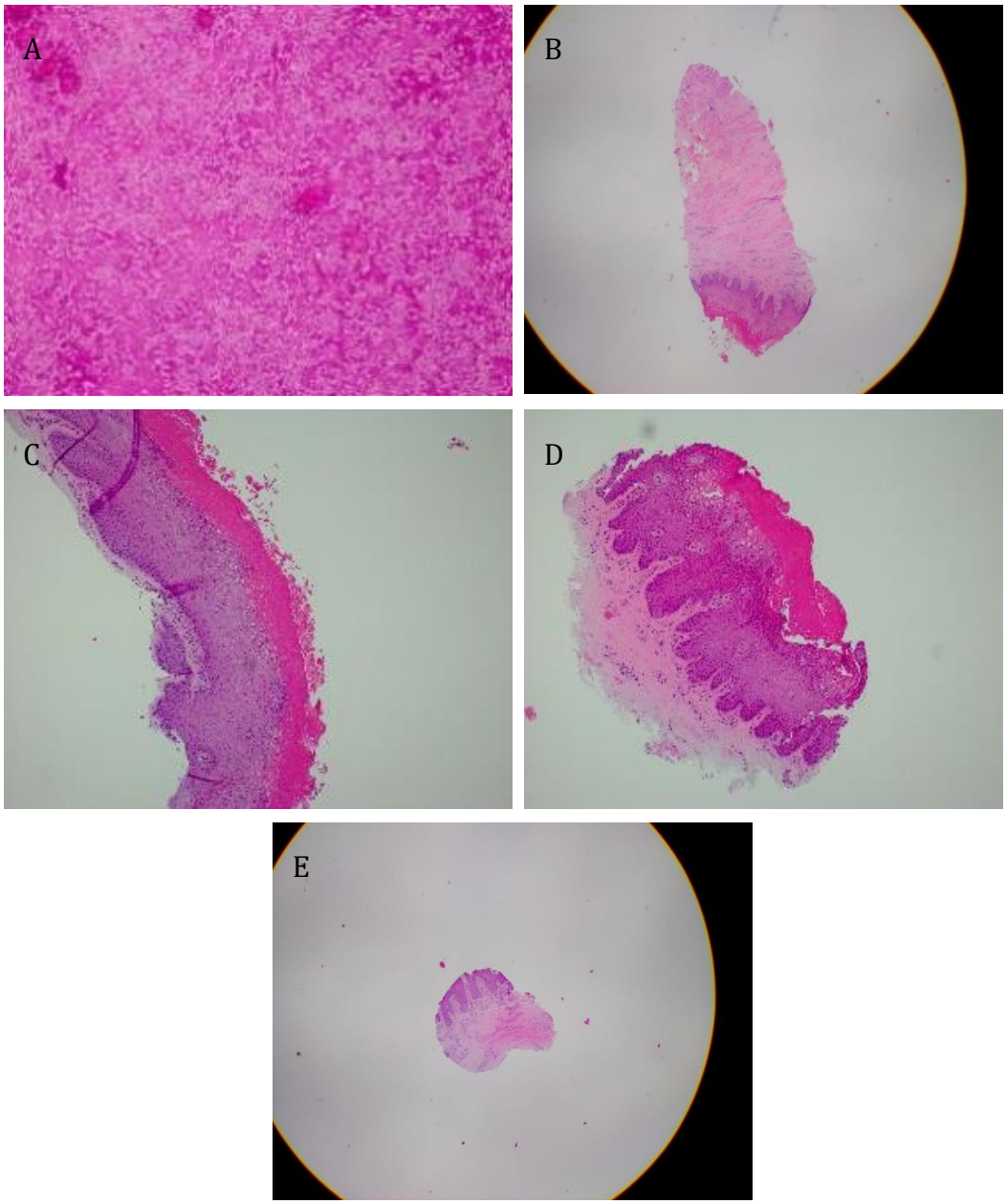


Figure 3.1 Photomicrographs of the histological sections obtained from the different biopsy instruments. A: cytobrush (4x) keratin with no appreciable tissue structure B: Achieve biopsy needle (4x) mainly muscular tissue observed C: tumour extractor (10x) four strata observable, but the sampling technique was not repeatable D: curette (10x) samples were well preserved in structure and repeatable E: punch (4x) some strata of the epithelial tissue are absent in the sample.

3.4.2 Study 2, in vivo: Curette and tumour extractor sampling- Inflammation and evaluation of healing

There was no statistical difference in the scores of the behavioural responses (discomfort, inflammation-oedema and wound) of the cows for the two techniques (tumour extractor and curette) during and after sampling. Only one cow scored 1 (discomfort) and all the others scored minimum levels of discomfort (Score 3). There was minimum variation between the scores obtained in the repeated measures analysis; hence, a fixed effect of day without adjusting for correlation within quarter was used.

The high variability of the size and quality of the tissue samples obtained with the tumour extractor on Day 0 forced the decision to not use the tumour extractor technique on Day 11 to safeguard the welfare of the cows. One D11-RS could not be obtained, after one attempt of sampling the teat (maybe as a result of a blunt curette) (Table 3.2). One quarter was diagnosed with mastitis on Day 11 (Cow 872 RR).

The inflammation and healing scores 13 days after the first sampling were similar for both techniques (Figure 3.2 A and B). Inflammation scores obtained from teats two days after sampling, were high ++++ (Figure 3.2- C).

Table 3.2 Design of in vivo Study 2 and histological scores obtained on Day 13. Cow: Cow ID, Teat: FL: front left, FR: front right, RL: rear left, RR: rear right, Day 0: Instrument used to obtain the sample on Day 0. Day 11: Instrument used to obtain the sample on Day 11. Inflammation scores on Day 13 were defined as No= not scored, ++++= very inflamed determined by the presence of a high number of neutrophils and corpora amylacea +++= moderate inflammation, ++= slight inflammation and signs of re-epithelization + = no inflammation observed. Scoring of histological slides on Day 13 was carried out only in teats sampled once (on Day 0) as the inflammation observed two days after sampling Day 11 was high and healing was not evident.

Cow	Teat	Day 0	Day 11	Inflammation Score Day 13
36	FL	Control	Curette	No
36	FR	T. extractor		++
36	RL	Curette	Curette	No
36	RR	Control		+
532	FL	Control		+
532	FR	Curette	Curette, no tissue in the sample	No
532	RL	Control	Curette	No
532	RR	T. extractor		++
547	FL	Curette		++
547	FR	T. extractor		++
547	RL	Control		+
547	RR	Control		+
603	FL	Curette	Curette	No
603	FR	Control		+
603	RL	Control	Curette	No
603	RR	T. extractor		++
617	FL	Control		+
617	FR	T. extractor		++++
617	RL	Curette		+
617	RR	Control		+
719	FL	Control	Mastitis	+
719	FR	Curette		+++
719	RL	T. extractor		+

719	RR	Control		+
784	FL	Curette		++
784	FR	T. extractor		+++
784	RL	Control		+
784	RR	Control		+
793	FL	T. extractor		++
793	FR	Control	Curette	No
793	RL	Control		+
793	RR	Curette	Curette	No
872[a]	FL	T. extractor		++
872	FR	Control		+
872	RL	Curette	Curette	No
872	RR	Control	Curette	No - Mastitis
TOTAL			10	26

[a] Cow 872 developed mastitis after the Day11 sampling and was euthanised and scored on Day 18 after sampling

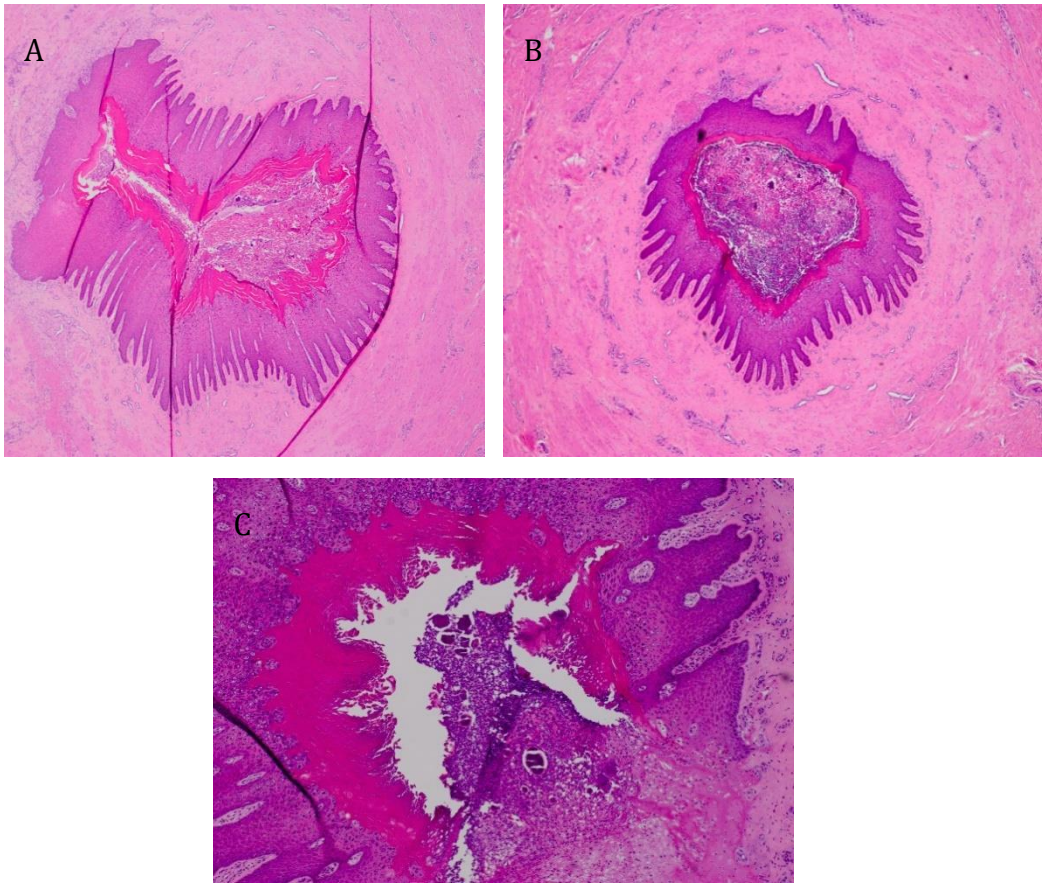


Figure 3.2 Top: Photomicrographs of the midsections of the teat canal, sampled on Day 0 with Curette 547 FL (A) and Tumour extractor 547 FR (B), at 13 days after sampling (retrieved at post-mortem examination) (4x). Both pictures showing healing score ++: slight inflammation and signs of re-epithelization. (C)Photomicrograph of teat 36 FL distal two days after sampling with the curette (10x). There are signs of high inflammation and corpora amylacea present in the injured tissue. Healing score: ++++

3.4.3 Study 2- Differential expression

The chosen samples had the highest average RIN mean (±SD) of 8.6 (±0.9) and were used to prepare the sequencing library. Two samples in the Day 11-S and one in the D11-RS groups were not analysed due to having a low RIN not ideal for Illumina analysis. RNA sequencing of the D11-S and D11-RS triplicate libraries resulted in an

average of 75.6 and 75.0 million paired-end reads per replicate, respectively. Following removal of reads with homology to sequencing adaptors and low-quality base calls (phred<10), between 94.3 and 95.6% of sequenced base-pairs were retained from the D11-S and D11-RS and passed the quality control (Table 3.3). A high percentage of quality-filtered reads successfully mapped to the *Bos taurus* genome, with the average mapping rates >90% for both time point triplicates. Based on the hisat2 mapping files, read counts were allocated to genes described in the ENSEMBL gene models for the cow.

The comparison between D11-S vs. D11 RS resulted in identifying 1167 upregulated and 300 downregulated genes out of 24615 mapped genes analysed (Figure 3.3). Highest levels of expression were achieved by genes codifying for collagen and elastin proteins (e.g. *COL1A1, COL1A2, SPARC, COL3A1, COL6A3, ELN*), inflammation (*C1S*), cornification of the epithelial tissue and extracellular matrix generation (*TGM3, FBN1*; Figure 3.4).

Table 3.3 QC results using BBduck with phred cut-off of 10 ("normal" QC reads) of the samples obtained on Day 11 from control teats (D11-S) and on Day 11 from teats that have been sampled previously on day 0 (D11-RS). Number of Pair-end reads: reads corresponding to the bovine genome. Base-pairs pass QC % total: percentage of base-pairs reads which passed the quality control. The chosen samples had the highest average RIN mean (±SD) of 8.6 (±0.9) and were used to prepare the sequencing library.

Sampled quarter	Group	Number of paired-end reads	Base-pairs pass QC % total
36 FL	D11-S	73620795	94.36
603 RL	D11-S	73677310	94.97
872 RR	D11-S	77675579	95.63
B36 RL	D11-RS	74477184	95.15
603 FL	D11-RS	85840451	94.26
B793 RR	D11-RS	74558987	95.21

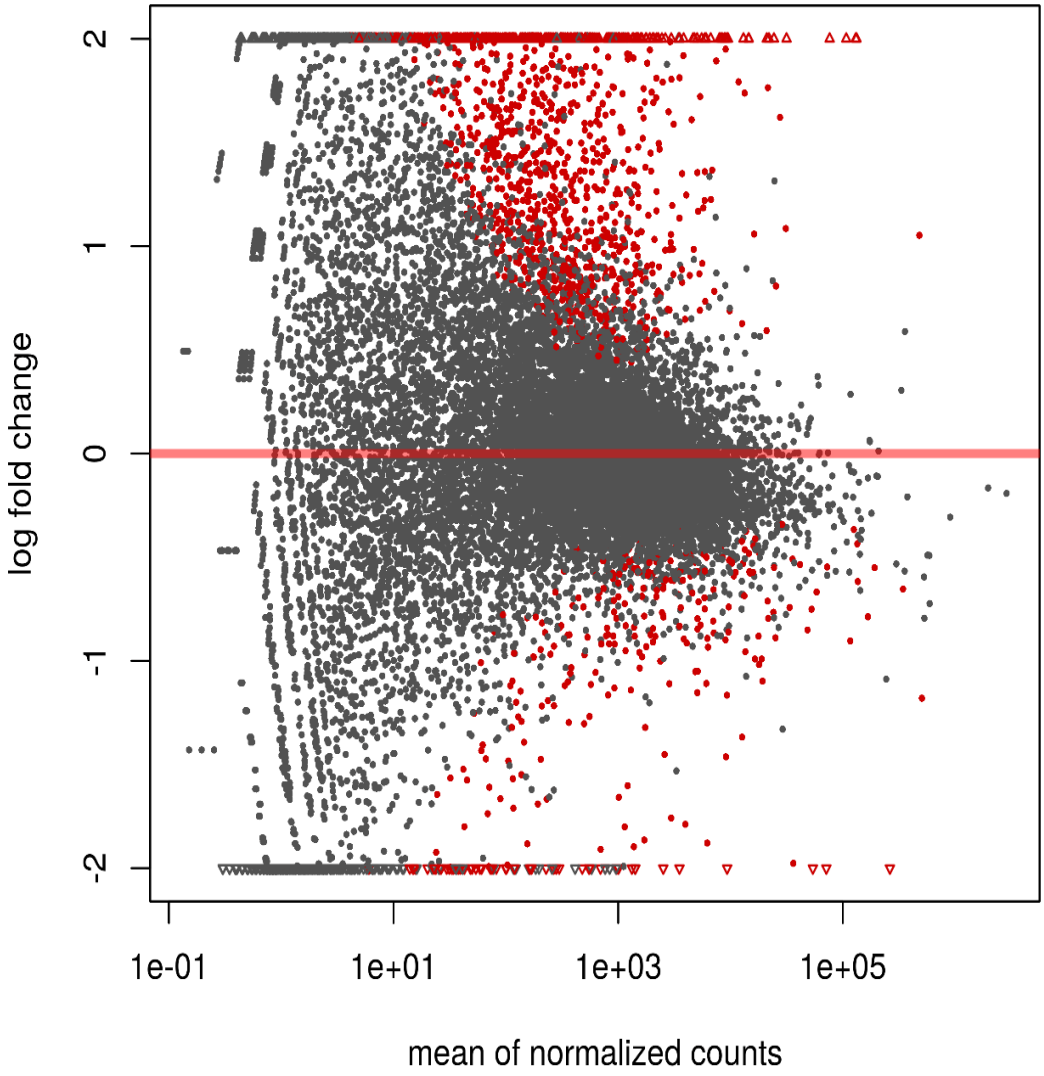


Figure 3.3 Volcano plot of the mean of normalised counts (X-axis) versus log fold change (Y-axis) showing the gene expression comparison between D11-S (control Day 11) and D11-RS (Day 11 re-sample). Genes showing significant differences are coloured in red (< 0.1 FDR). Log fold change above 0 = upregulated, below 0 = downregulated

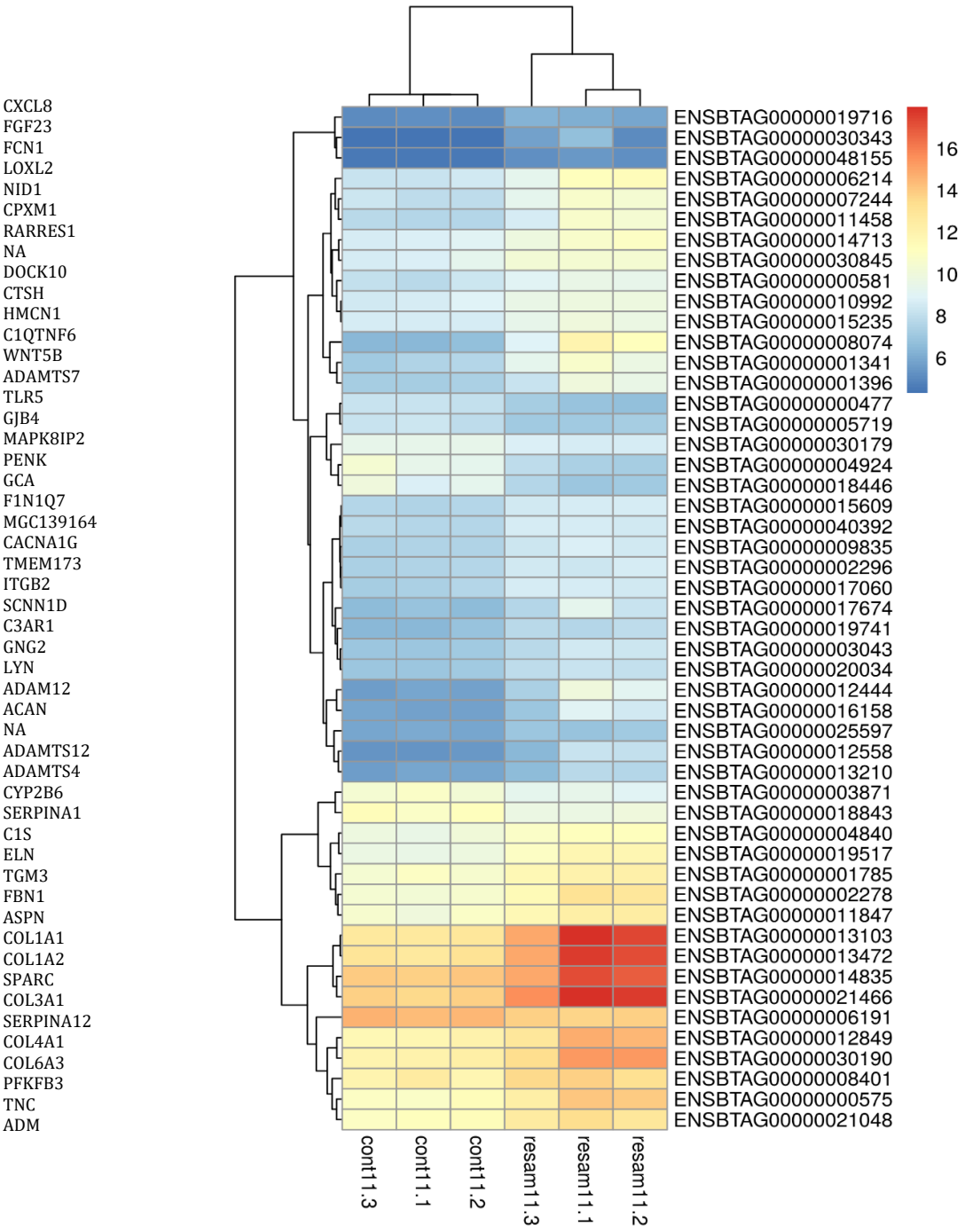


Figure 3.4 Heat map showing expression profiles of the 50 most significantly differentially expressed genes detected in the comparison between control D11-S (cont11.1- cont11.2 and cont11.3) and D11-RS (resam11.1- resam11.2 and resam11.3). Vertical dendrograms on the top and left correspond to the sample and gene hierarchical clustering respectively. Warmer colours are more highly expressed. Numbered scale on the right represents the log2 fold change. Gene names are presented on the left and ENSEMBLE numbers are presented on the right

3.4.4 Functional profiling

Functional profiles for the differentially expressed genes were represented for each of the GO categories: cellular component, molecular function, and biological process. Cellular component and biological process GO terms which exceeded the 1% representation are presented in Figure 3.5 (cellular components) and Table 3.4 (biological process), respectively. Between 5 and 25% of the differentially expressed genes belong to the extracellular matrix, the plasma membrane, and collagen trimmer in samples D11-RS. Cell adhesion, oxidation-reduction process, inflammatory response and immune response are the most represented biological processes. Molecular functions with the highest percentages were: calcium ion binding (7%), metalloendopeptidase activity (2.6%), heparin-binding (2.6%), serine-type endopeptidase activity (1.8%), GTPase activator activity (1.8%), protein binding (1.6%), extracellular matrix structural constituent (1.5%), carbohydrate-binding (1.5%), receptor binding (1.5%), iron ion binding (1.4%), identical protein binding (1.4%), growth factor activity (1.3%), extracellular matrix binding (1.1%), and scavenger receptor activity (1%). Gene biological processes related to proliferation of the epithelial tissue, “Regulation of cell proliferation” and “Cell proliferation”, were all highly upregulated in D11-RS when compared with D11 (p-value= 0.00017 and 0.045, respectively) (Table 3.4).

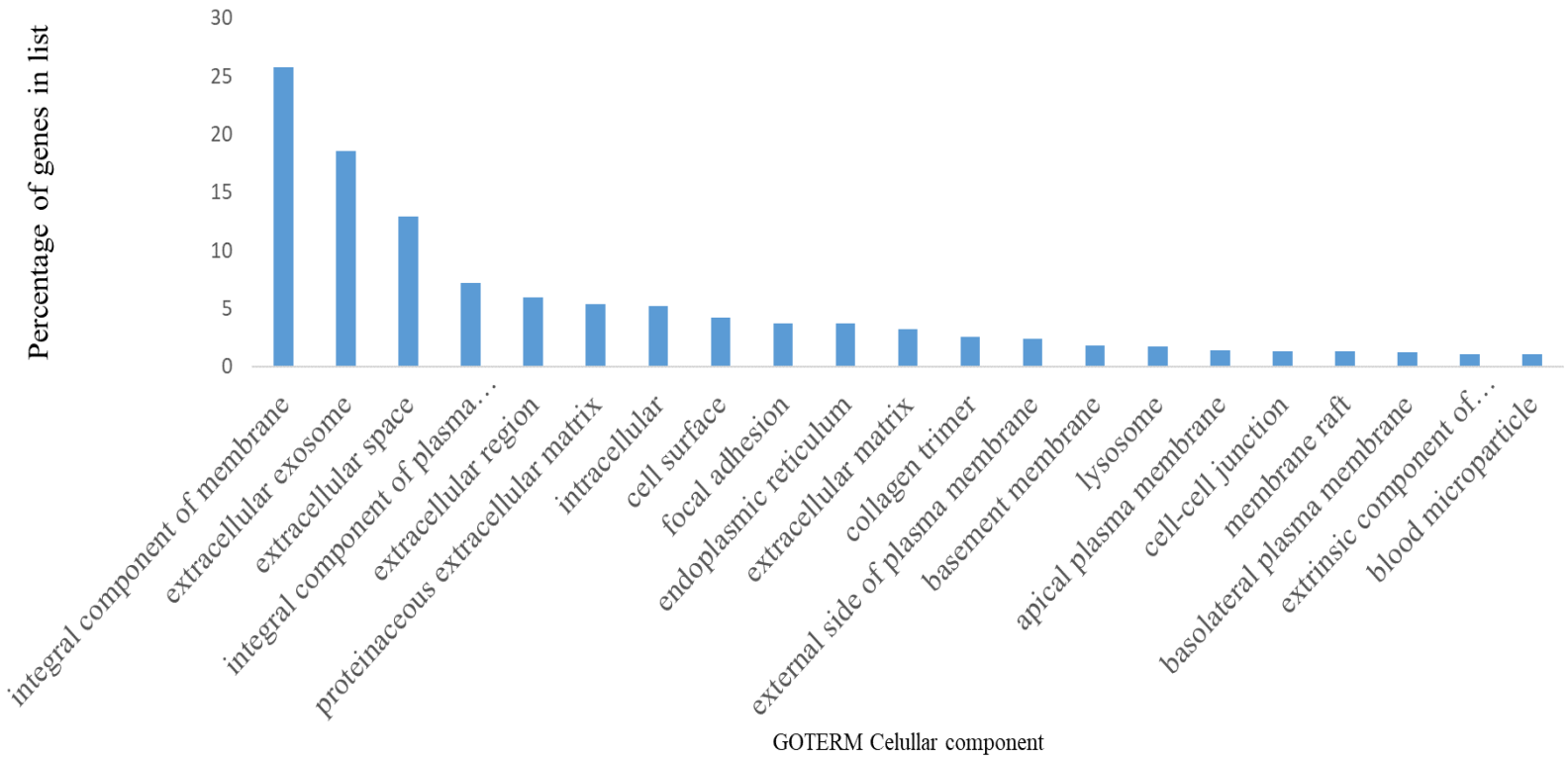


Figure 3.5 Cellular components GO annotations for the genes that were represented at higher than 1% when comparing RNA extracts from samples taken from the teat canal at D11-RS and D11-S

Table 3.4 List of biological process GO annotations of differentially expressed genes in descendent order of the comparison D11-S vs. D11-RS. Term: Biological process, Count: total of genes represented in this function, %: percentage representation of the function, Benjamini: p-value of the Benjamini test run to avoid over representations

Term	Count	%	Benjamini	p-Value
Cell adhesion	41	3.7	1.7E-09	6.4E-13
Oxidation-reduction process	34	3.1	0.26	0.0038
Inflammatory response	33	3	0.0018	0.0000066
Immune response	32	2.9	0.0044	0.000018
Proteolysis	29	2.6	0.0019	0.0000062
Angiogenesis	28	2.5	0.000056	0.000000082
Intracellular signal transduction	27	2.4	0.67	0.031
Positive regulation of cell migration	25	2.3	0.00057	0.0000014
Innate immune response	24	2.2	0.55	0.02
Positive regulation of cell proliferation	24	2.2	0.84	0.065
Cell differentiation	23	2.1	0.7	0.036
Cell migration	20	1.8	0.027	0.00017
Positive regulation of ERK1 and ERK2 cascade	20	1.8	0.069	0.0006
Regulation of cell proliferation	20	1.8	0.19	0.0024
Extracellular matrix organization	19	1.7	0.00015	0.00000034
Regulation of cell shape	19	1.7	0.02	0.00011
Small gtpase mediated signal transduction	19	1.7	0.88	0.087
Defense response to bacterium	17	1.5	0.01	0.000046
Positive regulation of gtpase activity	17	1.5	0.56	0.021
Endodermal cell differentiation	16	1.4	0.000000006	4.4E-12
Collagen fibril organization	16	1.4	0.000000034	3.7E-11
Cell proliferation	16	1.4	0.76	0.045
Integrin-mediated signalling pathway	15	1.4	0.0097	0.00005
Response to hypoxia	15	1.4	0.025	0.00019
Heart development	15	1.4	0.39	0.0096
Transmembrane receptor protein tyrosine kinase signalling pathway	14	1.3	0.026	0.00017

Defence response to virus	14	1.3	0.81	0.057
Positive regulation of angiogenesis	13	1.2	0.35	0.0077
Neutrophil chemotaxis	12	1.1	0.024	0.00014
Negative regulation of angiogenesis	12	1.1	0.025	0.00018
Skeletal system development	12	1.1	0.045	0.00037
Cellular response to lipopolysaccharide	12	1.1	0.28	0.0047
Total	1108			

3.5 DISCUSSION

A novel technique for obtaining serial epithelial samples from the TC was developed in these studies. The preferred technique was the curette, due to its repeatability, practicality, quality of the samples obtained and low levels of disturbance of the cows during and after sampling. This technique resulted in adequate and consistent size and quality of tissue samples that facilitates an adequate description of the changes on gene expression of the TC and keratin plug formation after drying off. The need for the development of such a technique had been posed repeatedly in the literature (Paulrud, 2005, Rainard and Riollet, 2006, Rinaldi et al., 2010). Different techniques have been used in the past to obtain samples from the TC keratin layer of lactating cows and assess the impact of the keratin plug on mastitis (Bitman et al., 1988, Bright et al., 1990, Hillerton and Lacy-Hulbert, 1995). Although these techniques allowed the study of different components of the TC lining, the methods were inadequate to determine factors such as the renewal status of the TC or the immune response to IMI. Other previous techniques for studying physiological aspects of the TC were IHC, Transmission Electron Microscopy, Scanning Electron Microscopy, and other histological techniques (Nickerson and Pankey, 1984, Molenaar et al., 2000, Smolenski, 2018). These studies provided valuable information about the structure of the tissue and allowed limited functional analysis of the TC and TS. The curette sampling method described herein allows researchers to conduct functional studies of the TC epithelium which opens the opportunity to analyse the dynamics of IMI and keratin plug formation in the future.

The biopsy method developed in this study induced minimal effect on cows' welfare during sampling, probably as a consequence of the local anaesthesia and Xylazine utilised in this protocol. However, a severe inflammatory response was noted in the photomicrographs in teats 2 days after sampling. Even though by day 13 after sampling the results showed evidence of advanced healing and recovery, the gene expression of the tissue of D-11S still showed signs of recovering as many genes related to extracellular matrix synthesis and inflammatory response were differentially expressed. This is relevant when planning future studies using the curette technique for sample retrieval, as it suggests that the results of RNA expression could potentially be affected if the biopsy samples are obtained too close to one another in time and position.

Previous gene expression studies of the TC epithelium focused mainly on proteins and peptides expressed in the TC that could serve as antimicrobials against mastitis-causing agents and possibly prevent bacterial infections (Regenhard et al., 2010, Rinaldi et al., 2010, Tetens et al., 2010). Other researchers presented gene expression analyses of the TC after exposure to bacterial challenge (Rinaldi et al., 2010, Kerro Dego et al., 2012). Recent studies have been published in other areas of udder health using transcriptomics NGS-tools to identify genetic mechanisms that are regulated by the host during mastitis (Asselstine et al., 2019). They mainly used mammary secretory tissue and the physiological response of the TC was not considered. Microarrays provided valuable information about both mammary gland and TC gene expression. However, the information obtained from microarrays was constrained by the limited list of selected genes. In addition, on many occasions, these methods required the slaughter of cows (Singh et al., 2008, Swanson et al., 2009,

Mitterhuemer et al., 2010, Lemay et al., 2013, Petzl et al., 2016) which could have caused modifications in the gene expression of the cells due to post-mortem changes. Other biopsy techniques of the mammary gland tissue have induced transient but undesirable health issues for cows such as bleeding, a decrease in milk production, or were surgically complex (Persson and Åström, 1989, Knight et al., 1992, Farr et al., 1996). The physiological status of the mammary tissue has effectively been described using RNA extractions from milk samples. The RNA originates from the somatic cells present in the milk and has shown to be as reliable as mammary gland tissue (Wickramasinghe et al., 2012, Lemay et al., 2013). However, using the latter approach the role of the TC in the immune response against mastitis was not analysed (Wickramasinghe et al., 2012). The TC has been characterised as being a highly proliferative tissue because of its thickness when compared with the teat skin or other epithelia in the cow (Paulrud, 2005). The transcriptomic analysis of previously unsampled teat canals (D11-S) compared to highly proliferating tissues of previously sampled teats (D11-RS) showed that the epithelium expressed different RNA sequences after sampling due to tissue proliferation. More than 1000 genes were upregulated in the highly proliferating tissue samples D11-RS compared to the control-TC sample D11-S. Genes related to synthesis of the extracellular matrix, cellular proliferation, cell adhesion and immune response are characteristics of healing and proliferation in epithelial tissues (Deonarine et al., 2007, Lisse et al., 2016).

The samples obtained in D11-S and D11-RS were obtained from the same area. Photomicrographs from day 13 showed that healing and re-epithelization occurs approximately 13 days from sampling. All the photomicrographs showed very low or no signs of inflammation and complete re-epithelization 11 days after sampling. These

results suggest that even though the healing procedure from the histological perspective was completed, from the cellular function and RNA expression perspective the process of complete regeneration of the TC epithelium could take longer than 13 days. Further research is necessary to make a conclusion on an appropriate sampling interval to avoid a modification of the gene expression induced by sampling.

Keratin plug formation has been suggested to be related to an individual ability of cows to "seal the TC" and to be indirectly related to milk production (Williamson, 2002, Dingwell et al., 2004, Pyorala, 2008). High producing cows are less likely to have a well formed keratin plug at the end of lactation and more keratin is lost by them during milking. Both of these factors predispose them to mastitis (Capuco et al., 1990, Schukken et al., 1993, Rajala-Schultz et al., 2005). Dingwell et al. (2004) clearly stated a need to investigate and identfy factors associated with the process of drying-off that influence the rate of TC closure. One of the factors that could influence TC closure is the physiological differences of cows at different production levels. However, before investigating factors affecting keratin plug formation, the nature of the process should be described.

Studies reported here suggest that keratin plug formation during the early dry period differs from a proliferative process in the TC epithelium when an involuting TC epithlial sample (Day 11-S) was compared with an artificially induced proliferative tissue (Day 11-RS). The knowledge of gene expression patterns of the TC might assist the development of new alternative interventions to induce keratin plug formation and thus avoid TC contamination by mastitis-causing agents during the early dry period. This is, as far as is known, the first study to characterise the gene expression in the TC by RNA-sequencing.

3.6 CONCLUSION

An effective technique to obtain biopsy samples from the TC was developed in these studies. The curette biopsy technique provided tissue samples of consistent size and quality that were useful for transcriptomic investigation of the TC epithelium physiology. The results of this study also showed that repeated samples from the TC should be greater than 11 days apart, to allow the TC to heal and gene expression to return to baseline expression. Further research would be necessary in order to assess if the gene expression of repeated samples obtained from other areas of the TC epithelium are less affected by the sampling technique.

4 Gene expression of the teat canal epithelium during the keratin plug formation and early lactation

A failure or delay in keratin plug formation at the end of lactation increases the risk of intramammary infections (IMI). In the study reported in this chapter mRNA sequencing was used to profile the transcriptome of the teat canal (TC) epithelium of dairy cows. Markers of cellular proliferation and immune response in the TC decreased during the early dry period and there was reactivation after calving. The decreased expression of genes related to proliferation and immune response could explain the higher incidence of intramammary infections observed in this period.

Gene expression of the teat canal epithelium during the keratin plug formation and early lactation

4.1 ABSTRACT

A keratin plug that forms in the teat canal (TC) at the end of lactation acts as a protective barrier to udder infections. However, the incidence of mastitis in the early dry period is relatively high compared to other stages of the lactation cycle. Changes in gene expression of the TC epithelium could provide insights into the physiology of the keratin plug formation in the early dry period, which remains largely unknown. mRNA-seq reads from TC biopsies sampled at different times in the early dry period and early lactation were analysed. In a first pilot study, the gene expression of samples from Day 0 (dry off day) and Day 11 of the dry period were analysed by transcriptomics (3 samples per time-point). Differentially expressed genes from Day 0 and Day 11 of the dry period and keratin-associated genes of interest were selected for further analysis. The relevant genes were analysed for variation of gene expression in a longitudinal study using NanoString nCounter. Repeated TC biopsy samples were taken from eight late lactating cows on Days 0, 7 and 21 of the dry period, and approximately 10 and 20 days after calving. Oral mucosa biopsy samples were taken at the same times to serve as controls. The pilot experiment resulted in 36 differentially expressed genes between samples of the TC epithelium taken at dry off (Day 0) and Day 11. Most of the genes (n= 22) were downregulated. Gene ontology enrichment analyses revealed that they were mainly related to cellular binding, adherence, and RNA transcription factors. Analysis of individual gene functions showed that they were genes involved in mitosis and immune response pathways.

Results from the longitudinal study showed different patterns of expression of genes between the TC and control oral tissue. Seven genes were significantly downregulated seven days after drying off whereas 12 genes presented differences only after calving. In a comparison between the results of both methods (Illumina and NanoString), the expression levels of the genes coincided and documented a decrease in gene expression related to TC cellular functions such as binding, adhesion, mitosis and immune response after drying off. These results could partially explain the higher incidence of mastitis observed during the early dry period.

4.2 INTRODUCTION

The incidence of intramammary infections (IMI) during the dry period is high (Dingwell, 2002, Green et al., 2002) and mostly caused by environmental bacteria (Oliver and Mitchell, 1983, Bryan et al., 2011). Absence of the flushing effect of milk and of post-milking daily teat sanitation might allow greater numbers of bacteria to persist in the TC and gain access to mammary tissue. Infections arising during or just before the dry period may extend until the next lactation and impact milk production (Smith et al., 1968, Green et al., 2002).

The epidermis of the TC consists of four well-defined strata: *stratum basale*, *stratum spinosus*, *stratum granulosum* and *stratum corneum* (Helmboldt et al., 1953, Comalli et al., 1984, Smolenski et al., 2015). The TC epithelium contains mainly keratinocytes, along with a minor population of dendritic and stem cells (Nickerson and Pankey, 1983, Paulrud, 2005, Smolenski, 2018). Dendritic, and Langerhans cells are antigen presenters and initiate a local immune response in the TC during bacterial invasion (Nickerson and Pankey, 1983). The *granulosum* and *corneum* strata of the TC

are thicker than in normal skin, probably because of the higher proliferation rates induced by the erosion of the *stratum corneum* by milking forces (Paulrud, 2005). At the end of lactation, the TC is sealed by an accumulation of keratin and cellular detritus called the "keratin plug". The openness of the TC and the thickness of the keratin layer (*stratum corneum*) influences the vulnerability of the mammary gland to mastitis (Murphy, 1944, Hillerton and Lacy-Hulbert, 1995, Davidov et al., 2011). Williamson et al. (1995) reported that 50 and 5% of teats had an incomplete keratin plug present 7 and 50 days into the dry period, respectively. Similarly, Dingwell et al. (2003) found 50 and 23% of teat ends were still "open" one and six weeks into the dry period. These studies presented an association between keratin plug formation and the susceptibility of the cows to mastitis, suggesting that changes in the TC epithelium at the end of lactation may impact the high incidence of mastitis observed in this period.

The physiology of the epithelial tissue covering the TC and its changes throughout the dry period have not been studied in detail. The advent of high throughput technologies to study genomics, proteomics and nutrigenomics provides an opportunity to study multiple levels of biological information to better understand animals' responses to changes in their environments. Closure of the TC at the end of lactation and keratin plug formation can be attributed to either the accumulation of keratin secondary to the absence of removal caused by the flushing effect of milk, the increased synthesis of keratin over the dry period, or both. The hypothesis of the two studies described herein is that changes in gene expression take place throughout the dry period in the TC epithelial tissue. To test this hypothesis, Next-generation sequencing (NGS) was undertaken to profile the transcriptome of the TC epithelium during late lactation and the early stages of the dry period in the first pilot study.

NanoString nCounter Technology was used in a longitudinal study to validate and extend the results obtained in the first experiment into the first stages of the next lactation. This appears to be the first study to investigate the changes in gene expression of the TC epithelium during this period.

4.3 MATERIALS AND METHODS

4.3.1 Pilot experiment- Study design

Teat canal biopsy samples from nine late lactating dairy cows enrolled in a larger study were extracted with a curette from one teat per cow on the last day of lactation (Day 0 of the dry period). From the remaining three teats, one was sampled with an alternative biopsy method and analysed as part of a different study and the other two teats were not sampled on Day 0. On Day 11, five cows were sampled with the curette from a teat that had served as control on Day 0. The study was approved by Massey University Animals Ethics Committee (MUAEC, Palmerston North, New Zealand), protocol number 16/126.

4.3.2 Pilot experiment- Sampling procedure

Sampling has been described by Notcovich et al. (2018). Briefly, after the morning milking on the day of dry off (Day 0), cows were held in a head bail and xylazine 2% in a dose of 0.025 mg/kg was administered intravenously into the caudal vein. Once the cows showed signs of sedation (drooping ears, slowed motion), the hind legs were loosely tied with a rope to the sides of a cattle chute as a precaution to avoid injuries to the operator and cows during the procedure. A teat end inspection was performed on all teats prior to sampling to assess skin condition of the teat and teat orifice. A tourniquet was placed at the base of the teat using an elastic bandage to prevent udder secretions from entering the teat cistern and diluting the local anaesthetic, to prevent the anaesthetic from diffusing up into the mammary gland, and to reduce bleeding from the site of sampling. The teat cistern was stripped to remove any accumulated secretion and the teat orifice was thoroughly cleaned using cotton balls moistened with methylated spirits. Local anaesthetic solution was infused into the teat cistern through the streak canal using a sterile disposable mastitis treatment syringe pre-filled with five millilitres of 2% lidocaine (Nopaine, Phoenix Pharm Distributors Ltd. Auckland, New Zealand). The local anaesthetic solution was then stripped from the cistern after approximately 30 seconds. The teat ends were cleansed again with a cotton swab moistened with methylated spirits and a sterile 3.5 mm diameter curette (Curette 160 mm, Moses pattern, [206689], Shoof International, 2017, Cambridge, New Zealand) was inserted through the TC. The teat was held with one hand and the curette was pulled out from the TC whilst applying pressure over the teat and the hand holding the teat. The sample of TC epithelium still attached to the teat end was grasped with forceps and rapidly detached from the teat by pulling, then

immediately placed in 1mL of "RNA later" (Life Technologies, Carlsbad, California, USA) for stabilization of the tissue. The samples obtained by the curette consist mainly of keratinocytes distributed across the four layers of the squamous epithelial tissue consisting of: *basal membrane*, *stratum spinosus*, *stratum granulosum*, and *stratum corneum*.

4.3.3 RNA extraction and RNA-sequencing

Total RNA was extracted from the teat tissue samples with mirVana miRNA Isolation Kit (Life Technologies, Carlsbad, California, USA), following the manufacturer's instructions. Extracted RNA was assessed for quality using an Agilent 2100 Bioanalyzer at Massey Genome Services (Massey University, Palmerston North, New Zealand). Samples of suitable quality were sent to New Zealand Genomics Limited (University of Otago, Dunedin, New Zealand) for sequencing. Three samples per time point with the highest RNA integrity number (RIN) were submitted for transcriptomic analysis (Day 0 n=3 and Day 11 n=3). The submitted RNA was made into libraries using Illumina TruSeq Stranded mRNA kit. Sequencing was performed on 3 lanes of Illumina HiSeq 2x 125 base PE v4 (Illumina, San Diego, CA, USA).

The raw reads were filtered for adaptor sequences and low-quality base pairs (phred<10) using BBduck (version 36.86) from the BBtools package Bushnell B (2016). The quality control (QC) filtered reads were aligned to the *Bos taurus* ARS-UCD1.2 (GCA_002263795.2) genome (ENSEMBL release 90) using HISAT2 (version 2.1.0) using the default settings, except –RNA-strandness set to 'RF' to incorporate strand information. The short-read alignment files were used to generate per gene read counts with htseq-count (version 0.9.1) using the 'union' mode (Anders et al., 2015) based on the ENSEMBL gene models. The resulting read counts were analysed

in R (version 3.3.1, R Core Team, 2015, R Foundation for Statistical Computing, Vienna, Austria) using DESeq2 (Version 1.4.5) (Love et al., 2014) as described in the packages vignette. Differentially expressed genes identified in the transcriptomic analysis were further analysed using the Panther Gene Ontology (GO) enrichment analysis tool (www. http://pantherdb.org/). A false discovery rate (FDR) corrected p-value of <0.1 was set for the identification of differentially expressed genes.

4.3.4 Longitudinal study- Study design and sample collection

Eight dairy cows of mixed ages (7 ± 2 years old) from Massey University Dairy unit Nº 4 at a similar stage of lactation at the study commencement (270 ± 16 days in milk, DIM) were selected for this study. The study was approved by MUAEC, protocol number 17/28. All animals included had no cracks or chaps in the teat skin and composite somatic cell counts (SCC) <200,000. Tissue samples from two contralateral TC (e.g. front left and rear right) were obtained using the sampling technique applied in the first pilot study at three different times of the early dry period, namely Day 0, 7, and 21. The quarters to be sampled were randomly assigned before the study commenced. Quarters sampled on Day 0 from the cranial aspect of the TC were again sampled on Day 21 from the caudal area of the TC. The two other quarters were sampled on Day 7 (Sampling schedule-Table 4.1). Previous pilot studies using the curette have shown that the gene expression of the sampled tissue does not return to baseline earlier than 13 days. As a precaution to avoid gene expression alteration due to sampling, the samples from the TC were taken 14 days apart (Day 7 to 21) in addition to relocating the sampling area of the teat from cranial to caudal areas for the second sample. Two more samples from the TC were obtained from all four quarters approximately 10- and 20-days post-calving (the last sample at the time of culling) and

these were processed in the same manner. Samples from the oral mucosa were obtained at Day 0, 7, 21 of the dry period, and approximately 10 days post-calving to be used as control epithelial tissues. Oral mucosa is a unique hairless stratified cornified epithelia that is easily accessible and is less likely affected by the milking machine, suckling, or hormones during the lactation cycle, and thus, it was chosen as a control tissue. Cows were held in a head bail, the head was immobilised using a halter, the left cheek was everted manually, and a cotton ball dampened with lidocaine applied to the area for approximately 30 seconds. One or two oral papillae were pulled from the cheek using forceps. At the end of each sampling, 0.5 mg/kg of an anti-inflammatory (Metacam, Boehringer Ingelheim Animal Health New Zealand Limited, Auckland New Zealand) and 5g-10g of penethamate hydriodide antibiotic (Penethaject, Bayer Animal Health Ltd., Auckland, New Zealand) were administered following manufacturer's instructions to avoid infections and pain as a result of the sampling. Teat canal and oral samples were immediately fixed in 1.5 mL of formaldehyde 4% for approximately 4 hours and were subsequently processed into a paraffin block by the histopathology service at Massey University. One cow (736) was euthanised after the second sampling due to a *Mortierella wolfii* infection found in the liver at post-mortem examination and unrelated to the study and was withdrawn from the study analysis.

Table 4.1 Teat canal sampling schedule for Experiment 2. FL: front left, FR: front right, RL: rear left, RR: rear right.

Cow ID	Quarter Sampled			10 days post-calving	20 days post-calving
	Day 0	Day 7	Day 21		
322	FL/RR	FR/RL	FL/RR	Four quarters	Four quarters retrieved from abattoir
506	FR/RL	FL/RR	FR/RL	Four quarters	Four quarters retrieved from abattoir
632	FR/RL	FL/RR	FR/RL	Four quarters	Four quarters retrieved from abattoir
633	FR/RL	FL/RR	FR/RL	Four quarters	Four quarters retrieved from abattoir
725	FL/RR	FR/RL	FL/RR	Four quarters	Four quarters retrieved from abattoir
736	FR/RL	FL/RR	Withdrawn from study		
854	FL/RR	FR/RL	FL/RR	Four quarters	Four quarters retrieved from abattoir
868	FL/RR	FR/RL	FL/RR	Four quarters	Four quarters retrieved from abattoir

4.3.5 RNA Extraction

Total RNA was isolated from the samples using total RNA isolation reagent kit NucleoSpin Total RNA FFPE (Macherey-Nagel GmbH & Co. KG, 52355 Düren, Germany). Three cuts of 10 µm of the paraffin block were obtained for mRNA extraction. Qubit RNA HS kit (Life Technologies, Carlsbad, California, USA) was used to measure the amount of RNA obtained in each sample, as per manufacturer's instructions.

A titration study was conducted to determine the adequate concentration of RNA required for this study. A PlexSet Titration kit was used to measure one high RNA yield random sample (sample 14 teat sample = 83 ng/µL), one with mid-RNA yield

(sample 18 oral sample= 44.4 ng/μL), and a third sample with less than two ng/μL (sample 58= teat sample); each one in three to five successive 1:10 dilutions. The samples were hybridised overnight for 22 hours with the probes containing the genes to be analysed in the study following NanoString manufacturer's instructions.

4.3.6 Gene expression analysis using NanoString

The samples were processed for NanoString analysis to measure the expression levels of 42 chosen genes at five different time-points of the lactation cycle (Table 4.1). Based on the titration study results, concentrations of 430 ng/μL of RNA from TC and 47 ng/μL RNA from oral samples were required for the study. The 42 genes of interest were selected based on the results obtained in the first pilot experiment plus nine keratin genes related to keratin expression in epithelial tissues. Results of the pilot experiment indicated that 36 genes had modifications (up or down regulation) throughout early stages of the dry period. Three genes from the original list of 36 genes were removed, as they were mainly genes codifying for intracellular transporters (*TTYH2, RCAN1* and *SLC38A5*). Keratin genes (*KRT14, KRT16, KRT17, KRT18, KRT2, KRT35, KRT4, KRT6C, KRT8*) were added to the analyses to assess their contribution in the process of keratin plug formation (Table 4.2).

Gene expression analysis from TC and oral samples was performed using the nCounter Analysis System (NanoString Technologies Inc., Seattle, WA). The use of NanoString technology enables direct RNA expression analysis from purified RNA extracted from samples without further amplification. Samples of RNA from the formalin fixed TC and oral samples were analysed using a CodeSet consisting of probes specific for the 42 selected genes designed from the *Bos taurus* reference genome ARS-

UCD1.2 (GCA_002263795.2). Two plates containing different gene combinations were analysed (Plates C and D). Additionally, 3 reference housekeeping genes (HK) (i.e. *YWHAZ, RPL15*, and *GUSB*) were included in each plate (Table 4.2) based on published recommendations (Heiser et al., 2018). Housekeeping genes are examples of regions in a genome that tend to be highly conserved, mainly due to their roles in the maintenance of basic cellular functions and are essential for the existence of a cell (Vandesompele et al., 2002).

Extracted RNA from FFPE samples was hybridized with the CodeSets according to the manufacturer's instructions (nCounter Gene Expression Assay User Manual; MAN-10079-01). Gene expression data was retrieved from the nCounter Analysis System as resource script files (RCC) and imported into nSolver Analysis Software v2.5 (http://www.NanoString.com/products/nSolver) to undergo the software's sample quality control. Samples that did not pass the quality control routine were excluded from analysis. Reference gene normalization was performed using the geometric mean of counts for the HK genes. The average of these geometric means across all lanes was used as the reference against which each lane was normalized. Statistical analysis of the expression data was performed using R software version 3.3.1 (R Core Team, 2015, R Foundation for Statistical Computing, Vienna, Austria). The data was first log 10 transformed, as they were not normally distributed. After conversion, most of the genes analysed showed a close to normal distribution and were analysed using a linear regression mixed model that included the random effect of cow and fixed effect of day of sampling. Oral and teat samples were analysed in two different data sets using the model stated above. Statistical significance was set at $p<0.05$.

Table 4.2 List of genes analysed with NanoString and descriptive analysis of the results. Number: NM number, Position: position in the genome, Plate: NanoString plate name, Mean, SD: Standard deviation, median, min: minimum value, max: maximum value, SE: Standard error.

Name	Number	Position	Plate	Mean	SD	median	min	max	SE
ATF3	NM_001046193.1	384-483	C	2.04	0.9	2.14	0.03	3.55	0.1
BAZ1A	NM_001192940.1	2683-2782	C	2.7	0.2	2.71	2.25	3.1	0
CSRP2	NM_001038183.1	488-587	C	2.93	0.2	2.95	1.97	3.5	0
FAM71A	NM_001099391.2	1871-1970	C	1.21	0.7	1.36	-0.38	2.2	0.2
FLRT3	NM_001192674.1	1657-1756	C	2.03	0.3	2.07	0.89	2.52	0
FOSB	NM_001102248.1	863-962	C	1.97	0.9	2.17	-1.28	3.31	0.1
GCLC	NM_001083674.1	728-827	C	2.59	0.2	2.59	2.22	3.16	0
HSPA1A	NM_203322.3	416-515	C	3.78	0.3	3.71	3.28	4.85	0
HSPA6	XM_002685850.4	1946-2045	C	1.53	0.8	1.49	0.19	3.68	0.1
HSPB8	NM_001014955.1	877-976	C	3.18	0.3	3.16	2.12	3.82	0
KIF23	NM_001098038.1	1362-1461	C	2.23	0.2	2.18	1.74	3.15	0
LOC107131159	XM_002686586.4	1609-1708	C	2.55	0.3	2.56	1.91	3.08	0
MKI67	XM_015460791.1	1886-1985	C	2.24	0.3	2.3	1.21	2.85	0
RFX1	XM_010806714.2	1906-2005	C	2.21	0.2	2.21	1.66	2.64	0
NR4A3	XM_015472563.1	2434-2533	C	2.01	0.8	2.15	-0.36	3.4	0.1

RFX2	NM_001102172.1	1487-1586	C	1.89	0.5	1.88	0.36	2.92	0.1
RNF182	NM_001077938.1	820-919	C	1.64	0.6	1.75	-1.49	2.62	0.1
SYT4	NM_001098108.1	208-307	C	2.74	0.2	2.74	2.09	3.28	0
TFPI2	NM_182788.1	287-386	C	3.09	0.5	3.16	1.27	3.86	0.1
TRPS1	NM_001192913.2	2697-2796	C	3.18	0.1	3.17	3.01	3.6	0
ZFAND2A	NM_001083762.1	207-306	C	2.59	0.3	2.55	2.01	3.41	0
IRX3	NM_001104996.2	877-976	D	2.38	0.2	2.41	1.79	2.99	0
KRT14	NM_001166575.2	1475-1574	D	4.37	0.3	4.46	3.49	4.85	0
KRT16	XM_003583602.1	352-451	D	0.72	0.4	0.85	0.17	1	0.2
KRT17	NM_001105322.1	185-284	D	3.83	0.5	3.98	2.72	4.63	0.1
KRT18	NM_001192095.1	1191-1290	D	1.42	0.6	1.5	0.37	2.68	0.1
KRT2	XM_001254015.1	778-877	D	2.55	0.8	2.54	0.05	4.02	0.1
KRT35	NM_001076073.1	1243-1342	D	1.13	0.7	1.12	-0.12	2	0.3
KRT4	NM_001098385.1	672-771	D	3.88	0.5	3.94	2.54	4.83	0.1
KRT6C	XM_002692256.4	679-778	D	3.72	0.4	3.73	2.74	4.56	0
KRT8	NM_001033610.1	1427-1526	D	2.43	0.8	2.6	0.04	3.95	0.1
MARCKS	NM_001076276.1	55-154	D	2.87	0.2	2.9	2.1	3.34	0
MT1E	NM_001078134.2	206-305	D	2.55	0.4	2.56	1.56	3.34	0
PADI1	NM_001101272.1	1593-1692	D	1.5	0.7	1.61	0.35	2.81	0.1

PENK	NM_174141.2	378-477	D	1.64	0.7	1.67	-0.63	2.95	0.1
PKIB	NM_001114518.2	239-338	D	2.26	0.4	2.26	1.06	3.52	0
RASSF9	NM_001193083.2	344-443	D	1.43	0.6	1.51	-0.8	2.29	0.1
RBBP8NL	XM_002692256.4	206-305	D	1.65	0.4	1.82	0.04	2.36	0.1
RND3	NM_001099104.1	873-972	D	2.26	0.3	2.24	1.63	3	0
SERPINA1	NM_173882.2	446-545	D	2.28	0.5	2.34	1.21	4.16	0.1
SYT17	NM_001193210.1	971-1070	D	1.86	0.5	1.93	0.52	2.79	0.1
TLR5	NM_001040501.1	1774-1873	D	1.58	0.7	1.74	-0.54	3	0.1
GUSB	NM_001083436.1	1815-1914	C-D						
RPL15	NM_001077866.1	554-653	C-D						
YWHAZ	NM_174814.2	302-401	C-D						

4.4 RESULTS

4.4.1 Pilot experiment- mRNA sequencing

Illumina based sequencing of the Day 0 and Day 11 RNA-seq triplicate libraries resulted in an average of 75.6 and 75.0 million paired end reads, respectively. Following removal of reads with homology to sequencing adaptors and low-quality base calls (phred<10), an average of 95% and 96.5% of sequenced base-pairs were retained from the Day 0 and Day 11 samples, respectively (Table 4.3). A high percentage of quality filtered reads successfully mapped to the *Bos taurus* genome,

with the average mapping rates >90% for both the Day 0 and Day 11 triplicates (Table 4.3).

Table 4.3 Summary of the quality control (QC) of the six samples analysed by Illumina. Base-pairs pass QC: base pairs that passed the quality control.

Sample	Group	Base-pairs pass QC (% total)	Overall alignment rate (% total)
A36RL	Day 0	17892809149 bases (94.52%)	94.88%
A793RR	Day 0	17892132553 bases (94.79%)	89.10%
617RL	Day 0	18111547639 bases (95.69%)	97.49%
36FL	Day 11	17366845459 bases (94.36%)	93.39%
603RL	Day 11	17492924833 bases (94.97%)	94.65%
872RR	Day 11	18570037048 bases (95.63%)	96.56%

4.4.2 Differential expression analysis

DESeq2 was used to identify differentially expressed genes in a comparison between Day 0 and Day 11 triplicate libraries. In comparison to the Day 0 epithelium samples, 14 genes were more expressed, and 22 genes were less expressed in the Day 11 epithelium (Figure 4.1). In general, genes were predominantly downregulated in the Day 11 sample compared to Day 0. Seven genes had greater than two log2 fold change in the downregulated group, versus one for those that were upregulated (Table 4.4). Seven downregulated genes are involved in pathways of cell proliferation and six intervene in immune response functions (Table 4.4). Among the 36 significantly differentially expressed genes (FDR corrected p-value <0.1), GO term enrichment

showed several transcription factor binding terms and heat shock and phosphatidylserine binding terms were enriched (Figure 4.2). Significant enrichments were also observed in the PANTHER GO-Slim cellular component category for terms related to organelles, vesicles, and the COP9 signalosome (Figure 4.2) which is a protein complex that regulates DNA-damage response, cell-cycle and gene expression. Interestingly, no significant difference in expression of keratin genes of epithelial tissues was observed between Day 0 and Day 11 epithelial tissues.

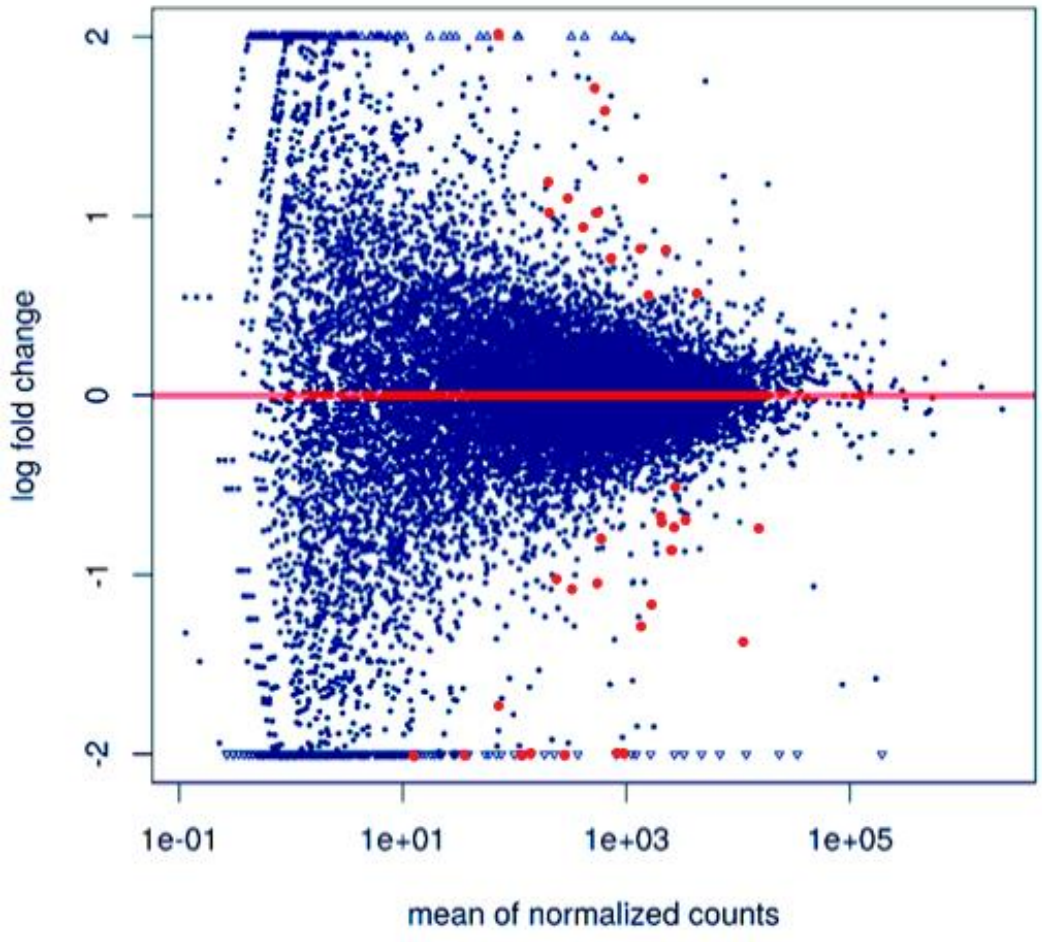


Figure 4.1 Volcano plot: The mean of normalized counts (X-axis) was plotted against the log2 of the fold change (Y-axis) of differentially expressed genes. Differential expression analysis identified 14 upregulated genes positioned above the red horizontal line and 22 downregulated genes situated below. Red dots indicate differentially expressed genes at a cut-off FDR <0.1

Table 4.4 Summary of results obtained in Illumina for the differentially expressed genes in teat canal biopsy samples. ENSBTAG= ensemble number, log_2FoldChange= fold change when comparing Day 0 and Day 11 samples (negative means downregulated), padj= p-value adjusted to the normalized means, Function= function of the gene and available pathways (source: www.genecards.org)

Ensembl ID	log_2 Fold Change	p-value	padj	gene symbol	Function
Cellular Proliferation					
ENSBTAG00000001864	-4.51234	6.51E-19	9.89E-15	*NR4A3*	Plays a role in the regulation of proliferation, survival, and differentiation of many different cell types and in metabolism and inflammation.
ENSBTAG00000002444	-0.7277	1.77E-06	0.005386	*MKI67*	Cellular proliferation
ENSBTAG00000001303	-1.37302	7.00E-06	0.014125	*HSPB8*	This gene appears to be involved in regulation of cell proliferation, apoptosis, and carcinogenesis
ENSBTAG00000013406	-0.8708	1.83E-05	0.023166	*CSRP2*	Maybe involved in regulating ordered cell growth
ENSBTAG00000009983	-0.79174	3.89E-05	0.03943	*KIF23*	This family includes microtubule-dependent molecular motors that transport organelles within cells and move chromosomes during cell division
ENSBTAG00000017694	-0.70116	0.000129	0.069779	*TRPS1*	Found in chondrocytes related to proliferation of chondrocytes
ENSBTAG00000002082	0.564755	0.000118	0.069779	*MARCKS*	The protein is thought to be involved in cell motility, phagocytosis, membrane trafficking, and mitogenesis
Immune response					
ENSBTAG00000039035	-2.18201	6.63E-08	0.000336	*HSPA6*	Antigen processing and presentation
ENSBTAG00000008545	-3.96754	1.46E-05	0.020196	*ATF3*	Integrin Linked Kinase signalling

ENSBTAG00000045884	-1.72975	0.000125	0.069779	*RNF182*	Among its related pathways are Class 1 MHC mediated antigen processing and presentation
ENSBTAG00000008854	-1.0306	3.59E-05	0.038934	*RFX1*	This transcription factor has been shown to regulate a wide variety of genes involved in immunity and cancer, including the MHC class II genes
ENSBTAG00000020035	-0.71488	0.000224	0.097119	*RCAN1*	The regulator of calcineurin 1 (RCAN1) inhibits nuclear factor kappaB signaling pathway which is involved in inflammatory responses
ENSBTAG00000000477	1.17367	8.29E-05	0.069141	*TLR5*	Plays a fundamental role in pathogen recognition and activation of innate immune responses.
Other cellular functions					
ENSBTAG00000015571	-1.28387	4.66E-07	0.001769	*GCLC*	Apoptosis-related network due to altered Notch3 in ovarian cancer
ENSBTAG00000001801	-1.15901	2.17E-06	0.005482	*SYT4*	Synaptotagmin 4, may serve as Ca^{+2} sensors in the process of vesicular trafficking and exocytosis
ENSBTAG00000021166	-7.30468	7.44E-06	0.014125	*FAM71A*	This gene encodes a protein that may be important for the integrity of the Golgi body
ENSBTAG00000011854	-2.05935	2.65E-05	0.030916	*SLC38A5*	This is an amino acid transporter
ENSBTAG00000017661	-2.57257	0.000118	0.069779	*RFX2*	It is a transcriptional activator
ENSBTAG00000020164	-0.51539	0.000188	0.087446	*BAZ1A*	Transcription regulator
ENSBTAG00000025441	-0.73844	0.000185	0.087446	*HSPA1A*	Has a pivotal role in protein quality control ensuring correct folding of proteins
ENSBTAG00000015844	-0.67767	0.000124	0.069779	*TFPI2*	Has been identified as a tumour suppressor in several types of cancer
ENSBTAG00000045685	-1.0745	0.000164	0.085895	*LOC107131159*	Uncharacterised protein
ENSBTAG00000007402	-1.05588	0.00019	0.087446	*ZFAND2A*	Zinc-finger AN1-Type Containing 2A

ENSBTAG00000002138	1.10204	1.26E-05	0.019172	*PADI1*	The type 1 enzyme is involved in the late stages of epidermal differentiation, where it deiminates filaggrin and keratin 1
ENSBTAG00000003319	0.564396	0.000211	0.094434	*FLRT3*	Involved in cell adhesion and/or receptor signalling
ENSBTAG00000039731	0.800611	8.72E-05	0.069141	*RND3*	Appears to act as a negative regulator of the cytoskeletal organization leading to loss of adhesion
ENSBTAG00000026966	2.051972	6.63E-05	0.059202	*RASSF9*	Membrane trafficking
ENSBTAG00000004924	1.580511	6.30E-05	0.059202	*PENK*	Involved in the synthesis of opioid type proteins
ENSBTAG00000018843	1.204053	9.11E-05	0.069141	*SERPINA1*	Inhibitor of serine proteases
ENSBTAG00000003163	1.02346	0.000102	0.069779	*RBBP8NL*	It is suggested that this gene might itself be a tumour suppressor
ENSBTAG00000012083	0.819737	9.66E-06	0.016297	*IRX3*	Regulator of energy metabolism
ENSBTAG00000011007	1.013314	0.000117	0.069779	*TTYH2*	Ion channel transporter
ENSBTAG00000004394	0.749964	0.000177	0.087446	*PKIB*	This gene may play a role in prostate cancer
ENSBTAG00000014229	0.93713	0.000232	0.097814	*SYT17*	Plays a role in dendrite formation by melanocytes

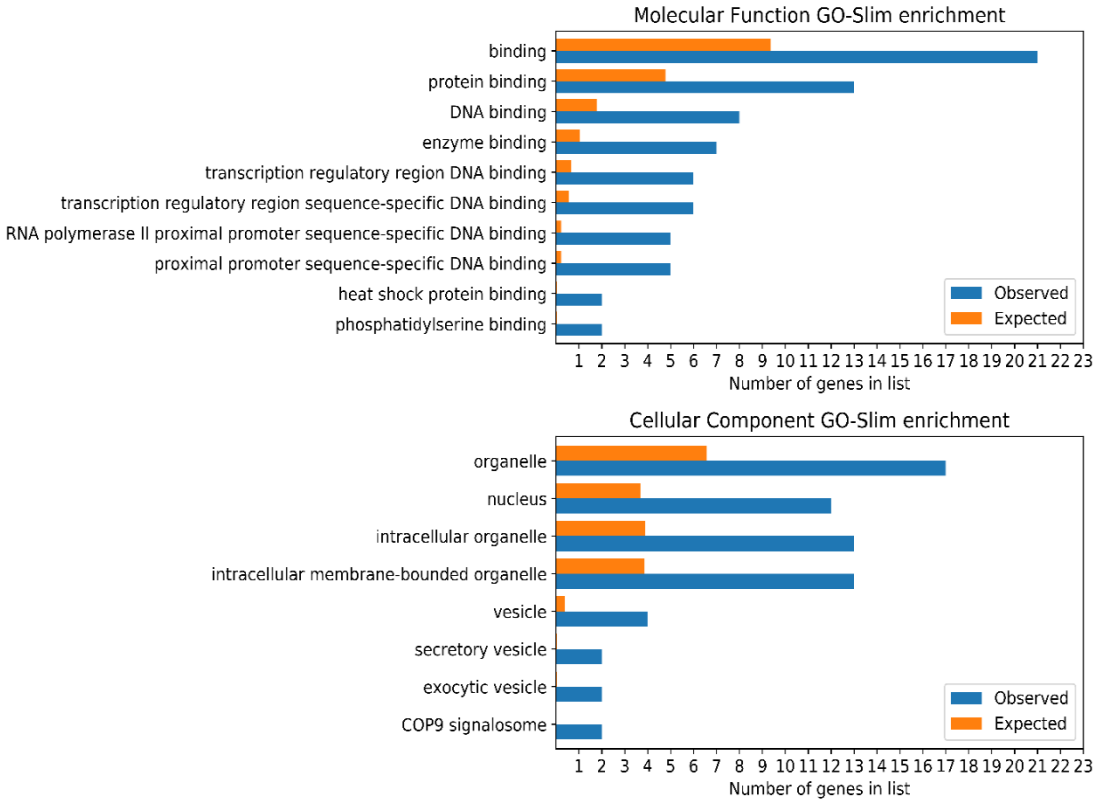


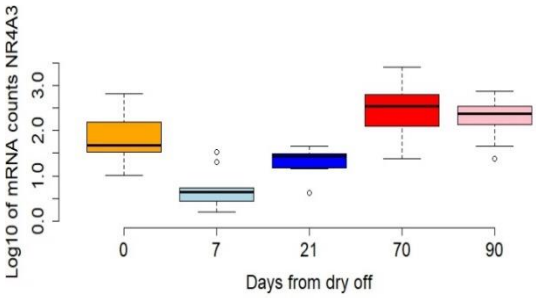
Figure 4.2 GO-Slim enrichment analysis for the molecular function (top panel) and cellular component (bottom panel) of the 36 differentially expressed genes found in Day 11 teat canal samples. The observed differentially expressed genes related to binding and expressed in the nucleus and intracellular organelles significantly outnumbered the expected number of genes when compared with the *Bos taurus* reference genome ARS-UCD1.2 (GCA_002263795.2)

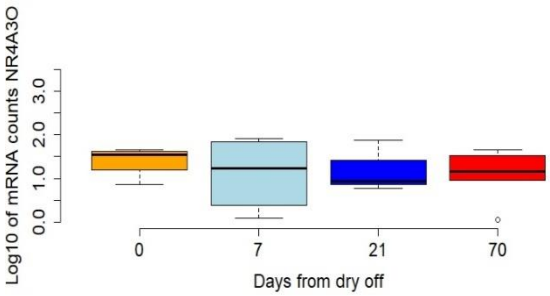
4.4.3 Longitudinal study- RNA yields and gene expression

RNA was extracted from 29 oral tissue and 100 TC tissue samples from a total of 135 samples obtained. The mean (±SD) total RNA yields of the samples was 30.69 (±15.6) ng/µL. Twelve samples yielded less than 10 ng/µL in two consecutive RNA extractions and these were excluded as they showed no expression levels for the targeted genes. Twenty-six samples from oral tissue and 63 from teat tissue passed the quality control and were analysed.

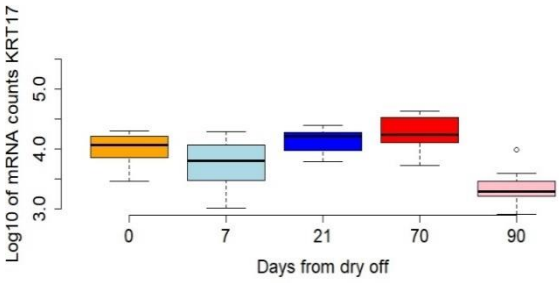
Genes showed different expression patterns in oral and TC tissue samples. Eight genes showed significantly reduced expression at Day 7 and 21 compared to Day 0 (last day of lactation), and a significant increase in expression after calving. This effect was observable in the TC samples, but not in the oral samples ($p<0.05$, Figure 4.3 and Table 4.5). Some of the genes were expressed in the TC but not expressed or expressed at very low levels in the oral tissue: *FAM71A* (this gene encodes a protein that may be important for the integrity of the Golgi body), *HSPA1A* (has a pivotal role in protein quality control ensuring correct folding of proteins), *HSPA6* (antigen processing and presentation), *KRT16* (marker of proliferation), *FOSB* (regulators of cell proliferation). In the TC, the genes that showed the minimal expression levels were *FAM71A*, *KRT16*, *KRT18* (mainly expressed in single layer epithelium) and *KRT35* (expressed in the hair cuticle of human hairs).

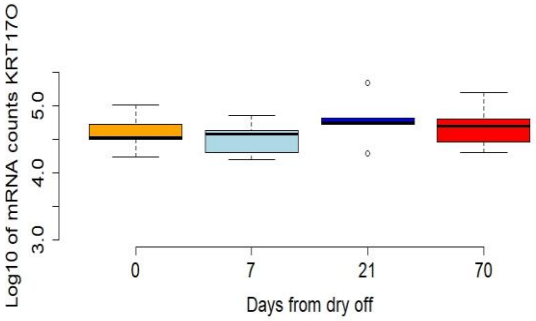
A)


Log10 of mRNA counts NR4A3
0.0 1.0 2.0 3.0
0 7 21 70 90
Days from dry off

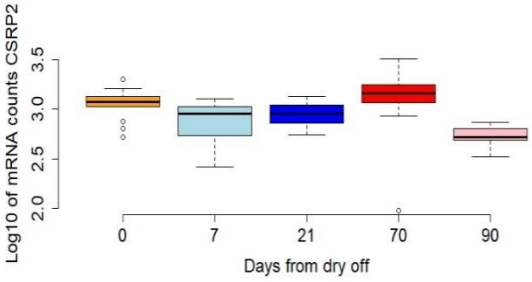

Log10 of mRNA counts NR4A3O
0.0 1.0 2.0 3.0
0 7 21 70
Days from dry off

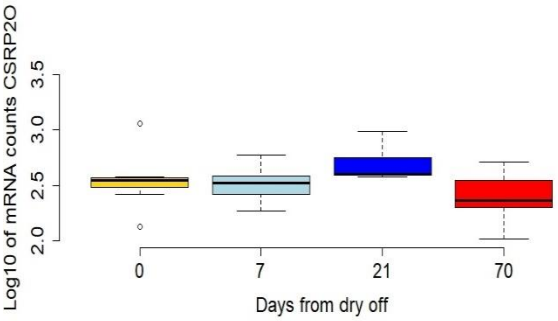
B)


Log10 of mRNA counts KRT17
3.0 4.0 5.0
0 7 21 70 90
Days from dry off

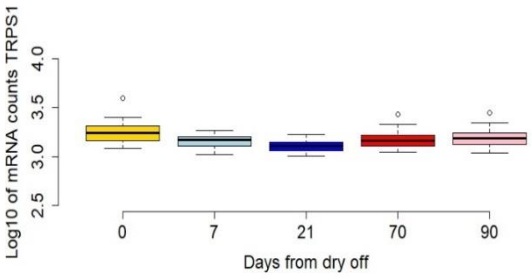

Log10 of mRNA counts KRT17O
3.0 4.0 5.0
0 7 21 70
Days from dry off

C)


Log10 of mRNA counts CSRP2
2.0 2.5 3.0 3.5
0 7 21 70 90
Days from dry off


Log10 of mRNA counts CSRP2O
2.0 2.5 3.0 3.5
0 7 21 70
Days from dry off

D)


Log10 of mRNA counts TRPS1
2.5 3.0 3.5 4.0
0 7 21 70 90
Days from dry off

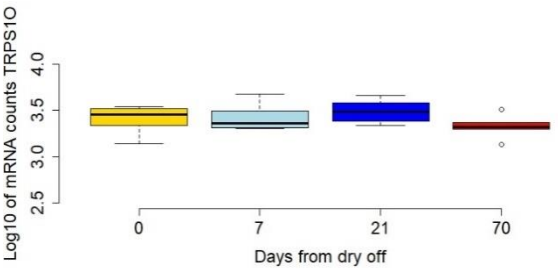

Log10 of mRNA counts TRPS1O
2.5 3.0 3.5 4.0
0 7 21 70
Days from dry off

Figure 4.3 Representation of the log10 of the median RNA copy numbers (±SD) in the teat canal (left panel) and oral samples (right panel) plotted against Days 0, 7, 21 after drying off, and 10 and 20 days after calving (Days 70 and 90) for the following genes: A) NR4A3, B) KRT17, C) CSRP2, D) TRPS1. The letter O added at the end of the gene name represent the ORAL origin of the sample. Analysis was performed in NanoString nCounter (NanoString Technologies Inc., Seattle, WA)

Table 4.5 Results of the mixed linear regression model showing the log10 of RNA counts of the teat canal samples at Day 0 of the dry off period (Day 0 DO) and the changes over the dry (DO) and after calving (AC) periods expressed as a percentage of change from the value of the intercept (Day 0 DO). – %: represents a downregulation in gene expression compared to Day 0, %: upregulation. NE: Not expressed. Significance levels set at p<0.05*, p<0.01, p<0.001*** **

Teat canal samples										Oral samples					
Gene symbol	Day 0 DO	Day 7 DO (%)		Day 21 DO (%)		Day 10 AC (%)		Day 20-25 AC (%)		Gene Symbol	Day 0 DO	Day 7 DO (%)		Day 21 DO (%)		Day 10 AC (%)	
NR4AR	1.8	-65.1	***	-30.4	*	34.6	***	26.9	**	*NR4AR*	1.2	-10.9		-3.4		-13.0	
KRT17	4	-6.6	**	3.6		6.3	**	-17.2	***	*KRT17*	4.6	-3.1		3.8		1.5	
CSRP2	3	-5.3	*	-3.2		2.5		-10.1	***	*CSRP2*	2.5	-1.4		4.8		-5.8	
KIF23	2.3	-6.6		-3.6		4.9		-2.8		*KIF23*	2.2	-3.0		5.1		-2	
TRPS1	3.3	-3.2	**	-4.4	***	-2.4	*	-2	*	*TRPS1*	3.4	0.3		2.4		-2.3	
LOC107131159	2.5	-7.7	*	-2.7		6.4	*	7.3	*	*LOC107131159*	2.4	-8.4		-9.3		-11.6	*
TFPI2	2.9	-12.1	**	-2.9		5.7		21.4	***	*TFPI2*	2.1	-21.3	*	-3.1		-4.0	
ATF3	1.3	-19.3		-21.9		106.7	***	87.1	***	*ATF3*	1.7	15.0		27.8		5.1	
FOSB	0.9	10.3		27.5		192.4	***	137.1	***	*FOSB*	1.2	-18.5		-26.3		-16.4	
HSPB8	3.1	-3.2		-4.6		12.1	***	3.3		*HSPB8*	3.0	-1.4		0.4		-7.6	*
HSPA1A	3.7	-2.1		-1.1		7.7	***	5.1	**	*HSPA1A*	3.5	-0.4		2.6		1.6	
HSPA6	1.2	-26.5		-30.4		70.4	**	12.9		*HSPA6*	1.0	-19.3		-42.0		-63.7	
X18	2.1	-0.6		5		9.3	**	15.3	***	*X18*	NE						
RFX2	1.8	-13		12.3		19.1	*	7.3		*RFX2*	1.2	5.9		-4.1		-5.2	***
SYT4	2.6	-0.1		-0.2		9.5	***	9.3	***	*SYT4*	2.1	5.8		-2.0		-3.5	

ZFAND2A	2.6	-1.4		-7.1	*	11	***	-6.7	*	*ZFAND2A*	2.2	-6.7	-10.6	*	-8.8	
MKI67	2.3	-4		-2.8		3.9		-9.5	*	*MKI67*	1.9	-14.7	-23.3		1.8	
KRT14	4.5	-0.8		0.2		1.5		-10.8	***	*KRT14*	5.3	-1.2	1.1		1.1	
GCLC	2.7	-2.5		-5.9	*	1.7		-6.3	**	*GCLC*	3.1	3.4	4.5		-2.1	
KRT6C	3.9	-0.9		-1.3		1.1		-12.2	***	*KRT6C*	1.5	-7.9	11.4		-13.6	
KRT8	2.2	-17.7		-0.8		7.6		28	*	*KRT8*	1.4	-27.1	29.5		4.9	
RNF182	1.6	-19.1		-23.9		8.7		8.3		*RNF182*	1.4	0.05	-13.4		0.3	
MT1E	2.7	-2.9		-9.9		-3.9		-7		*MT1E*	2.6	1.8	-1.8		16.0	
KRT18										*KRT18*	4.6	-3.1	3.8		1.5	
RASSF9	1.6	-15.7		-8.2		-30.7		-4.4		*RASSF9*	NE					
RBBP8NL	1.8	5.5		-0.4		-9.7		-33.2	***	*RBBP8NL*	2.0	-2.3	5.7		-13.2	
PKIB	2	10.8		2.3		3.7		23.8	***	*PKIB*	1.3	20.4	53.5	**	1.1	
MARCKS	2.7	2.8		5.3		4.3		8.2	***	*MARCKS*	2.2	3.8	6.3		12.5	***
TLR5	1.2	36.6		36.7		11		59.3	*	*TLR5*	0.7	26.9	120.9		55.6	
BAZ1A	2.7	1.3		1.1		6.2	***	-5.7	***	*BAZ1A*	2.7	-1.0	1.6		-3.3	
IRX3	2.4	6.1	*	1.6		0		-3.3		*IRX3*	2.7	-0.3	1.8		-2.3	
KRT4	4.1	2.6		-2.7		2.9		-15.7	***	*KRT4*	2.9	5.4	8.5		8.7	
PADI1	1.2	32.8		54.5	*	38.4		3.1		*PADI1*	3.9	-2.8	3.4		-2.1	
PENK	1.6	12.9		20.7		-10.4		-9.6		*PENK*	1.3	-5.3	-3.0		-43.7	
RND3	2	1.8		4.8		21.9	***	29.8	***	*RND3*	1.7	18.6	21.0	*	11.4	
SERPINA1	2.2	24.1	**	5.9		-2.4		-1.3		*SERPINA1*	2.7	-7.0	4.5		-6.4	
SYT17	2	2.7		4.4		3.5		-23.6	**	*SYT17*	2.1	-6.0	-0.4		-3.3	
FLRT3	NE									*FLRT3*	1.09	-18.5	-27.2		21.7	

KRT2	NE	*KRT2*	2.62	9.4	-0.1	-0.8	
KRT35	NE	*KRT35*	1.39	-102.6	18.0	-6.4	
FAM71A	NE	*FAM71A*	NE				
KRT16	NE	*KRT16*	NE				

4.5 DISCUSSION

These studies provide an unprecedented description of the gene expression of the bovine TC during the time of keratin plug formation in the early dry period. The introduction of transcriptomics to the study of the TC provides new insights into its physiology that may allow further development of preventative methods for mastitis. The results presented here identified differences in gene expression between the TC epithelial tissues during the early dry period relative to the TC epithelium during lactation. Thirty-six genes were differentially expressed before and after drying off in the first pilot study, representing differences between TC epithelia in lactating and dry cows. Genes were downregulated more than upregulated during the first days of the dry period suggesting that the involution observed in the mammary gland tissue (Wilde et al., 1997) may also occur in the TC. Some of the downregulated genes are related to mitosis and RNA transcription pathways. Although the total number of differentially expressed genes was low, these results support previous findings showing a decrease in proliferation rates of the TC in the early dry period and an increase in the cross-sectional area of the TC (Comalli et al., 1984, Oldham et al., 1991, Gleeson et al., 2003) . The downregulation of markers of proliferation in the TC of dairy cows after drying off reported herein supports the concept that the formation of the keratin plug results from an accumulation of dead cells due to the lack of milk flushing rather than an increase in the number of cells produced. Contrary to other findings reporting an inverse relationship between keratin synthesis in TC and milk production, (Lacy-Hulbert et al., 1996, Dingwell 2004), we show that after drying off the genetic messaging for epithelial proliferation in the TC (over the complete epithelium) declines. This could be related to modifications to the vascular perfusion

of the teat after drying off when machine milking ceases. It is known that milking machine forces as well as suckling by calves induce congestion, oedema and increased perfusion of blood into the teat end. All these stimuli disappear when lactation ends (Penry et al., 2017, Odorčić et al., 2019). It can be speculated that a reduction in blood supply to the teat end after drying off could lead to the observed downregulation of the genes in this study. The epithelial tissues of the TC, like the rest of the mammary gland, might be entering a stage of involution after drying off in which proliferation and the immune response to foreign agents are depressed.

RNA from genes coding for keratin synthesis were not significantly different between the last days of lactation and the early dry period in the experiments presented here. Studies have suggested that modifications in the assembly of intermediate filaments (keratins) could occur in response to environmental conditions without modifying the expression of genes (Herrmann et al., 2002). For example, the presence of calcium and modifications in pH can modify the alignment of keratins *in vitro* (Herrmann et al., 2002, Paulrud, 2005). Therefore, modifications in the composition of milk or the absence of milk in the TC during the early dry period could affect the structure in which the keratins align in the cytoplasm of epithelial cells during keratin plug formation without modifying keratin gene expression (Herrmann et al., 2002). However, some of the keratins analysed (*KRT14, KRT6C, KRT8*) were significantly downregulated after calving in the TC but not in oral tissue. Keratin 14 is mainly expressed in the basal epithelial tissue at a constant rate in most epithelia, while Keratin 6C and *KRT16* are induced during wound healing throughout the regenerative process of the skin (Pechter et al., 2012). Paulrud et al. (2005) suggested that the thickness of the TC epithelium could be due to its nature as a highly

proliferative tissue when compared with normal teat skin. In addition, some researchers found *KRT16* expressed in all the stratified epithelia, including oral and skin (Shamsher et al., 1995, Lessard et al., 2013). These reports do not align with the results presented herein. Gene expression of some genes associated with proliferation of the epithelial tissue like *KRT16* was very low or absent in TC and oral control tissues. However, *KRT6C* was expressed. *K6C* is expressed along with *KRT16* and *KRT17* in most stratified cornified epithelia during highly proliferative conditions (Paladini et al., 1996, Coulombe, 1997). These results suggest that the oral and the TC tissues are not highly proliferative or healing tissues as has been suggested by other authors (Paulrud, 2005) or at least are not proliferating at the same rate as a healing tissue would be, as it can be seen that keratin genes associated with cell proliferation were not upregulated during the early dry period during keratin plug formation.

In Experiment 2, *KRT2* was expressed in the oral tissue only. It has been suggested in previous studies, that *KRT2* is expressed in the oral epithelial tissue and mainly in mechanically stressed epithelia, and our results support this finding (Bragulla and Homberger, 2009). Interestingly, a study using mass spectrometry reported *KRT2* in the TC lining as well as in the external teat epithelial skin (Smolenski, 2018). Thus, the different forces induced by the milking machine could have led to this difference in gene expression between the two studies (Simhaee et al., 2009, Besier et al., 2016). *PADI1* gene was significantly upregulated in both current experiments after drying off. *PADI1* is a gene involved in filaggrin deamination, the process by which the keratinocytes enter the stage of cornification in the epithelium (Presland et al., 1992, Paulrud, 2005). *PADI1* upregulation in our study suggests that the process of cornification in the TC might occur at higher rates during the first stage of the dry

period, thus possibly being a key factor in increasing the thickness of the keratin layer in order to form the keratin plug.

The lower proliferation rate of the TC epithelium during the early dry period may make the lumen of the TC wider than during lactation, resulting in open teats. There is an association between openness of the TC and higher mastitis incidence during the early dry period in non-lactating cows and in pre-partum heifers (Williamson et al., 1995, Dingwell et al., 2004, Krömker and Friedrich, 2009). However, this might not be the only reason for the high susceptibility to mastitis observed in the non-lactating period.

Genes involved in the immune response were also downregulated during the early dry period in these experiments. Specific genes associated with antigen presentation and interleukin synthesis, like *HSPA6, RNF182* and *RFX1*, were downregulated in both. However, only in the first pilot experiment were those differences significant. Antigen presentation and initiation of the immune response in the TC at early stages of bacterial infection have been described in previous studies (Nickerson and Pankey, 1983, Smolenski, 2018). Heat shock proteins such as *HSPA6* and *HSPA1A* are also involved in maintaining the normal structure and morphology of the epithelial cells (Noonan et al., 2007). Downregulation of heat shock proteins, such as the ones reported here, have led to reduced cell viability in other studies (Noonan et al., 2007). The likely diminished immune response in the early dry period suggested by the results obtained herein agrees with findings describing an increase in intramammary infections during the early dry period and suggesting that bacteria might enter the TC more easily at this stage (Cousins et al., 1980, Oliver and Mitchell, 1983, Dingwell et al., 2004). However, more evidence is necessary to determine if the

reduced expression of the immune related genes observed in this study could predict a reduced immune response of the TC e.g. to a bacterial challenge during the early dry period.

The results presented in the pilot experiment must be interpreted with caution, as they originate from a small samples size (Day 0 n=3, Day 11 n=3). To increase confidence in the results there is a need to expand the number of samples in future studies. Another limitation is that the evidence about metabolic pathways related to some of the genes is scarce. Bovine genomic analyses are still incomplete; hence, most of the research performed to date and used as the basis for this study is derived from mouse or human information. The results of the longitudinal study support the findings of the first pilot experiment and expands the results, showing changes during the early dry period and after calving that occurred in most of the analysed genes expressed in the TC epithelium but not in the ones expressed in the oral tissue. The objective of setting the sampling of the longitudinal study on Day 7 and Day 21 instead of repeating Day 11 of sampling from the pilot experiment, was to determine with higher accuracy when the changes observed by Day 11 in the pilot study had occurred. These results from this study support the notion that modifications occur during the first 7 to 21 Days after drying off in the TC, in agreement with other reports showing changes in the mammary gland during involution (Oliver and Sordillo, 1988, Wilde et al., 1997, Pyorala, 2008).

4.6 CONCLUSIONS

The experiments reported herein use RNA-seq to demonstrate that the expression of genes related to cellular proliferation and immune response in the TC,

decreased during the early dry period. This was associated with a reactivation of gene expression after calving. These changes were not observed in other keratinised tissues in the same cows. Decreased cellular proliferation and immune response could be, among others, reasons for the higher incidence of intramammary infections observed in the early dry period. Further detailed studies on the physiology of the TC and keratin plug formation are warranted to develop strategies to improve the immune response of cows against mastitis in the early dry period and decrease intramammary infections.

5 Teat canal response to *Streptococcus uberis* challenge in the early dry period

In this Chapter, some of the mechanisms of invasion of the mammary gland by bacteria are addressed. However, the role of the teat canal (TC) and other natural protective structures in preventing intramammary infections (IMI) requires further investigation.

This chapter aims to describe the infection mechanism of a known strain of *S. uberis,* the immune response and histological changes occurring in the TC and teat sinus (TS) of dairy cows during bacterial challenge in the early dry period.

Teat canal response to *Streptococcus uberis* challenge in the early dry period

5.1 ABSTRACT

An experimental bacterial challenge was conducted in dairy cows to assess the progress of infection and the changes induced in the teat canal (TC) and teat sinus (TS) by a known strain of *S. uberis* (S325) during the early dry period. The hypothesis of this study was that *S. uberis* can develop a biofilm as an invading mechanism, induce histological modifications and an increase in cytokine synthesis in the TC and TS epithelia. Biofilm formation was assessed *in vitro* before the study and *in vivo* during the sampling period. The challenge model involved dipping the teats in a bacterial suspension of approximately 10^8 CFU/mL for 15 seconds in the morning and in the afternoon on the challenge day. Sixteen cows were allocated to four groups and challenged at different times, prior to and during the dry period (Day 0 = dry off). Group 1 was challenged on Day -7, Group 2 on Day 0, Group 3 on Day 7, and Group 4 on Day 25. Punch biopsy samples were obtained from TC and TS from cows euthanised one or five days after challenge in order to detect any bacteria ascending through the TC and any changes induced by the challenge. Bacteriology, histology, scanning electron microscopy (SEM), and concentration of cytokines IL6, IL-8, IL10, TNFα and IFNγ were analysed in the samples. No biofilm formation or *S. uberis* S325 bacterial presence was detected after *in vitro* culture of the samples or in the analysed SEM images. The challenge model was unsuccessful. Consequently, mixed linear model

analyses showed no difference between challenged and non-challenged tissue in all parameters evaluated and hence the results were pooled and analysed by Day after challenge. The thickness of the *stratum corneum* and *granulosum* decreased towards Day 25 as well as concentrations of IL-8 and TNFα ($p<0.05$) in all cows. Bacteria other than the challenge strain were found more frequently after culture in samples from Day 25 than in samples obtained earlier during the dry period. Teat canal tissue samples had higher concentrations of cytokines than TS samples ($p<0.05$). In conclusion, bacterial strain *S. uberis* S325 failed to colonise the TC or TS. Biofilms were not detected *in vivo* or *in vitro*. Nevertheless, some of the processes occurring in the teat tissue during the early dry period could be described. Insights into the changes observed over the first 25 days after drying off can be relevant for the development of new alternative tools to decrease colonization or possibly to modulate the synthesis of cytokines at the teat end in the early dry period.

5.2 INTRODUCTION

The teat canal (TC) is the first physical barrier that mastitis-causing bacteria cross in order to infect the mammary gland. The epidermis of the TC is an invaginated skin derived from the external skin of the teat, that consists of four well-defined strata: *stratum basale, stratum spinosum, stratum granulosum* and *stratum corneum* (keratin lining) (Comalli et al., 1984). The TC epithelium consists of keratinocytes, along with stem and dendritic cells (Nickerson and Pankey, 1983, Paulrud, 2005). Living bacteria that might be a source of intramammary infections (IMI) can survive in the keratin lining of the TC (Chandler et al., 1969, Forbes and Gehm, 2012) and the TC can remain infected for up to 9 days before an IMI develops (Murphy and Stuart, 1954). Some

studies suggest that significant bacterial growth occurred when the infection was confined to the TC (Forbes, 1968). Although several methods of infection have been hypothesised, there is a lack of understanding of the actual mechanisms by which bacteria traverse the TC. Bacteria may escape this natural physical barrier and first defence mechanism by propulsion into the TS by random vacuum fluctuations at the teat end during milking, or by multiplication of bacterial colonies ascending along the TC (Capuco et al., 1994, Lacy-Hulbert et al., 1996). Bacteria can also build biofilms in order to colonise different areas of the mammary gland tissue (Hensen et al., 2000, Fox et al., 2005).

The TC, especially the dorsal termination known as the Fürstenberg's rosette, is surrounded by a population of leukocytes which protect the mammary gland from ascending pathogens (Asti et al., 2011). Plasma cells, polymorphonuclear leukocytes and mononuclear phagocytes enter the TC lumen through the squamocolumnar junctions during bacterial infections. Thus, the Furstenberg's rosette is a migration point where lymphocytes transfer into the lumen during mastitis (Nickerson and Pankey, 1983, Rinaldi et al., 2010). Interleukins and other cytokine molecules participate in the early response of the mammary gland to infections (e.g. *S. uberis*) (Rambeaud et al., 2003). However, the role of various interleukins, the cellular response, and the whole "surveillance system" in place and operating during early stages of a bacterial infection in the TC are not known (Collins et al., 1986).

In many studies bacteria have been inoculated through the TC directly into the sinus to assess infection rates or early responses to infection after challenge (Rambeaud et al., 2003, Notcovich et al., 2016). Other studies have used a dipping technique to challenge the cows obtaining variable rates of infections (Galton, 2004,

Fernandez, 2007, Turner et al., 2013). Challenging the cows by dipping, causes lower infection rates than inoculating or injecting the bacterial suspension into the mammary gland through the TC, probably as a result of the protective mechanism exerted by the TC (Turner et al., 2013). However, the role of the keratin layer in the TC and other natural protective mechanisms that cows have in place are still to be investigated. It was hypothesised that *S. uberis* which is one of the main causes of clinical mastitis during the early dry period in New Zealand can attach to epithelial cells in the TC and develop a biofilm as an invading mechanism, eliciting an increase in cytokine response and modifications in the TC and TS epithelia. The objectives of this study are to describe 1) the progress through and changes induced by a known strain of *S. uberis* in the TC and TS after challenge; 2) the immune response of the TC to the bacterial challenge; 3) changes in the TC and TS epithelia through the early dry period.

5.3 MATERIALS AND METHODS

5.3.1 Animals

Twenty-four cows in late lactation were pre-selected for this study. Cows with four functional quarters, free of mastitis in the current lactation, no teat chaps or wounds, and a composite milk SCC of 51,000±36,000 cells/mL at the latest herd test were considered. Duplicate quarter milk samples were collected aseptically 10 days before drying off. All milk samples were cultured following National Mastitis Council Guidelines (Hogan, 1999). Only cows with negative results for major pathogens (*S. aureus, S. uberis, E.coli*), 215±13 DIM and 4.4 ± 0.4 body condition score (NZ Scale 1-9) at the study commencement were recruited (n= 16).

5.3.2 Bacterial identification

Streptococcus uberis strain S325 was obtained from the microbiology bacteria library in the Microbiology laboratory, School of Veterinary Science, Massey University, New Zealand. The strain was initially isolated by Douglas et al. (2000) and used previously for challenge models by dipping the teats of lactating cows at their last day of lactation (Fernandez, 2007, Petrovski et al., 2007). Bacterial strain S325 was kept at -80^{o} C, thawed, analysed by biochemical test to confirm purity, re-identified as *S. uberis* and tested for biofilm formation *in vitro* by two different tests.

Bacteria were tested for biofilm formation using the microtiter plate technique of Christensen et al. (1985). Briefly, the *S. uberis* isolate was cultured overnight at 37 °C in Trypticase Soy Broth (TSB). Sterile 96-well "Flat-Bottom" plates were inoculated with 200 µL of TSB containing 0.25% glucose and 20 µL of the bacterial suspension to create a 1:10 dilution and cultured statically at 37 °C for 24 h. The supernatant was discarded, and the wells were washed three times with 240 µL of sterile distilled water and air-dried at room temperature. Subsequently, the culture was fixed with 240 µL of methanol for 15 min. The liquid phase was poured off and the wells air-dried again. Two hundred microlitres of crystal violet 1% was added to the wells for five minutes. After rinsing three times with distilled water, the plate was inverted and air-dried. Finally, 240 µL of glacial acetic acid was added in order to re-solubilise the stain from the biofilm. Absorbance was measured at 570 nm in a plate reader (Spectrostar Nano, BMG Labtech, Gmbh, Jena, Germany) and was used to indicate the amount of biofilm that had formed on the microtitre plate surface. Un-inoculated wells containing TSB served as blanks. Blank-corrected absorbance values for the isolate were used for reporting biofilm production. Based on the absorbance of the bacterial films, the

isolates were classified into two categories: Biofilm Negative (BN) (optical density (OD) < 0.09), or Positive (P) (OD > 0.1). The cut-off optical density (ODco) was calculated as three standard deviations above the mean of the blank. Strains with OD > ODco were considered P. The assay was performed in triplicate.

Secondly, the ability of bacteria to form biofilms in ultra-high temperature (UHT) full fat milk and TSB was analysed by SEM. The bacterial strain was cultured overnight in one mL of sterile TSB, yielding approximately 10^8-10^9 CFU/mL. A sterile stainless-steel coupon was placed at the flat bottom of each of the wells of a 24 well plate (Sigma cell culture plate Sigma-Aldrich, Inc. Auckland, New Zealand). Ten wells were filled with TSB and ten wells with UHT full fat milk. Sterile distilled water was used as a negative control in four wells (two per culturing media). One hundred microliters of an overnight culture of the *S. uberis* strain S325 was placed in each well and cultured for 24 and 120 hours at 37 C^o (one and five days respectively). At 24 hours, five stainless steel coupons from wells of each culturing medium (UHT Milk and TSB) and two negative controls were removed. The coupons were washed three times with sterile distilled water and prepared for SEM observation in the Manawatu Microscope and Imaging Centre (Massey University, Palmerston North, New Zealand). The process was repeated after 120 hours with the remaining coupons.

5.3.3 Bacterial challenge

Sixteen cows were randomly allocated into four groups. Group 1 was challenged with a *S. uberis* suspension seven days before drying off (Day -7), Group 2 was challenged on Day 0 (dry off day), Groups 3 and 4 were challenged on Day 7 and Day 25 of the dry period. On the day of challenge, the teat ends were thoroughly cleaned with cotton balls moistened with methylated spirit. After evaporation of the

methylated spirit, the cows were challenged in two randomly assigned contralateral quarters (front right/rear left or front left/rear right; FR/RL or FL/RR) by dipping the teat ends (one cm) for 15 seconds in a *S. uberis* suspension (strain S325) with a concentration of 10^7- 10^8 CFU/ml (Table 5.1). The other two quarters remained as control unchallenged quarters and were dipped in an iodine-based teat disinfectant solution to avoid cross contamination of the teats. The challenge procedure was conducted in the morning and in the afternoon on the challenge days. Udders were visually examined and palpated regularly to detect mastitis for the duration of the study. Two cows within each group of four were euthanised each day at one or five days after challenge.

Table 5.1 Viable bacterial counts in challenge suspensions per Group and challenge times (morning and afternoon) of the challenge day

Group	Morning	Afternoon
1	7.8 x 10^7 CFU/ ml	>3 x 10^8 CFU/ ml
2	20 x 10^8 CFU/ ml	13 x 10^8 CFU/ ml
3	18.2 x 10^8 CFU/ ml	14.6 x 10^8 CFU/ ml
4	1.96 x 10^8 CFU/ ml	1.58 x 10^8 CFU/ ml

5.3.4 Sampling

After euthanasia, the udder was thoroughly disinfected. One sterile Kocher clamp was attached to the base of each teat. The teat barrel from all four quarters was removed using a scalpel cutting distal to the Kocher clamp. Each teat was then opened longitudinally with a scalpel blade from the base and through the TC. The teat was maintained open using four haemostatic clamps attached to each angle of the tissue (Figure 5.1). Samples from the TC and from the TS were taken for analysis as follows:

Intra-cisternal milk sample: Before removal of the teat barrel, an aseptic milk sample was obtained intra-cisternally using a sterile 21 G needle attached to a five mL sterile disposable syringe. The milk samples were stored for culture in pre-labelled sterile plastic containers. For culture, 100 µL of milk were spread onto a sheep blood agar (BA) plate and cultured at 37 C° for 24 hours. When three or more colonies of similar aspect were present, the quarter was considered infected, the presence of less than three colonies was recorded but the quarter considered non-infected. The presence of three or more different colonies was considered to be a contaminated sample according to the NMC guidelines (Hogan, 1999).

Bacteriology of the tissue: Using a sterile biopsy punch instrument (Biopsy punch 3 mm, Hallmark Surgical, New South Wales, Australia), samples were taken from the lower-left area of the TC and from the mid-left portion of the TS (Figure 5.1). The samples were immersed in one mL of sterile TSB and cultured for 24 hours at 37 C°. After 24 hours the TSB tube was vortexed, and 100 µL of the broth was plated in 5% supplemented aesculin sheep BA plates. The plates were cultured at 37 C° for 48 hours and observed every 24 hours. If bacteria were present in the plates, the colonies were sub-cultured in isolation and analysed by MALDI-TOF for full identification of the microorganisms.

Genome sequencing: All *S. uberis* isolates cultured from the biopsy samples were analysed and compared with the challenge strain S325 by full genome sequencing for identification of the source of infection. Genomic DNA was extracted from a single colony using the QIAamp DNA MiniKit (Qiagen, Germany). DNA was sequenced at Novogene (China) using Illumina HiSeq™ X sequencing (2x150base PE) after library preparation using the Nextera XT library kit (Illumina).

Histology: Three-millimetre biopsy samples were taken from the dorsal-left portion of the TC (at the Fürstenberg's rosette) and mid-left portion of the TS (Figure 5.1). Samples were fixed in 4% formaldehyde for approximately four hours, mounted in a paraffin block, and stained with Haematoxylin-Eosin for microscopy observation. The thickness of the TC and TS epithelium and the total number of neutrophils in the TS slides were recorded in the photomicrographs (cellSense Dimension 1.5, Olympus Corporation, Tokyo, Japan). The thickness was measured in micrometers at three different points in the slide (the thickest, the thinnest and a medium thickness point). Neutrophils found in the TS photomicrographs were counted and compared between challenged and nonchallenged quarters and between quarters challenged at different time-points.

SEM: Three mm diameter biopsy samples were taken from the ventral-right area of TC and from the mid-right region of TS (Figure 5.1). All samples were placed in primary Modified Karnovsky's fixative (3% gluteraldehyde 2% formaldehyde in 0.1M phosphate buffer (PBS), pH 7.2) and allowed to fix for at least eight hours at room temperature. The samples were washed three times (10-15 minutes each) in PBS (0.1M, pH 7.2) followed by dehydration in graded ethanol series (25%, 50%, 75%, 95%, 100%) for 10-15 minutes each and a final 100% ethanol wash for one hour. Samples were critical point dried using liquid CO2 as the critical point fluid and 100% ethanol as the intermediary (Polaron E3000 series II critical point drying apparatus). Then, they were mounted on to aluminium stubs using double sided tape and sputter coated with approximately 100nm of gold (Bal-tec SCD 050 sputter coater, Leica Microsystems, Vienna, Austria). Images were taken with FEI Quanta 200 Environmental Scanning Electron Microscope at an accelerating voltage of 25kV at

magnifications 50x 3000x, and 12000x in the Manawatu Microscope and Imaging Centre (Massey University, Palmerston North, New Zealand).

Cytokine/Chemokine Panel: Eight-millimetre diameter punch biopsy samples were taken from the dorsal right of the TC (Fürstenberg's rosette) and the centre of the TS (Biopsy punch Skin 8 mm, SMI AG, St. Vith, Belgium) (Figure 5.1). The samples were placed into microcentrifuge tubes and snap-frozen by submerging them in liquid nitrogen immediately after sampling. The samples were kept at -80°C until processing. Concentrations of IFN-γ, IL-10, IL-6, IL-8/CXCL8 and TNF-α were measured in all samples using the Bovine Cytokine/Chemokine Magnetic Bead Panel 1, 96-Well Plate Assay, BCYT1-33K (Merk, Millipore Corporation, Billerica, MA 01821 USA).

Tissue samples were removed from the freezer and homogenised in a two mL microcentrifuge tube with 600µL of PBS pH 7.4. Two sterile stainless-steel beads were added to the tube and the tissue was lysed in the Tissue Lyser II Qiagen in four cycles of one minute each with one-minute incubation in ice between cycles. Two hundred and fifty microlitres of the homogenate were transferred to a centrifuge tube and the tube placed in ice. Samples were then centrifuged to remove debris. In a 96-well plate, 25 µL of each Standard or Control buffers were added into the appropriate wells. Phosphate buffered saline pH 7.4 was used as a zero picograms standard (background). Twenty-five microlitres of PBS and 25 µL of undiluted samples were added into the appropriate wells. The premixed coated beads were vortexed and 25 µL of each premix added into each well. The plate was sealed with a plate sealer, wrapped and incubated with agitation on a plate shaker overnight (16-18 hours) at 2-8°C. Twenty-five microlitres of detection antibodies at room temperature were added into each well. The plate was sealed, covered and incubated with agitation on a plate

shaker for one hour. Streptavidin-Phycoerythrin was then added and the plate incubated with agitation on a plate shaker for 30 minutes at room temperature (20-25°C). The well contents were gently removed, and the plate washed three times. One hundred and fifty microlitres of delivery medium, was added to all wells. The beads were resuspended on a plate shaker for five minutes and the plate read on Bio-Plex® 200 and processed with the Bio-Plex Manager™ software.

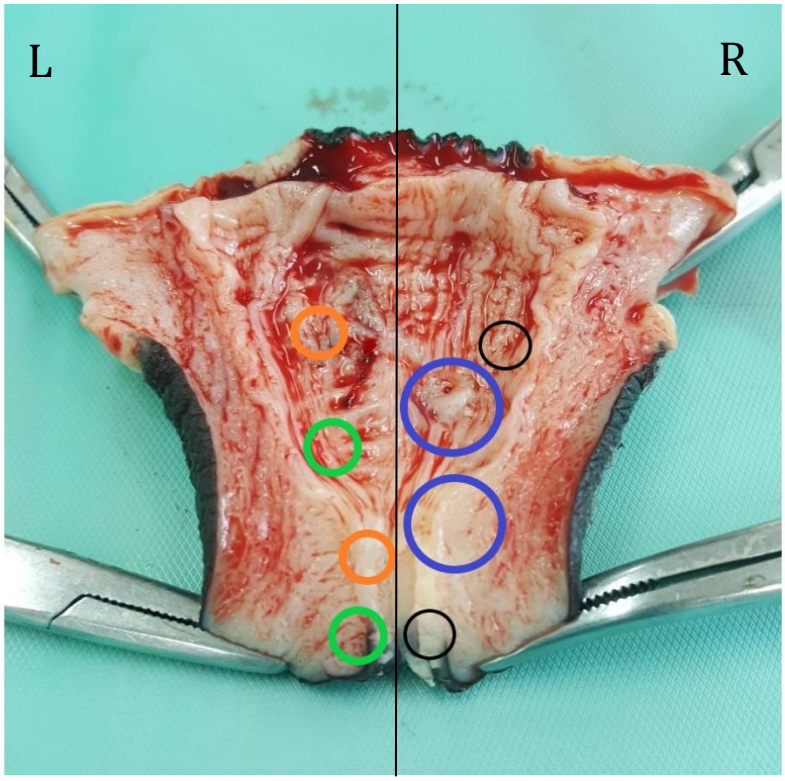


Figure 5.1 Diagram of an opened teat showing the sampling sites in the teat canal and teat sinus for bacteriology (green), histology (orange), SEM (black) and cytokine panel analysis (blue). L= Left, R= Right.

5.3.5 Statistical analysis

For the analysis of culture results, an Armitage test for trend (Margolin, 1988) was applied to binary outcomes of the overall infection rate with any bacterial species in order of the four sampling days (groups). Cultures with one or two colonies were classified as negative. To adjust for a possible dependence of infection rates in quarters within cow, the Z-score of the test for trend was divided by the square root of the variance inflation factor [1+(n-1)*rho] where n was the number of quarters within cow (n=4) and rho the intra-class correlation (ICC) based on the mean square between (MSB) and within (MSW) of an ANOVA model with infection as outcome and cow ID as a factor. Intra class correlation was calculated as (MSB-MSW) / (MSB + (n-1) *MSW). Data from the histological assessment (thickness of *granulosum* and *corneum strata* of the TC, thickness of mucosa of the TS) and cytokine concentrations were not normally distributed and therefore were converted by log10. A mixed linear model with a random effect for cow was used to detect differences in least square means of the histological measurements between challenged and non-challenged quarters at each time point (sampling day) (PROC MIXED, SAS 9.3 for Windows, SAS, 2018, SAS Institute Inc., Cary, NC, USA). In addition, the same models were tested for a trend over time using sampling-day as a continuous effect and its interaction with challenge and an additional model that included neutrophil presence to compare infected vs. non infected quarters. The mixed linear model for the cytokine analysis included fixed effects for days after challenge, tissue (TC and TS), an interaction between challenge, tissue, day after challenge (1 and 5) and a random effect for cow. Least square means and their 95% confidence intervals were computed to graphically illustrate the results. Where no exact p-values or other thresholds are stated, significance level was declared at $p< 0.05$.

5.4 RESULTS

5.4.1 Bacterial strain in vitro tests- Biofilm formation

Streptococcus uberis strain S325 was negative for biofilm formation when cultured *in vitro* in TSB with the microtiter plate technique. Scanning electron microscopy images from the second biofilm test showed no exopolysaccharide formation surrounding the chains of cocci in milk or in TSB either 24 or 120 h after culture (Figure 5.2).

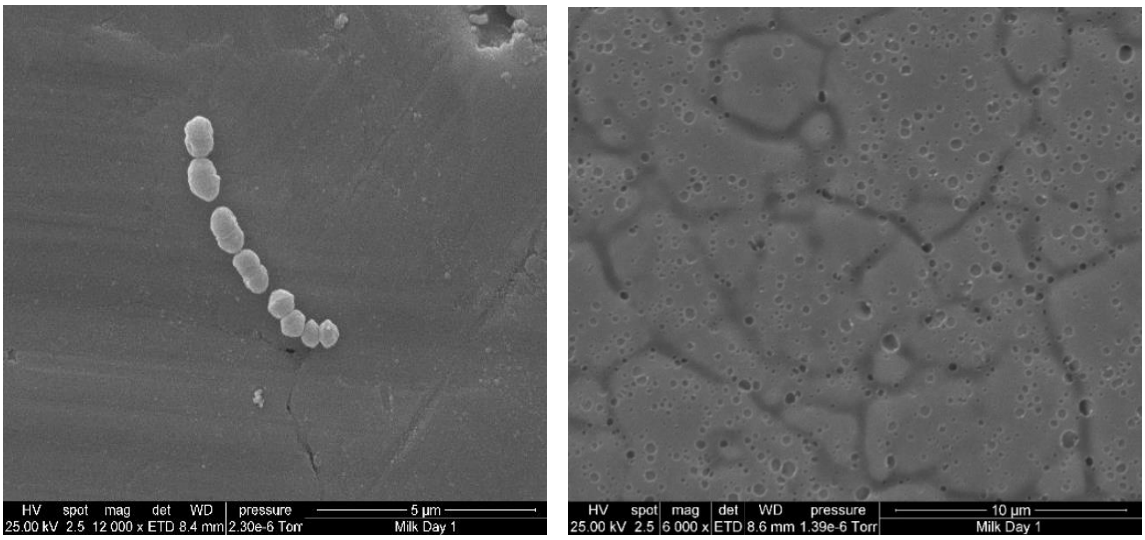


Figure 5.2 Electron microscopy scan of *Streptococcus uberis* strain S325 cultured on a stainless-steel coupon over 24 hours in a UHT milk media (left). Negative control (right).

5.4.2 Bacteriology of the milk pre-challenge

From the 64 quarters selected for the study, 41 were free from infection at the pre-screening culture (Day -10). Three quarters showed infection (more than two colonies) of minor pathogens (e.g. CNS, *C. bovis*); only one of them was challenged.

Seven quarters were contaminated in both duplicate samples and only one of them was challenged. Thirteen quarters presented one or two colonies of unidentified bacteria and six of them were challenged. Bacteriological results of the milk before challenge (Pre-challenge) from quarters that presented positive cultures after challenge are shown in Table 5.3.

5.4.3 Bacteriology of the tissue and milk after challenge

Of the 32 challenged quarters, only two were infected by *S. uberis*, both from Group 4 (Day 25 after drying off); however, the results of the DNA gene sequence analyses showed that the strains isolated from these quarters were different from the challenge strain S325 and very similar between them (Table 5.2). Only four quarters had bacteria isolated from milk. No quarters from Group 1 were found to be infected after culture of the tissue samples. Twenty-four biopsy samples showed bacterial growth after culture (Table 5.3). Thirteen of the quarters yielding bacteria were sampled on Day 25, and seventeen were TC samples. Most of the samples yielding bacteria were from groups sampled on Day 5 after challenge.

Mean overall infection rates did not differ between time points or challenge groups. However, there was a significant trend of increasing infection rates over time. Rates from TC and TS tissues respectively were 0% and 0% for Day -7, 31% and 6% for Day 0, 13% and 0% for Day 7, and 50% and 31% for Day 25.

Table 5.2 DNA sequencing results showing the larger size of the DNA chain of strain S325 (challenge strain) in bp (base pairs), base pairs which passed the quality control (ok) and the sample where the isolate was obtained from (Origin)

Isolate	Counting	bp	ok	Origin
SU325	17	1923113	1923113	Challenge strain
SU119	11	1886121	1886121	55 RL (TC)
SU120	13	1885956	1885956	55 RL (TS)
SU127	11	1885562	1885562	409 RL (TC)
SU128	10	1886988	1886988	409 RL (TS)
SU55RL	10	1887829	1887829	Milk isolate from quarter 55 RL

Table 5.3 Results of pre-challenge milk culture per quarter and the microorganisms isolated from the 3-mm tissue biopsy samples obtained from the teat canal (TC) and teat sinus (TS) which showed bacterial growth by group. ID= Cow ID, Quarter= FL: Front left, FR: Front right, RL: Rear left, RR: Rear right, Pre- challenge: before-challenge milk samples culture results: NG= No growth, D.NG: One sample was contaminated and the duplicate had no growth, Cont.: Contaminated sample, Infected: infected with a minor pathogen, CH: Challenged, Group: 1, 2, 3, 4 challenged -7, 0, 7 and 25 days from dry off, Milk: Milk Culture results, Biopsy: Tissue sampled in the biopsy, Micro ID: Results obtained from MALDI-TOF.

					Sample		
ID	Quarter	Pre-challenge	CH	Group	Milk	Biopsy	Micro ID
145	FL	NG	No	2	NG	TC	*Serratia liquefaciens*
145	FL	NG	No	2	NG	TS	*Bacillus licheniformis*
599	FR	1 colony	No	2	NG	TC	*Streptococcus lutetiensis*
599	RL	infected	No	2	NG	TC	*Escherichia coli* *Streptococcus parauberis*
413	FL	D. NG	No	2	NG	TS	Not in database
598	FR	NG	No	2	NG	TC	*Micrococcus luteus*
598	FL	1 colony	Yes	2	NG	TC	*Gram positive rods*
598	RR	NG	Yes	2	NG	TC	*Staphylococcus epidermidis*
598	RL	NG	No	2	NG	TC	*Gram positive cocobacillus*
447	FR	NG	Yes	3	NG	TC	*Probably Pseudomona family*
831	FR	NG	No	3	NG	TC	*Staphylococcus saprophyticus*
862	FR	NG	Yes	4	NG	TC	*Staphylococcus chromogenes*
862	FL	NG	No	4	Infected	TC	*Enterobacter cloacae*
862	FL	NG	No	4	Infected	TS	*Enterobacter cloacae*

268	FL	NG	Yes	4	NG	TC	*Sphingomonas parapaucimobilis*
55	RR	Cont	No	4	Infected	TC	*Staphylococcus chromogenes*
55	RR	D. NG	No	4	Infected	TS	*Staphylococcus chromogenes*
55	RL	NG	Yes	4	Infected	TC	*Streptococcus uberis*
55	RL	NG	Yes	4	Infected	TS	*Streptococcus uberis*
409	FR	NG	Yes	4	NG	TC	*Staphylococcus chromogenes and Staphylococcus xylosus*
409	FL	D. NG	No	4	NG	TS	*Brevundimonas diminuta*
409	RR	NG	No	4	NG	TC	*Staphylococcus chromogenes*
409	RL	NG	Yes	4	Infected	TC	*Streptococcus uberis*
409	RL	NG	Yes	4	Infected	TS	*Streptococcus uberis*

5.4.4 Histology

Histological measurements of the epithelial tissue (keratin layer, *stratum granulosum* and sinus mucosa) did not differ between challenged and non-challenged tissues (Appendix 1 and 2). No significant difference was found in the keratin layer (*stratum corneum*) throughout the sampling period. There was an overall decreasing thickness observed from day 0 to day 25 for *stratum corneum* (p=0.5) and *stratum granulosum* (p=0.2) of the TC, and the sinus mucosa was thicker at Day 25 when compared with Day -7 (p=0.01) (Figure 5.3). Thus, both the *corneum* and *granulosum strata* were thinning throughout the dry period. When compared among timepoints in

the *stratum granulosum*, thickness was significantly reduced at Day 7 and Day 25 when compared to Day -7 (Figure 5.3-A, B). The epithelial tissue thickness of the TS tended to increase in the early dry period (Figure 5.3-C). A significantly higher number of neutrophils was found in the infected quarters ($p=0.02$) in the TS micrographs.

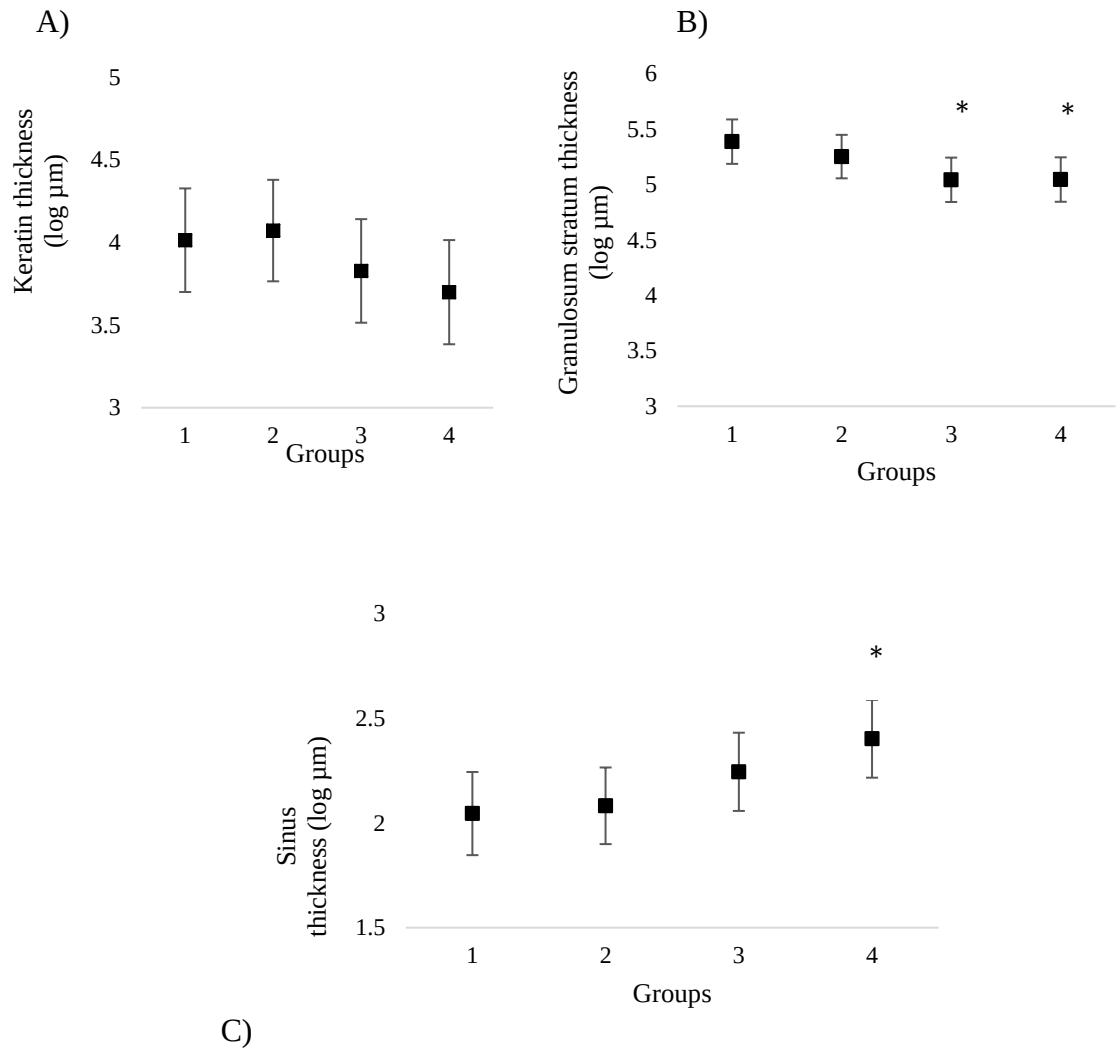


Figure 5.3 Least square means estimates of the log converted thickness of the A) Keratin layer thickness (*stratum corneum*), B) *stratum granulosum*, and C) sinus mucosa. (* indicates a significant difference between least square means compared to group 1, p<0.05). Y-axis show measurements taken histologically (in log scale) by Group challenged (X-axis) at different stages of the late lactation and early dry period. Group 1: challenged on day -7 to dry off, Group 2: day 0, Groups 3 and 4 days 7 and 25 after drying off, respectively

5.4.5 Scanning Electron Microscopy- SEM

The epithelial tissue from the TC samples presented a rough appearance with layers of keratin fragments on the surface and the presence of milk detritus (Figure 5.4, A-B). Teat sinus SE micrographs showed the mucosa with its characteristic microvilli, some cells bulging out from the epithelium and the presence of cells and

milk detritus (Figure 5.4, C-D). No major differences were observed in the aspect of the tissue from challenged and non-challenged teats. There was no increased squamous tissue in the TC or any difference cell shape in the TS. No planktonic bacteria or biofilm formation was observed in the images taken from the biopsies. Neutrophils and lymphocytes were identified on the images in challenged as well as in non-challenged quarters (Figure 5.5).

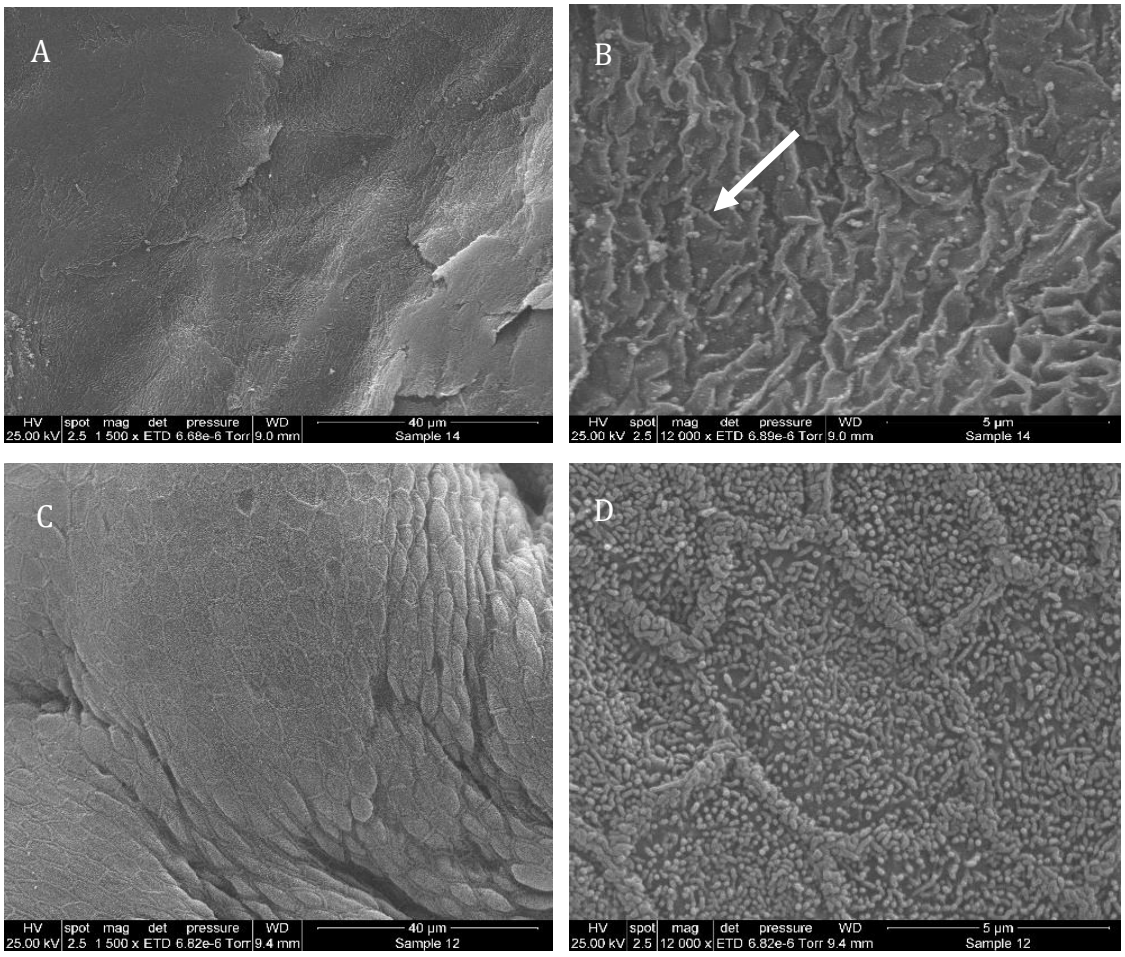


Figure 5.4 SEM images of a 3 mm punch biopsy sample from the teat canal (A and B) and teat sinus (C and D) from non-challenged teats. Magnification x1,500 and x12,000 for left and right micrographs, respectively. Arrow: milk detritus.

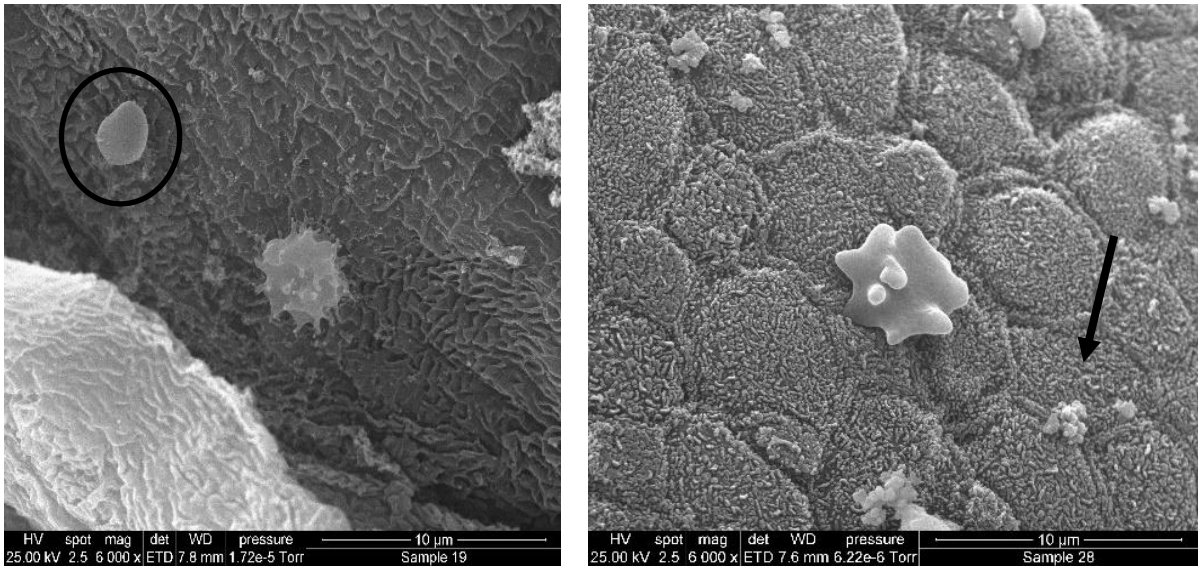


Figure 5.5 SEM image of neutrophils in a TC sample (Left) and a TS sample (right) taken from challenged cows. Magnification x 6,000. Arrows: milk detritus, circle: lymphocyte.

5.4.6 Cytokine panel analysis

There was no difference in cytokine levels between challenged and non-challenged quarters (Appendix 3 to 6). Hence, tissue samples were grouped by day from dry off for further analysis. The TC had higher concentrations of IL-8, IL-10 and TNF-α than the TS ($p<0.001$). Concentrations of IL-8 and TNF-α during lactation (Group 2= Day 7) were higher than later in the dry period (Group 4 = Day 25, Figure 5.6 $p<0.05$). Interferon-γ and IL-10 both showed a similar pattern of increasing concentrations during lactation (Day -7 to Day 0), then decreasing to the lowest levels a week after drying off, to increase again by Day 25. Interleukin-6 presented a very low to non-detectable concentration in both tissues except for one quarter (Cow 55 RL) from which *S. uberis* was isolated, which had 100 times the level of cytokines detected in the other quarters in both the TC and TS (data not shown).

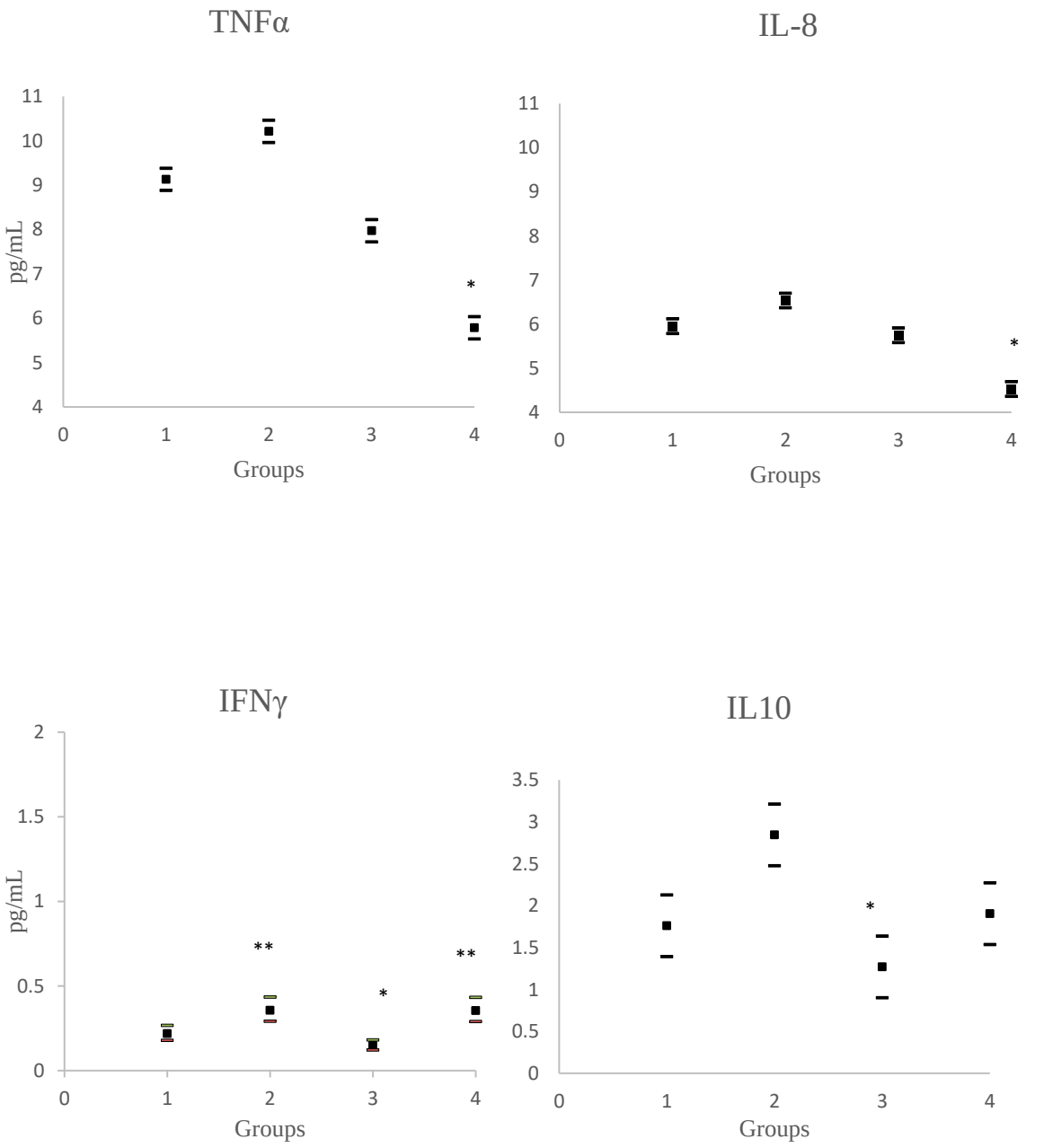


Figure 5.6 Cytokine concentrations of IFN-γ, IL-10, IL-8/CXCL8, TNF-α (95% CI) detected in teat canal and teat sinus biopsy samples collected from all four quarters of cows challenged at day -7 (Group 1), day 0 (Group 2), day 7 (Group 3) and day 25 (Group 4) from dry off. Asterisks mean p<0.05

5.5 DISCUSSION

This study assessed the ability of a *S. uberis* strain S325 to colonise the TC, to form biofilms *in vitro* and to induce a response *in vivo* in the epithelial tissue of the TC. As second and third objectives of this study, the changes in the TC after challenge and the changes occurring in the first 25 days of the dry period were investigated.

Strain S325 did not form biofilms *in vitro* in the microtiter assay (cultured in TSB) or when cultured in UHT milk and TSB. However, the ability of a bacterial strain to form biofilms *in vitro* is not a predictor of the biofilm formation *in vivo* (Tormo et al., 2007, Tassi et al., 2015). In many studies, the absence of biofilm formation was associated with increased virulence and infectivity (Baselga et al., 1993, Simojoki et al., 2012). In contrast, in this study the absence of biofilm formation found *in vitro* for *S. uberis* strain S325 was not a predictor of higher virulence and colonization ability. The absolute absence of infections of challenged quarters obtained in this study strongly suggests that strain S325 had a low ability to colonise the mammary gland, despite being unable to form biofilms *in vitro*. Strain S325 was a sub-culture of a clinical case obtained after a challenge study that resulted in 75% of dipped teats having clinical mastitis (Fernandez, 2007, Petrovski et al., 2007). In that study, the teats of the challenged cows were dipped twice on Day zero and Day four of the dry period using a similar concentration of 10^8 CFU/mL as was used in this study. In contrast to the methods presented herein, Fernandez's study did not include genetic analysis of all the strains retrieved from all the infected quarters (Fernandez, 2007, Petrovski et al., 2007). A DNA gene sequence analysis in the present study showed that the infections present in the *S. uberis* infected quarters were caused by a different strain from that utilised in the challenge model. Strain S325 obtained from an isolate from Fernandez's

study might not have been the original strain used to challenge the cows in that study as it was harvested from a clinical case from which the PFGE information was not accessible. This might be a field strain that was less virulent than the one used to challenge the cows in these cited studies. There is a large difference between host adapted and non- adapted strains of *S. uberis* in terms of the response they induce in the host (Tassi et al., 2013). Non-adapted strains tend to cause milder infections accompanied by a low host response. It is likely that strain S325 is a non-adapted host strain which had a low ability to colonise or did not colonise the teat tissue in this study and that is why did not induce an immune response in the cows in our study in agreement with other non-adapted strains in previous reports (Zadoks et al., 2005, Tassi et al., 2013). The bacterial species colonising the TC in this study (Micrococci, Staphylococci, Serratia, etc.) have been previously described as belonging to the normal microflora of dairy cows (Gill et al., 2006, Braem et al., 2012). The current results support previous studies suggesting that the flushing effect of milk has a protective effect against IMI (Capuco et al., 1994, Lacy-Hulbert et al., 1996). The infection trend found herein, indicates that TC and TS colonization by bacteria occur later during the dry period in Days 7 and 25 (Groups 3 and 4), but not often during lactation in which there were no isolated microorganisms from Group 1 (Day -7).

The histological tissue thickness was similar in challenged and non-challenged quarters. This was presumably a result of the lack of infection induced by strain S325. In the current study, the number of neutrophils in the TS was an indicator of infection but was not influenced by the challenge.

Histological measurements of the TC and TS showed that the keratin layer and the *stratum granulosum* thickness decreased through the early dry period. These

results agree with data showing a physiological atrophy of the *stratum granulosum* and the decrease of the keratin content in the TC after drying off (Comalli et al., 1984, Gleeson et al., 2003). Measurements of micrographs of the *stratum corneum* of the TC taken in this study in dry cows ranged from 60 to 250 micrometres whereas measurements taken during lactation in other studies were wider, ranging between 30 and 436 micrometres (Davidov et al., 2011). No histological difference between dry and lactating cows was found in a study (Collins et al., 1986). Taken together, these studies support the concept that there is a decrease in the *corneum* and *granulosum strata* during the dry period. The present study showed that 50% of the TC and 31% of the TS samples were colonised by bacteria by Day 25 after drying off. The decrease in the keratin and *stratum granulosum* thickness, and the concomitant increase in the rate of TC colonization, agree with previous studies reporting a significantly higher incidence of infection in quarters with a low wet weight (< 1.8 mg) of removable keratin compared with those that contained high (> 1.8 mg) amount of removable keratin (Capuco et al., 1992, Hillerton and Lacy-Hulbert, 1995). Other studies also showed an increase in neutrophils and SCC in quarters with a TC containing a thinner keratin layer (Davidov et al., 2011). However, more evidence is necessary to draw a conclusion from these data.

Teat duct colonization might not only be related to the thickness of keratin in the TC lining, but also to the irrigation of the tissue and the nutrients available for the epithelial cells, both of which are reduced during the early dry period compared to the lactating state. Congestion of the teat end and blood stasis with oedema in the teat end during and after machine-milking might increase the risk of teat colonization (Zecconi et al., 1992, Capuco et al., 1994). This might also apply for teat ends during the early

dry period where blood circulation of the teat end is less than in lactation (Jankus and Baumann, 1986, Hamann et al., 1994).

Cytokine response to challenge was not detected in the TC and TS examined in this study. Previous studies also recorded that non-adapted *S. uberis* strains caused minimal response of cytokines in all the cytokines analysed. These non-adapted strains also caused a minor transient response in IL-8 and did not colonise the mammary gland, although they were able to grow *in vitro* in the milk of challenged cows (Tassi et al., 2013). In other challenge studies where the *S. uberis* strains were inoculated directly into the TC, the incidence of clinical mastitis and the cytokine response were high (Rambeaud et al., 2003, Notcovich et al., 2016). Tumour Necrosis Factor α, IL-8, and IL-1β have all been found in high concentration in milk from infected glands after challenge, whereas no IL-8 was detected in challenged, uninfected mammary glands or in unchallenged control mammary glands (Rambeaud et al., 2003). However, the challenge technique used in the current study was dipping the teat into a bacterial broth, and then assessing response in the TC and TS, but not in mammary tissue. Interleukin 6, IL-8 and TNFα are pro-inflammatory cytokines that increase the response of the tissue to bacterial infection or tissue damage, and act as chemo-attractants for neutrophils (Riollet et al., 2002). In this study, due to the failure of the challenge strain colonization, a response in cytokines levels in the analysed tissue could not be assessed. Interestingly, the levels of IL-8 and TNFα in the current study showed a significant decrease in concentration after 25 days from dry off. In contrast, an increased number of macrophages and dendritic cells have been reported at dry off in previous studies on the physiology of the TC (Smolenski, 2018). These results suggest that even though they could appear in higher numbers in the TC at dry

off, there could be a functional decrease in those cells that impairs their ability to synthesise high amount of cytokines (Smolenski, 2018). This reduced cytokine response of the TC and TS might be one of the reasons why the TC became more susceptible to infection by bacteria other than the challenge strain by Day 25 of the dry period (Green et al., 2002). The decrease in IL-8 and TNFα synthesis could also be a consequence of the reduced irrigation described above due to the cessation of machine milking during the early dry period (Zecconi et al., 1992). It would be interesting to investigate if the increase in IL-8 and TNFα during the early dry period affects bacterial colonization of the TC. The patterns observed for concentration of cytokines in the TC and TS showed that a week after drying off, the concentration of cytokines IFNγ and IL-10 were lowest, increasing again by Day 25. This later increase could occur as a response to the increased bacterial colonization observed in the tissue. Interleukin 10 is a cytokine with anti-inflammatory properties that plays a role in limiting host immune response to pathogens (Iyer and Cheng, 2012). The current study also revealed that the cytokine concentrations in the TC and TS differ. The TC had higher levels of IFNγ, IL-8, IL-10 and TNFα, possibly supporting the "sentinel function" that has been proposed previously in other studies where it was suggested that the increase of cytokines in the TC occurs earlier than in other parts of the mammary tissue (Rainard and Riollet, 2006, Rinaldi et al., 2010).

5.6 CONCLUSIONS

The current study indicates that *S. uberis* strain S325 failed to induce IMI when challenging udders by twice dipping the teats of 16 dairy cows in late lactation. Strain S325 did not form biofilms *in vitro* or *in vivo*. The TC was more frequently colonized

by non-pathogenic bacteria during the first 25 days after drying off than when lactating (Day -7 or 0). Taken together, the results of the study indicate that the decrease in thickness of the TC epithelium concomitant with a decreased synthesis of pro-inflammatory cytokines during the early dry period could be related to an increased TC and TS colonization by non-pathogenic bacteria frequently observed at this stage of the lactation cycle.

6 Effect of bismuth subnitrate on *in vitro* growth of major mastitis pathogens

Bismuth subnitrate-based formulations are used as internal teat sealants (ITS) on the last day of lactation in dairy cows. Internal teat sealants are defined by the World Health Organization as a physical, non-pharmacological barrier that hinders bacterial invasion over the dry period. However, several studies have shown an inhibitory effect of bismuth subnitrate-based products when used for treating several bacterial diseases in humans. The results in this Chapter support the concept that bismuth subnitrate slows bacterial growth. These results could change our perspective on how to prevent mastitis over the dry period and allow the development of new approaches to minimize the disease.

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Effect of bismuth subnitrate on *in vitro* growth of major mastitis pathogens.

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Effect of bismuth subnitrate on *in vitro* growth of major mastitis pathogens

6.1 ABSTRACT

The mode of action of bismuth subnitrate in teat sealant formulations as a preventative for intramammary infections during the dry period is unknown. Although previous studies proposed a mechanism of action consisting of creating a physical barrier in the teat canal (TC) to prevent bacterial invasion, this has not been proven experimentally. The hypothesis that bismuth subnitrate has an inhibitory effect on bacterial growth *in vitro* was tested. The objective of this study was to assess the effect of bismuth subnitrate on bacterial growth of major mastitis-causing agents. A strain of *S. uberis* (SR115), two strains of *S. aureus* (SA3971/59 and SA1) and a strain of *E. coli* (P17.14291) were tested *in vitro* for their ability to grow in the presence or absence of bismuth subnitrate. Disk diffusion testing, impedance measurement and evaluation of bacterial growth in shaking conditions were the methods used. A reduction of growth in the presence of bismuth subnitrate occurred for all the bacterial strains tested ($p<0.001$). However, strain and species variations were observed in the extent of growth inhibition. These results suggest that an inhibitory effect on bacterial growth by bismuth subnitrate could partially explain the efficacy of bismuth-based formulations for preventing intramammary infections (IMI) over the dry period. Further research is required to test the effect of teat sealant formulations on bacterial growth.

6.2 INTRODUCTION

The late lactating and early dry periods are stressful stages for the anatomy and physiology of dairy cows, particularly their mammary glands. Remodelling, apoptosis, and cell dedifferentiation are processes occurring in mammary glands during the first two weeks after milk removal ceases (Cousins et al., 1980, Wilde et al., 1997). This time-specific physiological stress is manifested by high susceptibility of cows to new intramammary infections (IMI) (Green et al., 2002). Thus, methods to treat and to prevent IMI during the dry period have been available for many years. These treatments consist mainly of antimicrobials infused into mammary glands after the last milking before drying off and result in a reduction of new IMI during the dry period in treated cows compared to non-treated individuals (Smith 1966, Pearson and Wright, 1969, Schukken et al., 1993, Bryan et al., 2011). Dry cow therapy (DCT) has been used on all cows in a herd regardless of their infection status with the objective of eliminating subclinical infections and preventing new infections during the dry period (Williamson et al., 1995, Hassan et al., 1999). However, due to concerns regarding increasing antimicrobial resistance as a global issue and the need for responsible use of antimicrobials, there is a trend towards investigating the use of selective DCT and non-antimicrobial alternatives (Oldham and Daley, 1991, Ryan et al., 1998, Hoernig et al., 2016, Vanhoudt et al., 2018). The current and most used alternatives to antimicrobials at the time of dry-off are ITS that contain bismuth subnitrate, a heavy metal compound, in a paraffin-based excipient. They have proven to be effective at preventing new dry period infections in challenge models as well as in natural exposure studies (Meaney, 1977, Woolford et al., 1998, Bhutto et al., 2011). The mechanism of action of bismuth-based products remains undocumented,

although creating a physical barrier has been suggested in many reports as the main method of protection (Meaney, 1977, Woolford et al., 1998, Berry and Hillerton, 2002, Kabera et al., 2018). Internal teat sealants are also commonly used in combination with DCT, in order to prevent and treat IMI during the dry period (Godden et al., 2003). However, although there is an increase in the efficacy of preventing IMI using both products in combination, this increase is non-significant (Rabiee and Lean, 2013).

Bismuth formulations used as internal teat sealants (ITS) are defined as "medical devices", a non-pharmacological barrier (Codex, 2016). Assisting the TC in forming a better keratin plug and creating a physical barrier against bacteria in the TC are proposed mechanisms through which teat sealants exert their function (Woolford et al., 1998); however, none of these hypothesized modes of action has been scientifically proven. Other studies testing different physical barriers, such as wax plugs or intramammary polyethylene devices, were unsuccessful in the long-term protection of cows against IMI and mastitis. Even though intramammary devices elicited some immune response and a seemingly initial protective effect (Poutrel et al., 1983, Paape et al., 1988, Nickerson et al., 1990, Serna-Cock and Pabón-Rodríguez, 2016), in the long term these devices resulted in an increase in IMI. It is therefore hypothesized that creating a physical barrier against bacteria traversing the TC is not the sole mode of action providing the efficacy observed for bismuth-based formulations.

Studies have shown an inhibitory effect of bismuth-based products when tested *in vitro* against bacterial species that are associated with disease in humans (Phillips et al., 2000, Folsom et al., 2011, Vega-Jiménez et al., 2012). Inhibition of bacterial growth by bismuth salts (mainly bismuth subsalicylate) has been studied in the

treatment of stomach ulcers, traveller's diarrhoeas and colitis caused by bacteria such as *Helicobacter pylori* and *Campylobacter pyloridis* (Marshall et al., 1987, Fine and Lee, 1998).

In this study, the hypothesis is that bismuth subnitrate inhibits the *in vitro* growth of bacteria which are associated with the colonization of the mammary gland during the dry period and the development of new IMI. The objective of the study was to assess the effect of bismuth subnitrate on the growth of mastitis-causing bacterial strains *in vitro* by using three different methods, namely disk diffusion testing, impedance measurement and evaluation of bacterial growth in shaking conditions.

6.3 MATERIALS AND METHODS

6.3.1 Bacterial Strains

Streptococcus uberis strain SR115, two strains of *Staphylococcus aureus* (SA3971/59 and SA1) and an *Escherichia coli* strain P17.14291 were used in this study. The source of the *S. aureus* and *S. uberis* strains was the bacteria library in the Microbiology Laboratory, School of Veterinary Science, Massey University, New Zealand and they were originally isolated by veterinary diagnostic laboratories from clinical mastitis cases that occurred in New Zealand at various stages of lactation. *Escherichia coli* was isolated from an early lactation clinical case of mastitis that occurred in August 2017 at Massey University Dairy Unit number 4. For complete identification, the *S. uberis* and *S. aureus* isolates were cultured on Trypticase Soy Agar (TSA) plates and incubated in aerobic conditions at 37^{o}C for 24 h. In order to confirm strain purity, one colony from each strain was selected from the culture plate, transferred onto a new TSA plate, and incubated as above. The isolates were re-

identified phenotypically using biochemical tests. *Streptococcus uberis* SR115 was confirmed by positive Gram stain, catalase-negative, aesculin and inulin positive reactions, and negative growth in buffered azide glucose glycerol broth. Positive results for Gram stain, catalase reaction, and coagulase rabbit plasma test confirmed isolates as being *S. aureus* SA3971/59 and SA1. *Escherichia coli* P17.14291 was cultured on MacConkey agar.

6.3.2 Experiment 1: Bacterial growth in agar- Disk diffusion test

To test the hypothesis that bismuth subnitrate can inhibit the growth of bacteria on agar media, bacterial strains were cultured overnight at 37°C in 1 mL of Trypticase Soy Broth (TSB). One hundred microliters of bacterial suspensions were spread on TSA plates. These bacterial strain suspensions were cultured overnight to contain approximately 10^8 to 10^9 CFU/mL. Sterile bismuth subnitrate powder was suspended in sterile distilled water at three different concentrations (65 mg/mL, 195 mg/mL, and 390 mg/mL) and vortexed thoroughly to create a uniform suspension. Concentrations were chosen to represent 10, 30, and 60% of the 650 mg/g present in a current teat sealant product (drySeal, Bayer Animal Health, New Zealand). Higher concentrations induced precipitation of the bismuth subnitrate in the tubes and were therefore not used. Sterile six mm paper disks were immersed in the bismuth subnitrate suspensions, placed on the plates and the plates were incubated for 24 h at 37°C. One extra disk was immersed in sterile distilled water and used as a control. After incubation, the plates were observed for zones of inhibition and changes around the disks at the different concentrations and the diameters of the zones were measured with a 20 cm calliper. The experiment was performed in five plates for each strain.

6.3.3 Experiment 2: Bacterial growth in a fluid medium- Impedance standardization

The hypothesis of this experiment was that bismuth subnitrate inhibits the growth of bacteria in a fluid medium. During the growth phase, bacterial metabolism breaks down proteins and lipids in the medium and transforms uncharged or weakly charged compounds of the culture medium into highly charged compounds that change the electrical properties of the medium. Impedance in microbiology is the ability of a microorganism to change (reduce) the resistance to flow of an electric current as it passes through a conducting material (Silley and Forsythe, 1996). Impedance was measured using the BacTrac 4300 microorganism growth analyser (SyLab, Purkersdorf-Vienna, Austria). The BacTrac 4300 measures impedance in the medium (M-value) and impedance in the electrode (E-value). In this experiment, the E-value generated by BacTrac was used, as it has been shown to be positively associated with bacterial growth (Wang et al., 2016). Bacterial strains were cultured overnight in 1 mL of TSB at 37^{o}C. Bismuth subnitrate (BIS) in a final concentration of 32.5 mg/mL (5% of the 650 mg/g contained in teat sealant product) was added to sterile BacTrac vials containing 9.9 mL of TSB which were vortexed. Control vials containing 9.9 mL of sterile TSB (C) were filled with 0.325 mL of sterile distilled water to correct for volume and vortexed to mix. One hundred microliters of bacterial strain suspension cultured overnight, containing approximately 10^8 to 10^9 CFU/mL, was added to all vials. The vials were cultured for 24 h at 37^{o}C in the BacTrac. The E-value was monitored every 20 min for 24 hours and a curve expressing the increase in impedance over time was drawn compared to the starting point at Time 0. The experiment was performed in triplicate, in four repeats per strain (12 vials per treatment).

Before and after culture in the BacTrac, viable bacteria were counted for validation of the results using the pour plate technique. Briefly, a 100µL sample from the BacTrac vials (six per strain) was transferred into 1.5 mL sterile tubes containing 900 µL of TSB. These suspensions were serially diluted 10^{-3} to 10^{-7} and transferred into sterile empty Petri dishes. Trypticase soy agar was poured over the plates containing the bacterial suspension and the plates were incubated at 37°C for 24 h. The total number of viable colonies (CFU) was counted after incubation of the plates.

6.3.4 Experiment 3: Bacterial growth in a shaking fluid medium

The hypothesis of this experiment was that by shaking the media, bismuth subnitrate particles would come in closer contact with bacterial cells and therefore increase the inhibition of bacterial growth. Bacterial strains were cultured overnight in one mL of TSB at 37^{o}C. Erlenmeyer glass flasks were filled with 9.9 mL of sterile TSB in triplicate. Bismuth subnitrate was added at a final concentration of 32.5 mg/mL (BIS). Control flasks without bismuth (C) received 0.325 mL of sterile distilled water to correct for volume. One hundred microliters of the overnight bacterial culture (approximately 10^{8} to 10^{9} CFU/mL) was added to all the flasks. Two trials were run with this method to observe the effect of longer incubation time on bacterial growth. In Trial 1, the flasks were placed on a shaking platform at 200 rpm for 24 h for culturing. In Trial 2, the flasks were cultured in shaking mode for 48 h. Aliquots of 100 µL of the culture media were taken at multiple time points to count the number of viable colonies: immediately after inoculation (0 h), at 3, 5, 24 and at 0, 3, 5, 24 and 48 h after inoculation for Trials 1 and 2, respectively. The 100-µL aliquots were serially diluted ten-fold in 0.9 mL of TSB. These serial dilutions were processed for CFU count using the poured plate technique described above.

6.3.5 Statistical analysis

For Experiment 1, the outcome 'diameter of dark area around the disk' had an approximately normal distribution. A standard ANOVA with post hoc Tukey-Kramer adjustment for multiple paired comparisons was used to compare the diameter (in millimetres) associated with each strain at different concentrations. For Experiment 2, results are presented as least square means estimates of the E-value ±95% CI by Time. Statistical differences were based on Tukey-Kramer adjusted pairwise comparison per strain. The repeated measure model included the fixed effects of Treatment (BIS vs C), Time (0 to 24 in 20 minutes intervals) and the interaction between Treatment and Time. Vials with incomplete curves due to a malfunction of the electrodes were dropped from the analysis. Seven vials of *S. uberis* SR115, 9 of *S. aureus* SA3971/59, 6 of *S. aureus* SA1 and 8 of *E. coli* P17.14291 were removed from the analysis for this reason. Viable CFU were not normally distributed and were therefore converted to $\log_2$ CFU values. Paired Student T-test was used to compare the difference of $\log_2$-CFU before (Hour 0) and after culturing (Hour 24) for bismuth (BIS) and for control (C) groups. Student T-test was used to compare the mean difference of the $\log_2$-CFU between groups (BIS and C) at each time point. For Experiment 3, the CFU data were right-skewed but an e-log conversion of the CFU/10000 counts showed an approximately normal distribution as evaluated using density plots. The outcome was the mean of triplicate plate counts per treatment (BIS, C) per strain showing between 30 and 300 CFU. Four repeated measures models, one for each strain (PROC MIXED, SAS 9.3 for Windows, SAS, 2018, SAS Institute Inc., Cary, NC, USA) were developed to determine the effect of bismuth supplementation on bacterial growth at each time point (0, 3, 5, 24 and 48 h) in shaking culture for both trials. The model included the fixed effect of time (0, 3, 5, 24, 48), trial (1 vs 2), treatment (BIS vs C) and interaction

of time and treatment. To account for the correlation of repeated measures within group, the model for a cluster effect of group was nested within trial (1, 2) and a 1st-order autoregressive correlation structure assuming that measurements closer in time were correlated more strongly. This structure resulted in the lowest Akaike Information Criterion (AIC) as a measure of overall model fit. The statistical significance level for all statistical tests was set at $p<0.05$. All statistical analysis was done in SAS 9.3 for Windows, (SAS, 2018, SAS Institute Inc., Cary, NC, USA).

6.4 RESULTS

6.4.1 Experiment 1: Bacterial growth in agar- Disk diffusion test

An area of seven to 10 mm diameter of complete inhibition (which coincided with the spread of the suspended bismuth subnitrate when wet disks were placed on the agar) was present around disks in the *S. uberis* SR115 cultures in the three different concentrations (Figure 6.1A). Control (NC) disks showed no inhibition for any of the strains tested.

The two *S. aureus* strains (SA3971/59 and SA1) and *E. coli* P17.14291 on agar plates presented an area of partial inhibition (less dense colony growth around the disks) (Figure 6.1B- *E. coli*) and a dark brown area around the disks that was larger in higher concentrations (Figure 6.2), although complete inhibition was not observed for these bacteria. Brown areas around the disks varied with concentration within each *S. aureus* strain (SA1 [65 mg/mL] vs. SA1 [390 mg/mL] p= 0.0048, SA [65 mg/mL] vs. SA [390 mg/mL], p= 0.0186). However, paired comparisons showed no difference between strains.

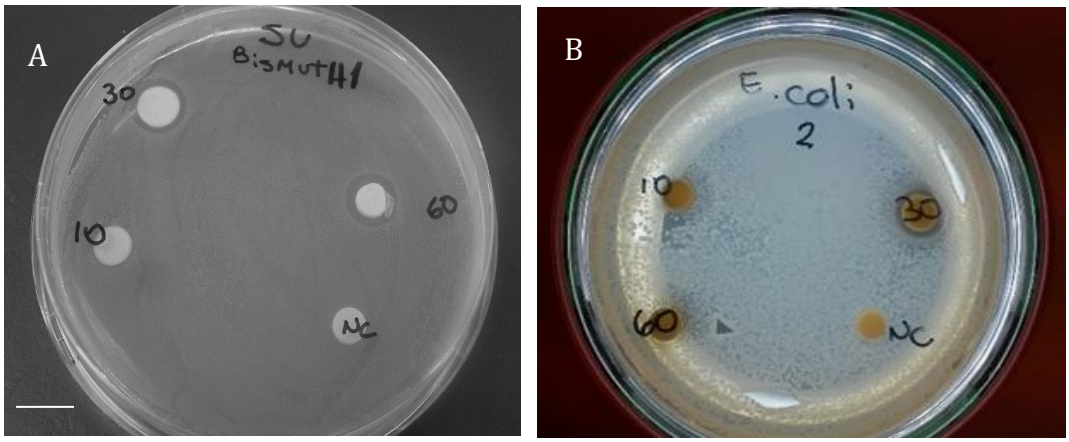


Figure 6.1 Experiment 1. Panel A: Complete inhibition of growth of 7-10 mm around the disks of a TSA plate cultured with *Streptococcus uberis*. Panel B: E. coli P17.14291 showing an area of less dense colony growth around the disks. 10= 65 mg/mL, 30= 195 mg/mL, and 60= 390 mg/mL of bismuth subnitrate, NC= disks immersed in sterile distilled water. Reference line showing 10 mm

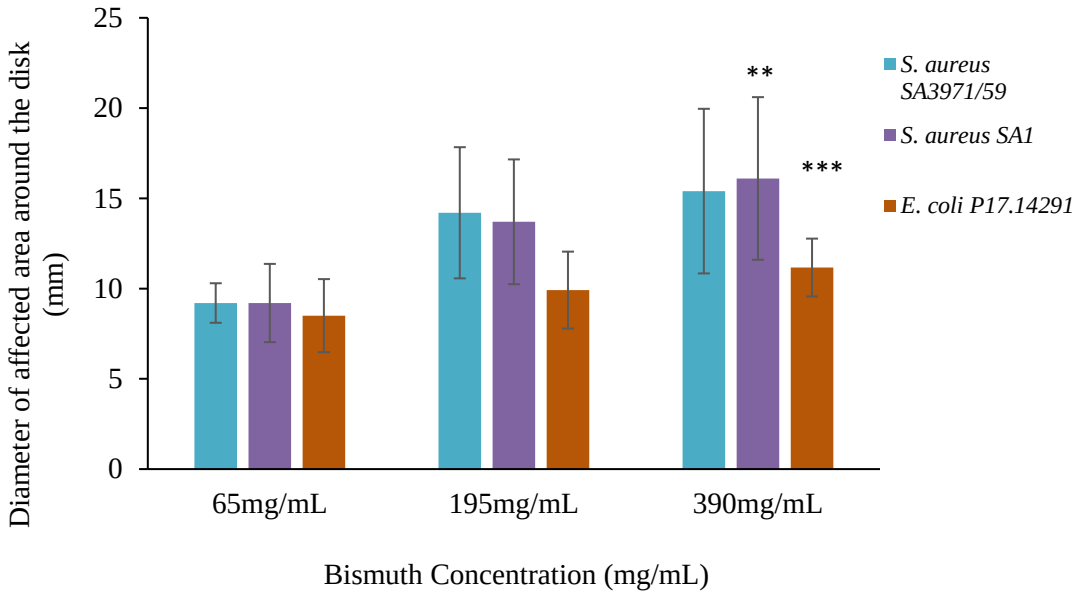
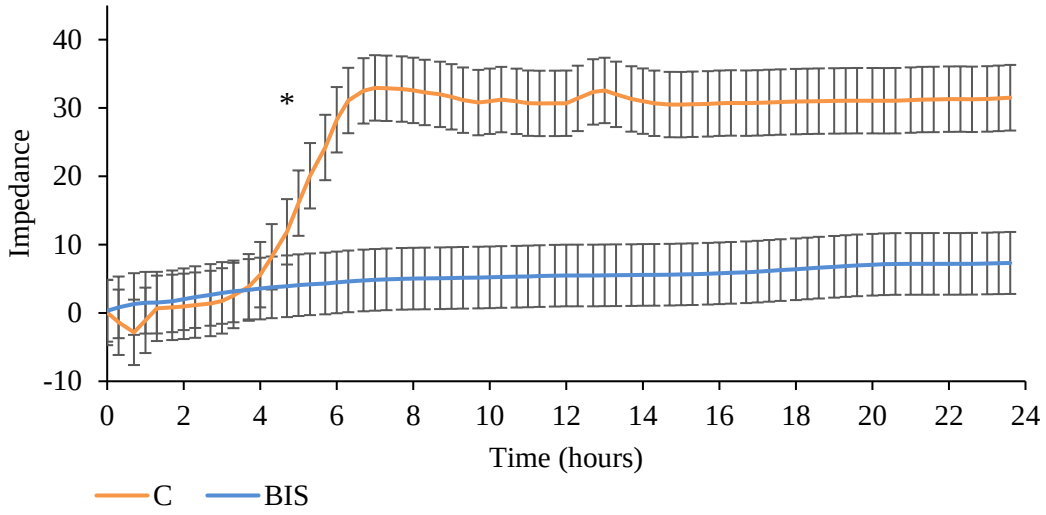


Figure 6.2. Experiment 1: Diameter of the area (± SD) affected by bismuth subnitrate in the six mm disk diffusion test. *S. aureus* SA3971/59, SA1 and *E. coli* P17.14291. Results represent areas of dark coloration around the disk. n= 5. Dark areas around the disks varied with concentration within each *S. aureus* strains (p<0.01= **, p<0.001=*)**

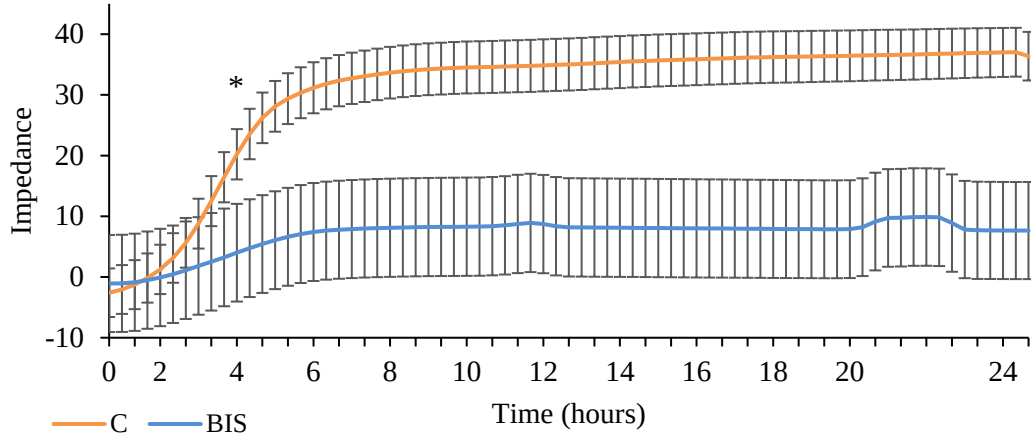
6.4.2 Experiment 2: Bacterial growth in a fluid medium

The impedance of the media was measured using the BacTrac 4300 microbiological growth analyser. Growth curves showed lower impedance levels for all the strains cultured in a TSB medium when supplemented with 32.5 mg/mL bismuth subnitrate as compared to the control growth curve (Figure 6.3). Vials that showed incomplete reads due to having out of range values during the 24 hours culture were removed from the experiment. From the 24 vials per strain analysed, seven vials of *S. uberis* SR115, nine of *S. aureus* SA3971/59, six of *S. aureus* SA1 and eight of *E. coli* P17.14291 were removed from the analysis. The results of the impedance test showed that inhibition of bacterial growth was not complete. Changes in impedance levels occurred indicating bacterial growth. There was evidence of growth in the first 10 hours of culture in the BIS treatment group, although lower than C vials for the duration of the experiment. Significant differences between BIS and C vials were detected after four hours in SA and SA1, five hours in SR115, SA3971/59 and after 10 hours in *E. coli* P17.14291 (Figure 6.3). Colony-forming unit counts ±SD were assessed before (0 h) and after (24 h) incubation for six selected vials per strain (Table 6.1). Plate counts indicated that there was reduced bacterial growth in the presence of bismuth subnitrate at the provided concentrations after 24 hours of culture for the four bacterial strains tested as compared to the non-bismuth supplemented group (t-test, $p<0.001$).

A)


40
30
20
10
0
-10
Impedance
*
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (hours)
C
BIS

B)


40
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Impedance
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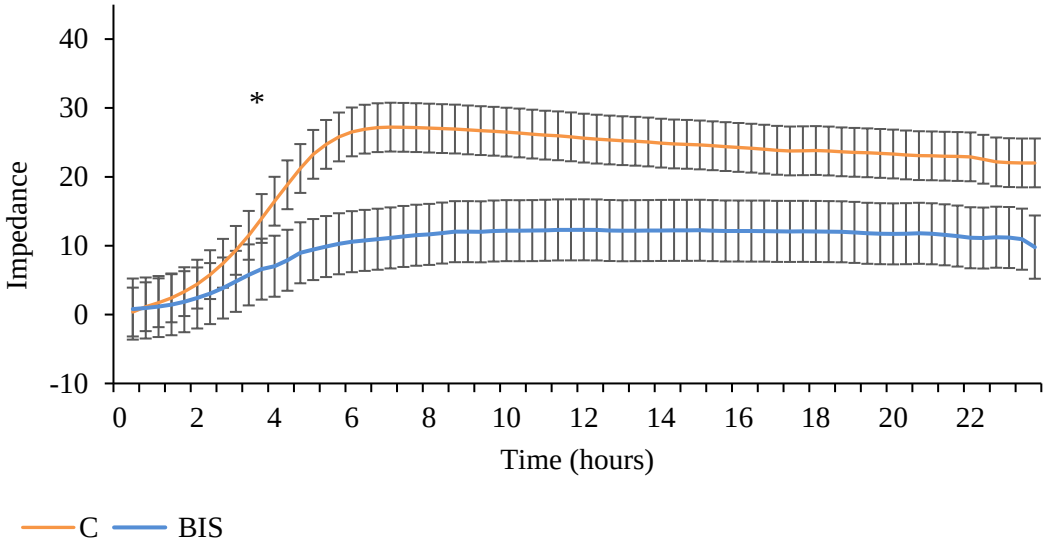
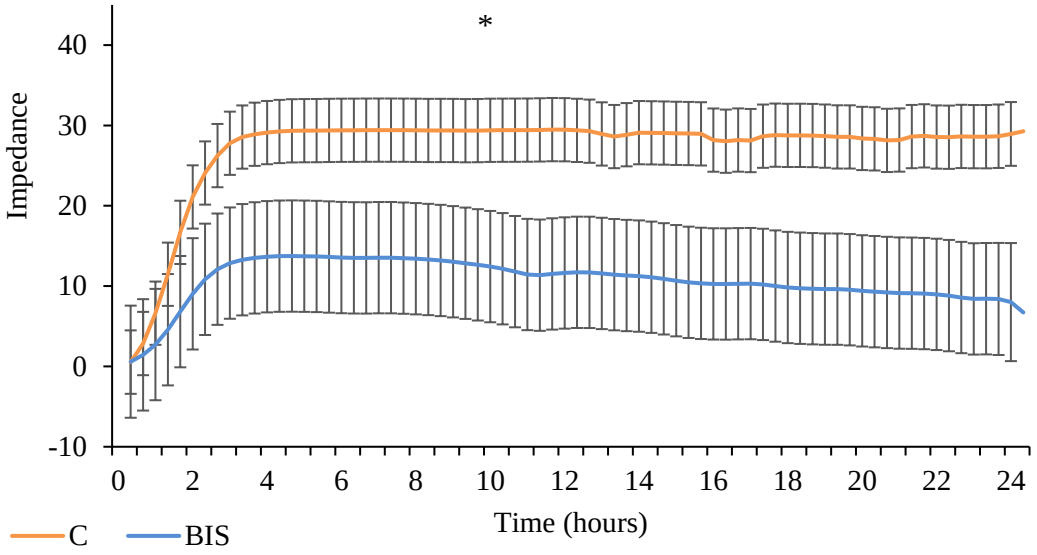
Time (hours)
C
BIS



C)



D)

Figure 6.3. Experiment 2: Least square means estimates of the impedance (± 95%CI) measured in BacTrac 4300. Bacteria were cultured in TSB with (BIS) and without bismuth (C) for 24 h at 37° C in four repeats of triplicates per treatment. A) *S. uberis* SR115 (BIS n= 9, C n= 8); B) *S. aureus* SA3971/59 (BIS n= 6, C n= 9); C) *S. aureus* SA1 (BIS n= 7, C n= 11); D) *E.coli* P17.14291 (BIS n=7, C n= 9). N= number of vials which had readable results from BacTrac 4300. Asterisks indicate the first time-point of difference (p< 0.05).

Table 6.1 Experiment 2: Comparison of the mean (±SD) of the log2 conversion of colony-forming units (CFU)/mL counts for bismuth (BIS) vs. control (C) vials before (0 h) and after (24 h) incubation at 37° C for 24 h in BacTrac 4300. N= 6 per strain

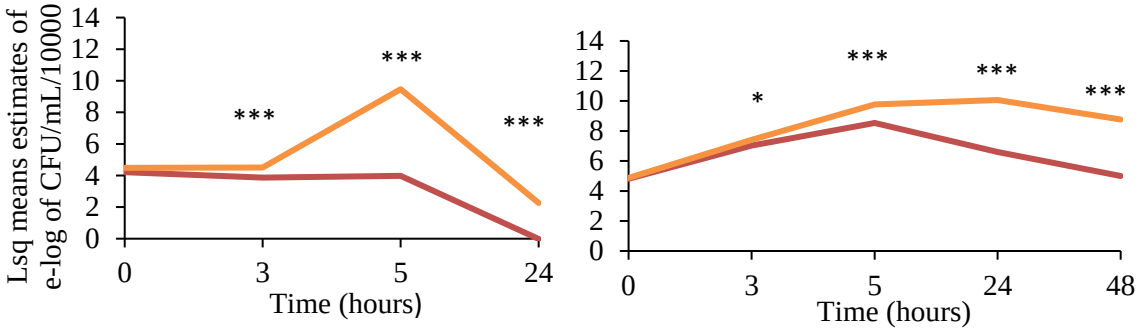
Strain	Treatment	0 h	24 h	Mean diff (±SD)	Paired t-test p-value
S. uberis SR115	BIS	18.8 (±0.5)	12.6 (±1.7)	-6.2 (±0.8)	0.0015
	C	19.9 (±0.1)	26.4 (±0.4)	6.5 (±0.37)	<0.0001
	T-test p-value			<0.0001	
S. aureus SA3971/59	BIS	19.97 (±0.1)	22.18 (±0.3)	2.2 (±0.5)	0.0002
	C	20.08 (±0.4)	27.15 (±0.2)	7.03 (±0.4)	<0.0001
	T-test p-value			<0.0001	
S. aureus SA1	BIS	20.19 (±0.09)	18.24 (±2.15)	-1.95 (±2.2)	0.08
	C	20.12 (±0.09)	25.62 (±0.2)	5.49 (±0.1)	<0.0001
	T-test p-value			<0.0001	
E. coli P17.14291	BIS	20.06 (±0.6)	18.44 (±0.7)	-1.61 (±0.7)	0.0036
	C	20.24 (±0.2)	26.52 (±0.4)	6.28 (±0.5)	<0.0001
	T-test p-value			<0.0001	

6.4.3 Experiment 3: Bacterial growth in a shaken fluid medium

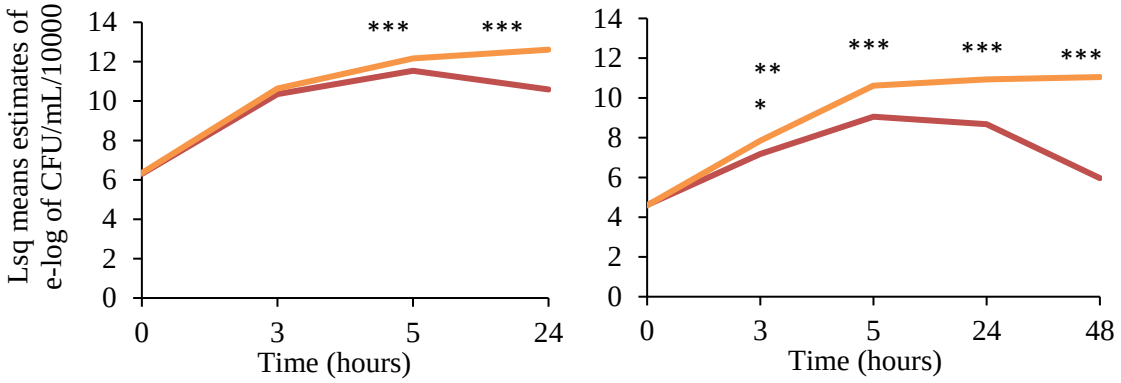
Colony-forming units in TSB containing bismuth (BIS) and control (C) groups were counted at four and five-time points over a 24 and 48 h period for Trials 1 and 2, respectively. Results showed significant growth inhibition by bismuth subnitrate from 3 h after the commencement of the culturing period (Figure 6.4). Trials 1 and 2 showed a very similar response to bismuth with increased inhibition after 24 hours for strains SR115, SA3971/59 and *E. coli* but a resumption in growth for strain SA1

(Figure 6.4, panel C). The difference between BIS and C flasks remained significant for most of the experiment in both trials.

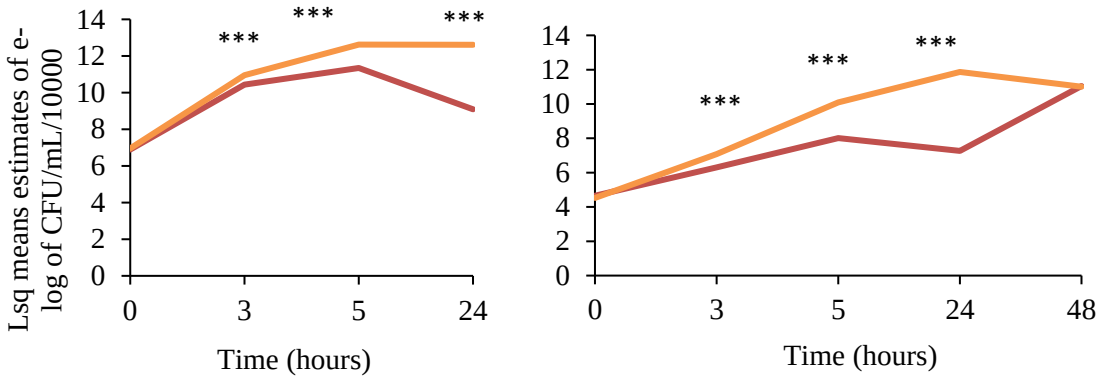
A)



B)



C)



D)

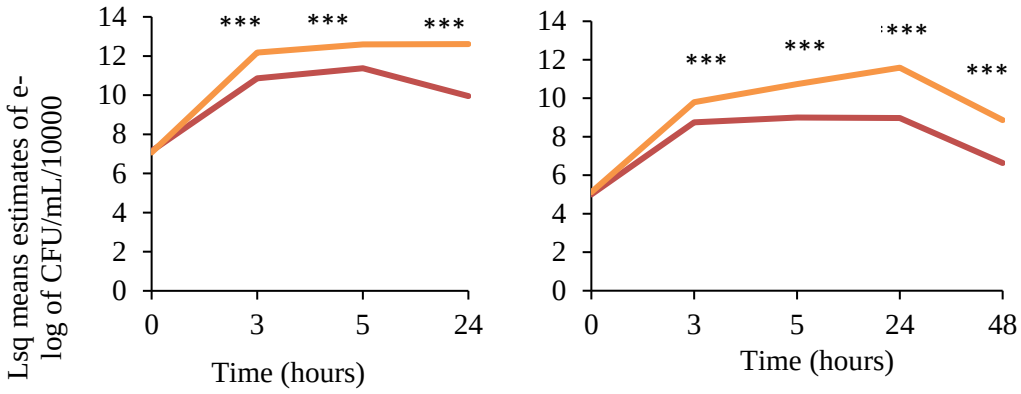


Figure 6.4. Least square means estimates of e-log CFU/mL/10.000 for Trial 1 (left) and 2 (right) after culture at 37° C in shaking mode at 200 rpm for 24 and 48 hours, respectively. A) *S. uberis* SR115, B) *S. aureus* SA3971/59, C) *S. aureus 1*, D) *E. coli* P17.14291. Significance level p< 0.05*; p< 0.0001*. — Bismuth supplemented flasks (BIS), — non-supplemented flasks (C).**

6.5 DISCUSSION

Teat sealants containing bismuth subnitrate are described as inert non-antibiotic products that act as a physical barrier to the colonization of bacteria through the TC during the dry period (Meaney et al., 2001, Codex, 2016). However, our results support the concept that bismuth subnitrate slows bacterial growth *in vitro*. This is, to our knowledge, the first description of a potential inhibitory effect of bismuth subnitrate on mastitis-causing agents relevant to the dairy industry.

Streptococcus uberis is one of the main mastitis causing microorganisms during the dry period, particularly in New Zealand (Williamson et al., 1995, McDougall, 2003). The results of the disk diffusion test in the present study (Experiment 1) show that *S. uberis* SR115 appeared to be more susceptible to bismuth subnitrate than *S. aureus* (SA3971/59 and SA1) or *E. coli* (P17.1429), demonstrated by an area of complete inhibition. In the same test, *S. aureus* and *E. coli* presented an apparent concentration-

dependent dark area around the disks, but not complete inhibition as for *S. uberis*. The dark area observed for *S. aureus* and *E. coli* could be explained by the fact that both strains are able to hydrolyse sulphide (Tomasova et al., 2016). The hydrolysis of sulphide with the addition of bismuth, forms bismuth-3 sulphide, a black pigment produced by some bacteria in the mouth and lower intestines (Ioffreda et al., 2001). There are other examples in which bacteria in conjunction with bismuth can form bismuth-3 sulphide. The "black spot defect" found in cheddar cheese is produced by bismuth-3 sulphide and has been linked to the presence of bismuth subnitrate in the milk (Lay et al., 2007). The production of bismuth-3 sulphide could hence have caused the dark area around the disk in the disk diffusion test of this study. However, the presence of sulphide in the TSA plates was not tested. The disk diffusion test in this study was used as a screening tool; other traditional methods for antimicrobial testing such as the use of Muller Hinton plates that could be used in dose-dependent antimicrobial testing for bismuth subnitrate were not utilized.

Bismuth subnitrate is a heavy metal compound with low solubility that precipitates in fluid media, thus it does not readily diffuse in agar. After observing the results of the disk diffusion test, two more experiments were designed to reassess the inhibitory effect of bismuth subnitrate that could have been masked by the minimum spread of the bismuth suspension observed on the surface of the agar plates in Experiment 1. Due to marked precipitation of bismuth subnitrate observed in Experiment 2, the third experiment was added. In the BacTrac and shaking media studies (Experiments 2 and 3), bacterial growth was significantly reduced by the presence of bismuth in the culture media. There was no complete inhibition or killing of bacteria, but the results demonstrate a suppression of bacterial growth. Even

though a low number of BacTrac vials could not be included in the analysis due to a fault in some of the electrodes that showed out of range readings, the results presented in this study show a significant difference in bacterial growth between control and bismuth-supplemented vials. No physical explanation was found for the out of range readings. Bismuth precipitation over the base of the tube where the electrodes are placed was hypothesised to affect functionality, and this was a reason to design Experiment 3 (shaking media). However, literature reflects that it is not unusual to find out of range measurements when measuring impedance (Colvin and Sherris, 1977). Ideal curve shapes that are meaningful for the purposes of the study were needed and determined before the study (Chen and Chang, 1994). *Streptococcus uberis* was the strain most influenced by the effect of bismuth subnitrate in the 3 experiments presented here. In Experiments 1 and 3, *S. uberis* showed complete inhibition after the 24-hour culture with bismuth subnitrate. This could be due to its high sensitivity to bismuth subnitrate, or to inconsistent growth rates found *in vitro* in some *S. uberis* strains that are highly dependent on nutrient availability (Leigh and Field, 1991).

In Experiment 3, significant differences between supplemented and non-supplemented flasks were observed after three hours of culturing for all the strains. Experiment 2 produced significant differences after four or five hours for most of the strains except for *E. coli*. For the latter, significant differences between BIS and C vials were found 10 hours after the culture began. The different nature of the studies and the differences in the statistical analyses performed for each of the experiments might have produced these time differences within the *E. coli* strain. The different growth rates of the four strains observed in this study support the concept of high variability in growth patterns and response to treatment of different pathogens (Keane, 2019).

Staphylococcus aureus is usually associated with persistent IMI that may be present throughout the dry period (Pankey et al., 1982, Barkema et al., 2006). In contrast, *E.coli* and *S. uberis* tend to induce IMI or clinical mastitis cases that either cure spontaneously or for which the use of antimicrobials at dry off is usually effective (Todhunter et al., 1995, Keane, 2019). *Staphylococcus aureus* 1 resumed growth after 24 hours in Experiment 3, the origin of this resumption in growth could not be determined. This *in vitro* behaviour may suggest adaptability of this strain to an adverse environment that could be relevant to the *in vivo* situation. This is the reason why identification of the pathogen and a treatment that targets the bacterial agent (selective DCT) could improve cure rates during the dry period (Bradley and Green, 2001, Barkema et al., 2006). A limitation of this study is the absence of a positive control for the inhibitory effect. Experiments 2 and 3 would have benefited from having the impedance curve and CFU counts obtained for vials with a known inhibitory agent.

The current results are in line with human medical and dentistry *in vitro* research studies showing the inhibitory effect of bismuth salts (mainly bismuth subsalicylate, but also bismuth subnitrate) (Domenico et al., 1997, Athanikar, 1998, Lin et al., 2011, Vega-Jiménez et al., 2012). Bismuth-based products are used to treat different diseases: syphilis, gastric ulcers, traveller's diarrhoea, and other pathologies caused mainly by bacteria (Marshall et al., 1987, Slikkerveer and de Wolff, 1989, Phillips et al., 2000).

The mechanism of action by which bismuth subnitrate slows bacterial growth is unknown. A few different hypotheses could explain this effect including: an oxidizing effect, interfering with bacterial metabolism by reducing ATP synthesis, enzyme inhibition, and inhibition of biofilm formation, among others (Sox and Olson, 1989,

Zhang et al., 2006, Folsom et al., 2011). As an oxidizing heavy metal, the action bismuth might be like that of other metals such as silver, zinc oxide (ZnO) or copper, which are known for their antimicrobial and antifungal effects (Kim et al., 2007, Pasquet et al., 2014). For example, the antimicrobial effect of ZnO is due to the production of reactive oxygen species, the destabilization of microbial membranes when in contact with ZnO particles, and the antimicrobial properties of $Zn2^{+}$ ions released by ZnO in an aqueous medium (Pasquet et al., 2014). ATP synthesis reduction in bacterial cells could be due to either a direct effect on bacterial metabolism or interference with the extracellular membrane function inhibiting the transport of nutrients (Sox and Olson, 1989, Sadler et al., 1999). Bismuth has also been shown to inhibit ureases, which are essential in some bacteria, and thus, it interferes with vital bacterial metabolism that causes bacterial death. However, *S. uberis* is a urease negative bacterium and showed high susceptibility to bismuth in the present study, suggesting that the action mechanism of bismuth, in this case, could be different. Bismuth compounds were also found to be effective at inhibiting and disrupting biofilm formation (Folsom et al., 2011, Hernandez-Delgadillo et al., 2012). The four strains utilized here were non-biofilm formers when cultured in TSB (Sarah Chia Jia Ning, personal communication); hence, this might not be the reason for the inhibition of growth observed in this study. It is not known whether biofilm inhibition by bismuth subnitrate teat sealants is an action mechanism that happens *in vivo.*

The *in vitro* results are in part supported by the findings of several *in vivo* studies. In a study of nulliparous heifers, the infusion of bismuth subnitrate before parturition reduced the prevalence of IMI after calving when compared to the pre-treatment infection levels, suggesting that bacterial growth might have slowed after

contact with bismuth-based teat sealants (Parker et al., 2007). Another study also reported a lower prevalence of coagulase-negative staphylococci (CNS) infections post-calving in a group of cows treated with ITS (Berry and Hillerton, 2002). Bismuth subnitrate in the ITS could have inhibited the growth of bacteria in the TC to undetectable levels, which would assist in preventing bacterial infection over the dry period. However, this requires experimental testing.

Other mechanisms of mastitis prevention by the bismuth-based teat sealants currently under investigation are stimulation of the immune response, formation of a physical barrier, and prevention of biofilm formation. Most studies comparing the efficacy of teat sealants with antibiotic DCT showed no difference in clinical mastitis incidence during the dry period and after calving (Woolford et al., 1998, Huxley et al., 2002). Interestingly, these studies state that the bismuth-based internal seal was as effective as an antibiotic in preventing new IMI over the dry period (Huxley et al., 2002, Bhutto et al., 2011, Rabiee and Lean, 2013). The current results show inhibition of bacterial growth by bismuth subnitrate that might at least partially explain these observations. The inhibitory effect of bismuth could prevent the colonization of the TC by mastitis pathogens during the dry period. However, in current studies, bismuth subnitrate was tested in a powder form and not within a teat sealant formulation that includes other excipients.

The results of this study provide impetus for the reconsideration of the definition of ITS as “inert”. The word inert might not accurately reflect all mechanisms contributing to the efficacy of the bismuth subnitrate based products. Further *in vitro* experiments are required before the observations reported herein can be applied *in vivo*. It would be important to assess the effect of bismuth subnitrate within the teat

sealant formulations on bacterial growth at different concentrations and with different pathogens, such as CNS and different *Streptococci* as these organisms are highly prevalent throughout the dry period in dairy cows and heifers (Green et al., 2002, Parker et al., 2008).

6.6 CONCLUSION

The results obtained in these *in vitro* experiments show that bismuth subnitrate slowed bacterial growth of four major mastitis-causing agents to different degrees. This suggests that the efficacy of bismuth subnitrate-based teat sealants in preventing clinical mastitis may at least in part be due to a reduction in growth of the major mastitis-causing agents, particularly *S. uberis*, in the TC, during the early stages of IMI in the dry period. In addition to the barrier effect described in previous works, the presence of an inhibitory effect in bismuth subnitrate formulations might be crucial for their proven high efficacy. It may also indicate that new intramammary methods to prevent mastitis over the dry period will require inhibitory substances in addition to barrier components.

6.7 ACKNOWLEDGMENTS

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7 Cellular response of neutrophils to bismuth subnitrate and micronized keratin products *in vitro*

Various mechanisms of mastitis prevention by the bismuth-based teat sealants are currently under investigation. In this Chapter, the effect of bismuth subnitrate and micronized keratin on bovine neutrophils was assessed *in vitro*, with the hypothesis that recruitment and activation of neutrophils into the teat canal (TC) and sinus are one of the likely mechanisms of action of these teat sealant formulations.

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Cellular response of neutrophils to bismuth subnitrate and micronized keratin products *in vitro*. S. Notcovich, N.B. Williamson, J. Yapura, Y.H Schukken, C. Heuer

Cellular response of neutrophils to bismuth subnitrate and micronized keratin products *in vitro*

7.1 ABSTRACT

The aim of this study was to assess the effect of bismuth subnitrate and micronized keratin on bovine neutrophils *in vitro*. The hypothesis is that recruitment into the teat canal (TC) and sinus (TS) and activation of neutrophils are the mechanisms of action of bismuth subnitrate and a novel keratin-based teat sealant formulation. To test this, a chemotaxis assay (Experiment 1) and a myeloperoxidase (MPO) assay (Experiment 2) were conducted *in vitro*. Blood was sampled from 12 mid-lactation dairy cows of variable ages. Neutrophils were extracted and diluted to obtain cell suspensions of approximately 10^6 cells/mL. In Experiment 1, test substances were placed in a 96-well plate, separated from the cell suspension by a 3µm pore membrane and incubated for 3 hours to allow neutrophils to migrate through the membrane. In Experiment 2, neutrophils were exposed to the test products and the amount of MPO released was measured by optical density. Results showed that neutrophils were not activated by bismuth or keratin products ($p<0.05$) in both tests performed. These results suggest that the mechanisms of action of bismuth subnitrate and keratin-based teat sealants do not rely on neutrophil recruitment and activation in the TC and TS after treatment.

7.2 INTRODUCTION

Methods to treat and prevent mastitis in the dry period have been available since the middle of the last century (Pearson and Wright, 1969, Wilkinson, 1969). The current and most used methods are teat sealants and dry cow antibiotic therapy. The use of these formulations reduced the incidences of intramammary infections in the dry period and early lactation in treated compared to non-treated quarters (Huxley et al., 2002). Teat sealants containing bismuth subnitrate were developed in the 1970s and are effective at preventing new dry period infections in challenge and natural exposure studies (Meaney, 1977, Woolford et al., 1998). The proposed mechanism of action of bismuth-based products is the creation of a physical barrier that prevents the passage of mastitis pathogens into the teats, but this has not been shown experimentally despite their proven efficacy.

The TC is the duct that connects the lumen of the udder with the external environment. It is lined by modified teat skin. Macrophages, Langerhans, plasma, and dendritic cells were described under the basal membrane in the TC epithelial tissue. They are dedicated cells that take up, process, and present antigens to T-cells in their major histocompatibility complex (MHC) receptors during infections (Collins et al., 1986, Bassel and Caswell, 2018, Smolenski, 2018). Neutrophils are polymorphonuclear cells (PMN), which are the first active line of defence against pathogens entering the mammary gland. The surveillance role carried out by PMN consists mainly of their ability to migrate to the site of infection (chemotaxis), respiratory burst, and myeloperoxidase (MPO) release. During phagocytosis, neutrophils increase their oxygen consumption through the activity of NADPH-oxidase, and via successive electron reductions generate superoxide anion O_2^- and

hydrogen peroxide (H_2O_2) (Dahlgren and Karlsson, 1999, Van Acker and Coenye, 2017). This process is known as the respiratory burst. These oxygen metabolites activate additional reactive oxygen species (ROS) that are strongly anti-microbial, for example, MPO. Myeloperoxidase is an enzyme present in azurophilic granules in neutrophils and performs a vital role in destroying phagocytosed microorganisms. During mastitis, neutrophils migrate from blood at the Fürstenberg's rosette and TS into the teat lumen, following chemotactic signals released from these antigen presenter cells, bacterial invaders or damaged tissue (Nickerson and Pankey, 1984, Paape et al., 2002). The presence of high somatic cell counts (SCC) in milk has a protective effect against mastitis due to major pathogens (Erskine et al., 1988, Rainard and Poutrel, 1988, Lam et al., 1997). The presence of neutrophils in milk is considered valuable in preventing infection by major mastitis pathogens, especially during the early dry period and after treatment with internal teat sealants (ITS). However, most of the reviewed literature shows that an increase in SCC (PMN, macrophages, and desquamating epithelial tissue) are signs of intramammary infection (Dohoo and Leslie, 1991, Deluyker et al., 1993). A chemotactic effect of bismuth subnitrate based teat sealant has not been experimentally demonstrated. Based on this, another proposed mechanism of action of bismuth subnitrate based teat sealants is the generation of a local cellular immune response after treatment that protects against, and hypothetically, cures subclinical mastitis (Kimbrell et al., 2016). Similarly, a novel keratin-based teat sealant product under development showed, in preliminary studies, an increase in SCC after treatment suggestive of a mechanism of action related to the recruitment of neutrophils into the TC and TS (Williamson, 2012).

The hypothesis of this study is that bismuth subnitrate and keratin-based teat sealants induce chemotaxis and activation of neutrophils in the TC and TS and hence protect the mammary gland from mastitis infections during the early dry period. In order to test this hypothesis, the objective was to evaluate the ability of bismuth subnitrate and micronized keratin to induce a cellular response (migration and activation) *in vitro*.

7.3 MATERIALS AND METHODS

7.3.1 Blood collection and neutrophil preparation from peripheral blood

The study protocol was approved by the Massey University Animal Ethics Committee, protocol number 18/02.

This study compared the *in vitro* activation of neutrophils in response to bismuth subnitrate and micronized keratin to a negative control of media only. Fifty-millilitre blood samples were collected into EDTA tubes (BD-vacutainer K2E EDTA, BD-Plymouth, PL6 7BP, UK) by coccygeal venipuncture of 12 dairy cows at mid-lactation. Neutrophils were isolated from blood and the number of neutrophils per sample was determined using a TC20 automated cell counter, (Bio-Rad Laboratories, California 94547, USA). Neutrophil suspensions were used to complete chemotaxis assays and myeloperoxidase tests *in vitro*. Within one hour of blood collection, neutrophils were separated from whole blood samples. Briefly, approximately 50 mL of blood was transferred into two 50-mL conical tubes and centrifuged at 1,500 g for 30 min at room temperature. After centrifugation, the plasma, buffy coat layer, and top layer (approximately 1/3) of packed red blood cells were removed by aspirating with a Pasteur pipette. Approximately seven mL of blood were left in each tube and 38 mL

of Milli-Q (MQ) water was added to lyse the red blood cells. The tube was rotated for five seconds. Then, five mL of 10× concentrated PBS pH 7.4 was added and the tube immediately rotated again to restore osmotic balance for the cells. The tubes were centrifuged at 330 g for 10 min at room temperature (20-22 °C) and the supernatant discarded, leaving a neutrophil-rich red pellet at the bottom of each tube. The pellet was washed with 10 mL of 1× PBS pH 7.4 and vortexed to mix. The tubes were centrifuged for five min at 670 g at room temperature 20-22 °C, the supernatant was discarded, and the cells re-suspended in two mL of the media required in the procedures for each performed assay. Cell suspensions prepared using this procedure had ≥ 90% pure populations of living neutrophils confirmed by cell counting with trypan blue.

7.3.2 Chemotaxis assay (Experiment 1)

A chemotaxis assay was performed using a CytoSelect 96-well Cell Migration Assay (3 µm, Fluorometric Format, Cell Biolabs. Inc., San Diego, CA 92126, USA). A cell suspension containing approximately five x 10^6 cells/mL was prepared in serum free-media (RPMI 1640, R8758-500ML, Sigma, Christchurch, New Zealand) containing 0.5% bovine serum albumin (BSA). Foetal bovine serum (FBS) 10% was used as a chemotactic agent for positive control (PC) and 0.5% BSA-RPMI was used as the negative control (NC). Keratin and bismuth subnitrate suspensions were prepared as follows to create high and low concentrations (3% and 1.5%, respectively). These concentrations were chosen as 3% was the maximum concentration of bismuth subnitrate that could be manipulated with minimal precipitation of the suspension. For the keratin and bismuth suspensions in high concentrations (KH and BH treatments), 1.2 g of sterile micronized keratin or bismuth subnitrate were added to

40mL of RPMI. Keratin and bismuth at low concentrations (KL and BL treatments) were prepared with 0.6 g of micronized keratin or bismuth subnitrate in 40 mL of RPMI. In a cell culture chamber, 150 µL of KH, BH, KL, BL, the positive control and RPMI with 0.5% BSA used as the negative control (NC) were loaded in the feeder tray (bottom plate) in triplicates. A membrane chamber was placed on top of the feeder tray. One hundred microlitres of the cell suspension were added to each well and the plate was incubated in a cell culture incubator at 37ºC for 3 hours. The cell suspension from inside the membrane chamber was carefully discarded by inverting the plate and the insert chamber was transferred to a clean 96-well plate containing 150µL of pre-warmed Cell Detachment Solution. This plate was incubated for 30 minutes at 37^{o}C. Cells were completely dislodged from the underside of the membrane by gently tilting the insert several times in the Detachment Solution, and the insert was removed and discarded. Seventy-five microlitres of media from the feeder tray were combined with 75 µL of the Detachment Solution in a clean 96-well plate. Fifty microlitres of 4X Lysis Buffer/CyQuant GR dye solution was added to each well (already containing 150 µL of Cell Detachment Solution). This step combines cells that migrated through the membrane and into the medium and migratory cells that were detached from the bottom side of the membrane by the Cell Detachment Solution. The plate was incubated for 20 minutes at room temperature. One hundred and fifty µL of the mixture was transferred to a 96-well plate suitable to measure fluorescence. Fluorescence was measured using a fluorescence plate reader (Flexstation 3 by Molecular Devices, Biostrategy, Auckland, New Zealand) at 480 nm/520 nm (SoftMaxPro version 5.4.1 Molecular Devices, Biostrategy, Auckland, New Zealand). Fluorescence levels in this study were obtained as a result of the lysis of migrated cells in the wells at the bottom of the analysed plate.

7.3.3 Myeloperoxidase Assay (Experiment 2)

Myeloperoxidase (MPO) release was measured in a 96-well plate from Phorbol 12-myristate 13-acetate (PMA, positive control), keratin, or bismuth-stimulated neutrophils, from lysed neutrophils to account for the total amount of MPO contained within the neutrophils and from a negative control (NC) buffer. Isolation of neutrophils was performed as described above obtaining 2.5 x10^7 cells/mL of Hanks' Balanced Salt Solution (HBSS) [14025092, Life Technologies NZ Ltd, Auckland, New Zealand]. A 96-well plate (ELISA Microplate 96F, GR655061, Grenier Bio-one, International GmbH, Kremsmünster, Austria) was seeded with 50 µL/well of lysis reagent, 50 µL/well of stimulation reagent or 50 µL/well of bismuth or keratin in high concentration (3% BH, KH). Negative control wells contained 50 µL/well of HBSS. The lysis reagent contained 0.02% hexadecyltrimethylammonium bromide (Sigma) in MQ water. Stimulation reagent was prepared by mixing 1 part of CaI stock solution [50 µg/mL calcium ionophore A23187 (Sigma) in HBSS], 1 part of cytochalasin B stock solution [50 µg/mL cytochalasin B (Sigma) in HBSS], 1 part of PMA stock solution [20 µg/mL PMA (Sigma) in HBSS], and 7 parts of HBSS. Plates were incubated at 30°C for 60 min. 3,3′,5,5′-Tetramethylbenzidine (Becton Dickinson, Auckland, New Zealand) and hydrogen peroxide were mixed 1:1, and 100 µL of the mixture was added to each well. Colour was developed at room temperature for approximately 2 min and 50 µL of 2 N H_2SO_4 was added as a stop reagent. Plates were centrifuged at 600 × g for 5 min at room temperature, and 150 µL/well was transferred into a new plate. Optical density at 450 nm was determined using a microtiter plate spectrophotometer (Versamax, Molecular Devices, Sunnyvale, CA, USA).

7.3.4 Statistical analysis

The data from these studies were not normally distributed, so they were transformed by log2 transformation. Mixed linear models included treatment as a fixed effect and cow as a random effect in SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Statistical significance was set at $p<0.05$. Statistical differences were based on Tukey-Kramer adjusted pairwise comparison per product tested against the negative control.

7.4 RESULTS

7.4.1 Chemotaxis assay (Experiment 1)

Neutrophils did not show signs of increased chemotaxis when treated with B or K and compared to the negative control (Figure 7.1). Bismuth subnitrate in high (BH) and low concentrations (BL) showed significantly lower levels of chemotaxis than the negative control.

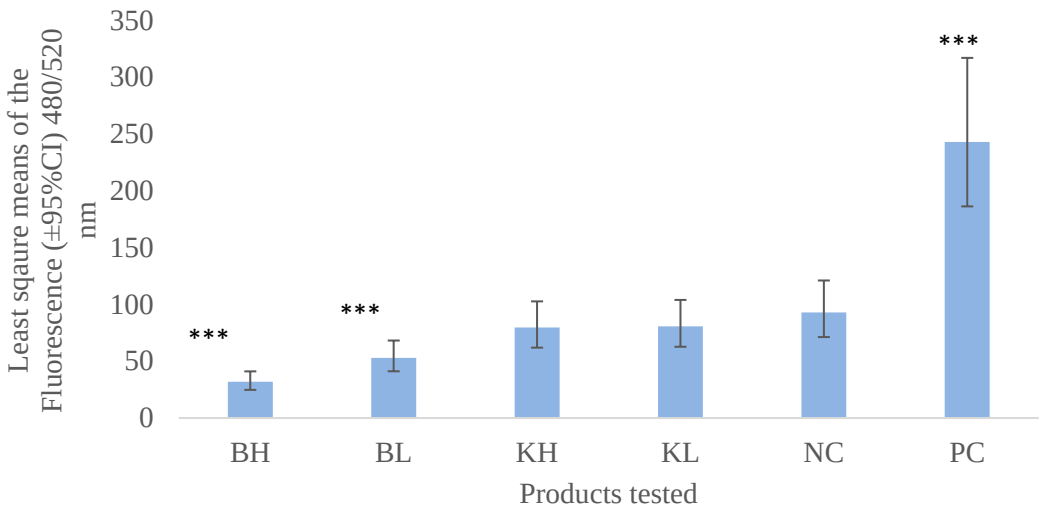

Figure 7.1 Least square means (± 95% CI) of the fluorescence values obtained from chemotaxis assay measured at 480/520 nm wavelength. BH = bismuth high [3%w/v], BL = bismuth low [1.5%w/v], KH = keratin high [3%], KL = keratin low [1.5%], NC = negative control [0.5% BSA-RPMI], PC = positive control (FBS). Asterisks show the significance levels of the comparison with the negative control. * means p-value <0.001.**

7.4.2 Myeloperoxidase Assay (Experiment 2)

Bismuth and keratin induced a lower release of MPO than the negative control ($p<0.001$, Figure 7.2). Negative control (NC) values represent approximately 50% of the values obtained by the total lysis of the neutrophils and their total MPO content (Figure 7.2, PC(lysis).

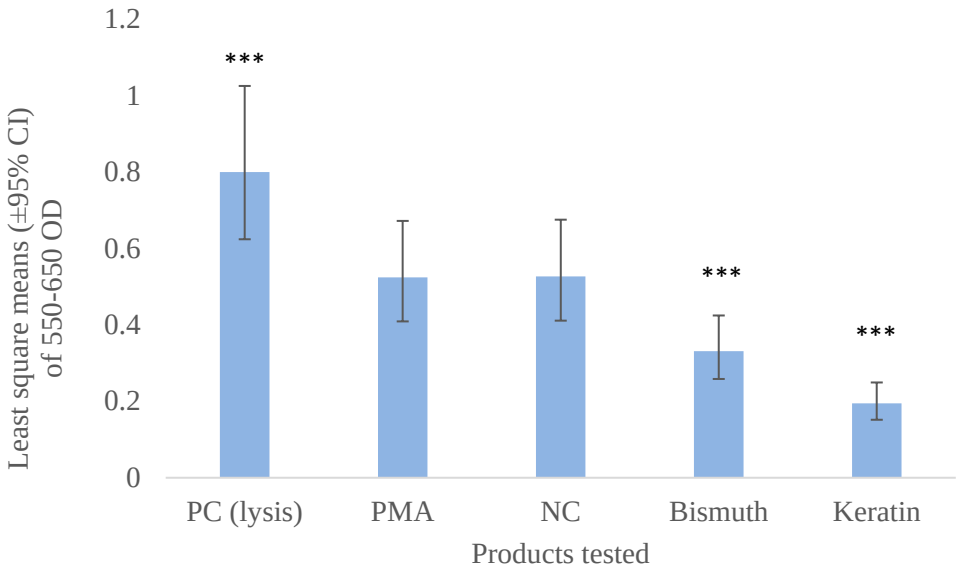

Figure 7.2 Least square means (±95% CI) of 550-650 optical density of neutrophils exposed to bismuth 3% and keratin 3%. HBSS was used as a negative control (NC) and 1 part of 20 µg/mL of PMA, 1 part of 50 µg/mL calcium ionophore, 1 part of 50 µg/mL cytochalasin B with 7 parts of HBSS was used as the positive control (PC-lysis). The lysis buffer contained 0.02% hexadecyltrimethylammonium bromide. * means p-value <0.001.**

7.5 DISCUSSION

In this study, the response of bovine neutrophils to bismuth subnitrate and micronized keratin products *in vitro* was investigated. The hypothesis that these products activate neutrophils by inducing chemotaxis and MPO release *in vitro* was rejected. The results show that neutrophils were not activated by the test compounds *in vitro*. This appears to be the first published study to evaluate *in vitro* the cellular response as a potential mechanism of action of bismuth subnitrate and the novel micronized keratin compound.

The objective of Experiment 1 was to evaluate *in vitro* the chemotactic effect of bismuth subnitrate and micronized keratin on bovine neutrophils. Previous studies have proposed that the mechanism of action of bismuth subnitrate might be the

activation of immune cells in the TC (Kimbrell et al., 2016 [non-peer reviewed], Lanctôt et al., 2017). Bismuth subnitrate used in ITS, with or without concomitant antimicrobial treatment, prevented and cured mastitis by generating an increase in neutrophil concentration and cytokine expression in the mammary gland (Kimbrell et al., 2016). These results showed that bismuth subnitrate treated teats had an increased number of neutrophils in mammary gland secretions after treatment and a significant increase in the TNF-α:IL-8 ratio. However, care must be taken when analysing these results as the study had its limitations. The increase in neutrophils occurred in all treated teats whether treated with DCT or bismuth. Hence, there was no evidence to conclude that bismuth subnitrate induced neutrophil migration. In addition, the study did not describe how teat sealants were applied to teats. Any substance infused into the mammary gland may induce an immune response mainly involving PMN. Constricting the teat sinus at the base of the teat when infusing ITS is important, as it avoids the products being infused into the mammary tissue and inducing a large cellular response. The application of a chitosan-based teat sealant product, when compared with a bismuth-based teat sealant and an untreated control, did not show differences in neutrophil concentration or in markers of inflammation in the mammary gland secretions of treated cows (Lanctôt et al., 2017). This supports a lack of chemotactic effect *in vitro* from bismuth subnitrate and chitosan as was shown in the current study for bismuth subnitrate and micronized keratin. Current results align with others who found that chemotaxis of macrophages was inhibited by tripotassium dicitrato bismuthate (a bismuth-based treatment for stomach ulcers), thus showing a putative anti-inflammatory effect of bismuth-based formulations (Soutar and Coghill, 1986).

A small percentage of neutrophils migrated through the 3 μm membrane (NC was different from zero). This could have been caused by random migration through the membrane or simply by gravity, which has been reported previously (Roth et al., 2001). The significantly lower values of chemotaxis shown by the bismuth-exposed neutrophils compared to the NC could be an indication that bismuth subnitrate prevented or inhibited the migration of neutrophils through the membrane, potentially explained by bismuth subnitrate being a charged molecule. Chemotaxis starts by depolarization of the neutrophil's membrane followed by a period of hyperpolarization (Gallin, 1980). Bismuth subnitrate charged molecules may interact with the neutrophil membranes to prevent depolarization or prolong the hyperpolarization period, thus impeding the passage of the neutrophils through the 3 μm pores. It is also possible that bismuth subnitrate particles blocked the pores, as in our study BH (bismuth in high concentration, (3%) showed lower levels of chemotaxis than BL (1.5%). In further research, a different experimental design would be required to demonstrate such mechanisms. There was no difference between the results of keratin in high and low concentrations and the negative control in this study, suggesting that keratin did not induce chemotaxis *in vitro*. This is contrary to what was reported in a previous *in vivo* study, where an increase in SCC was present after introducing a micronized keratin product into the TC (Williamson, 2012 [non-peer reviewed]).

Myeloperoxidase release from neutrophils exposed to bismuth and keratin was significantly lower than from the NC that were not exposed. A major limitation of this study was that the proposed positive control (PMA) failed to activate the neutrophils. Exposure of neutrophils to cytochalasin B, a fungal metabolite, blocks the

polymerization of contractile microfilaments and, consequently, facilitates degranulation (Follin et al., 1991). However, even though neutrophils were treated with cytochalasin B and PMA in this study, they did not show signs of MPO being released in the positive control wells. Although in this study PMA-stimulated cells were not significantly different from the NC, the MPO value of the cells that were lysed (thus releasing 100% of the MPO content in the cells) was considered a valid positive control. All the other values, therefore, can be presented as a proportion of this 100% MPO content since all showed significantly lower levels of MPO release (Bachoual et al., 2011). Since bismuth and keratin presented significantly lower levels of MPO release than the PC-lysis and NC it can be confidently concluded that there was no increased activation of neutrophils. The lack of reaction to the PMA could be due to an early activation and exhaustion of MPO within the cells prior to the conduct of the study due to manipulation as has been reported in previous studies (Easmon et al., 1980, Washburn et al., 1982).

There is evidence that bismuth compounds (specifically bismuth subsalicylate and bismuth subgallate) have anti-inflammatory effects. Bismuth subgallate inhibited nitric oxide production in macrophages by inhibiting the mRNA expression and stability of the enzyme nitric oxide synthases (Lin et al., 2004). In addition, a reduction in mononuclear cells and neutrophilic inflammation was observed in the histological analysis of patients with microscopic colitis after treatment with bismuth subsalicylate (Fine and Lee, 1998). This could explain the low values of MPO observed in this study for the bismuth exposed neutrophils. Historically, bismuth subnitrate was used by intramuscular injection for the treatment of syphilis (Hopkins, 1923). If bismuth subnitrate had induced chemotaxis and activation of neutrophils when injected

intramuscularly, the site of injection would have been swollen and inflamed. However, no reports occur on bismuth subnitrate causing an inflammatory reaction at the injection site although this was not included as an endpoint of interest by the authors (Ghadially and Yong, 1976). In agreement with the results obtained in this *in vitro* study where bismuth subnitrate did not induce chemotaxis or MPO release and activation after treatment, bismuth subnitrate is classified as an "inert product" when inserted into the teat sinus within a teat sealant product during the early dry period (Codex, 2016).

Studies on the effect of keratin products on the TC are lacking. Micronized keratin is currently used in the human cosmetic industry and there have been no reports of inflammatory reactions after treatment of skin with micronized or hydrolysed keratin. Furthermore, in various studies conducted with micronized wool keratin applied to human skin, researchers found that the keratin formulations reinforced the skin barrier integrity and improved its water-holding capacity (Fischer et al., 2001, Barba et al., 2008). This supports our findings of low levels of activation and lack of chemotaxis for keratin exposed neutrophils.

The results obtained in this study should be complemented by *in vivo* experiments in which chemotaxis and activation of neutrophils within the TC are assessed. Differences between *in vivo* and *in vitro* study results cannot be ignored, especially when dealing with inflammatory cell responses such as the mechanisms of activation of neutrophils that were addressed in this study (Persson et al., 1993).

These results provide impetus for the continuing investigation of the mechanism of action of teat sealant treatments. For example, "what are the effects of bismuth subnitrate and keratin *in vivo* when inserted in the TC?" and "can bismuth or

keratin prime neutrophils?" i.e. can bismuth or keratin take neutrophils to a more responsive state without activating them? These questions remain unanswered. The results of this study strongly suggest that a mechanism of action of bismuth subnitrate and micronized keratin is not the activation of a local immune response in the TS and TC.

7.6 CONCLUSIONS

Results of the described *in vitro* experiments indicated that bismuth and keratin products did not induce chemotaxis nor activate neutrophils. Further *in vivo* research is recommended to address questions on the mechanisms of action of bismuth subnitrate and micronized keratin when utilized as ITS for prevention of intramammary infections.

7.7 ACKNOWLEDGMENTS

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8 Effects of bismuth and keratin-based teat sealant formulations on the epithelia of the teat canal at dry off

Evidence that teat sealants do not act solely as physical barriers to the invasion of mastitis pathogens has been presented in Chapters 6 and 7. The putative action of teat sealants on improving natural keratin plug formation in the teat canal (TC) has been proposed in previous studies and it is tested herein.

In this chapter the effect of the intramammary application of bismuth subnitrate and keratin-based teat sealant formulations on gene expression of markers of proliferation and other physiological characteristics of the TC epithelium were examined.

Effects of bismuth and keratin-based teat sealant formulations on the epithelia of the teat canal at dry off

8.1 ABSTRACT

The objective of this study was to evaluate the effect of administration of two internal teat sealant (ITS) formulations on the closure of the teat canal (TC) and keratin plug formation. Eighty quarters (20 cows) on Day 0 of the dry period were randomly assigned to treatments (bismuth subnitrate-based teat seal, keratin-based formulation, and non-treated control) with or without biopsy sampling (6 groups in total). Curette biopsy samples from TC were obtained immediately before (Day 0) and 7 days after treatment (Day 7). On Day 7 of the dry period, the teats were scored for closure of the TC (0= closed; 1= open) before biopsy sampling. RNA was extracted from the biopsy samples and gene expression was compared between ITS-treated and non-treated control samples using Nanostring (NanoString Technologies Inc., Seattle, WA). The thickness of the *stratum granulosum* and occlusion percentage of the TC with endogenous keratin were measured and compared histologically using TC samples obtained post-mortem on Day 21. Two genes related to proliferation pathways were upregulated after treatment. However, the closure of the TC, thickness of the *stratum granulosum* and keratin coverage of the lumen of the TC did not differ between treatments. In conclusion, bismuth subnitrate and keratin-based ITS induced the gene expression of genes related to proliferative functions; however, an enhanced natural keratin plug formation was not observed during the early dry period in this study.

8.2 INTRODUCTION

The incidence of intramammary infections (IMI) and clinical mastitis over the early dry period have been reported to be up to 10 times the incidence during lactation (Smith et al., 1985, Bradley and Green, 2004) and delayed keratin plug formation might facilitate their occurrence (Dingwell, 2002). Bismuth-based teat sealants have long been used to prevent mastitis during the dry period (Meaney, 1977, Huxley et al., 2002, Dufour et al., 2019). The use of internal teat sealants (ITS) achieved 27 to 89% reduction in the incidence of IMI at calving when applied to glands uninfected at drying off (Meaney, 1977, Berry and Hillerton, 2002). Teat sealants are registered as physical barriers to the entry of mastitis pathogens, but this mechanism of action has not been tested experimentally.

Neuropathies, osteoarthropathies and other pathological conditions have been associated with toxicity from bismuth subnitrate and other bismuth-based salts ingested in high doses in humans (Bes et al., 1976, Murray, 1979, Emile et al., 1981). This suggests that the wide use of bismuth subnitrate in the dairy industry might not be innocuous for human or animal health. Bismuth subnitrate has also been identified to cause faults in the industrial processing of milk by inducing a “black spot defect” in cheddar cheeses (Lay et al., 2007). However, due to its high efficacy in preventing IMI and its non-antibiotic nature, bismuth subnitrate based teat sealants are currently one of the few alternatives to antimicrobial use to reduce the incidence of IMI during the dry period and early lactation.

It has been suggested that teat sealants improve natural keratin plug formation in the teat canal (TC), which has been used to promote the use of the product

(Woolford et al., 1998). Products to induce keratin plug formation would be desirable if they reduced the time to seal the TC after drying off and thus reduced the incidence of IMI during that period. Keratin has been used in dressings and creams for wound healing and skin restoration in human medicine (Barba et al., 2008, Loan et al., 2016). Exogenous keratins applied to injured skin accelerated healing in epithelial tissues and induced the expression of genes markers of proliferation (Pechter et al., 2012, Wang et al., 2012). However, the stimulation of keratin plug formation in the TC by teat sealant has not been tested experimentally. In this study the primary hypothesis was that keratin and bismuth subnitrate can accelerate the natural keratin plug formation. The objective of this study was to evaluate the proliferation status of the TC epithelium and keratin plug formation in response to treatment with bismuth and keratin-based teat infusions in the early dry period. A novel teat infusion formulation based on keratin was investigated in the current work as a natural alternative to bismuth subnitrate.

8.3 MATERIALS AND METHODS

8.3.1 Study design

Thirty-nine non-pregnant dairy cows destined for culling participated in the pre-screening procedure of this study 14 days before it commenced. The study was conducted at the Massey University Dairy Number 4, Palmerston North, New Zealand. Body condition and udder palpation scores, rapid mastitis test (RMT), body temperature and aseptically collected milk samples obtained for culturing were assessed in the pre-selected cows. Twenty cows were selected, and all 80 quarters were randomly assigned to six groups. Clinical examination and a second milk

sampling for culture of the 20 selected cows was carried out seven days before study commencement to confirm the health status of the quarters. Cows selected for the study had four functional quarters, no chaps or cracks in the teats, no antimicrobial therapy in the 14 days before commencement of the study, had a mean (±SD) composite milk somatic cell count (SCC) 37.600 (±27.000) cells/mL at the latest herd test and a mean score 0-1 in the RMT performed on Day 0 of the study (drying off day). The mean (±SD) body condition score of the cows participating in the study was 4.2 (± 0.3, NZ BCS scale). All cows selected for the study had negative cultures, or isolation of minor pathogen bacteria e.g. coagulase negative staphylococci (CNS) from all quarters.

Sample size was calculated based on the mean and SD of the normalised total counts of the expression of seven genes, and from the thickness of the *stratum granulosum* obtained in previous pilot studies. Calculations assumed a 95% confidence limit ($\alpha = 0.05$) with 80% power ($\alpha = 20\%$). The critical range (i.e. the difference 'L' to be shown significant) and the SD were read from these pilot data. L and SD were approximately equal resulting in a required sample size of 13-14 teats per treatment. The 80 teats of the 20 cows selected for the study were assigned by systematic random allocation to the following six groups: bismuth (B), keratin (K), non-treated negative control (NC), plug control-keratin (PC-K), plug control-bismuth (PC-B), and plug control-negative control (PC-NC) using online randomising list software (https://www.random.org/lists/). Plug control teats were not sampled using a curette, whereas the other groups were biopsied on Day 0 and Day 7 (Table 8.1). All quarters had an equal chance to be allocated to any group. No treatment was assigned to more than one quarter within a cow. In addition, the participating cows were randomly assigned to one of two replicates: Replicate 1 (n=10) and Replicate 2 (n=10)

using the same online tool. The sampling procedure was completed over two days, processing one replicate per day.

Table 8.1 Treatment groups. All four teats of the 20 cows participating in the study were randomly assigned to one of six groups.

Group Abbreviation	Group	Curette sampling on Days 0 and 7	Treatment
K	Keratin	YES	Keratin
B	Bismuth	YES	Bismuth
NC	Negative Control	YES	No treatment
PC-K	Plug control- Keratin	NO	Keratin
PC-B	Plug control- Bismuth	NO	Bismuth
PC-NC	Plug control- Negative control	NO	No treatment

8.3.2 In vivo sampling 1

A complete clinical examination was conducted before sampling, approximately one hour after the morning milking on the last day of lactation (Day 0). Each replicate of cows (n=10) in its sampling day were tranquilized via injection of 0.01 mg/kg of Xylazine 2% into the caudal vein. The teat ends were thoroughly cleaned, rubber tourniquets were applied to the base of the teat barrel and five millilitres of local anaesthetic solution (2% lidocaine) infused into the teat cistern through the streak canal using pre-filled mastitis treatment syringes. The local anaesthetic solution was stripped from the cistern after approximately 30 seconds and the teat ends were disinfected with a cotton swab moistened with methylated spirit. A sample of the cranial aspect of the TC epithelium (±1.5 mm width and ±5 mm long) was obtained with a curette (Volkman curette, Veterinary instrumentation, [782205], UK), from the B, K, and NC assigned teats. The samples were then immersed in 1.5 mL

of an aqueous, non-toxic tissue and cell storage reagent that stabilizes and protects cellular RNA in intact, unfrozen tissue and cell samples until processed for RNA extraction (RNA later- Sigma, Merk, Auckland, New Zealand). Immediately after sampling, all four quarters were cleaned with methylated-spirit dampened swabs and were treated with a cloxacillin-based intramammary dry cow therapy (DCT) (DryClox DC, Bayer Animal health, Auckland NZ). The product was massaged upward in the udder following manufacturer's instructions. After DCT administration, the teat ends were disinfected again and one of the intramammary study treatments applied into each teat following the treatment allocation list. Syringes of K and B products were infused into their assigned quarters with one hand whilst the other hand of the operator constricted the base of the teat to avoid the product being inserted beyond the teat sinus (TS). After application, the operator slightly pressed the teat until a drop of product was observed at the teat orifice. This action aimed to assure that the product was covering the TC as well as the lower portion of the TS. Quarters were treated in the following order: front right (FR), front left (FL), rear right (RR), and rear left (RL). After treatment, all the teats were immersed in an iodine-based teat dip (Kontact, De Laval Ltd, Hamilton, New Zealand). General health assessment and udder condition scoring were carried out 3-4 days after sampling and teat dip reapplied. Cows scoring two or more in the udder palpation score were treated with 2.5 mL/100 kg of an anti-inflammatory subcutaneously (Metacam, Boehringer Ingelheim Animal Health, Georgia, USA).

8.3.3 Teat closure scoring

On Day 7, all teats were examined to assess the formation of a keratin plug and scored using a binomial scoring scale. The assessment method consisted of applying light pressure to the TS using a gentle milking action with the thumb and index finger. If secretion was expelled from the orifice, the teat was classified as open (Score 1). If no secretion was observed, the teat was classified as closed (Score 0) (Williamson et al., 1995). The worker scoring the teats was blinded to treatment.

8.3.4 In vivo sampling 2

After scoring on Day 7, teats in groups K, B and NC were biopsied again, this time from the caudal aspect of the TC, using the technique described above. Sampling was followed by an iodine-based teat dip application onto all four teats.

8.3.5 Histological analysis of ex vivo samples

At the end of the DCT withholding period (21 days), all cows were slaughtered on-farm. The base of the teats was constricted using tightly pulled plastic seals to avoid leakage of milk, and the lower third of the teats were detached using a scalpel blade and snap frozen in liquid nitrogen to be processed for histology. Only teats from PC-NC, PC-K, and PC-B were included in this analysis as they had not been sampled with the curette and the epithelial tissue was intact. Five μm thickness sections from the snap-frozen tissue were mounted on slides and stained with haematoxylin-eosin. The slides were photographed using cellSense Dimension 1.5 software (Olympus Corporation, Tokyo, Japan). The thickness of the *stratum granulosum* of the TC was measured in μm. Measurements were obtained from four locations of the TC

circumference: up, down, left, right in the micrographs. Six different slides were prepared from each teat.

The coverage percentage of the TC lumen was measured using a photo editor (https://www.photopea.com/) and calculated as follows:

$$C=TA - (No\text{-}Cov_1 + No\text{-}Cov_2 + No\text{-}Cov_3 + No\text{-}Cov_n)$$

$$CP= (C/TA)*100$$

Where C is the area covered by keratin in the TC, TA is the total area of the TC, $No\text{-}Cov_n$ are the non-covered areas, CP is the percentage of the TC covered by keratin. All the parameters were measured in pixels.

8.3.6 RNA- Nanostring analysis

RNA from the TC biopsy samples was extracted using RNA easy Mini kit, (Qiagen, Cat. No. 74104, Venlo, Netherlands) following the manufacturer's instructions and preserved at -80^{o}C until processed. Eighty samples were processed for gene expression analysis using the nCounter Analysis System (NanoString Technologies Inc., Seattle, WA) with the objective of measuring the expression levels of 42 chosen genes at two time-points: before and seven days after treatment. The use of NanoString technology enabled direct RNA expression analysis from purified RNA extracted from freshly collected samples without further amplification. Samples were analysed using a CodeSet consisting of probes specific for 42 genes from *Bos taurus*. Two plates (Teat canal C and D) containing different gene combinations were analysed. Additionally, three reference housekeeping genes (HK) genes were included in each plate (i.e. *YWHAZ, RPL15*, and *GUSB*) based on published recommendations (Heiser et al., 2018). Teat canal C contained the following genes: *ATF3, BAZ1A, CSRP2, FAM71A, FLRT3,*

FOSB, GCLC, HSPA1A, HSPA6, HSPB8, KIF23, LOC107131159, MKI67, RFX1, NR4A3, RFX2, RNF182, SYT4, TFPI2, TRPS1, ZFAND2A. Teat canal D contained: *IRX3, KRT14, KRT16, KRT17, KRT18, KRT2, KRT35, KRT4, KRT6C, KRT8, MARCKS, MT1E, PADI1, PENK, PKIB, RASSF9, RBBP8NL, RND3, SERPINA1, SYT17, TLR5.*

8.3.7 Statistical analysis

The effect of treatment on keratin plug formation by teat scoring on Day 7 was assessed as a binary response (1= open; 0= closed) and analysed by logistic regression (PROC GENMOD, SAS 9.3; SAS for Windows, SAS, Cary, CA, 2018). The model included the fixed effect of group production of milk solids per day and accounted for the variability of the repeated measures per cow using Generalised Estimating Equations (GEE) in SAS. Milk production had no effect on the results and was removed from the final model.

The measurement of *stratum granulosum* thickness was replicated six times for each teat and converted to a natural log. The effect of treatment over the thickness of the *stratum granulosum* in the TC was analysed by a linear mixed model with treatment as fixed and treatment nested in cow as random effects.

The percentage of occlusion of the TC lumen (cov_perc) was measured six times per teat for each treatment. Data were not normally distributed and thus were logit transformed using the formula: log(cov_perc/100/(1-cov_perc/100)). This transformed data set was analysed using a mixed linear model with treatment nested in cow as random effect to assess the effect of treatment on the coverage percentage of the TC lumen.

A mixed linear model was used to assess the effect of treatment over the difference in gene expression before and after treatment. Firstly, the arithmetic difference between the gene expression of each gene before and after treatment was calculated as diff= gene.exp 2 (after treatment) - gene.exp 1 (before treatment). The first model included the difference between treatments and the difference in least square means calculated. The second model included the fixed effect of time and treatment, the random effect of cow and the interaction between treatment and time. Statistical significance was set at $p<0.05$ for all tests.

8.4 RESULTS

8.4.1 Teat score day 7

The teats of all cows were scored on Day 7 to assess keratin plug formation and the closure status of the TC after treatment. The binomial scoring scale was set as 0 = closed teat, no secretion emerging from the teat orifice, and 1 = open teat, milk secretion emerging from the orifice. Results are summarised in Table 8.2. When the treatments were evaluated independently from cow and quarter, more closed teats were found after treatment with bismuth subnitrate (n= 13/26) than with keratin (7/28) or non-treated teats (n= 7/26).

Table 8.2 Summary of the number of closed (Score 0) or open teats (Score 1) seven days after treatment

Treatment	Closed	Open	%open	Total
Bismuth	13	13	50%	26
Keratin	7	21	75%	28
Negative control	7	19	73%	26
Total	27	53	66%	80

The analysis of the results also showed that the probability of finding an open teat seven days after treatment was highly influenced by biopsy sampling for all the teats independently of the treatment. Teats sampled by curette on Day 0 were 22% more likely to be open than teats that were not sampled (mean 77% sampled vs. mean 55% non-sampled Table 8.3). Bismuth treatment reduced the probability of having open teats, however non-significantly. No significant difference was observed between treatments.

Table 8.3 Least square means estimates of teat canal scoring on Day 7 after treatment. Scoring scale: 0 = Closed teat, no secretion emerging from the teat. 1= Open teat, milk secretion emerging from the teat orifice. %-open = Likelihood of finding an open teat

Group	Sample	Estimate	Standard Error	Z Value	Pr > z|	%-open
Bismuth	Yes	0.53	0.58	0.93	0.3548	63%
Keratin	Yes	1.69	0.71	2.37	0.0179	84%
Negative Control	Yes	1.68	0.75	2.23	0.0257	84%
PC-B	No	-0.47	0.57	-0.83	0.4039	38%
PC-K	No	0.57	0.56	1.02	0.3066	64%
PC-NC	No	0.52	0.57	0.92	0.3591	63%

8.4.2 Granulosum stratum thickness and occlusion percentage

The thickness of the *granulosum stratum* was measured in the photomicrographs of the teats that were not sampled by curette (PC-NC, PC-K and PC-B). The average thickness of the *granulosum stratum* was calculated from the average of up, down, left and right measurements from the six slides produced from the frozen teat samples (Figure 8.1-A). Mean thickness of the *granulosum stratum* was 222.6 µm,

ranging between 126.5 and 347.5 µm. The *granulosum stratum* thickness did not differ between treatments.

Mean occlusion percentage was 89.81% and ranged from 46.18 to 100%. Occlusion percentage did not differ between the treatments evaluated (Figure 8.1-B).

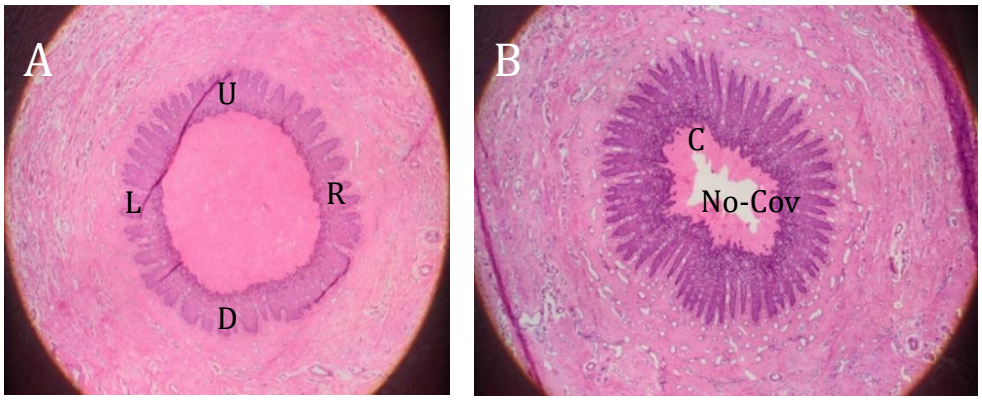

Figure 8.1 Photomicrographs of the TC slides (4x magnification). A) Measurement of the thickness of the teat canal *granulosum strata* U = up, D = down, R = right, L = left. B) Area covered by keratin= C and non-covered = No-Cov.

8.4.3 Gene expression

Gene expression of the TC before and after treatment was compared between B, K and NC samples. Genes KRT17 and KRT6A showed modified expression levels after treatment with K and B were. The latter one only showed modification in the expression levels after treatment with B (Table 8.4). Specific genes that were slightly but not significantly upregulated after treatment with K were of interest as they are markers of proliferation and immune response (*MKI67*), determine transcription factors (*BAZA1*) or participate in the keratinization process of the epithelium (*PADI1* and *PENK*).

Table 8.4 Estimated least square means difference of KRT6A and KRT17 (markers of proliferation) before and after treatment. NC: No treatment, B: Bismuth, K: Keratin-based teat sealants. Asterisks mean *: p<0.05, **: p<0.01.

Gene	TX	TX	Estimate difference	SE	DF	P-value	
KRT6A	B	K	0.09692	0.22	31	0.6626	
KRT6A	B	NC	0.4975	0.2201	31	0.0309	*
KRT6A	K	NC	0.4006	0.2151	31	0.072	
KRT17	B	K	0.0275	0.1347	31	0.8396	
KRT17	B	NC	0.3842	0.1347	31	0.0077	**
KRT17	K	NC	0.3567	0.1317	31	0.0109	**

8.5 DISCUSSION

Two teat sealants were tested to assess their effects on the epithelial tissue of the TC and on keratin plug formation. Teat canal closure, the thickness of the TC and sinus epithelia, keratin coverage, and expression levels of genes markers of proliferation were measured before and after treatment. Keratin and bismuth subnitrate teat sealant formulations did not enhance the formation of an endogenous keratin plug or TC closure, but upregulated some genes related to cell proliferation pathways.

The closure of the TC early in the dry period reduces the risk of new IMI in the dry and early lactation periods (Dingwell et al., 2004). Internal teat sealants use has increased during the last 30-50 years (Meaney, 1977). Without any interventions, 50 percent of TC remain open 6 weeks after drying off (Dingwell et al., 2004). Artificially sealing the TC has the advantage of preventing IMI during the pre-milking and early lactation times (Huxley et al., 2002, Parker et al., 2007). Although teat sealants

products are placed mainly in the TS and not in the TC, it has been suggested that teats treated with ITS formed a natural keratin plug after treatment (Woolford et al., 1998). Others found treated teats with DCT were more frequently closed than non-treated teats (Williamson et al., 1995). Our results contrast with those suggestions, as overall, no significant difference was detected between treated and non- treated teats when keratin plug formation and gene expression of markers of proliferation were measured, except for the upregulation of KRT6A and KRT17 after treatment. Bismuth-based treatment appeared to have impacted improving the closure of the TC based on scoring closure on Day 7 of this study, if the treatments were considered independently. However, the sample size (n= 13 per treatment) provided insufficient power to evaluate whether this difference was statistically significant when accounting for the random effect of cow.

Sample size calculation in this study was based on the thickness of the *granulosum stratum* of the TC and the gene expression of certain markers of proliferation obtained in previous studies (Chapter 3 and 4). Therefore, the sample size selected may not have been suitable for all the variables analysed in the study regarding treatment and teat closure. Similarly, in the current study, milk production did not affect keratin plug formation. However, other studies have demonstrated that milk production level influences the formation of a protective plug during dry off (Dingwell et al., 2004, Rajala-Schultz et al., 2005). Again, sample size or the low variation in milk production of the cows in the study could have limited these results.

Thickness of the *stratum granulosum* was described as a proxy to assess the proliferation status of an epithelial tissue, and it was used in this study to compare treatments (Chandler et al., 1969, Comalli et al., 1984, Paulrud, 2005). There is high

variability in the distribution of keratin (*corneum stratum*) through the TC lumen (Chandler et al., 1969). The results obtained in this study reflects that variability. Cows selected for the study were 3 to 13 years old and age might have affected the ability to form a keratin plug after drying off. Generally, older cows tend to have thinner *stratum corneum* in the TC than heifers (Gleeson et al., 2003). In addition, the cows in this study were non-pregnant cows. The influence of hormones during the formation of the keratin plug remains unknown.

Some genes appeared to have changes in expression after treatment (genes downregulated prior to treatment appeared to be upregulated after treatment). *KRT17* and *KRT6A*, which were upregulated after treatment are markers of proliferation. *KRT17* is a marker of proliferation as well as a regulator of the immune response of epithelial tissue (DePianto et al., 2010). However, most of the genes evaluated in this study remained unchanged, it cannot be assessed if the modification of expression of a pair of genes would have any clinical impact. The expression levels of *KRT16* was not modified by any treatment in this study. However, in a previous study (Pechter et al., 2012) keratin treatment induced and accelerated healing by increasing the expression of *KRT16* and *KRT6*. Both keratin genes are wound inducible keratins and change during cicatrisation (DePianto et al., 2010). Contrary to what has been found in studies on the involuting mammary gland, which showed a threefold increase in expression of *ATF3* when cows were shifted from a twice daily milking regime to once daily milking (Littlejohn et al., 2010), the current study showed that *ATF3* gene expression (which is a marker of cell growth and proliferation) is downregulated in the TC epithelium of an involuting TC and the treatments tested in this study did not affect this.

8.6 CONCLUSIONS

Bismuth and keratin-based treatments did not induce modifications in the TC epithelium that could be associated with stimulation of keratin plug formation. Although bismuth-treated quarters showed a decrease in being open after treatment, this was not different from keratin or untreated controls. The modification of gene expression observed in this study suggests that although the treatments induced modifications in two genes out of 42, this was insufficient to induce a phenotypic expression that could be assessed during the study. These results support previous studies that suggest that keratin plug formation is the result of an accumulation of keratin due to a lack of the flushing effect of milk rather than an active process of increased keratin synthesis.

9 General discussion

9.1 OVERVIEW

Previous research states that the keratin plug formed in the teat canal (TC) at the end of lactation acts as a physicochemical barrier throughout the dry period and obstructs bacterial colonization (Bitman et al., 1988, Bright et al., 1990). The physiological mechanisms involved in forming the keratin plug are still uncertain. The incidence of intramammary infections (IMI) in dairy cows during the early dry period is one of the highest of the lactation cycle, impacting significantly on the milk production of the following lactation (Green et al., 2002, Bradley and Green, 2004, Green et al., 2007). The main objectives of this thesis were to describe some of the physiological characteristics of the TC and keratin plug formation during the early dry period and to evaluate how they associate with the occurrence of IMI. A second objective was to make inferences, through *in vitro* and *in vivo* tests, about the probable alternative modes of action of current mastitis preventative methods (bismuth subnitrate based teat sealants) and a novel compound (micronized keratin) currently under investigation as a possible teat seal for preventing IMI during the early dry period.

Briefly, Chapters 3, 4 and 5 focus on the physiology and functions of the TC epithelium. Chapter 3 describes the development of a biopsy method and assesses the genetic makeup of the epithelial tissue in the TC after sampling in the early dry period by next generation sequencing (NGS). Chapter 4 examined the physiological characteristics of the TC during early involution and early lactation by two different gene sequencing methods. Chapter 5 reports on the physiology of the TC in the early

dry period after cows were challenged by dipping in a bacterial culture. The later study aimed to assess the response of the TC and TS epithelia to bacterial challenge and infection. Chapters 6 to 8 focussed on the mechanisms of action of some of the constituents of current teat sealants and of a novel formulation. In Chapter 6 the hypothesis that bismuth subnitrate has an antimicrobial effect was tested. Chapter 7 reported studies on the ability of both bismuth subnitrate and a novel micronized keratin product to induce a cellular immune response in bovine neutrophils. Chapter 8 evaluated the effect of the intramammary application of bismuth subnitrate and keratin-based internal teat sealants (ITS) formulations on gene expression, the thickness of the epithelial tissue and other characteristics of the TC. The objectives of the following sections are to report the general outcomes, conclusions and implications of the experiments conducted in this thesis and to integrate them with the current literature. Limitations are considered and future research recommendations are made. Finally, the main conclusion of this work is presented.

9.2 PHYSIOLOGY OF THE TEAT CANAL

A novel biopsy method developed as part of this work allowed serial samples of the TC to be obtained, yielding good quality mRNA which was analysed by genome sequencing. Downregulation of the gene pathways related to tissue proliferation and immune response during the early dry period was the main finding. These results agree with observations made in previous studies which describe a decrease in the mitotic index and thickness of the *stratum granulosum* in the TC after drying off (Comalli et al., 1984, Gleeson et al., 2003). The results also suggest that after undergoing a process of involution that includes the downregulation of certain

markers of proliferation and immune related metabolic pathways there is a reactivation of some of these genes after calving. Opposing these results, other studies examining the genetic makeup of the secretory tissue during early involution found the expression of some of the genes related to immune response pathways to be upregulated after drying off and downregulated after calving (Wilde et al., 1997, Finucane et al., 2008, Singh et al., 2008). Taken together, these observations suggest that the physiology of the TC and the mammary secretory tissues may differ in their physiology throughout the lactation cycle. The TC could be reducing its ability to prevent IMI; however, the mammary secretory tissue could be increasing its responsiveness to invading agents, possibly compensating the absence of the "cleaning function" of the flushing effect of milk or, alternatively, the increased expression of immune related genes observed could be related to the remodelling of the tissue during involution and not directly to the response to invading agents.

The biopsy method developed in this thesis, has advantages over previously developed methods to obtain samples from the TC (Bitman et al., 1988, Bright et al., 1990, Capuco et al., 1990), as it obtained epithelial tissue samples instead of lipid/proteinaceous desquamating tissue, while preserving animal wellbeing and inducing minimal harm to the TC. Other existing biopsy methods focus on samples of the mammary secretory tissue (Knight et al., 1992, Farr et al., 1996, De Vries et al., 2010, Cánovas et al., 2014) or from the udder and teat cisterns (Tulleners and Hamir, 1990). This is the first effective biopsy method developed exclusively for the TC. This novel method will allow the generation of new information that could be applied in the development of novel approaches to IMI prevention and treatments.

The physiological response of the TC to bacterial infections during early involution could not be determined in this thesis, as the bacterial strain utilised in the challenge model did not infect any of the challenged quarters. This removed the opportunity to observe differences in infected versus non-infected quarters; but since spontaneous infections with other pathogens did occur, the data from the study allowed hypotheses to be evaluated about the efficiency of the TC and the keratin plug to protect the mammary gland against bacterial infection and to report on the presence of normal bacterial microflora in the TC. It is possible that the strain utilised in the challenge model was not sufficiently virulent to cause mastitis because it probably was a non-host adapted bacteria (Tassi et al., 2013). However, further research would be necessary to confirm that. Previous research showed that the normal microbiota, also called microbiota reduces the growth of mastitis-causing agents (Woodward et al., 1987, Rainard, 2017). The cultures obtained from the TC showing non-pathogenic microorganisms, and in two quarters *S. uberis* different from the original strain, would support the notion that bacteria present in the TC during the early dry period might have intervened in the mechanisms of invasion of the challenge strain (Bouchard et al., 2018). The changes observed throughout the early dry period were mainly a reduction in the thickness of the *stratum granulosum* of the TC, colonization by non-pathogenic bacteria in the TC and teat sinus (TS) at Day 25, and a variation in the synthesis of cytokines that coincided with the presence of bacteria in the TC at different time points in the early dry period. These results support previous studies showing an increased susceptibility of the TC to bacterial colonization during the first days after drying off (Cousins et al., 1980, Bradley and Green, 2004). The present results showed that in addition to changes occurring in the secretory areas of the mammary gland observed by others (Sordillo et al., 1987, Sordillo and Nickerson, 1988, Wilde et al., 1997),

histologic, anatomic and physiological changes occur in the TC during involution (e.g. the thickness of the *granulosum* and *corneum stratum* decrease with time after drying off). It could be hypothesised that this is a phenotypical result of the genetic downregulations shown in previous chapters (Chapters 3 and 4).

9.3 ACTION MECHANISMS OF TEAT SEALANT FORMULATIONS

The mechanism of action of bismuth subnitrate-based ITS remains unknown. Thus far, a physical barrier effect against bacterial colonization is alleged to be the only mode of action of these formulations. Alternative formulations of teat sealants and intramammary devices developed previously exerted a physical barrier effect, elicited some immune response and a seemingly initial protective effect; however, they were unsuccessful in the long-term protection of cows against IMI and resulted in an increase in IMI (Poutrel et al., 1983, Paape et al., 1988, Nickerson et al., 1990, Serna-Cock and Pabón-Rodríguez, 2016). In order to find an answer to this incongruity, three *in vitro* tests were conducted to assess the effect of bismuth subnitrate on bacterial growth. Bismuth subnitrate inhibited the growth of environmental and contagious bacterial strains (Chapter 6). These results provide a different perspective on how new products to protect the mammary gland from IMI are to be developed in the future. An inhibitory effect might be necessary in future formulations to obtain a similar efficacy in IMI prevention observed with bismuth-based formulations. This study was conducted using *S. uberis*, *Staph. aureus*, and *E. coli* isolates which all originated from clinical cases occurring in NZ. The results obtained differ from peer reviewed studies and some of the experiments that support the patents of the ITS products, which mention the lack of inhibitory effect from ITS without presenting scientific evidence

on this statement (Berry and Hillerton, 2002, Huxley et al., 2002, Ahmadi et al., 2010, Bhutto et al., 2011, Compton et al., 2014). Most of the bismuth-based ITS formulations are patented as being "anti-infective free formulations" (e.g. Patent US 6,254,881 B1; Bradley 2007). The results obtained herein also suggest that in the case that ITS is confirmed as an inhibitor of bacterial growth in the TC, their registration might need to be reviewed in the future. In the study presented in this thesis, bismuth subnitrate showed to be inhibiting bacterial growth and this was proposed as a plausible mechanism of action for ITS formulations, however, this does not dismiss the possibility of alternative modes of action.

The induction of a local immune response in the teat after ITS treatment has been postulated by other authors, suggesting that bismuth-based ITS could protect cows against new infections by the recruitment and activation of neutrophils (Kimbrell et al., 2016). This hypothesis was tested *in vitro,* and the results showed that there was no cell response after exposing bovine neutrophils to bismuth subnitrate or keratin products (Chapter 7). Both bismuth and keratin products were unsuccessful attracting or activating bovine neutrophils *in vitro*. Previous i*n vitro* and *in vivo* studies using different animal models support our findings reporting a mild anti-inflammatory effect of bismuth salts in intestine mucosa of pigs and mice at different concentrations (Ericsson et al., 1990, Lin et al., 2004) and the absence of an inflammatory response to micronized keratin (Fischer et al., 2001, Barba et al., 2008, Hobbs et al., 2012). In another study, the TS response to bismuth-based ITS treatment compared with chitosan-based products was assessed. Bismuth-based ITS showed no effect on markers of inflammation or in the migration of inflammatory cells into the milk analysed after treatment (Lanctôt et al., 2017). In addition to the proposed

mechanisms of action of bismuth subnitrate mentioned so far, it has been suggested that a further mechanism of action of bismuth could be to stimulate keratin plug formation (Woolford et al., 1998). Many publications on keratin-derived formulations being applied to healing tissues reported to accelerate and improve wound healing (Loan et al., 2016, Nayak and Gupta, 2017). Hence, it was hypothesised in this work that bismuth subnitrate and keratin ITS formulations could modify the expression levels of genes markers of proliferation and consequently to enhance and accelerate the keratin plug formation at the end of lactation. However, bismuth subnitrate and keratin ITS formulations did not induce significant changes in gene expression for indicators of proliferation. Markers of proliferation and some keratin codifying genes of the TC epithelial tissue have demonstrated to be downregulated during the early dry period (Chapters 3 and 4). There were no differentially expressed genes in those studies that suggested that the TC epithelium tissue was comparable with a healing (proliferating) tissue. The lack of response of the TC epithelium to keratin and bismuth subnitrate (Chapter 8), could be explained by the fact that the TC was in a state of involution instead of proliferation unlike other tissues tested for the effect of keratin and other products on healing epithelial tissues (Kim et al., 2006, Patel et al., 2006, Poranki et al., 2014, Park et al., 2015). However, the expression of *KRT6A* and *KRT17* were stimulated by bismuth and keratin treatment. This could indicate an opportunity to investigate other substances that could modify the genetic message expressed by epithelial cells (Migliario et al., 2013, Prakoso et al., 2020). Inducing upregulation of genes related to cell proliferation and promoting cell growth in the TC epithelium, could result in the development of formulations that could serve as non- antimicrobial alternatives to the current teat sealant therapies in order to accelerate keratin plug formation and the sealing of the TC in dairy cows. The induction of certain functions

and protein expression is currently possible by mRNA therapy, as reviewed by Sahin et al. (2014) and could be used in the future for enhancing proliferation in the TC.

Bismuth subnitrate and keratin-based formulations were not able to modify the physiological characteristics of the TC epithelial tissue to a degree that could be phenotypically observed histologically or by the formation of a larger keratin plug at the end of lactation. Therefore, previously proposed mechanisms such as forming a physical barrier effect and the bacterial inhibitory effect of bismuth subnitrate confirmed in this thesis could be the main mechanism of action of ITS. Further *in vivo* experiments with the objective of testing commercial formulations are necessary to assess the effect of the currently used products.

9.4 METHODOLOGICAL CONSIDERATIONS

Statistical power is defined as the likelihood of a study to detect an effect when there is a real effect to be detected. The statistical power is affected by the effect size and sample size of an experiment. Effect size is a variable that is inherent to the biological mechanisms underlying the experiments and in many occasions is unknown before the experiment is performed, however, sample size is the variable that the researchers have influence on (Watson and Aviva, 2006). In Chapter 3, three TC biopsy samples from Day 0 and three from day 11 were analysed by NGS in Illumina. More replicates were desirable for this study but the cost per replicate made this prohibitive. However, with three biological replicates the differences between gene expression detected for the two sampling days had sufficient power to support the conclusions (Chapter 3) which were confirmed by an additional transcriptomics method, the Nanostring nCounter (Chapters 4 and 8). Another example of the importance of having

a proper sample size calculated based on previous data, are the results obtained in Chapter 8. Sample size for closeness of the TC, and gene expression, was calculated based on the results of the *stratum granulosum* thickness and the gene expression of 7 genes obtained in previous Chapters. Teat canal closure as measured in Chapter 8 has been demonstrated to have insufficient power for valid conclusions. Three hundred cows were assessed to determine the closure of the TC using a method like the one used in this study (Williamson et al., 1995, Dingwell et al., 2004). However, the inclusion of a larger number of cows in this study was technically and economically beyond the resources available for this study. Choosing the correct sample size for each study is usually a combination of financial, practical, ethical and statistical considerations (Watson and Aviva, 2006).

The virulence of a bacterial strain is critical in the development of a challenge model in mastitis research area (Notcovich et al., 2016). Considering that the bacterial strain utilised in Chapter 5 did not induce observable changes or colonisation of the TC of the challenged cows, performing a pilot study to assess the virulence of the bacterial strain before conducting the study reported in that chapter would have been beneficial. A model that was developed in NZ could have been repeated with different *S. uberis* strains, and by dipping teats in high and low bacterial concentrations (Notcovich et al., 2016). The assessment of the ability of different strains to colonise the mammary gland before carrying out the study would have increased the chances of selecting a strain with higher infectivity. A different and more effective method of challenging the cows could have also been used; however, inoculation into the TC (by the Newbold technique, consisting in inoculating the bacterial suspension in the lowest three mm of the TC) or into the mammary gland (by injecting the bacterial

suspension through the TC) are methods that bypass the TC barrier and would have not been useful for the purpose of this study. Considering that there was no confirmation that strain S325 utilised in this challenge model, was the same challenge strain utilised in Fernandez's study, other tests should have been performed to assess virulence and colonizing ability of strain S325. In addition to the methods mentioned above, DNA strain typing is recommended to assess the origin of the strain during the development of future challenge models.

Transferring *in vitro* test results to the *in vivo* environment is always challenging. Despite the inhibitory effect of bismuth subnitrate on bacterial growth being shown in this thesis *in vitro*, bismuth subnitrate should be evaluated *in vivo* and within an ITS formulation with the current paraffin excipient compound before firm conclusions about the mechanism of action of the current products can be made. However, the results contained in this thesis are valid as a potential explanation of the high efficacy obtained when using bismuth-based ITS products to prevent IMI infections during the early dry and early lactation periods. The inhibitory effect that bismuth subnitrate exhibited in our *in vitro* studies, differed from the reports that had shown that bismuth subnitrate, when included in the ITS products, did not demonstrate an inhibitory effect (US 6,254,881 B1, US6340469B1, Bradley 2007 non-peer reviewed). It is interesting to observe that the studies supporting most of the patents for bismuth-based teat sealants were performed using the ITS formulation (which include bismuth subnitrate and paraffin) in a disk diffusion test or a well diffusion test. From the experiments in Chapter 6 of this thesis, it was observed that bismuth subnitrate tends to precipitate and has little to no ability to diffuse. It is probably worth revisiting the inhibitory effect of bismuth-based ITS using other

culturing methods like the ones presented in Chapter 6 (Sections 6.3.3 and 6.3.4). Fluid media used in Chapter 6 do not rely on diffusion of the bismuth subnitrate to contact the formulation with the bacterial strain and could be useful in re-assessing the *in vitro* effect of ITS whole formulation in bacterial growth.

Studies involving manipulation of neutrophils present some technical difficulties, as the life spam of neutrophils is short once they are isolated from plasma and they are very susceptible to cell death and alteration in cellular functions (Kuijpers et al., 1991, Youssef et al., 1995, Tsinti et al., 2018). Two different groups of 12 cows were chosen for each experiment. Ideally, the same 12 cows should be sampled in two consecutive weeks for these studies, in order to reduce between-cow variation. This was not possible because the Cytoselect chemotaxis kit was faulty on delivery and needed to be replaced. This study was repeated 3 times in total. The first unsuccessful time was due to a failure in the neutrophil extraction protocol technique, which yielded a low number of neutrophils per millilitre. Once the protocol was modified, the Cytoselect chemotaxis kit was faulty on arrival and the results of the MPO study (that originally used a human-based kit) failed to provide reliable results. Finally, it was decided to repeat the MPO study with a laboratory-based protocol to avoid the use of a marketed kit. Phorbol Myristate Acetate (PMA) was used as a positive control to reveal the differences in MPO release by neutrophils after exposure to different substances; however, the positive control did not show significant differences from the negative control (buffer only). It is possible that the dose of PMA would need to be adjusted for any future studies. It was demonstrated that among the many changes induced by manipulation of neutrophils in *in vitro* studies, there is a measurable increase in resistance of neutrophils to PMA due to a modification in the expression of

certain cell membrane receptors and epitopes (Youssef et al., 1995). Also, it is possible that what was considered a negative control value was in fact a value obtained from neutrophils that were already activated during manipulation of the blood or during the preparation of the cell suspensions and hence they did not show an increase in response when undergoing the MPO assay (as they may have already exhausted their NADPH reserves) (Easmon et al., 1980, Washburn et al., 1982). In this study a significant difference between the positive and negative controls was not observed, but the results were still considered of value, as the MPO concentration measured by optical density (OD) of the treatments was compared with the Lysis value (the total amount of MPO contained in the neutrophils) and this allowed the validation of the results. In both tests, (chemotaxis and MPO) bismuth and keratin showed lower values of fluorescence and OD than the negative control respectively, suggesting that there was no increase in chemotaxis or activation of the bismuth and keratin treated cells.

9.5 RECOMMENDATIONS FOR FUTURE RESEARCH

The experiments reported in this thesis focused on two main areas: 1) the physiology of the TC during the early dry period and during bacterial challenge. 2) the action mechanism of bismuth and keratin-based teat sealants. Previous research proposed that physiological characteristics of individual cows (e.g. production levels, age, days in milk, etc.) could have an impact in their ability to seal the teat orifice at the end of lactation (Dingwell et al., 2004, Paulrud, 2005, De Prado-Taranilla et al., 2020). Because of the development of the novel biopsy sampling technique and the description of genomic changes observed in this thesis, the effect of some of these physiological characteristics on the keratin plug formation, can now be compared

between individual cows. In addition, the flushing effect of milk which has been hypothesised in many researches as detrimental to keratin plug formation (Bright et al., 1990, Capuco et al., 1992) can also be assessed in future studies.

The presented results support the notion that the flushing effect of milk induces keratin plug formation and its absence during the dry period diminishes it. This could be due to erosion of the most superficial layer of the epithelial tissue. However, more evidence is needed to support conclusions on this. Repeating the RNA sequencing following the model developed here and adapting the design to the needs of future questions could allow the discovery of functions or molecules affecting the keratin plug formation in the TC. For example, PADI1 is a gene that was upregulated during the early dry period in the several gene expression analysis presented in this work (Chapters 3, 4 and 8). The main cellular function of PADI1 is to deaminates filaggrin, it is involved in the late stages of epidermal differentiation and maintains hydration and function of the stratum corneum (Chavanas et al., 2006). This suggests that PADI1 could be considered as a marker of keratin plug formation for dairy cows and if confirmed as such, maybe would allow to identify cows with better ability to seal the TC after drying off and use it as a selection method in the future.

The biopsy method developed in this thesis can be applied to other research areas. Studies of the effect of the milking machine on the epithelial tissue of the teat, the study of the physiology of hyperkeratosis of the teat ends and observing which modifications occur in the TC in the process of hyperkeratosis, evaluation of factors interfering with keratin plug formation, could all benefit from the existence of this biopsy method in addition to the gene expression analyses presented herein. Due to the high quality of samples obtained and the anatomical areas that are sampled by the

curette sampling technique, studies that focus on the TC rather than on the Fürstenberg's rosette and TS will benefit most.

New management techniques and novel formulations may be developed to induce the generation of a more effective keratin plug that would protect the mammary gland from IMI. For example, high vacuum forces exerted over an epithelial tissue can induce hyperkeratosis (Neijenhuis et al., 2000, Zoche-Golob et al., 2015, Upton et al., 2016). Thus, vacuum could be used in a controlled manner to modify the TC epithelial tissue and to induce a better and faster formation of the keratin plug. It was demonstrated that the milking machine altered functioning (e.g. high vacuum, high pressure in the C-D phases), can also induce a modification of the keratin lining of the TC (hyperkeratosis) (Neijenhuis et al., 2000, Zoche-Golob et al., 2015). In human medicine, vacuum is utilised as a tool to accelerate healing and decrease infections in extensive wounds (Mu et al., 1997, Argenta et al., 2006, Morykwas et al., 2006). Studies that induce modification of the keratin lining of the TC utilising the vacuum of the milking machine, could be design and closely controlled by the sampling method and mRNA information generated in this thesis.

Modification of gene markers of proliferation was not induced in the TC by infused substances used in this thesis. However, having revealed that some genes showed modification in their expression patterns after treatment is promising, and now, many other substances, that could modify gene expression on some markers of proliferation, could be tested in the TC (Prakoso et al., 2020). For example, Celery extracts (*Apium graveolens*) have been demonstrated to have antibacterial activity and to have an effect inducing the gene expression of cytokeratin-17 in wound healing tissue (Prakoso et al., 2020). Aloe vera extracts have also been tested for their effect

on inducing upregulation of genes related to wound healing in rat models and are under investigation for teat sealant development as well (Tabandeh et al., 2014, Serna-Cock and Pabón-Rodríguez, 2016). Nanostring was demonstrated to be a technique that will allow further research to be undertaken on the TC. Gene expression analysis using the NanoString nCounter System resulted in a robust and highly reproducible method for detecting mRNA gene expression in a single reaction. It detects mRNA molecules of interest digitally using color-coded probe pairs. It does not require the conversion of mRNA to cDNA by reverse transcription or the amplification of the resulting cDNA by PCR, making the protocols simpler and faster to complete (Kulkarni, 2011). It is reported that only 36 genes were differentially expressed in the TC epithelium of lactating cows when compared with cows during the early dry period, suggesting that there are not marked differences in the gene expression patterns between these two time points (Chapter 4). Processes other than the modification of gene expression could be involved in keratin plug formation. Post-translational modifications or reorganization of the keratin filaments within cells may initiate the formation of the keratin plug after drying off (Omary et al., 1998, Herrmann et al., 2002).

Expanding on the information obtained in this study, other objectives could be assessed such as post-translation modifications or alternative splicing, and using different methods like immunoprecipitation, western blot, and mass spectrometry. The inclusion of multivariate analysis of transcript splicing (MAT) of the mRNA obtained at different sampling times would also be of high value. Multivariate analysis of transcript is a robust and specific statistical analysis used to detect alternative splicing in paired replicates studies like the one presented in this thesis. Alternative

splicing is a cellular process that enables the mRNA to be translated into different isoforms of proteins with different cellular functions or properties (McManus and Graveley, 2011, Pohl et al., 2013). In the epithelial tissue is very common to find post-translational modifications and alternative splicing, as the skin is exposed to the environment and it adapts to different conditions (Robert et al., 2009, Knöbel et al., 2015, Terajima et al., 2019). These techniques could be applied to the study of the keratin plug formation in order to find alternative routes to the sealing of the TC.

Based on the results obtained in the challenge model described in Chapter 5, recommendations can be made regarding the need of a pilot study conducted earlier than a challenge model with the objective of identifying the virulence of the putative bacterial strains to be used. If conducted, a pilot study, with careful selection of the challenge strain and multiple controls in place could provide answers about the colonization mechanism that bacteria use to invade the mammary gland and aid in the selection of an adequate strain to be used for the challenge. If using a biofilm forming bacterial strain, with the same study design, biofilms might be detected attached to the walls of the TC or TS by SEM and possibly differences in the histological aspects of the sinus mucosa between challenged and non-challenged teats could be detected.

Whether the excipients of teat sealants modify the inhibitory effect shown by bismuth subnitrate when tested in its powder form needs to be investigated. This would be very relevant to the industry, as bismuth subnitrate has been used until now as a non-anti-infective product (Woolford et al., 1998, Huxley et al., 2002, Dufour et al., 2019). If the evidence presented is supported by *in vivo* studies performed with the entire formulation, our perspectives on how to protect the cows against IMI during the dry period should then be modified. The results obtained in this thesis, if supported by

in vivo studies could re-orient the search for alternative products back into the anti-infective world and maybe more natural anti-infective substances (e.g. plant-based substances) (Bakri and Douglas, 2005, Mullen et al., 2014, Prakoso et al., 2020) and retreat from alternative barrier products. The two neutrophil tests (chemotaxis and MPO) conducted *in vitro* and reported in Chapter 7 could be complemented with *in vivo* studies to determine the effect of bismuth subnitrate and keratin products on neutrophils when applied to the TC. Alternatively, the same study could be repeated to overcome the identified abnormal finding of the positive control, as it is acknowledged that having controls in scientific studies is a foundation for any experimental studies. In future studies, immunohistochemistry could be used in *in vivo* experiments to identify neutrophils in histological sections of TC treated with bismuth and keratin formulations for example, 2 and 24 hours after treatment. Antibody CH138A could be used in these immunohistochemistry studies to identify neutrophils in bovine TC in formalin fixed sections. Other alternative method proposed to this project that was considered while designing the experiments of this thesis was metabolomics. A study was design to obtain metabolome information from bovine neutrophils activated by PMA used as a positive control (Chokesuwattanaskul et al., 2018) which would have given a clearer picture on the response of neutrophils stimulated by bismuth subnitrate and micronized keratin products. This study was put off due to cost and time.

9.6 OVERALL SUMMARY AND CONCLUSIONS

This thesis examined the whole transcriptome of the TC during the early dry period and early lactation stages and developed a biopsy technique to obtain samples

from the TC that could be useful in a wide range of applications. It also explored the action mechanism of two products intended for use in teat sealant formulations: bismuth subnitrate and keratin.

The development of the biopsy method allowed elucidation of some of the physiological mechanisms involved in the formation of the keratin plug. To our knowledge, transcriptomics has never been used before in this epithelial tissue due to the inaccessibility of the tissue to be sampled. The analysis of gene expression revealed that there are some genes involved in immune response pathways, that when downregulated, could be among the many causes for the increase in IMI infections and clinical mastitis cases observed during the early dry period.

Opportunity may arise from the results of these studies to develop alternative formulations to prevent IMI in the future. For example, the bacterial growth inhibiting mechanism of action of bismuth subnitrate may inform in the selection of new non-antimicrobial inhibitory substances that could serve as an effective alternative to bismuth. Ideally, formulations that could inhibit the growth of bacteria in a natural manner without compromising human or animal health could be used to reduce the use of heavy metals like bismuth subnitrate in the food chain. In addition, based on the results presented in this project, showing a slight modification in the gene expression of some genes after treatment with teat sealants, could aid in the development of new alternatives to prevent mastitis over the dry period that could induce the production on keratin in the TC. The findings of the studies presented herein have led to the following conclusions:

- Biopsy samples from the TC of dairy cows can be obtained using a bone curette. The curette method is the first biopsy method developed exclusively for the TC.

It has demonstrated to be safe for the animals, and provided quality, repeatable samples for mRNA extraction and transcriptomics analysis.

- In the process of keratin plug formation in dairy cows, there was a downregulation of the genes related to multiplication of cells and decreased expression of immune related genes. No signs of increased keratin synthesis or epithelial multiplication were observed.

- It was confirmed that there are signs of a reduction in the expression genes related to cell multiplication and immune response pathways during the first 25 days of the dry period in the TC epithelium. Increased presence of bacterial strains belonging to the teat microbiota was also observed by Day 25 after drying off.

- Bismuth subnitrate inhibits the growth of bacteria *in vitro*. This could open a wide range of opportunities for the development of new alternative products with inhibitory non-antimicrobial barrier substances.

- Bismuth subnitrate and keratin failed to show activation of neutrophils *in vitro*, suggesting that the promotion of an immune response might not be the main mechanism of action of bismuth subnitrate or keratin when applied into the TC.

- Bismuth subnitrate and keratin increased the expression of some of the genes in TC epithelial cells although not at the expected levels. Bismuth and keratin did not induce an increase of the thickness of the *stratum granulosum* or *stratum corneum* nor inducing better keratin plug formation when given *in vivo*.

This thesis has added to the knowledge of mechanisms involved in the formation of the keratin plug in the TC at the end of lactation. They are important

findings as they can be used in the development of new alternatives for treatment or preventative measures for IMI, an issue that causes enormous economic losses in the dairy sector as well as animal welfare concerns.

10 Appendices

10.1 Appendix 1

Table 10.1 *Stratum corneum* (keratin lining) estimates and 95% CI of the teat canal per Tissue, Challenge, challenge time with respect to dry off (Challenge time), and days from challenge to sampling. No differences between challenge and non-challenged quarters.

Tissue	Challenge Time	Days from challenge to sampling	Challenge	Mean of the Keratin measurements	Lower 95% CI	Upper 95% CI
Canal	-7	1	control	75.61	44.03	129.84
Canal	-7	1	challenge	77.91	45.37	133.79
Canal	-7	5	control	68.41	42.82	109.28
Canal	-7	5	challenge	50.49	31.60	80.66
Canal	0	1	control	76.33	44.45	131.07
Canal	0	1	challenge	52.53	32.88	83.92
Canal	0	5	control	109.47	68.53	174.88
Canal	0	5	challenge	46.48	29.10	74.26
Canal	7	1	control	64.57	37.60	110.87
Canal	7	1	challenge	40.29	25.22	64.36
Canal	7	5	control	68.34	42.78	109.18
Canal	7	5	challenge	42.09	26.35	67.24
Canal	25	1	control	64.79	40.56	103.50
Canal	25	1	challenge	46.17	28.90	73.76
Canal	25	5	control	50.44	31.58	80.58
Canal	25	5	challenge	40.53	25.37	64.75

10.2 Appendix 2

Table 10.2 *Stratum granulosum* estimates and 95% CI of the teat canal per Tissue, Challenge, challenge time with respect to dry off (Challenge time), and days from challenge to sampling.

Tissue	Challenge Time	Days from challenge to sampling	Challenge	Mean of granulosa measurements	Lower CI	Upper CI
Canal	-7	1	control	273.59	188.35	397.40
Canal	-7	1	challenge	197.11	135.70	286.31
Canal	-7	5	control	257.75	185.45	358.25
Canal	-7	5	challenge	191.73	137.95	266.49
Canal	0	1	control	248.20	170.87	360.52
Canal	0	1	challenge	222.43	160.04	309.16
Canal	0	5	control	193.33	139.10	268.71
Canal	0	5	challenge	212.18	152.66	294.90
Canal	7	1	control	164.51	113.26	238.96
Canal	7	1	challenge	169.86	122.21	236.09
Canal	7	5	control	224.96	161.85	312.67
Canal	7	5	challenge	178.98	128.77	248.76
Canal	25	1	control	203.33	146.29	282.61
Canal	25	1	challenge	142.10	102.24	197.51
Canal	25	5	control	170.65	117.48	247.87
Canal	25	5	challenge	161.10	115.91	223.91

10.3 Appendix 3

Table 10.3 Interferon Gamma concentrations estimates and 95% CI of the teat canal per Tissue, Challenge, challenge time with respect to dry off (Challenge time), and days from challenge to sampling.

Tissue	Challenge time	Days from challenge to sampling	Challenge	Mean of IFNγ	Lower CI	Upper CI	Superscript
Canal	-7	1	control	0.023	0.008	0.066	ab
Canal	-7	1	challenge	0.024	0.009	0.069	ab
Canal	-7	5	control	0.053	0.019	0.150	ab
Canal	-7	5	challenge	0.036	0.013	0.101	ab
Canal	0	1	control	0.135	0.048	0.383	ab
Canal	0	1	challenge	0.208	0.073	0.588	a
Canal	0	5	control	0.129	0.046	0.366	ab
Canal	0	5	challenge	0.140	0.049	0.396	ab
Canal	7	1	control	0.023	0.008	0.064	ab
Canal	7	1	challenge	0.017	0.006	0.047	ab
Canal	7	5	control	0.010	0.004	0.028	b
Canal	7	5	challenge	0.010	0.004	0.028	b
Canal	25	1	control	0.078	0.028	0.221	ab
Canal	25	1	challenge	0.039	0.014	0.111	ab
Canal	25	5	control	0.165	0.058	0.466	ab
Canal	25	5	challenge	0.131	0.046	0.370	ab
Sinus	-7	1	control	0.084	0.027	0.265	ab
Sinus	-7	1	challenge	0.027	0.010	0.076	ab
Sinus	-7	5	control	0.015	0.005	0.042	ab
Sinus	-7	5	challenge	0.028	0.010	0.079	ab
Sinus	0	1	control	0.055	0.020	0.157	ab
Sinus	0	1	challenge	0.074	0.026	0.209	ab
Sinus	0	5	control	0.029	0.010	0.082	ab
Sinus	0	5	challenge	0.095	0.033	0.268	ab
Sinus	7	1	control	0.010	0.004	0.028	b
Sinus	7	1	challenge	0.016	0.006	0.046	ab
Sinus	7	5	control	0.010	0.004	0.028	b
Sinus	7	5	challenge	0.010	0.004	0.028	b

Sinus	25	1	control	0.066	0.023	0.188	ab
Sinus	25	1	challenge	0.073	0.026	0.206	ab
Sinus	25	5	control	0.156	0.055	0.441	ab
Sinus	25	5	challenge	0.106	0.037	0.299	ab

10.4Appendix 4

Table 10.4 Interleukin-8 concentrations estimates and 95% CI of the teat canal per Tissue, Challenge, challenge time with respect to dry off (Challenge time), and days from challenge to sampling.

Tissue	Challenge Time	Days from challenge to sampling	Challenge	Mean	Lower CI	Upper CI
Canal	-7	1	control	71.40	38.35	132.92
Canal	-7	1	control	39.88	21.42	74.25
Canal	-7	1	control	111.46	59.87	207.49
Canal	-7	1	control	49.99	26.85	93.07
Canal	-7	5	challenge	73.96	39.73	137.69
Canal	-7	5	challenge	39.18	21.05	72.94
Canal	-7	5	challenge	94.95	51.00	176.76
Canal	-7	5	challenge	43.26	23.24	80.54
Canal	0	1	control	156.03	83.81	290.46
Canal	0	1	control	74.92	40.24	139.47
Canal	0	1	control	102.00	54.79	189.89
Canal	0	1	control	24.56	13.19	45.72
Canal	0	5	challenge	166.23	89.29	309.45
Canal	0	5	challenge	67.58	36.30	125.80
Canal	0	5	challenge	92.77	49.83	172.70
Canal	0	5	challenge	34.32	18.44	63.90
Sinus	7	1	control	79.03	42.45	147.13
Sinus	7	1	control	43.27	23.24	80.55
Sinus	7	1	control	71.37	38.34	132.86
Sinus	7	1	control	36.65	19.69	68.23
Sinus	7	5	challenge	89.35	48.00	166.34
Sinus	7	5	challenge	45.32	24.34	84.37
Sinus	7	5	challenge	78.94	42.40	146.96
Sinus	7	5	challenge	34.44	18.50	64.12
Sinus	25	1	control	33.92	18.22	63.15
Sinus	25	1	control	20.78	11.16	38.69
Sinus	25	1	control	40.02	21.50	74.51
Sinus	25	1	control	38.82	20.85	72.28

Sinus	25	5	challenge	44.81	24.07	83.42
Sinus	25	5	challenge	33.78	18.14	62.88
Sinus	25	5	challenge	42.42	22.79	78.98
Sinus	25	5	challenge	17.40	9.35	32.39

10.5 Appendix 5

Table 10.5 Interleukin 10 concentrations estimates and 95% CI of the teat canal per Tissue, Challenge, challenge time with respect to dry off (Challenge time), and days from challenge to sampling.

tissue	Challenge Time	Days from challenge to sampling	Challenge	Mean	Lower CI	Upper CI
Canal	-7	1	control	3.69	1.18	11.54
Canal	-7	1	challenge	1.23	0.39	3.85
Canal	-7	5	challenge	33.51	10.72	104.78
Canal	-7	5	control	1.48	0.47	4.62
Canal	-7	1	control	6.93	2.22	21.66
Canal	-7	1	challenge	1.01	0.32	3.16
Canal	-7	5	challenge	20.71	6.62	64.76
Canal	-7	5	control	1.02	0.33	3.20
Canal	0	1	challenge	63.96	20.46	199.98
Canal	0	1	challenge	13.16	4.21	41.14
Canal	0	5	challenge	14.92	4.77	46.64
Canal	0	5	challenge	0.70	0.22	2.19
Canal	0	1	control	67.41	21.56	210.76
Canal	0	1	challenge	17.57	5.62	54.94
Canal	0	5	control	17.12	5.48	53.53
Canal	0	5	challenge	1.30	0.42	4.07
Sinus	7	1	challenge	3.29	1.05	10.29
Sinus	7	1	control	0.92	0.29	2.86
Sinus	7	5	challenge	3.80	1.21	11.88
Sinus	7	5	challenge	0.57	0.18	1.78
Sinus	7	1	control	4.79	1.53	14.96
Sinus	7	1	control	0.82	0.26	2.57
Sinus	7	5	challenge	5.63	1.80	17.61
Sinus	7	5	challenge	0.57	0.18	1.78
Sinus	25	1	control	4.20	1.34	13.15
Sinus	25	1	control	3.52	1.13	11.02
Sinus	25	5	control	9.11	2.91	28.48
Sinus	25	5	challenge	6.13	1.96	19.17

Sinus	25	1	challenge	3.20	1.02	10.00
Sinus	25	1	control	3.41	1.09	10.66
Sinus	25	5	control	3.07	0.98	9.60
Sinus	25	5	challenge	5.15	1.65	16.11

10.6 Appendix 6

Table 10.6 Interferon alpha concentrations estimates and 95% CI of the teat canal per Tissue, Challenge, challenge time with respect to dry off (Challenge time), and days from challenge to sampling.

tissue	Challenge Time	Days from challenge to sampling	Challenge	Mean	Lower CI	Upper CI
Canal	-7	1	control	241.55	107.59	542.29
Canal	-7	1	challenge	350.83	156.27	787.65
Canal	-7	5	control	817.90	364.31	1836.25
Canal	-7	5	challenge	518.32	230.87	1163.68
Canal	0	1	control	941.02	419.15	2112.67
Canal	0	1	challenge	955.65	425.67	2145.52
Canal	0	5	control	442.08	196.91	992.50
Canal	0	5	challenge	377.14	167.98	846.70
Canal	7	1	control	263.39	117.32	591.33
Canal	7	1	challenge	334.97	149.20	752.02
Canal	7	5	control	243.33	108.38	546.30
Canal	7	5	challenge	300.61	133.90	674.89
Canal	25	1	control	106.17	47.29	238.36
Canal	25	1	challenge	85.45	38.06	191.84
Canal	25	5	control	56.99	25.38	127.95
Canal	25	5	challenge	67.53	30.08	151.61
Sinus	-7	1	control	37.18	16.56	83.47
Sinus	-7	1	challenge	69.58	30.99	156.22
Sinus	-7	5	control	79.43	35.38	178.33
Sinus	-7	5	challenge	67.10	29.89	150.64
Sinus	0	1	control	130.74	58.23	293.52
Sinus	0	1	challenge	145.18	64.66	325.94
Sinus	0	5	control	26.29	11.71	59.02
Sinus	0	5	challenge	52.16	23.23	117.09
Sinus	7	1	control	51.99	23.16	116.72
Sinus	7	1	challenge	50.05	22.29	112.37

Sinus	7	5	control	51.53	22.95	115.70
Sinus	7	5	challenge	47.09	20.97	105.71
Sinus	25	1	control	20.59	9.17	46.22
Sinus	25	1	challenge	50.07	22.30	112.42
Sinus	25	5	control	79.60	35.45	178.70
Sinus	25	5	challenge	38.71	17.24	86.90

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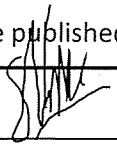
Candidate's Signature:	
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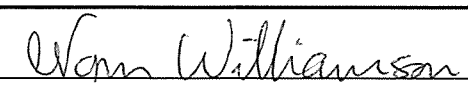
Primary Supervisor's Signature:	
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Effect of bismuth subnitrate on in vitro growth of major mastitis pathogens

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ABSTRACT

The mode of action of bismuth subnitrate in teat sealant formulations as a preventative for intramammary infections during the dry period is unknown. Although previous studies proposed an action mechanism—creating a physical barrier in the teat canal to prevent bacterial invasion—it has not been proven experimentally. We hypothesized that bismuth subnitrate has an inhibitory effect on bacterial growth, in addition to its barrier effect. The objective of this study was to assess the effect of bismuth subnitrate on bacterial growth of major mastitis-causing agents. A strain of *Streptococcus uberis* (SR115), 2 strains of *Staphylococcus aureus* (SA3971/59 and SA1), and a strain of *Escherichia coli* (P17.14291) were tested in vitro for their ability to grow in the presence or absence of bismuth subnitrate. Disk diffusion testing, impedance measurement, and evaluation of bacterial growth in shaking conditions were the methods used to test this hypothesis. A reduction of growth in the presence of bismuth subnitrate occurred for all the strains tested. However, we observed strain and species variations in the extent of growth inhibition. These results suggest that an inhibitory effect on bacterial growth by bismuth subnitrate could partially explain the efficacy of bismuth-based formulations for preventing intramammary infections over the dry period. Further research is required to test the effect of teat sealant formulations on bacterial growth.
Key words: bismuth subnitrate, mastitis, teat sealant, dry cow

INTRODUCTION

The late lactating and early dry periods are stressful stages for the anatomy and physiology of dairy cows, particularly their mammary glands. Remodeling, apoptosis, and cell dedifferentiation are processes occurring in mammary glands during the first 2 wk after milk removal ceases (Cousins et al., 1980; Wilde et al., 1997). This time-specific physiological stress is manifested by high susceptibility of cows to new IMI (Green et al., 2002). Thus, methods to treat and prevent IMI during the dry period have been available for many years. These treatments consist mainly of antimicrobials infused into mammary glands after the last milking before drying off, and they result in a reduction of new IMI during the dry period in treated compared with untreated cows (Pearson and Wright, 1969; Schukken et al., 1993; Bryan et al., 2011). Dry cow therapy (**DCT**) has been used on all cows in a herd, regardless of their infection status, with the objective of eliminating subclinical infections and preventing new infections during the dry period (Williamson et al., 1995; Hassan et al., 1999). However, due to global concerns regarding increasing antimicrobial resistance and the need for responsible use of antimicrobials, there is a trend toward investigating the use of selective dry cow therapy and nonantimicrobial alternatives (Oldham and Daley, 1991; Ryan et al., 1998; Hoernig et al., 2016; Vanhoudt et al., 2018). The current and most commonly used alternatives to antimicrobials at the time of dry-off are internal teat sealants that contain bismuth subnitrate, a heavy metal compound, in a paraffin-based excipient. They have proven to be effective at preventing new dry-period infections in challenge models, as well as in natural exposure studies (Meaney, 1977; Woolford et al., 1998; Bhutto et al., 2011). The mechanism of action of bismuth-based products remains undocumented, although creating a physical barrier has been suggested in many studies as the main method of protection (Meaney, 1977; Woolford, et al., 1998; Berry and Hillerton 2002; Kabera et al., 2018). Internal teat sealants are also commonly used in combination with DCT, to prevent and treat IMI during the dry period (Godden et al., 2003). However, although there is an increase in the efficacy of preventing IMI using both products in combination, this increase is nonsignificant (Rabiee and Lean, 2013).

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Bismuth formulations used as internal teat sealants are defined as "medical devices," a nonpharmacological barrier (Codex Alimentarius, 2016). The proposed mechanisms through which teat sealants exert their function include assisting the teat canal in forming a better keratin plug and creating a physical barrier against bacteria in the teat canal (Woolford et al., 1998); however, none of these hypothesized modes of action has been scientifically proven. Other studies testing different physical barriers, such as wax plugs or intramammary polystyrene devices, were unsuccessful in the long-term protection of cows against IMI and mastitis. Even though intramammary devices elicited some immune response and a seemingly initial protective effect (Poutrel et al., 1983; Paape et al., 1988; Nickerson et al., 1990; Serna-Cock and Pabón-Rodríguez, 2016), in the long term these devices resulted in an increase in IMI. We hypothesize, therefore, that creating a physical barrier against bacteria traversing the teat canal is not the sole mode of action providing the efficacy observed for bismuth-based formulations.

Studies have shown an inhibitory effect of bismuth-based products when tested in vitro against bacterial species that are associated with disease in humans (Phillips et al., 2000; Folsom et al., 2011; Vega-Jiménez et al., 2012). Inhibition of bacterial growth by bismuth salts (mainly bismuth subsalicylate) has been studied in the treatment of stomach ulcers, traveler's diarrheas, and colitis caused by bacteria such as *Helicobacter pylori* and *Campylobacter pyloridis* (Marshall et al., 1987; Fine and Lee, 1998).

In this study, we hypothesized that bismuth subnitrate inhibits the growth of bacteria associated with the colonization of the mammary gland during the dry period and the development of new IMI. The objective of this study was to assess the effect of bismuth subnitrate on the growth of mastitis-causing bacterial strains in vitro.

MATERIALS AND METHODS

Bacterial Strains

Streptococcus uberis strain SR115, 2 strains of *Staphylococcus aureus* (SA3971/59 and SA1), and an *Escherichia coli* strain (P17.14291) were used in this study. The source of the *Staph. aureus* and *Strep. uberis* strains was the bacteria library in the Microbiology Laboratory, School of Veterinary Science, Massey University, New Zealand, and they were originally isolated by veterinary diagnostic laboratories from clinical mastitis cases that occurred in New Zealand from various stages of lactation. *Escherichia coli* was isolated from an early lactation clinical case of mastitis that occurred in August 2017 at Massey University Dairy Unit number 4. For complete identification, the *Strep. uberis* and *Staph. aureus* isolates were cultured on trypticase soy agar plates (**TSA**) and incubated in aerobic conditions at 37°C for 24 h. To confirm strain purity, one colony from each strain was selected from the culture plate, transferred onto a new TSA plate, and incubated as above. The isolates were reidentified phenotypically using biochemical tests. *Streptococcus uberis* SR115 was confirmed by positive Gram stain, catalase-negative, esculin and inulin positive reactions, and negative growth in buffered azide glucose glycerol broth. Positive results for Gram stain, catalase reaction, and coagulase rabbit plasma test confirmed isolates as being *Staph. aureus* SA3971/59 and SA1. *Escherichia coli* P17.14291 was cultured on MacConkey agar.

Experiment 1: Bacterial Growth in Agar-Disk Diffusion Test

To test the hypothesis that bismuth subnitrate can inhibit the growth of bacteria on agar media, bacterial strains were cultured overnight at 37°C in 1 mL of trypticase soy broth (**TSB**). One hundred microliters of each bacterial suspension was spread on TSA plates. These bacterial strain suspensions were cultured overnight to contain approximately 10^8 to 10^9 cfu/mL. Sterile bismuth subnitrate powder was suspended in sterile distilled water at 3 different concentrations (65, 195, and 390 mg/mL) and vortexed thoroughly to create a uniform suspension. Concentrations were chosen to represent 10, 30, and 60% of the 650 mg/g present in current teat sealant products (drySeal, Bayer Animal Health, New Zealand). Higher concentrations induced precipitation of the bismuth subnitrate in the tubes and were therefore not used. Sterile 6-mm paper disks were immersed in the bismuth subnitrate suspensions and placed on the plates, and the plates were incubated for 24 h at 37°C. One extra disk was immersed in sterile distilled water and used as a control. After incubation, the plates were observed for zones of inhibition and changes around the disks at the different concentrations and the diameters of the zones were measured with a 20-cm caliper. The experiment was performed in 5 plates for each strain.

Experiment 2: Bacterial Growth in a Fluid Medium—Impedance Standardization

The hypothesis of this experiment was that bismuth subnitrate inhibits the growth of bacteria in a fluid medium. During the growth phase, bacterial metabolism breaks down proteins and lipids in the medium and transforms uncharged or weakly charged compounds

of the culture medium into highly charged compounds that change the electrical properties of the medium. Impedance in microbiology is the ability of a microorganism to change (reduce) the resistance to flow of an electric current as it passes through a conducting material (Silley and Forsythe, 1996). Impedance was measured using the BacTrac 4300 microorganism growth analyzer (SyLab, Neupurkersdorf, Austria). The BacTrac 4300 measures impedance in the medium (*M*-value) and impedance in the electrode (*E*-value). In this experiment, the *E*-value generated by BacTrac was used, as it has been shown to be positively associated with bacterial growth (Wang et al., 2016). Bacterial strains were cultured overnight in 1 mL of TSB at 37°C. Bismuth subnitrate in a final concentration of 32.5 mg/mL (**BIS**, 5% of the 650 mg/g contained in teat sealant product) was added to sterile BacTrac vials containing 9.9 mL of TSB, which were vortexed. Control vials containing 9.9 mL of sterile TSB (**CON**) were filled with 0.325 mL of sterile distilled water (to correct for volume) and vortexed to mix. One hundred microliters of bacterial strain suspension cultured overnight, containing approximately 10^8 to 10^9 cfu/mL, was added to all vials. The vials were cultured for 24 h at 37°C in the BacTrac. The *E*-value was monitored every 20 min for 24 h, and a curve expressing the increase in impedance over time was drawn, compared with the starting point at time 0. The experiment was performed in triplicate, in 4 repeats per strain (12 vials per treatment).

Before and after culture in the BacTrac, viable bacteria were counted for validation of the results using the pour plate technique. Briefly, a 100-μL sample from the BacTrac vials (6 per strain) was transferred into 1.5-mL sterile tubes containing 900 μL of TSB. These suspensions were serially diluted 10^{-3} to 10^{-7} and transferred into sterile empty petri dishes. Trypticase soy agar was poured over the plates containing the bacterial suspension, and the plates were incubated at 37°C for 24 h. Viable colonies were counted after incubation of the plates.

Experiment 3: Bacterial Growth in a Shaking Fluid Medium

The hypothesis of this experiment was that by shaking the medium, bismuth subnitrate particles would come in closer contact with bacterial cells and therefore increase the inhibition of bacterial growth.

Bacterial strains were cultured overnight in 1 mL of TSB at 37°C. Glass Erlenmeyer flasks were filled with 9.9 mL of sterile TSB in triplicate. Bismuth subnitrate was added at a final concentration of 32.5 mg/mL (BIS). Control flasks without bismuth (CON) received 0.325 mL of sterile distilled water to correct for volume. One hundred microliters of the overnight bacterial culture (approximately 10^8 to 10^9 cfu/mL) was added to all the flasks. Two trials were run with this method to observe the effect of longer incubation time on bacterial growth. In trial 1, the flasks were placed on a shaking platform at 200 rpm for 24 h for culturing. In trial 2, the flasks were cultured in shaking mode for 48 h. Aliquots of 100 μL of the culture medium were taken at multiple time points to count the number of viable colonies: immediately after inoculation (0 h), at 3, 5, and 24 h for trial 1, and at 0, 3, 5, 24, and 48 h after inoculation for trial 2. The 100-μL aliquots were serially diluted 10-fold in 0.9 mL of TSB. These serial dilutions were processed for counting (cfu) using the pour-plate technique described above.

Statistical Analysis

For experiment 1, the outcome "diameter of dark area around the disk" had an approximately normal distribution. A standard ANOVA with post hoc Tukey-Kramer adjustment for multiple paired comparisons was used to compare the diameter (in millimeters) associated with each strain at different concentrations. For experiment 2, results are presented as LSM estimates of the *E*-value ± 95% CI × time. Statistical differences were based on Tukey-Kramer adjusted pairwise comparison per strain. The repeated measure model included the fixed effects of treatment (BIS vs. CON), time (0 to 24 h in 20 min intervals) and the interaction between treatment and time. Vials with incomplete curves due to a malfunction of the electrodes were dropped from the analysis. Seven vials of *Strep. uberis* SR115, 9 of *Staph. aureus* SA3971/59, 6 of *Staph. aureus* SA1, and 8 of *E. coli* P17.14291 were removed from the analysis for this reason. Viable counts (cfu) were not normally distributed and were therefore converted to $\log_2$-cfu values. Paired Student's *t*-test was used to compare the difference of $\log_2$-cfu before (0 h) and after culturing (24 h) for BIS and CON groups. Student's *t*-test was used to compare the mean difference of the $\log_2$-cfu between groups (BIS vs. CON) at each time point. For experiment 3, the count data were right-skewed but an e-log conversion of the colony-forming units/10,000 counts showed an approximately normal distribution, as evaluated using density plots. The outcome was the mean of triplicate plate counts per treatment (BIS, CON) per strain showing between 30 and 300 cfu. Four repeated-measures models, one for each strain (PROC MIXED, SAS 9.3 for Windows; SAS Institute Inc., Cary, NC), were developed to determine the effect of bismuth supplementation on bacterial growth at each time point (0, 3, 5, 24, and 48 h) in shaking culture for both trials. The model included the fixed effect of time (0, 3,

5, 24, 48h), trial (1 vs. 2), treatment (BIS vs. CON) and interaction of time and treatment. To account for the correlation of repeated measures within group, the model for a cluster effect of group was nested within the experiment (1, 2) and a first-order autoregressive correlation structure, assuming that measurements closer in time were correlated more strongly. This structure resulted in the lowest Akaike information criterion as a measure of overall model fit. The significance level for all statistical tests was set at $P < 0.05$. All statistical analysis was done in SAS 9.3.

RESULTS

Experiment 1: Bacterial Growth in Agar-Disk Diffusion Test

An area 7- to 10-mm in diameter of complete inhibition, which coincided with the spread of the suspended bismuth subnitrate when wet disks were placed on the agar, was present around disks in the *Strep. uberis* SR115 cultures in 3 different concentrations (Figure 1A). Control disks showed no inhibition for any of the strains tested.

The 2 *Staph. aureus* strains (SA3971/59 and SA1) and *E. coli* P17.14291 on agar plates presented an area of partial inhibition (less-dense colony growth around the disks; Figure 1B), and a dark brown area around the disks that was larger for higher concentrations (Figure 2), although complete inhibition was not observed for these bacteria. Brown areas around the disks varied with concentration within each *Staph. aureus* strain [SA1 (65 mg/mL) vs. SA1 (390 mg/mL), $P = 0.0048$; SA3971/59 (65 mg/mL) vs. SA3971/59 (390 mg/mL), $P = 0.0186$]. However, paired comparisons showed no difference between strains.

Experiment 2: Bacterial Growth in a Fluid Medium

The impedance of the medium was measured using the BacTrac 4300 microbiological growth analyzer. Growth curves showed lower impedance levels for all strains cultured in TSB medium when supplemented with 32.5 mg/mL bismuth subnitrate, compared with the control growth curve (Figure 3). Vials that showed incomplete reads due to having out-of-range values during the 24-h culture were removed from the study. Of the 24 vials per strain analyzed, 7 vials of *Strep. uberis* SR115, 9 of *Staph. aureus* SA3971/59, 6 of *Staph. aureus* SA1, and 8 of *E. coli* P17.14291 were removed from the analysis. The results of the impedance test showed that inhibition of bacterial growth was not complete. Changes in impedance levels occurred, indicating bacterial growth. There was evidence of growth in the first 10 h of culture in the BIS treatment group, although lower than in CON vials for the duration of the study. Significant differences between BIS and CON vials were detected after 4 h in SA3971/59 and SA1, after 5 h in SR115, and after 10 h in *E. coli* P17.14291 (Figure 3). Counts (cfu) ± SD were assessed before incubation (0 h) and 24 h after incubation for 6 selected vials per strain (Table 1). Plate counts indicated that there was reduced bacterial growth in the BIS group after 24 h of culture for the 4 bacterial strains tested compared with the CON group (*t*-test, $P < 0.001$).

Experiment 3: Bacterial Growth in a Shaken Fluid Medium

Colony-forming units in BIS and CON groups were counted at 4 and 5 time points over 24- and 48-h periods for trials 1 and 2, respectively. Results showed significant growth inhibition by bismuth subnitrate

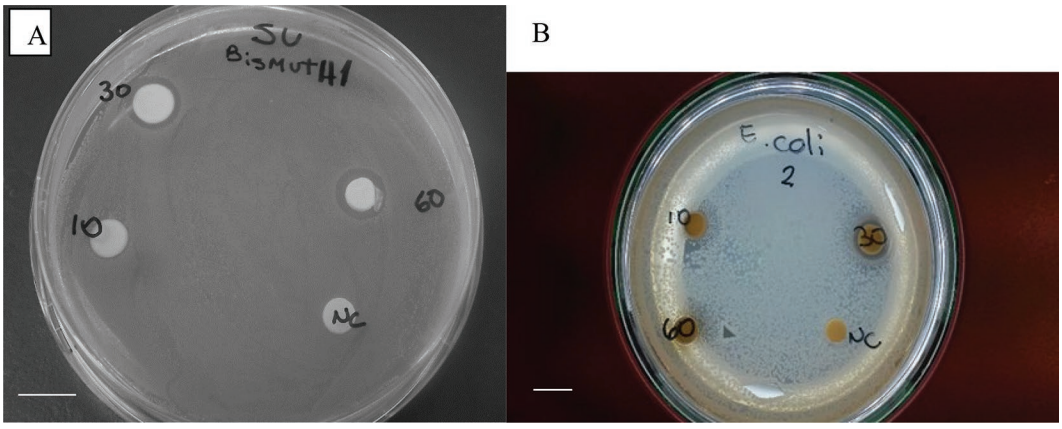

Figure 1. (A) Experiment 1: Complete inhibition of growth of 7 to 10 mm around the disks of a trypticase soy agar plate cultured with *Streptococcus uberis* SR115. (B) *Escherichia coli* P17.14291 showing an area of less dense colony growth around the disks; 10 = 65 mg/mL, 30 = 195 mg/mL, and 60 = 390 mg/mL of bismuth subnitrate, NC = disks immersed in sterile distilled water. Reference line shows 10 mm.

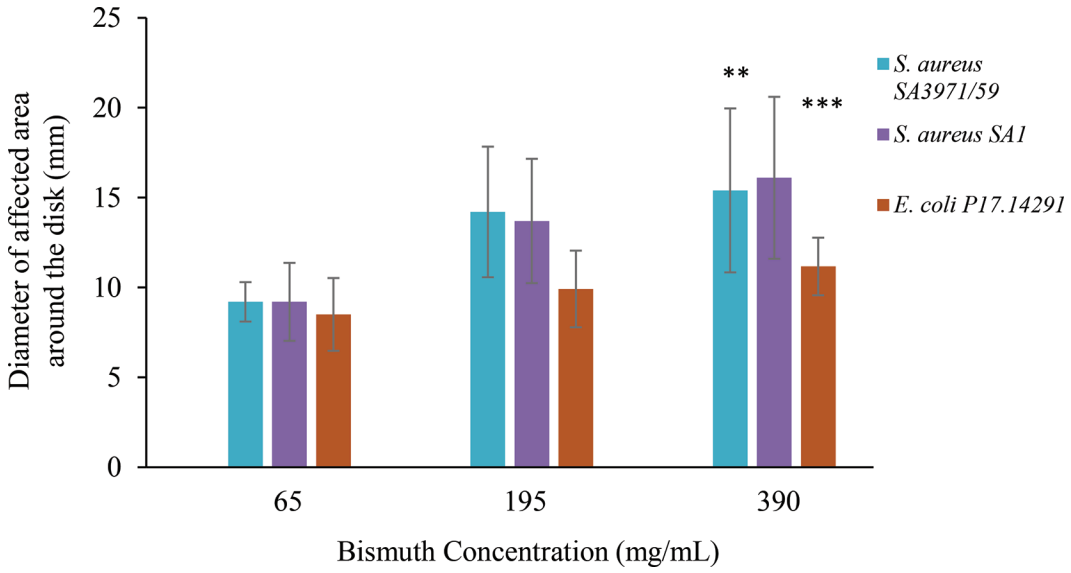

Figure 2. Experiment 1: Diameter of the area (±SD) affected by bismuth subnitrate in the 6-mm disk diffusion test using *Staphylococcus aureus* SA3971/59, *Staph. aureus* SA1, and *Escherichia coli* P17.14291. Bars represent areas of dark coloration around the disk; n = 5. Dark areas around the disks varied with concentration within each *Staph. aureus* strain; $^{**}P < 0.01$, $^{***}P < 0.001$.

from 3 h after the commencement of the culturing period (Figure 4). Trials 1 and 2 showed a very similar response to bismuth, with increased inhibition after 24 h for strains SR115, SA3971/59, and *E. coli*, but a resumption in growth of strain SA1 (Figure 4 C). The difference between BIS and CON flasks remained significant for most of the study in both trials.

DISCUSSION

Teat sealants containing bismuth subnitrate are described as inert nonantibiotic products that act as a physical barrier to the colonization of bacteria through the teat canal during the dry period (Meaney et al., 2001; Codex Alimentarius, 2016). However, our results support the concept that bismuth subnitrate slows bacterial growth in vitro. This is, to our knowledge, the first published assessment of a potential inhibitory effect of bismuth subnitrate on mastitis-causing agents in the dairy industry.

Streptococcus uberis is one of the main mastitis-causing microorganisms during the dry period, particularly in New Zealand (Williamson et al., 1995; McDougall, 2003). The results of the disk diffusion test in the present study (experiment 1) show that *Strep. uberis* SR115 appeared to be more susceptible to bismuth subnitrate than *Staph. aureus* (SA3971/59 and SA1) or *E. coli* (P17.1429), demonstrated by an area of complete inhibition. In the same test, *Staph. aureus* and *E. coli* presented an apparent concentration-dependent dark area around the disks, but not complete inhibition as for *Strep. uberis*. The dark area observed for *Staph. aureus* and *E. coli* in this study could be explained by the fact that both strains have the ability to hydrolyze sulfide (Tomasova et al., 2016). The hydrolysis of sulfide with the addition of bismuth forms bismuth (3) sulfide, a black pigment produced by some bacteria in the mouth and lower intestines (Ioffreda et al., 2001). There are other examples in which bacteria in conjunction with bismuth can form bismuth 3 sulfide. The "black spot defect" found in cheddar cheese is produced by bismuth 3 sulfide and has been linked to the presence of bismuth subnitrate in the milk (Lay et al., 2007). The production of bismuth 3 sulfide, therefore, could have caused the dark area around the disk in the disk diffusion test of this study. However, the presence of sulfide in the TSA plates was not tested. The disk diffusion test in this study was used as a screening tool; other traditional methods for antimicrobial testing such as the use of Mueller Hinton plates that could be applied in dose-dependent antimicrobial testing for bismuth subnitrate were not used.

Bismuth subnitrate is a heavy metal compound with low solubility that precipitates in fluid medium, thus it does not diffuse easily in agar. After observing the results of the disk diffusion test, 2 more experiments were designed to reassess the inhibitory effect of bismuth subnitrate that could have been masked by the minimum spread of the bismuth suspension observed on the surface of the agar plates in experiment 1. Due to marked precipitation of bismuth subnitrate observed in experiment 2, the third experiment was added. In

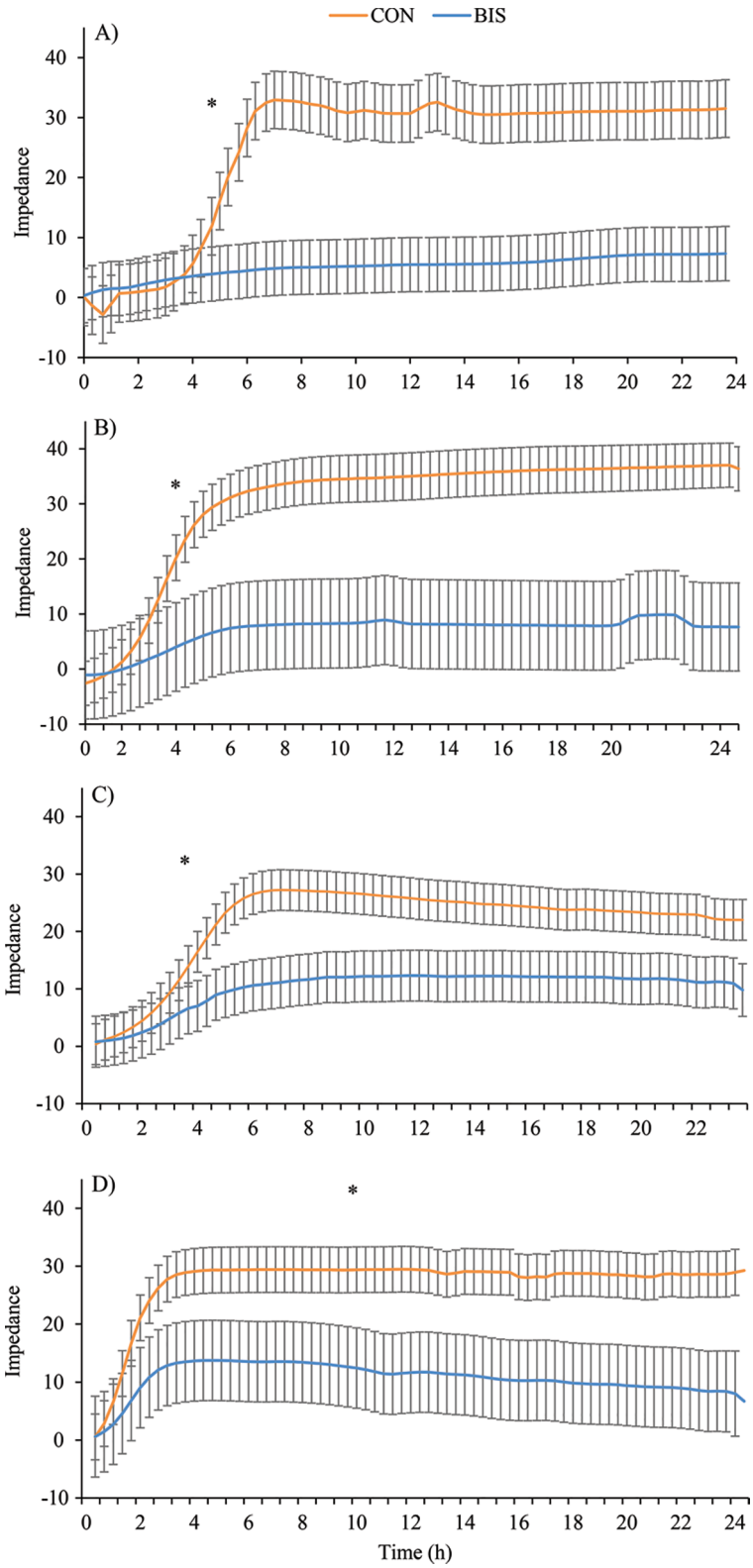

Figure 3. Experiment 2: Least squares means estimates of the impedance (±95%CI) measured in BacTrac 4300 (SyLab, Neupurkersdorf, Austria). Bacteria were cultured in trypticase soy broth with (BIS) and without bismuth (CON) for 24 h at 37°C in 4 repeats of triplicates per treatment. (A) *Streptococcus uberis* SR115 (BIS n = 9, CON n = 8); (B) *Staphylococcus aureus* SA3971/59 (BIS n = 6, CON n = 9); (C) *Staph. aureus* SA1 (BIS n = 7, CON n = 11); (D) *Escherichia coli* P17.14291 (BIS n = 7, CON n = 9). n = number of vials that had readable results from BacTrac 4300; *first time point of difference ($P < 0.05$).

Table 1. Experiment 2: Comparison of the mean (±SD) of the $\log_2$ conversion of counts (cfu/mL) for bismuth subnitrate (BIS) versus control (CON) vials before (0 h) and after (24 h) incubation at 37°C[1]

Strain	Treatment	0 h	24 h	Mean difference (±SD)	Paired *t*-test *P*-value
Streptococcus uberis SR115	BIS	18.8 (±0.5)	12.6 (±1.7)	−6.2 (±0.8)	0.0015
	CON	19.9 (±0.1)	26.4 (±0.4)	6.5 (±0.37)	<0.0001
	t-test *P*-value			<0.0001	
Staphylococcus aureus SA3971/59	BIS	19.97 (±0.1)	22.18 (±0.3)	2.2 (±0.5)	0.0002
	CON	20.08 (±0.4)	27.15 (±0.2)	7.03 (±0.4)	<0.0001
	t-test *P*-value			<0.0001	
Staph. aureus SA1	BIS	20.19 (±0.09)	18.24 (±2.15)	−1.95 (±2.2)	0.08
	CON	20.12 (±0.09)	25.62 (±0.2)	5.49 (±0.1)	<0.0001
	t-test *P*-value			<0.0001	
Escherichia coli P17.14291	BIS	20.06 (±0.6)	18.44 (±0.7)	−1.61 (±0.7)	0.0036
	CON	20.24 (±0.2)	26.52 (±0.4)	6.28 (±0.5)	<0.0001
	t-test *P*-value			<0.0001	

[1]Incubation in BacTrac 4300 (SyLab, Neupurkersdorf, Austria); n = 6 per strain.

the BacTrac and shaking medium studies (experiments 2 and 3), bacterial growth was significantly reduced by the presence of bismuth in the culture medium. There was no complete inhibition or killing, but the results clearly show a suppression of bacterial growth. Even though a low number of BacTrac vials could not be included in the analysis, due to a fault in some of the electrodes giving out-of-range readings, the results presented in this study show a significant difference in bacterial growth between BIS and CON vials. No explanation was found for the out-of-range readings. We hypothesized that bismuth precipitation over the base of the tube where the electrodes are placed had an effect, and this is one of the reasons that lead to the design of study 3 (shaking medium). However, literature reflects that it is not unusual to find out-of-range measurements when measuring impedance (Colvin and Sherris, 1977). Ideal curve shapes that are meaningful for the purposes of the study need to be determined before the study (Chen and Chang, 1994), and we did this. *Streptococcus uberis* was the most susceptible strain to the effect of bismuth subnitrate in the 3 experiments presented here. In experiments 1 and 3, *Strep. uberis* showed complete inhibition after the 24-h culture with bismuth subnitrate. This could be due to its high sensitivity to bismuth subnitrate, or to inconsistent growth rates found in vitro in some *Strep. uberis* strains that are highly dependent on nutrient availability (Leigh and Field, 1991).

In experiment 3, significant differences between BIS and CON flasks were observed after 3 h of culturing for all the strains. Experiment 2 produced significant differences after 4 to 5 h for most of the strains except for *E. coli*. For the latter, significant differences between BIS and CON vials were found 10 h after the culture began. The different nature of the studies and the differences in the statistical analyses performed for each of the experiments might have produced these time differences within the *E. coli* strain. *Staphylococcus aureus* SA1 resumed growth after 24 h in experiment 3, but the origin of this resumption in growth could not be determined. The different growth rates of the 4 strains observed in this study support the concept of the presence of high variability in growth patterns and response to treatment of different pathogens (Keane, 2019). *Staphylococcus aureus* is usually associated with persistent IMI that may be present throughout the dry period (Pankey et al., 1982; Barkema et al., 2006). In contrast, *E. coli* and *Strep. uberis* tend to induce IMI or clinical mastitis cases that either cure spontaneously or for which the use of antimicrobials at dry off is usually effective (Todhunter et al., 1995; Keane, 2019). In this study, one of the *Staph. aureus* strains showed a resumption in growth after 48 h of exposure to bismuth subnitrate. This in vitro behavior may suggest adaptability of this strain to an adverse environment that could be relevant to the in vivo situation. This is why identification of the pathogen and a treatment that targets the bacterial agent (selective DCT) could improve cure rates during the dry period (Bradley and Green, 2001; Barkema et al., 2006). A limitation of this study is the absence of a positive control for the inhibitory effect. Experiments 2 and 3 would have benefited from having the impedance curve and counts obtained for vials with a known inhibitory agent.

Our results are in line with human medical and dentistry in vitro research studies showing the inhibitory effect of bismuth salts (mainly bismuth subsalicylate, but also bismuth subnitrate; Domenico et al., 1997; Athanikar, 1998; Lin et al., 2011; Vega-Jiménez et al., 2012). Bismuth-based products are used to treat different diseases, such as syphilis, gastric ulcers, traveler's

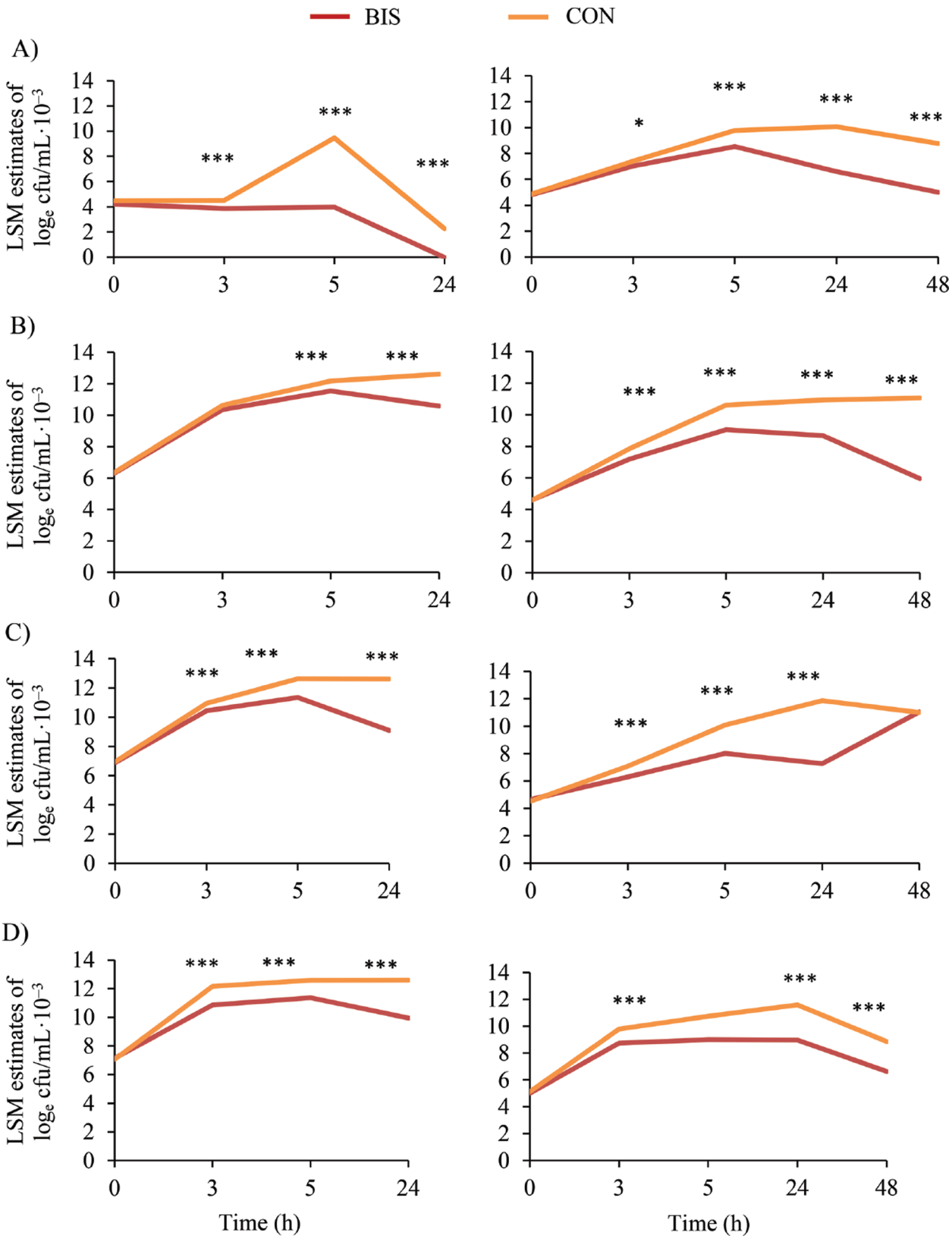

Figure 4. Least squares means estimates of e-log (cfu/mL)/10,000 for trial 1 (left) and trial 2 (right) after culture at 37°C in shaking mode at 200 rpm for 24 and 48 h, respectively. (A) *Streptococcus uberis* SR115, (B) *Staphylococcus aureus* SA3971/59, (C) *Staph. aureus* SA1, and (D) *Escherichia coli* P17.14291. *$P < 0.05$; ***$P < 0.0001$. Vials did (BIS) or did not (CON) contain bismuth subnitrate.

diarrhea, and other pathologies caused mainly by bacteria (Marshall et al., 1987; Slikkerveer and de Wolff, 1989; Phillips et al., 2000).

The mechanism of action by which bismuth subnitrate slows bacterial growth is unknown. A few different hypotheses could explain this effect: oxidation, interference with bacterial metabolism by reducing ATP synthesis, enzyme inhibition, and inhibition of biofilm formation, among others (Sox and Olson, 1989; Zhang et al., 2006; Folsom et al., 2011). As an oxidizing heavy metal, the action mechanism of bismuth might be similar to that of other metals such as silver, zinc oxide (ZnO) or copper, which are known for their antimicrobial and antifungal effects (Kim et al., 2007; Pasquet et al., 2014). For example, the antimicrobial effect of ZnO is due to the production of reactive oxygen species, the destabilization of microbial membranes when in contact with ZnO particles, and the antimicrobial properties of Zn^{2+} ions released by ZnO in an aqueous medium (Pasquet et al., 2014). Reduction of ATP synthesis in bacterial cells could be due to either a direct effect on bacterial metabolism or interference with the extracellular membrane function, inhibiting the transport of nutrients (Sox and Olson, 1989; Sadler et al., 1999). Bismuth has also been demonstrated to inhibit ureases, which are essential in some bacteria, and thus, it interferes with vital bacterial metabolism that culminates in bacterial death. However, *Strep. uberis* is a urease-negative bacterium and showed high susceptibility to bismuth in the present study, suggesting that the action mechanism of bismuth, in this case, could be different. Bismuth compounds were also found to be effective inhibiting and disrupting biofilm formation (Folsom et al., 2011; Hernandez-Delgadillo et al., 2012). The 4 strains used here were non-biofilm formers when cultured in TSB (Sarah Chia Jia Ning, School of Chemical Engineering and Food Technology, Singapore Institute of Technology; personal communication). Hence, this might not be the reason for the inhibition of growth observed in this study. It is not known whether biofilm inhibition by bismuth subnitrate teat sealants is an action mechanism that happens in vivo.

Our in vitro results are in part supported by the findings of several in vivo studies. In a study using nulliparous heifers, the application of bismuth subnitrate before parturition reduced the prevalence of IMI after calving compared with pretreatment infection levels, suggesting that bacterial growth might have slowed after being in contact with bismuth-based teat sealants (Parker et al., 2007). Another study also reported a lower prevalence of CNS infections post-calving in a group of cows treated with internal teat sealant (Berry and Hillerton, 2002). Bismuth subnitrate in the internal teat sealant could have inhibited the growth of bacteria in the teat canal to undetectable levels, which could assist in preventing bacterial invasion over the dry period. However, this requires experimental testing.

Other mechanisms in mastitis prevention of the bismuth-based teat sealants currently under investigation are the stimulation of the immune response, formation of a physical barrier, and prevention of biofilm formation, among others. Most studies comparing the efficacy of teat sealants with antibiotic DCT showed no difference in clinical mastitis incidence during the dry period and after calving (Woolford et al., 1998; Huxley et al., 2002). Interestingly, these studies state that the bismuth-based internal seal was as effective as an antibiotic in preventing new IMI over the dry period (Huxley et al., 2002; Bhutto et al., 2011; Rabiee and Lean, 2013). Our results showing inhibition of bacterial growth by bismuth subnitrate might at least partially explain these observations. The inhibitory effect of bismuth could be avoiding the colonization of the teat canal by mastitis pathogens during the dry period. However, in our studies, bismuth subnitrate was tested in a powder form and not within a teat sealant formulation with other excipients.

The results of this study provide impetus for the reconsideration of the definition of internal teat sealants as "inert." The word "inert" might not accurately reflect all mechanisms contributing to the efficacy of the bismuth subnitrate-based products. Further in vitro experiments are required before the observations reported herein can be applied in vivo. It would be important to assess the effect of bismuth subnitrate within the teat sealant formulations on bacterial growth at different concentrations and with different pathogens, for example, CNS and different streptococci. These organisms are highly prevalent throughout the dry period in dairy cows and heifers (Green et al., 2002; Parker et. al., 2008).

CONCLUSIONS

The results obtained in these in vitro studies show that bismuth subnitrate slowed bacterial growth in 4 major mastitis-causing agents to different degrees. This suggests that the efficacy of bismuth subnitrate-based teat sealants in preventing clinical mastitis may at least in part be due to a reduction in growth of the major mastitis-causing agents, particularly *Strep. uberis*, in the teat canal during the early stages of IMI in the dry period. In addition to the barrier effect described in previous works, the presence of an inhibitory effect in bismuth subnitrate formulations might be crucial for their high efficacy as documented. It may also indicate

that new intramammary methods to prevent mastitis over the dry period will require inhibitory substances in addition to barrier components.

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Article

Cellular Response of Neutrophils to Bismuth Subnitrate and Micronized Keratin Products In Vitro

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Abstract: The aim of this study was to assess the effect of bismuth subnitrate and micronized keratin on bovine neutrophils in vitro. We hypothesized that recruitment and activation of neutrophils into the teat canal and sinus are the mechanisms of action of bismuth subnitrate and keratin-based teat sealant formulations. To test this, a chemotaxis assay (Experiment 1) and a myeloperoxidase (MPO) assay (Experiment 2) were conducted in vitro. Blood was sampled from 12 mid-lactation dairy cows of variable ages. Neutrophils were extracted and diluted to obtain cell suspensions of approximately 10^6 cells/mL. In Experiment 1, test substances were placed in a 96-well plate, separated from the cell suspension by a 3 µm pore membrane and incubated for 3 h to allow neutrophils to migrate through the membrane. In Experiment 2, neutrophils were exposed to the test products and the amount of MPO released was measured by optical density. Results showed that neutrophils were not activated by bismuth or keratin products ($p < 0.05$) in all of the tests performed. These results suggest that the mechanisms of action of bismuth subnitrate and keratin-based teat sealants do not rely on neutrophil recruitment and activation in the teat canal and sinus after treatment.

Keywords: teat sealants; bismuth subnitrate; keratin; neutrophils; teat canal; immune response

1. Introduction

Methods to treat and prevent mastitis in the dry period have been available since the middle of last century [1,2]. The current and most commonly used methods are teat sealants and dry cow antibiotic therapy. The use of these formulations reduced the incidences of intramammary infections in the dry period and early lactation in treated compared to non-treated quarters [3]. Teat sealants containing bismuth subnitrate were developed in the 1970s and are effective at preventing new dry period infections in challenge and natural exposure studies [4,5]. The proposed mechanism of action of bismuth-based products is the creation of a physical barrier that prevents passage of mastitis pathogens into the teats, but this has not been shown experimentally despite their proven efficacy.

The teat canal is the duct that connects the lumen of the udder with the external environment. It is lined by modified teat skin. Macrophages, Langerhans, plasma, and dendritic cells have been described as being present under the basal membrane in the teat canal epithelial tissue. They are dedicated cells that take up, process, and present antigens to T-cells in their major histocompatibility complex (MHC) receptors during infections [6,7]. Neutrophils are polymorphonuclear cells (PMN), which are the first active line of defense against pathogens entering the mammary gland. The surveillance roles conducted by PMN consist mainly of their ability to migrate to the site of infection (chemotaxis),

a respiratory burst and myeloperoxidase (MPO) release. During phagocytosis, neutrophils increase their oxygen consumption through the activity of nicotinamide adenine dinucleotide phosphate oxidase (NADPH-oxidase), and via successive electron reductions generate superoxide anion O_2 and hydrogen peroxide (H_2O_2) [8,9]. This process is known as the respiratory burst. These oxygen metabolites activate additional reactive oxygen species (ROS) that are strongly anti-microbial, for example MPO. Myeloperoxidase is an enzyme present in azurophilic granules in neutrophils and performs a vital role in destroying phagocytosed microorganisms. In mastitis, neutrophils migrate from blood at the Fürstenberg's rosette and teat sinus into the teat lumen, following chemotactic signals released from these antigen presenter cells, bacterial invaders or damaged tissue [10,11]. The presence of high somatic cell counts (SCC) in milk has a protective effect against mastitis induced by major pathogens [12–14]. The presence of neutrophils in milk could be considered valuable in preventing infection by major mastitis pathogens, especially during the early dry period and after treatment with internal teat sealants. However, most of the reviewed literature shows that an increase in somatic cell counts (PMN, macrophages, and desquamating epithelial tissue) are signs of intramammary infection [15,16]. A chemotactic effect of bismuth subnitrate based teat sealant has not been experimentally demonstrated. Based on this, another proposed mechanism of action of bismuth subnitrate based teat sealants is the generation of a local cellular immune response after treatment that protects and, hypothetically, cures subclinical mastitis [17]. Similarly, a novel keratin-based teat sealant product under development showed, in preliminary studies, an increase in SCC after treatment suggestive of a mechanism of action related to the recruitment of neutrophils into the teat canal and sinus [18].

The hypothesis of this study is that bismuth subnitrate and keratin-based teat sealants induce chemotaxis and activation of neutrophils in the teat canal and sinus and hence protect the mammary gland against mastitis infections during the early dry period. In order to test this hypothesis, our objective was to evaluate the ability of bismuth subnitrate and micronized keratin to induce a cellular response (migration and activation) in vitro.

2. Materials and Methods

2.1. Blood Collection and Neutrophil Preparation from Peripheral Blood

The study protocol was approved by the Massey University Animal Ethics Committee, protocol number 18/02, on the 26 February 2018 Fifty-milliliter blood samples were collected into EDTA tubes (BD-vacutainer K2E EDTA, BD-Plymouth, PL6 7BP, UK) by coccygeal venipuncture of 12 dairy cows at mid-lactation. This study compared the in vitro activation of neutrophils in response to bismuth subnitrate and micronized keratin against a negative control using media only.

Neutrophils were isolated from blood and the number of neutrophils per sample was determined using a TC20 automated cell counter, (Bio-Rad Laboratories, Hercules, CA 94547, USA). Neutrophil suspensions were used to complete chemotaxis assays and myeloperoxidase tests in vitro. Within 1 h of blood collection, neutrophils were separated from whole blood samples. Briefly, approximately 50 mL of blood was transferred into two 50 mL conical tubes and centrifuged at 1500× *g* for 30 min at room temperature. After centrifugation, the plasma, buffy coat layer and top layer (approximately 1/3) of packed red blood cells were removed by aspirating with a Pasteur pipette. Approximately seven milliliters of blood were left in each tube and 38 mL of MQ water was added to lyse the red blood cells. The tube was rotated for 5 s. Then, 5 mL of 10× *g* concentrated PBS pH 7.4 was added and the tube immediately rotated again to restore osmotic balance for the cells. The tubes were centrifuged at 330× *g* for 10 min at room temperature (20–22 °C) and the supernatant discarded, leaving a neutrophils-rich red pellet at the bottom of each tube. The pellet was washed with 10 mL of 1 × PBS pH 7.4 and vortexed to mix. The tubes were centrifuged for 5 min at 670× *g* at room temperature 20–22 °C, the supernatant was discarded, and the cells re-suspended in 2 mL of the media required in the procedures for each performed assay. Cell suspensions prepared using this procedure had ≥90% pure populations of living neutrophils confirmed by cell counting with trypan blue.

2.2. Chemotaxis Assay (Experiment 1)

A chemotaxis assay was performed using a CytoSelect 96-well Cell Migration Assay (3 μm, Fluorometric Format, Cell Biolabs. Inc., San Diego, CA 92126, USA). A cell suspension containing approximately 5×10^6 cells/mL was prepared in serum free-media (RPMI 1640, R8758-500ML, Sigma Aldrich, Inc. Christchurch, New Zealand) containing 0.5% bovine serum albumin (BSA). Fetal bovine serum 10% was used as a positive control chemoattractant. Fetal bovine serum 10% was used as a chemotactic agent for the positive control (PC) and 0.5% BSA-RPMI was used as the negative control (NC). Keratin and bismuth subnitrate suspensions were prepared as follows to create high and low concentrations (3% and 1.5%, respectively). These concentrations were chosen, as 3% was the maximum concentration of bismuth subnitrate that could be manipulated with minimal precipitation of the suspension. For the keratin and bismuth suspensions in high concentrations (KH and BH treatments), 1.2 g of bismuth subnitrate or sterile micronized keratin were added to 40 mL of RPMI. Keratin and bismuth at low concentrations (KL and BL treatments) were prepared with 0.6 g of bismuth subnitrate or micronized keratin in 40 mL of RPMI. In a cell culture chamber, 150 μL of KH, BH, KL, BL, the positive control and RPMI with 0.5% BSA used as the negative control (NC) were loaded in the feeder tray (bottom plate) in triplicates. A membrane chamber was placed on top of the feeder tray. One hundred microlitres of the cell suspension were added to each well and the plate was incubated in a cell culture incubator at 37 °C for 3 h. The cell suspension from inside the membrane chamber was carefully discarded by inverting the plate and the insert chamber was transferred to a clean 96-well plate containing 150 μL of pre-warmed Cell Detachment Solution. This plate was incubated for 30 min at 37 °C. Cells were completely dislodged from the underside of the membrane by gently tilting the insert several times in the Detachment Solution, and the insert was removed and discarded. Seventy-five microlitres of media from the feeder tray were combined with 75 μL of the Detachment Solution in a clean 96-well plate. Fifty microlitres of 4X Lysis Buffer/CyQuant GR dye solution was added to each well (already containing 150 μL of Cell Detachment Solution). This step combines cells that migrated through the membrane and into the medium and migratory cells that were detached from the bottom side of the membrane by the Cell Detachment Solution. The plate was incubated for 20 min at room temperature. One hundred and fifty μL of the mixture was transferred to a 96-well plate suitable for fluorescence measurement. Fluorescence was read using a fluorescence plate reader (Flexstation 3 by Molecular Devices, Biostrategy, Auckland, New Zealand) at 480 nm/520 nm (SoftMaxPro version 5.4.1, Molecular Devices, Biostrategy, Auckland, New Zealand). Fluorescence levels in this study were obtained as a result of the lysis of migrated cells in the wells at the bottom of the analyzed plate.

2.3. Myeloperoxidase Assay (Experiment 2)

Myeloperoxidase release was measured in a 96-well plate from Phorbol 12-myristate 13-acetate (PMA, positive control), keratin, or bismuth-stimulated neutrophils and from lysed neutrophils to account for the total amount of MPO contained within the neutrophils. Isolation of neutrophils was performed as described above obtaining 2.5×10^7 cells/mL of HBSS (14025092, Life Technologies NZ Ltd. Auckland, New Zealand). A 96-well plate (ELISA Microplate 96F, GR655061, Grenier Bio-one, International GmbH, Kremsmünster, Austria) was seeded with 50 μL/well of lysis reagent, 50 μL/well of stimulation reagent or 50 μL/well of bismuth or keratin in high concentration (3% BH, KH). Negative control (NC) wells contained 50 μL/well of HBSS. The lysis reagent contained 0.02% hexadecyltrimethylammonium bromide (Sigma) in MQ water. Stimulation reagent was prepared by mixing 1 part of CaI stock solution (50 μg/mL calcium ionophore A23187 (Sigma) in HBSS), 1 part of cytochalasin B stock solution (50 μg/mL cytochalasin B (Sigma) in HBSS), 1 part of PMA stock solution (20 μg/mL PMA (Sigma) in HBSS, and 7 parts of HBSS. Plates were incubated at 30 °C for 60 min. 3,3′,5,5′-Tetramethylbenzidine (Becton Dickinson Ltd. Auckland, New Zealand) and hydrogen peroxide were mixed 1:1, and 100 μL of the mixture was added to each well. Colour was allowed to develop at room temperature for approximately 2 min and 50 μL of 2 N H_2SO_4 was added as a stop reagent. Plates were centrifuged at 600× *g* for 5 min at room temperature, and 150 μL/well

was transferred into a new plate. Optical density at 450 nm was determined using a microtiter plate spectrophotometer (Versamax, Molecular Devices, Sunnyvale, CA, USA).

2.4. Statistical Analysis

The data from these studies were not normally distributed, so they were transformed by log2 transformation. Mixed linear models included treatment as a fixed effect and cow as a random effect in SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Statistical significance was set at $p < 0.05$. Statistical differences were based on Tukey-Kramer adjusted pairwise comparison per product tested against the negative control.

3. Results

3.1. Chemotaxis Assay (Experiment 1)

Bismuth subnitrate and keratin products did not show signs of increased chemotaxis (Figure 1). Bismuth subnitrate in high (BH) and low concentrations (BL) showed significantly lower levels of chemotaxis than the negative control.

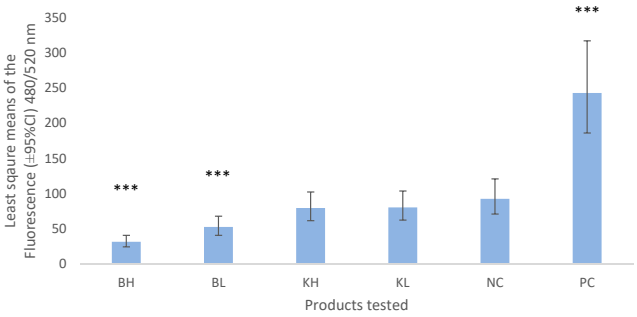

Figure 1. Least square means (±95% CI) of the fluorescence values obtained from chemotaxis assay measured at 480/520 nm wavelength. BH = bismuth high (3% *w/v*), BL = bismuth low (1.5% *w/v*), KH keratin high (3%), KL = keratin low (1.5%), NC = negative control (0.5% BSA-RPMI) PC = positive control (FBS). Asterisks show the significance levels of the comparison with the negative control. *** means p-value < 0.001.

3.2. Myeloperoxidase Assay (Experiment 2)

Bismuth and keratin induced a lower release of MPO than the negative control ($p < 0.001$, Figure 2). Negative control (NC) values represent approximately 50% of the values obtained by the total lysis of the neutrophils and their total MPO content (Figure 2, Lysis).

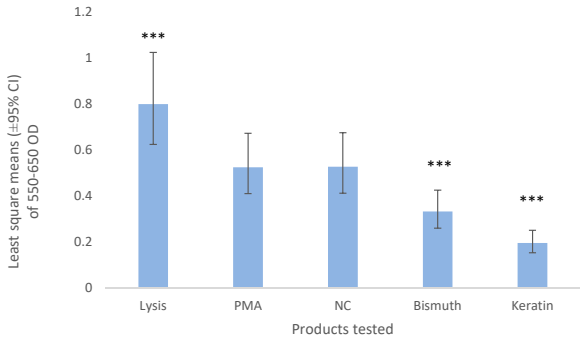

Figure 2. Least square means (±95% CI) of 550–650 optical density of the neutrophils exposed to bismuth 3% and keratin 3%. HBSS was used as a negative control (NC) and 1 part of 20 µg/mL of PMA, 1 part of 50 µg/mL calcium ionophore, 1 part of 50 µg/mL cytochalasin B with 7 parts of HBSS was used as the positive control (PMA). The lysis buffer contained 0.02% hexadecyltrimethylammonium bromide. *** means p-value < 0.001.

4. Discussion

In this study we investigated the response of bovine neutrophils to bismuth subnitrate and micronized keratin products in vitro. We hypothesized that these products activate neutrophils inducing chemotaxis and MPO release. However, the results show that neutrophils were not activated by the test compounds in vitro. Most of the research on the mechanism of action of bismuth focuses on the mechanical effect of bismuth subnitrate intramammary teat sealants in preventing bacterial infections by blocking the teat canal and teat sinus to bacterial colonization [3,19]. This appears to be the first published study to evaluate in vitro the cellular response as a potential mechanism of action of bismuth subnitrate and the novel micronized keratin compound.

The objective of Experiment 1 was to evaluate in vitro the chemotactic effect of bismuth subnitrate and micronized keratin on bovine neutrophils. Previous studies have proposed that the mechanism of action of bismuth subnitrate might be the activation of immune cells in the teat canal [17,20]. Bismuth subnitrate used in internal teat sealants, with or without concomitant antimicrobial treatment, prevented and cured mastitis by generating an increase in neutrophil concentration and cytokine expression in the mammary gland [17]. These results showed that bismuth subnitrate treated teats had an increased number of neutrophils in milk after treatment and a significant increase in the TNF-α:IL-8 ratio. However, care must be taken when analyzing these results as the study had its limitations. The increase in neutrophils occurred in all treated teats whether treated with dry cow therapy or bismuth. Hence, there was no evidence to conclude that bismuth subnitrate induced neutrophil migration. In addition, the study did not describe how teat sealants were applied to teats. Any substance infused into the mammary gland may induce an immune response mainly involving PMN. Constricting the teat sinus at the base of the teat when infusing internal teat sealants is important, as it avoids the products being infused into the mammary tissue and inducing a large cellular response. The application of a chitosan-based teat sealant product, when compared with a bismuth-based teat sealant and an untreated control, did not show differences in neutrophil concentration or in markers of inflammation in the milk of treated cows [20]. This supports a lack of chemotactic effect in vitro from bismuth subnitrate and chitosan as was shown in the current study for bismuth subnitrate and micronized keratin. Our results align with others who found that chemotaxis of macrophages was inhibited by tripotassium dicitrato bismuthate (a bismuth-based treatment for stomach ulcers), thus showing a putative anti-inflammatory effect of bismuth-based formulations [21].

We found that a small percentage of neutrophils migrated through the 3 µm membrane (NC was different from zero). This could have been caused by random migration through the membrane or simply by gravity, which has been reported previously [22]. The significantly lower values of chemotaxis shown by the bismuth-exposed neutrophils compared to the negative control could be an indication that bismuth subnitrate prevented or inhibited the migration of neutrophils through the membrane. This could be explained by bismuth subnitrate being a charged molecule. Chemotaxis starts by depolarization of the neutrophil's membrane followed by a period of hyperpolarization [23]. Bismuth subnitrate charged molecules may interact with the neutrophil membranes to prevent depolarization or prolong the hyperpolarization period, thus impeding the passage of the neutrophils through the 3 µm pores. It is also possible that bismuth subnitrate particles blocked the pores, as in our study BH (bismuth in high concentration, 3%) showed lower levels of chemotaxis than BL (1.5%). In further research a different experimental design would be required to demonstrate such mechanisms. There was no difference between the results of keratin in high and low concentrations and the negative control in this study, suggesting that keratin did not induced chemotaxis in vitro. This is opposite to what has been reported in previous in vivo studies, where an increase in SCC was present after introducing a micronized keratin product into the teat canal [18].

Myeloperoxidase release from neutrophils exposed to bismuth and keratin was significantly lower than from the negative controls that were not exposed. One of the biggest limitations of this study was that the positive control failed to activate the neutrophils. Exposure of neutrophils to cytochalasin B, a fungal metabolite, blocks the polymerization of contractile microfilaments and,

consequently, facilitates degranulation [24]. However, even though neutrophils were treated with cytochalasin B and PMA in this study, they did not show signs of MPO being released in the positive control wells. Although in this study PMA-stimulated cells were not significantly different from the NC, the MPO value of the cells that were lysed (thus releasing 100% of the MPO content in the cells) was considered a valid positive control. All the other values, therefore, can be presented as a proportion of this 100% MPO content since all showed significantly lower levels of MPO release [25]. Since bismuth and keratin presented significantly lower levels of MPO release than the positive and negative control we can confidently conclude that there was no increased activation of neutrophils. The lack of reaction to the PMA could be due to an early activation and exhaustion of MPO within the cells prior to the conduct of the study due to manipulation as this has been reported in previous studies [26,27].

There is evidence that bismuth compounds (specifically bismuth subsalicylate and bismuth subgallate) have anti-inflammatory effects. Bismuth subgallate inhibited nitric oxide production in macrophages by inhibiting the mRNA expression and stability of the enzyme nitric oxide synthases [28]. In addition, a reduction in mononuclear cells and neutrophilic inflammation was observed in the histological analysis of patients with microscopic colitis after treatment with bismuth subsalicylate [29]. This could explain the low values of MPO observed in this study for the bismuth exposed neutrophils. Historically, bismuth subnitrate was used by intramuscular injection for treatment of syphilis [30]. If bismuth subnitrate had induced chemotaxis and activation of neutrophils when injected intramuscularly, the site of injection would have been swollen and inflamed. However, no reports occur on bismuth subnitrate causing an inflammatory reaction at the injection site [31]. In agreement with the results obtained in this in vitro study where bismuth subnitrate did not induce chemotaxis or MPO release and activation after treatment, bismuth subnitrate is classified as an "inert product" when inserted into the teat sinus within a teat sealant product during the early dry period [32].

Studies on the effect of keratin products on the teat canal are lacking. Micronized keratin is currently used in the human cosmetic industry and there have been no reports of inflammatory reactions after treatment of skin with micronized or hydrolyzed keratin. Furthermore, in various studies conducted with micronized wool keratin applied to human skin, researchers found that the keratin formulations reinforced the skin barrier integrity and improved its water-holding capacity [33]. This supports our findings of low levels of activation and lack of chemotaxis for keratin exposed neutrophils [33,34].

The results obtained in this study should be complemented by in vivo experiments in which chemotaxis and activation of neutrophils within the teat canal are assessed. A reason for this is that differences between in vivo and in vitro study results exist, especially when dealing with inflammatory cell responses such as the mechanisms of activation of neutrophils that were addressed in this study [35].

These results provide an impetus for the continuing investigation of the mechanism of action of teat sealant treatments. For example, "what are the effects of bismuth subnitrate and keratin in vivo when inserted in the teat canal?" and "can bismuth or keratin prime neutrophils?" i.e., can bismuth or keratin take neutrophils to a more responsive state without activating them? These questions still remain unanswered. The results of this study strongly suggest that mechanisms of action of bismuth subnitrate, and micronized keratin are not the activation of a local immune response in the teat sinus and teat canal.

5. Conclusions

Results of the described in vitro experiments indicated that bismuth and keratin products did not induce chemotaxis nor activate neutrophils. Further in vivo research is recommended to address questions on the mechanisms of action of bismuth subnitrate and micronized keratin when utilized as internal teat sealants for prevention of intramammary infections.

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