Investigation of palpable udder defects in non-dairy ewes

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Abstract

Udder defects have been associated with low survival rates and reduced live weight gains in pre-weaned lambs and increased pre-mature culling of ewes. However, there are gaps in our understanding of the pathology of udder defects and changes that may occur over time. To address these issues three studies were undertaken. Chapter 4 describes the effect of palpable udder defects (hard or lump) on milk production and composition, while Chapter 5 assesses changes in udder half defects over time. In chapter 6, aerobic bacterial species were identified, and in Chapter 7 the gross and histological lesions associated with palpable udder defects were described.

Milk yield was reduced by more than 50% in defective udder halves; however, the whole udder milk yield depended on the persistence and type of the defect and contralateral udder half compensatory increase. Udder half defects changed over time in lactation and during the non-lactating period. Udder halves categorised as hard or lump at pre-mating were more likely (Relative risk ratio (RRR= 6.8-1440) to be defective at some time in the future, within the same year or in the following season, compared to the udder halves categorised as normal.

Mannheimia haemolytica and *Streptococcus uberis* were identified as dominant bacteria from defective udder halves while *S. aureus* was isolated from both defective and normal udder halves in similar proportions. Among several bacterial species identified during lactation, *Mannheimia haemolytica, Staphylococcus xylosus, and Streptococcus pluranimalium* were more stable over time. Udder halves categorised as hard was described as moderately enlarged and contained a single large abscess on dissection while typical histology showed the presence of pyogranuloma with extensive chronic inflammation and granulation tissue. The pathological changes in udder halves categorised as lump varied from normal appearance to severe chronic inflammation with a purulent exudate.

In conclusion, due to an increased risk of future recurrence and long-term damage to the mammary tissue associated with udder halves categorised as hard, ewes with such defects should be culled. In contrast, udder halves categorised as lump showed marked pathological variation; therefore, additional studies are needed to determine more accurately, by sub-categorising and/or use of methods other than udder palpation (e.g., ultrasonography).

Keywords: Bacteria, Change Over Time, Gross pathology and Histology, Milk Composition, Milk Yield, Palpable Udder Defect, Somatic Cell Counts.

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Key Abbreviations

BCS	Body Condition Score
CML	Cranial Midline Lump
DCC	DeLaval Cell Counter
FTIRS	Fourier Transform Infrared Spectroscopy
Ig	Immunoglobulin
IMI	Intra-mammary Infection
IMM	Intra-mammary Mass
LSM	Least Squares Mean
MALDI-ToF	Matrix-assisted Laser Desorption/Ionization Time-Of Flight
PMN	Polymorphonuclear Neutrophils
Pp.	Predicted probability
RRR	Relative Risk Ratios
SCC	Somatic Cell Count
SEM	Standard Error of the Mean
SNF	Solids Non-Fat
Spp.	Species

Chapter 1: Overall Introduction

The major changes in the New Zealand sheep flock over the past 30 years have included increased fecundity and increased emphasis on pre-weaning lamb growth rates (Beef and Lamb New Zealand, 2019a; Morris, 2009). Lambs are born with limited energy reserves and no passive immunity (Abdel-Salam *et al.*, 2019; Lerias *et al.*, 2014; Nowak & Poindron, 2006). Therefore, gaining rapid access to a healthy and fully functional udder is essential for new-born lambs to ingest colostrum to ensure they survive and thrive. Lamb survival rate and pre-weaning growth rates are highly dependent on their dam's milk supply and milk composition (Arsenault *et al.*, 2008; Danso *et al.*, 2016; Lerias *et al.*, 2014; Ramsey *et al.*, 1994; Watson & Buswell, 1984), which is directly affected by udder defects (Burriel, 1997; Hayman *et al.*, 1955; Tančin *et al.*, 2017).

Lamb survival to weaning is lower in lambs suckling ewes with defective udders (Arsenault *et al.*, 2008; Griffiths *et al.*, 2019a; Hayman *et al.*, 1955). In addition, udder defects have been associated with a reduced pre-weaning lamb growth rate (Fthenakis & Jones, 1990b; Griffiths *et al.*, 2019b; Hayman *et al.*, 1955; McLaren *et al.*, 2018). Udder defects have been shown to be important causes of premature culling in different breeds of ewes in both New Zealand (Flay *et al.*, 2021; Peterson *et al.*, 2017; Quinlivan, 1968a) and internationally (Arsenault *et al.*, 2008; Grant *et al.*, 2016; Huntley *et al.*, 2012; Madel, 1981). However, high rates of premature culling of ewes result in a reduction in farm productivity and profitability and increases costs via the need for retaining or purchasing more replacement ewe lambs (Farrell *et al.*, 2019; Flay *et al.*, 2021).

Udder assessment is a useful tool to identify defective udders of ewes to provide timely selective treatment or make culling decisions (Bergonier & Berthelot, 2003; Grant *et al.*, 2016). Udder assessment is usually performed by visual inspection and/or palpation by farmers or veterinarians (Blagitz *et al.*, 2014; Grant *et al.*, 2016; Saratsis *et al.*, 1998). Applying this management tool for selecting ewes can have a direct impact on reducing the risk of udder defects or improving the rearing ability of ewes (Blagitz *et al.*, 2014; Casu *et al.*, 2006; Griffiths *et al.*, 2019a). Udder defects in dairy ewes can be more easily detected because farmers check the udder during regular daily milking events (Fragkou *et al.*, 2014) whereas, in non-dairy ewes, udder defects may only be identified once or twice a year at events such as weaning or prior to mating, when farmers are selecting ewes for the next breeding season (Saratsis *et al.*, 1998).

Udder defects are abnormalities of the udder, the causes of which can range from non-mechanical injury to infectious agents, which can be detected by palpation or visual inspection with different consistency and size such as lumps in different locations of the gland or diffuse hardness of the tissue (Hayman *et al.*, 1955; Madel, 1981). Acute mastitis and other udder defects including chronic mastitis with palpable udder defects have been reported to be from 0 to 10% internationally (Arsenault *et al.*, 2008; Barber *et al.*, 2006; Cooper *et al.*, 2016; Grant *et al.*, 2016; Onnasch *et al.*, 2002; Saratsis *et al.*, 1998). Studies in New Zealand indicate that the prevalence of udder defects in mixed-age ewes is up to 7.5% (Clark, 1980; Griffiths *et al.*, 2019a; Peterson *et al.*, 2017; Quinlivan, 1968a, 1968b).

Palpable udder defects can be defined as diffuse hard consistency of an udder half or any palpable or detectable mass of different consistency from the rest of the gland tissue upon palpation (Griffiths *et al.*, 2019a). In New Zealand, more than 75% of farmers assess their ewes' udders once a year, presumably to make culling decisions

(Corner-Thomas *et al.*, 2016). Recently, studies of udder defects in Romney ewes in New Zealand showed that lambs reared by dams with palpable udder defects (diffusely hard udder or intramammary masses (IMM) of various sizes and consistency) had lower odds of survival to weaning (Griffiths *et al.*, 2019a) and reduced growth rates when compared to lambs suckling ewes with non-defective udders (Griffiths *et al.*, 2019b). However, there is a paucity of data on the relative importance of various udder defects, and the best time to examine udders, making it difficult for farmers to make more accurate culling decisions.

Furthermore, there is a scarcity of information on how defects change over time. Repeated udder examinations have shown varying percentages of ewes from 5.0 to 7.5% with palpably defective udders across a year in New Zealand (Griffiths *et al.*, 2019a) and from 3.1 to 15.8% variation in two years in the United Kingdom (Grant *et al.*, 2016). Further, 2.4% of ewes developed new palpable udder defects within four to six weeks after initial normal palpation score at weaning (Ridler *et al.*, 2021). Overall, there are gaps in our understanding of the change over time of palpable udder defects in non-dairy ewes. So, it is crucial to better understand the dynamics of udder defects over time and their impact on mammary function.

Bacterial culture from mastitic milk and intra-mammary abscesses have identified numerous bacterial species in both dairy and non-dairy ewes (Arsenault *et al.*, 2008; Bergonier & Berthelot, 2003; Peterson *et al.*, 2017; Ridler *et al.*, 2021; Smith *et al.*, 2015; van den Crommenacker-Konings *et al.*, 2021). *Mannheimia haemolytica (M. haemolytica)* and staphylococcus species are the most commonly found isolates in non-dairy ewes (Gelasakis *et al.*, 2015; Mørk *et al.*, 2007; Ridler *et al.*, 2021). However, there are limited studies on bacterial isolation from udders with palpable defects, especially under New Zealand conditions (Peterson *et al.*, 2017; Quinlivan, 1968b; Ridler *et al.*, 2021). Additionally, most studies evaluating bacterial culture from ewes with udder defects have been done at a single point in time and there has been no investigation of changes over time. Hence, it is essential to identify bacterial species from ewe milk or abscess samples and study the stability or change over time.

Udder defects cause gross enlargement of the mammary glands with hemorrhage and multifocal reddening of the cut surface, forming a mottled appearance (el-Etreby & Abdel-Hamid, 1970; El-Masannat *et al.*, 1991; Watkins & Jones, 2007). Bacterial invasion, multiplication, and toxins in mammary epithelium are associated with initial neutrophilic inflammation (Akers & Nickerson, 2011; Arteche-Villasol *et al.*, 2022; Cifrian *et al.*, 1996; Zhao & Lacasse, 2008). At later stages, microscopic changes include epithelial cell death and parenchymal tissue damage with extensive mononuclear cell infiltration and development of fibrotic tissue at the late stages (Burriel, 1998). Single or multiple accumulation of abscess or purulent exudate may occur within the mammary gland with palpable udder defects (Smith *et al.*, 2015; Watkins & Jones, 2007). Gross and histological changes associated with the dynamics of palpable udder defects are not known. Conventionally, these microbiological and pathologic changes of mammary tissue have been considered to decrease milk production, alter milk composition, and increase milk somatic cell count (SCC) (Burriel, 1998; Gelasakis *et al.*, 2015; Leitner *et al.*, 2003a), but no detailed information exists on palpable udder defects in ewes.

In summary, udder defects cause premature culling of ewes and compromise the welfare and survival of their lambs. A systematic assessment of udders using a standardized udder scoring system is essential to monitor the changes and the relative importance of udder defect types in a season and across seasons. Bacterial identification, gross pathology, and histology are instrumental to understanding the biology related to either infection or tissue changes in the mammary gland and how these alter over time in association with milk production, and milk composition. Overall, in-depth understanding of udder defects can provide information to aid in making scientifically justified and accurate culling decisions, subsequently improving survival and weight gain of pre-weaned lambs.

This thesis comprises eight chapters. Chapter 1 emphasizes the general overview of the thesis and defines the aim of the thesis. Chapter 2 reviews the literature available on causes, mechanisms, and effects of udder defects in ewes. Chapter 3 describes the general methodology of all three studies undertaken, with a special focus on shared materials and methods by all the experimental chapters (chapters 4-7). Each of these four experimental chapters was organized to address specific objectives, and comprise an introduction, materials, and methods (chapter-specific), findings, discussion, and conclusions. The last chapter (chapter 8) discusses the combined summary of the main findings and draws general conclusions.

The overall aims of this thesis, therefore, were to assess effect, change over time, microbiology and pathology of palpable udder defects in non-dairy (Romney) ewes. The specific objectives were to:

- Determine changes in milk yield, composition, and somatic cell counts (SCC) in ewes with various udder half defects (**Chapter 4**)
- Assess the changes in udder half defect status over time, and predict the risk of future udder half defect occurrence (**Chapter 5**)
- Identify aerobic bacteria species from palpably hard, lump, and normal udder halves at different physiological times (**Chapter 6**)

- Describe or characterise gross and histological lesions of palpably defective (hard or lumps) udder halves (**Chapter 7**)

Chapter 2: Literature Review

2.1 Overview of Sheep Production in New Zealand

In New Zealand, sheep and beef cattle are often farmed together (Morris, 2017). The beef and lamb sector is the second largest in the export goods in the country (Beef and Lamb New Zealand, 2019a). For the last three decades, the export sector has grown and diversified with exports to more than 120 countries. Currently, New Zealand exports 80% of beef and 95% of sheep meat produced (Beef and Lamb New Zealand, 2018). Based on land mass coverage, sheep and beef production farms cover one-third of the country's land mass (9.3 million hectares) (Beef and Lamb New Zealand, 2018). Sheep and beef cattle farming systems have been classified into eight farm types ranging from extensive high or hill country to lowland intensive sheep farms as described in Table 2.1 (Beef and Lamb New Zealand Economic Service, 2019b). Sheep and beef farming in New Zealand is extensive in nature with pasture as the main diet at an average of 6.3 stocking rate per hectare with no supplementary feed and no housing (Beef and Lamb New Zealand Economic Service, 2019b).

Table 2.1 Beef and sheep farm classes and estimated number of commercial sheep and beef farms in each class in New Zealand.

	Sheep and beef farm class	Estimated farms
1	South Island High Country	200
2	South Island Hill Country	620
3	North Island Hard Hill Country	920
4	North Island Hill Country	3,055
5	North Island Intensive finishing	1,045
6	South Island Finishing breeding	1,820
7	South Island Intensive finishing	1,040
8	South Island Mixed finishing	465
	Total all classes	9,165

Source: (Beef and Lamb New Zealand Economic Service, 2019b)

Sheep production in New Zealand has been a significant contributor to the national economy since the 19th century (Wickham & McDonald, 1982) and the main focus has been the export of meat (mutton or lamb) and wool. The country is the largest exporter of lamb in the world (Cranston *et al.*, 2017). More recently, however, there is a small developing dairy sheep production industry, which is in line with the increasing demand for sheep milk and cheese internationally (Cranston *et al.*, 2017; Gosling *et al.*, 1997).

Over the past 30 years, New Zealand's total number of sheep has approximately halved from 57.9 million in 1990/91 to 27.5 million in 2017/18. However, over the same time period, lamb production has only decreased by 5% (Beef and Lamb New Zealand, 2019a) due to increases in lambing percentage, fast growth rate of growing sheep (carcass weight) and mating of ewe hoggets (Beef and Lamb New Zealand, 2018). Lambing percentage increased from one lamb per ewe in 1990 to 1.3 lambs per ewe in 2017 which has resulted from an improved understanding of, and addressing, multiple aspects of sheep production including nutrition, growth and reproduction, genetics and health concerns across the industry (Morris, 2013; West *et al.*, 2018).

A large number of breeds of sheep exist in the country. Nevertheless, dual-purpose (meat and coarse wool) straight-bred Romney and their derivatives (Perendale and Coopworth) are the dominant breeds (Beef and Lamb New Zealand Economic Service, 2019a; Morris, 2013). Fine wool-producing Merino sheep and their crosses account for less than 7% of the total population. Many sheep are composites of two, three or four breeds including Romney, Coopworth, Perendale, East Friesian, Finn and Texel (Morris, 2013).

2.2 Udder Health and Culling in Ewes

The mammary gland is an accessory reproductive organ that secretes milk to nourish the new-born (Hurley & Loor, 2011). The gland undergoes repeated cycles of structural development, functional differentiation, and regression (Lerias *et al.*, 2014) to fulfil a variety of physiological, immunological and biochemical functions (Oliver & Sordillo, 1989). Change in physiological status has been associated with intramammary infection (IMI) which might be a possible reason for the gland's susceptibility to infection (Oliver & Sordillo, 1988). IMI leads to elevated Somatic Cell Counts (SCC) (Ariznabarreta *et al.*, 2002), decreased milk yield (Gonzalo *et al.*, 2002), change in milk quality (Leitner *et al.*, 2004) and mammary tissue damage (Burriel, 1997) in sheep. Mammary gland inflammation, also known as mastitis, is a multidimensional disease condition that affects animal welfare, decreases milk production and composition, and has negative economic consequences (Leitner *et al.*, 2003; Persson *et al.*, 2017).

A healthy udder contributes a crucial role for the survival of the new-born lamb by providing colostrum which contains immunoglobulins (Igs) (Abdel-Salam *et al.*, 2019; Lerias *et al.*, 2014) and milk as the sole food source until the lamb's rumen is capable of digesting pasture which can take a few weeks. It also plays an important role for body weight gain and survival of lambs throughout their suckling life (Clark, 1980; West *et al.*, 2018). However, the ewe's ability to produce adequate milk and rear lambs depends on nutrition, genetics, number of lambs being reared and absence of mammary impairment (Hayman *et al.*, 1955).

Generally, ewes are culled at the age of six or seven years, but some farmers keep older ewes even longer (Farrell *et al.*, 2019; Flay *et al.*, 2021; McGregor, 2011). Premature culling is the removal of ewes from the flock prior to the potential end of their productive lifespan and in New Zealand commercial flocks, a higher proportion of ewes are prematurely culled for various reasons than those that are culled for age (Flay *et al.*, 2021). Poor ewe reproductive performance such as failure to conceive and failure to rear lambs post-lambing are typically the main drivers of premature culling in New Zealand due to the seasonal pattern of production (Cranston *et al.*, 2017; Garrick, 1998).

Results from a survey showed that the majority of farmers (>75%) in New Zealand examined teeth and udders annually, most likely for culling decisions (Corner-Thomas *et al.*, 2016). Teeth examination and subsequent culling due to teeth problems has been well established on sheep farms for many years (McGregor, 2011; West *et al.*, 2009). Udder defects, such as abscesses and diffuse hard consistency of udders have been associated with mortality and reduced growth of the lambs (Bruce *et al.*, 2013; Madel, 1981; Menzies & Ramanoon, 2001). Culling of ewes with intramammary masses (IMM), those with a SCC >400,000 cells/ml milk or reduced milk production has also been suggested because these issues result in slower growth rates in lambs (Arsenault *et al.*, 2008; Huntley *et al.*, 2012). The economic cost of udder abnormalities (mastitis) in ewes has been associated with ewe and lamb mortality, treatment costs, reduced milk production, discarded milk and reduced lamb growth (Bergonier & Berthelot, 2003; Leitner *et al.*, 2004) and premature culling (Persson *et al.*, 2017; Watson & Buswell, 1984).

2.3 The Role of Ewe's Milk for Lambs During Early Life

Colostrum is the initial post-partum mammary secretion (Nickerson & Akers, 2011). It is a mixture of substances including fat, lactose, Igs and others components such as vitamins, minerals, hormones, growth factors, cytokines and enzymes (Castro *et al.*, 2011). Colostrum consumption in the first 24 to 48 hours is critical for the survival of the new-born (Nowak & Poindron, 2006) as it is the sole source of initial acquired immunity for ruminant neonates (Stelwagen *et al.*, 2009). Placental passive immunity transfer takes place in species like humans, but it does not occur in ruminants due to their synepitheliochorial placenta which impedes transfer (Wooding, 1992). In addition, the immune system of new-born ruminants does not totally or adequately produce its own Igs during the first weeks of life (Castro-Alonso *et al.*, 2009; Logan & Penhale, 1971). Apart from the defensive role, colostrum regulates body temperature, activates peristalsis, is involved in meconium ejection and participates in protection against bacterial infection of the intestinal mucosa of neonates (Dwyer & Morgan, 2006; Lérias *et al.*, 2014).

Subsequent to colostrum consumption, in early life lambs profoundly depend on ewe milk for survival and growth from birth to weaning including while the lamb is consuming forages (Clark, 1980; Hayman *et al.*, 1955; Lérias *et al.*, 2014). Even though the lamb's rumen can fully digest pasture at the age of three weeks, lambs weaned before six weeks are likely to suffer because they can't make up for the lost milk by suddenly increasing pasture intake (Beef and Lamb New Zealand, 2019b). Ewe's milk contains all essential nutrients for the survival and initial growth of the new-born including protein, lactose, fat, minerals, vitamins, electrolytes, Igs and water. Sheep milk has higher percentages of protein and fat compared to other ruminants (Morand-Fehr *et al.*, 2007; Wohlt *et al.*, 1981). Mammary infection decreases milk yield and modifies (increase or decrease) the main milk composition (Marti De Olives *et al.*, 2013). It is, therefore, essential for lambs to be reared with adequate ewe's milk yield and composition for proper growth and survival (Danso *et al.*, 2016; Lérias *et al.*, 2014; Ramsey *et al.*, 1994).

2.4 Mammary Gland Structure and Milk Production

Studies of anatomy and morphology of the mammary gland in domestic animals including sheep have been conducted for many years (Turner, 1952) as have the morphological characteristics in relation to physiological state (pregnancy, lactation and involution), milk production, machine milking, genetics and mastitis resistance (de la Fuente *et al.*, 1996; Fernández *et al.*, 1997). Udder morphology traits and udder assessment protocols have been developed and recommended for selection and breed improvement programs in dairy sheep (Casu *et al.*, 2006); however, limited studies have been conducted in non-dairy sheep (Rovai *et al.*, 2004).

The two mammary glands in ovine species are considered as a single mass called the udder, which is located at the ventral abdomen between the thighs of the hind legs (Dyce *et al.*, 2010). The udder is suspended and supported to the body wall by medial elastic and non-elastic lateral suspensory ligaments. The ligaments are connected to the abdominal cavity via the inguinal canal that allows passage of blood vessels, nerves and lymphatics. One half of the udder is totally independent of the other having a separate secretory unit, duct system, blood supply, nerve supply, suspensory apparatus and teat (Fails & Magee, 2018). The median suspensory ligament also distinctively splits the two halves (into right and left halves), forming an intramammary groove. Both the halves are covered by a unique skin bag and underneath the skin each gland is wrapped by connective tissue. Teats are covered by fine hair in sheep, unlike most other domestic animals (Nickerson & Akers, 2011; Rovai *et al.*, 2004).

The alveolus is a milk-secreting single layer of cuboidal to columnar epithelial cells (Dyce *et al.*, 2010) which structurally appears globe-like or flattened depending on the volume of milk in the lumen associated with suckling or milking. Myoepithelial cells are contractile smooth muscle cells that surround alveoli and ducts and are responsible for milk let down in response to oxytocin (Akers, 2016). The smallest excretory ducts that drain alveoli combine several branching ducts by draining lobules and lobes of the gland and finally form the large sinus at the lower part of the gland near the junction with the lactiferous sinus which comprises gland and teat cisterns (Fails & Magee, 2018).

After secretion at the level of the alveolus, milk is stored in either the alveolar lumen or gland cistern, with a higher proportion in the alveoli, until suckling or milking (Fails & Magee, 2018). The gland cistern comprises a collection of folds and pockets which are effaced when the cistern is full of milk. The gland cistern ventrally empties to the teat cistern that distally continues as the teat canal; circular smooth muscle bundles at the tip called a sphincter assist in closure of the canal (Dyce *et al.*, 2010). The sphincter prevents leakage of milk or entrance of bacteria from the external environment and skin. Desquamation of teat canal luminal cells leads to the accumulation of keratin that seals the teat orifice between milkings (Akers & Nickerson, 2011).

The mammary gland comprises two types of mammary tissues: parenchyma and stroma. The parenchyma is composed of tubular and alveolar components whereas stroma comprises non-cellular components like blood vessels, lymphatics and nerves (Akers, 2016; Thibault & Levasseur, 2001). A small unit that comprises several alveoli surrounded by connective tissue is called a lobule and a group of lobules surrounded by connective tissue form a lobe (Fails & Magee, 2018). Blood vessels, lymphatics and nerve supply are supported by interstitial connective tissue throughout the gland and teat. The ability of ruminant mammary glands to produce milk is determined by the number of cells secreting milk and their level of activity (Boutinaud *et al.*, 2004). The ratio of growth rate of udder tissues (parenchyma to stroma) before and at puberty may affect the future milking capacity (Boutinaud & Jammes, 2002; Thibault & Levasseur, 2001).

2.4.1 Morphological and functional patterns of the mammary gland

Understanding the dynamics of the mammary gland throughout gestation and lactation is crucial because the gland is one of few body tissues that repeatedly undergo growth, functional differentiation, and regression (Lerias *et al.*, 2014). As a result of these cyclic events, the mammary gland goes through several changes in volume, internal structure and composition in an animal's lifetime that may affect the milk quantity and quality (Akers & Denbow, 2013) . The mammary gland pattern and proportion of parenchyma to stroma changes during the different physiological periods. Physiologic mammary gland patterns include proliferation, secretion, and involution. Proliferation involves multiplication of secreting mammary tissues during pregnancy and early lactation, while secretion begins in late pregnancy and continues during the lactation period (Knight & Peaker, 1984). Involution, on the other hand, takes place around the end of lactation or post-weaning (Petridis & Fthenakis, 2019).

Mammogenesis is the development of both structural and biochemical changes in the parenchyma of the mammary gland which begins during the fetal stage of an animal's life (Akers & Denbow, 2013). The ductal system of the gland grows (isometric) with the body until puberty with the well-differentiated glandular cistern and teats present at birth. The ducts and stroma of the glands grow quickly (allometric growth) at puberty and in late pregnancy under the influence of sex hormones and are associated with physiological changes. These developmental stages of the mammary gland are critical because they may impair subsequent milk production if overgrowth of stromal, relative to parenchymal, tissue takes place (Rovai *et al.*, 2004). The mammary alveolar tissue increases from 10 to 90% during late gestation and in early lactation by replacing the adipose tissue (Akers & Denbow, 2013) . In the ewe, which has a gestation period of approximately 146 days, mammogenesis during pregnancy is clearly seen between days 95 and 100 of gestation which is followed by the start of lactogenesis. This results in an increase of udder size during late pregnancy to early lactation (Thibault & Levasseur, 2001).

The massive structural developments that take place during puberty, pregnancy and early lactation, along with appropriate onset of milk production at parturition and maintenance of milk production, require complex physiological regulatory activity in mammary development (mammogenesis), onset of lactation (colostrogenesis or lactogenesis) and maintenance of milk secretion (galactopoiesis) (Akers, 2016; Brandon *et al.*, 1971; Neville *et al.*, 2002). Proper mammary structural development and metabolism are essential for milk production and suckling to occur (Akers, 2016). Milk yield is positively correlated with udder size or volume (Boutinaud *et al.*, 2004; Fernandez *et al.*, 1995) because the gross mammary volume increases, during pregnancy and early lactation, due to an increase of the milk secretery tissue (Lerias *et al.*, 2014). However, udder structure can be influenced by other factors including genotype, lactation stage and parity (Fernandez *et al.*, 1995; Milerski *et al.*, 2006).

The mammary secretory unit starts to reduce progressively through lactation until it quickly regresses after weaning by the process called involution. Involution takes place by omission of cells while keeping the basic architecture and parenchymal integrity of the gland (Tatarczuch *et al.*, 1997) which causes a decrease in the size of the udder (Lérias *et al.*, 2014; Rovai *et al.*, 2008). After weaning, the mammary gland has proportionately more stroma than parenchyma compared with the lactating stage where the parenchyma grows extensively and constitutes the bulk of the tissue (Fails & Magee, 2018; Lérias *et al.*, 2014; Rovai *et al.*, 2008). In dairy ewes, mammary gland involution is initiated when milking is stopped or gradual involution happens when milking frequency decreases progressively over several days or weeks (Petridis *et al.*, 2014). In non-dairy ewes, removal of lambs from the dams at weaning initiates involution (Sargison, 2009).

2.5 Lactation, Milk Yield and Milk Composition in Sheep

2.5.1 Milk yield

Milk yield in the ewe increases from parturition and then reaches a maximum, before declining gradually until the ewe dries off (Akers, 2017; Turner, 1926). The lactation curve can be defined as a pattern of milk yield throughout lactation and designates the peak and persistency of lactation (Pulina *et al.*, 2007). A desirable lactation curve is characterized by a lactation peak followed by a moderate decline (Grossman *et al.*, 1999; Scatà *et al.*, 2010; Sölkner & Fuchs, 1987). A lactation curve can be affected by several factors such as genetics, hormones, udder morphology, season, management, mastitis, nutrition, stress, age, live weight and size of ewes, and other factors (Glover, 1972; Peterson, 2016; Pulina *et al.*, 2007). Due to these differences and a considerable number of subjective assumptions by researchers, it is usually difficult to compare milk yields between different studies (Scales, 1968). Milk synthesis and secretion is controlled by both local factors in the mammary gland and systemic hormones like prolactin and oxytocin which maintain lactation (Snowder & Glimp, 1991). A high level of milk secretion during early lactation is due to an increase in secretory cell function while the decline in milk yield with advancing lactation is driven by decreased cell number (Capuco *et al.*, 2003).

Lactation curves and milk yield differ between dairy and non-dairy breeds of sheep. They are also affected by the number of lambs reared and the age of ewe. In dairy ewes, there is a relatively lesser lactation peak with persistent milk yield (Figure 2.1)

(Pulina *et al.*, 2007). In contrast, in non-dairy ewes, the lactation peak is relatively higher and is followed by a more rapid decline in milk yield as the lambs increase forage intake. This results in a shorter lactation that lasts about three months (Peterson, 2016; Pulina *et al.*, 2007). In non-dairy Romney ewes, Peterson (2016) reported that daily milk yield peaked at 10-12 days of lactation which halved the peak values by eight weeks. Similarly, Scales (1968) reported Romney, Corriedale, and Merino ewes' milk yield peaked in the second week of lactation and then reduced at a constant rate until the twelfth week. Lactation curves for the pooled data from four different non-dairy breeds (Rambouillet, Columbia, Polypay, and Suffolk ewes) found a 33 and 28% decline in milk yield from 28 to 56 days for ewes with singles and twins, respectively (Snowder & Glimp, 1991).

A higher number of lambs suckling (twins and triplets) has been associated with increased milk yield (Bencini & Pulina, 1997; Geenty, 1979; Loerch *et al.*, 1985; Peterson *et al.*, 2005; Peterson *et al.*, 2006; Snowder & Glimp, 1991) due to an increase in frequency and duration of suckling (Hinch, 1989). Geenty (1979) demonstrated that twin-rearing ewes generally have a greater peak than single-rearing ewes, with both showing a gradual decline in milk yield (Figure 2.2). However, the number of fetuses carried during pregnancy did not influence milk yield (Loerch *et al.*, 1985). Hoggets (young ewes which are usually approximately 1-year-old at parturition) have a lower peak than mature ewes with a lower decline rate (Figure 2.3) (McMillanan *et al.*, 2014; Peterson, 2016).

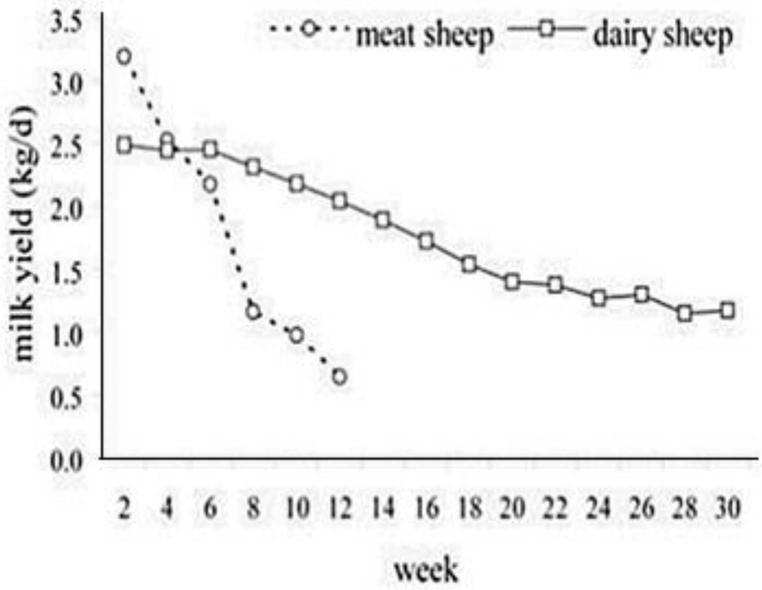

Figure 2.1 Lactation curves of dairy and meat-wool ewes (Cappio-Borlino *et al.*, 1997; Pulina *et al.*, 2007; Snowder & Glimp, 1991)

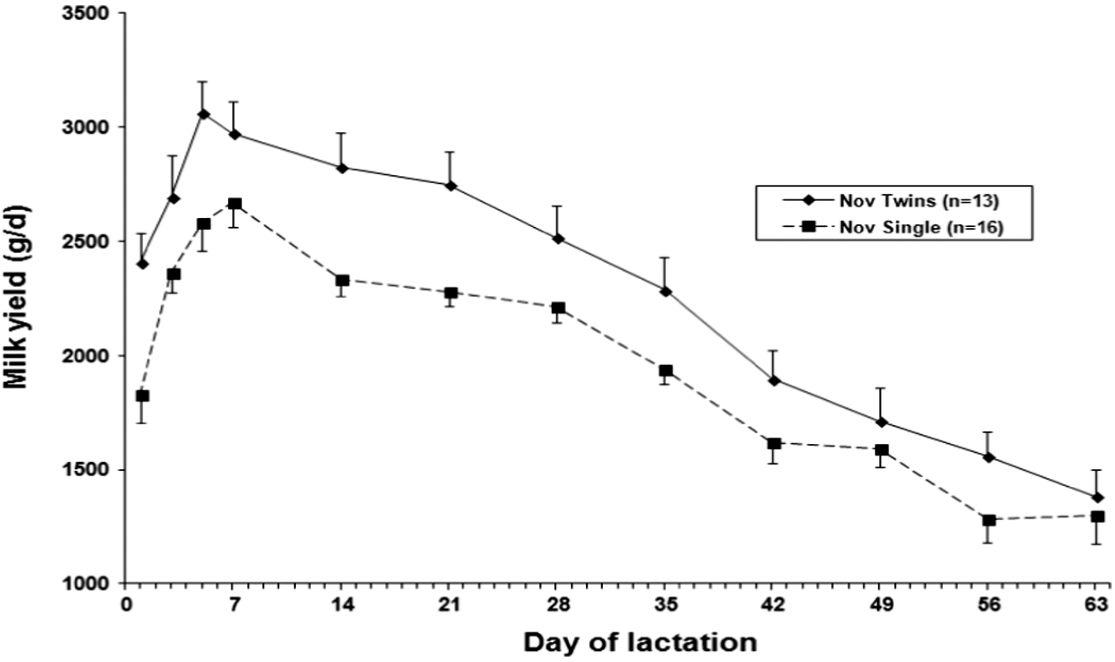

Figure 2.2 Milk yields (g/d) of non-dairy East Friesian ewes with single and twin lambs born in November and grazing pasture in Manawatu, New Zealand (Petersen, 2016)

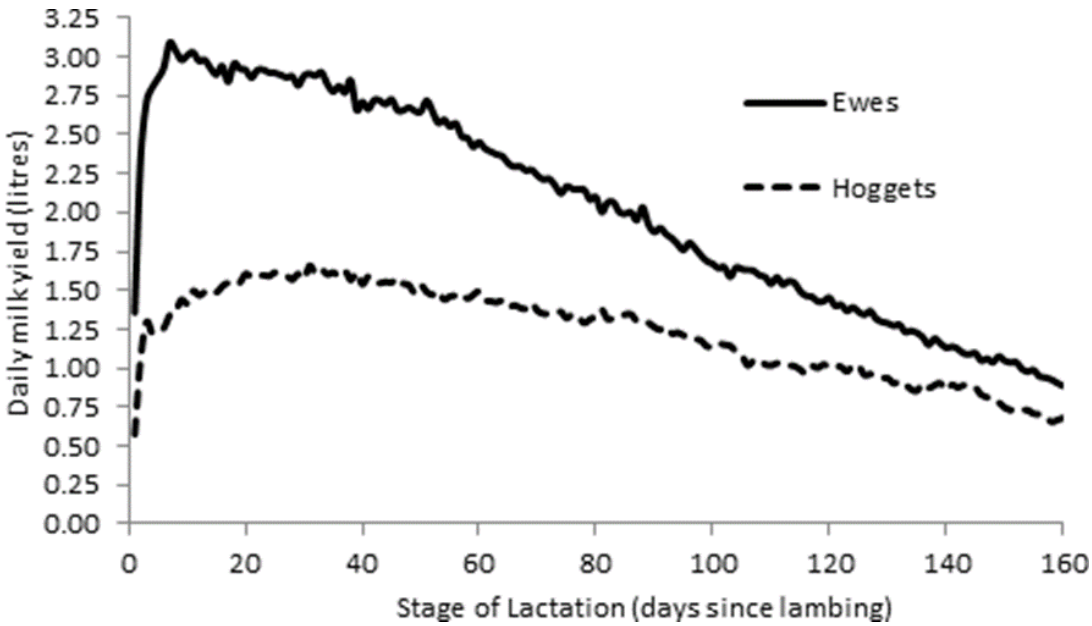

Figure 2.3 Commercial East-Friesian-cross mean daily milk yield in the spring (litres). Includes only ewes and hoggets producing at least 300 and 175 litres to Day 160, respectively (McMillanan *et al.*, 2014; Peterson, 2016).

2.5.2 Milk composition

Water is the main constituent of milk, accounting for more than 80% of its volume, with the remainder (17–18%) consisting of solids including major solids such as proteins, fat, and lactose (Barlowska *et al.*, 2011; Park *et al.*, 2007). Milk also includes minerals, enzymes, cells, hormones, Igs and vitamins (Lérias *et al.*, 2014; Pulina *et al.*, 2005). Sheep milk contains higher total solids and major contents such as crude protein, fat, ash and total solids than goat and cow milk (Table 2.2) (Balthazar *et al.*, 2017; Barlowska *et al.*, 2011; Dario *et al.*, 2008; Hadjipanayiotou, 1995; Park *et al.*, 2007). The normal concentration range of fat, protein and lactose in sheep milk from different breeds of sheep range from 4.10 to 9.30, 3.35 to 6.60 and 3.7 to 5.21%, respectively (Table 2.2) (Hadjipanayiotou, 1995; Kremer *et al.*, 1996; Peterson, 2016; Pulina *et al.*, 2005).

The composition of sheep milk can be affected by genetics (breed), stage of lactation, parity, season, environmental temperature, lactation efficiency, animal age, nutrition and diseases of the udder (Park *et al.*, 2007; Snowder & Glimp, 1991). Total solids, solids non-fat (SNF), fat and protein increase with the advancement of lactation (Broadhurst, 2016; Kuchtík *et al.*, 2008). However, fat is more variable than the other components in milk (Broadhurst, 2016).

Table 2.2 Comparison of sheep milk composition with cow and goat milk.

Species	Parameters	Protein %	Fat (%)	Lactose (%)	Solids-not-fat (%) (Park *et al.*, 2007)
Sheep	Mean	5.73	6.99	4.75	12.0
	SD	0.61	1.25	0.35	
	Min	3.35	4.10	3.70	
	Max	6.60	9.30	5.21	
Cow	Mean	3.42	4.09	4.82	9.0
	SD	0.35	0.46	0.21	
	Min	2.54	3.23	4.40	
	Max	4.19	5.34	5.33	
Goat	Mean	3.26	4.07	4.51	8.9
	SD	0.46	0.76	0.26	
	Min	2.38	3.06	4.08	
	Max	4.43	6.02	5.09	

Source: (Barlowska *et al.*, 2011; Hadjipanayiotou, 1995)

2.5.3 Milk yield and composition correlation

Correlations between fat and protein percentages and sheep milk yield are negative (Ashton *et al.*, 1964; Pulina *et al.*, 2005) due to a dilution effect (Pulina *et al.*, 2005). Production of a higher milk volume results in an increase in lactose synthesis compared with fat and protein (Pulina *et al.*, 2005). The negative relationship between yield and composition of milk may explain why twin-rearing ewes, who produce more milk than singleton-rearing ewes, have lower concentrations of fat and protein in the milk (Bencini & Pulina, 1997).

2.5.4 Effect of milk yield and composition on lamb growth relationship

In healthy ewes, early postpartum milk yield is adequate for the maintenance and growth of lambs (Danso *et al.*, 2016; Scales, 1968; Snowder & Glimp, 1991). The strongest correlation between ewe milk yield and lamb growth rate occurs up to 4-6 weeks of age and then weakens with age as the herbage intake of the lamb increases (Burgos-González *et al.*, 2018; Geenty, 1979; Morgan *et al.*, 2007; Scales, 1968). Nevertheless, growth established in the first 6-8 weeks of lactation affects the later live weight (Gibb & Treache, 1980). Scales (1968) reported a high correlation (coefficient > 0.7) between milk yield and lamb growth up to 12 weeks of age in Romney lambs. The correlation is higher in twin lambs compared with single-born or reared lambs (Morgan *et al.*, 2007; Snowder & Glimp, 1991). Although ewe milk yield explains the greatest variation in lamb live weight gain in the first six weeks of life, milk composition (fat, protein, lactose and SNF) and other factors also play significant roles (Danso *et al.*, 2016; Scales, 1968), but reports on their impact have been inconsistent (Danso *et al.*, 2016; Scales, 1968).

2.5.5 Milk yield estimation in ewes

Milk yield can be estimated by different methods, but accurate estimates of either milk yield by the ewe or milk intake by the lamb are difficult because most of the methods interfere with the natural behaviour of lambs and ewes (Treache & Caja, 2002; van der Linden *et al.*, 2010). This may result in errors with underestimation or overestimation of the lamb intake and/or ewe milk yield (Dove, 1988). Weighing lambs before and after suckling, measuring milk secretion rate, indirect methods of measuring live weight change of the lamb, ewe udder dimensions measurement and marker-based techniques have all been used for estimation of milk yield or milk intake (Aboul-Naga *et al.*, 1981; Treache & Caja, 2002; van der Linden *et al.*, 2010).

Injecting the ewe with oxytocin, followed by hand milking or stripping, stimulates milk ejection or complete expulsion from mammary alveoli and cistern and is more efficient, compared with hand milking (Nezamidoust *et al.*, 2015). The "oxytocin method" followed by machine and/or hand milking is a commonly reported research method for estimating milk yield (Glover, 1972; Morrissey *et al.*, 2007; Paten *et al.*, 2013; Peterson *et al.*, 1997). The use of a low dose of oxytocin (1IU) has been suggested as it is sufficient to ensure milk ejection in ewes and there was no significant difference compared with higher doses (2-5 IU) (Bencini, 1995; De Azambuja Ribeiro *et al.*, 2004).

2.6 Effect of Mastitis and Udder Defects on Milk Yield and Composition

Clinical mastitis, sub-clinical mastitis, or udders with palpable defects such as hardness or lumps can cause mild to serious damage to the mammary gland in ewes (Fthenakis & Jones, 1990b; Madel, 1981; Mavrogianni *et al.*, 2005). These either hinder the synthesis capacity of epithelial cells and/or increase the permeability of blood components into the milk (Burriel, 1997; Harmon, 1994). This may result in reduced milk yield or alter milk composition (Marti De Olives *et al.*, 2013).

In dairy ewes, studies on the effect of mastitis on milk yield and composition have been conducted for the purpose of quantifying milk losses and assessing milk quality for cheese production (Arias *et al.*, 2012; Burriel, 1997; Gonzalo *et al.*, 2002; Leitner *et al.*, 2003). Globally, cheese production is the main form of sheep milk consumption with limited consumption in liquid form (Bencini & Pulina, 1997). In contrast, in non-dairy ewes the focus of mastitis studies (Cooper *et al.*, 2016; Huntley *et al.*, 2012; Martí-De Olives *et al.*, 2020) or udder defects (Fthenakis & Jones, 1990b; Grant *et al.*, 2016; Griffiths *et al.*, 2019a; Hayman *et al.*, 1955) has been on association with the survival and growth of pre-weaned lambs. Mastitis alters the major components of milk in both dairy and non-dairy ewes (Bencini & Pulina, 1997; Martí-De Olives *et al.*, 2020). Further detailed summary of milk yield loss with a causal agent, SCC and udder half or whole udder in dairy and non-dairy ewes are available in a recent review by Martí-De Olives *et al.* (2020).

Clinical mastitis has a significant negative impact on milk yield in ewes (Fthenakis & Jones, 1990b; Gonzalo *et al.*, 2004; Mørk *et al.*, 2007; Smith *et al.*, 2015). Additionally, the milk from affected glands usually contains contaminants including blood, flakes, pus or other abnormalities and there are associated changes in the milk composition (Fthenakis & Jones, 1990b). The milk cell population also significantly changes due to clinical mastitis (Bergonier *et al.*, 2003). Milk from clinically mastitic ewes may not contain adequate nutrients for lamb growth and/or survival in the early post-partum period.

Several authors have quantified the impact of subclinical mastitis on milk yield and composition, although the clinically undetectable nature of the infection and the compensatory effect of the gland make it more difficult to estimate the effects, as reviewed by Martí-De Olives *et al.* (2020). The effect of subclinical mastitis on milk yield depends on whether both or single udder-halves are involved (Gonzalo *et al.*, 2002), the type of causative agent (Gelasakis *et al.*, 2015; Gonzalo *et al.*, 2002) and the severity of infection (Arias *et al.*, 2012), among many other factors. Production loss due to mastitis is proportionally larger in higher producing ewes (Marti De Olives *et al.*, 2013).

Bulk tank, whole udder (individual ewe) and udder half milk sampling approaches have been implemented to diagnose mastitis, quantify the milk yield loss and assess milk composition in ewes (Fragkou *et al.*, 2014; Martí-De Olives *et al.*, 2020; Marti

De Olives *et al.*, 2013). For many years assessment of bulk tank milk has been used as a principal tool to assess and monitor flock udder health in dairy ewe flocks (Gonzalo *et al.*, 2005). While individual ewe assessment of the whole udder has been used to quantify mastitis-associated milk loss in individual dairy or non-dairy ewes (Leitner *et al.*, 2003; Madel, 1981; Saratsis *et al.*, 1999). Considering the anatomic, functional and infection independency of the two udder-halves, and the compensatory effects in both unilateral and bilateral infection, assessment of the whole udder has been associated with huge individual variation (Gonzalo *et al.*, 2004). This variation poses possible risks for underestimation of the impact in sheep or the need for large sample sizes (Gonzalo *et al.*, 2005; Marti De Olives *et al.*, 2013). Hence, milk loss due to mammary infection is better studied by the assessment of individual udder-halves (Martí-De Olives *et al.*, 2020).

In unilateral mammary infection, the response time of milk yield decrease in the infected udder half, and compensatory increase by the uninfected half is rapid following infection (Marti De Olives *et al.*, 2013). These milk yield responses in the mammary gland to an infection (milk reduction and compensation) remain constant for weeks regardless of the time of infection or whether mastitis was present at lambing or occurred after lambing (Marti De Olives *et al.*, 2013; Pulina *et al.*, 2007). Infection prior to the time of peak lactation leads to a reduction in peak lactation followed by a shorter lactation persistency (Pulina *et al.*, 1993). Unilateral infection results in a higher compensatory increase in milk yield by the contralateral udder half unlike bilateral infection (Malek dos Reis *et al.*, 2013). Consequently, bilateral infection results in a severe milk yield loss in ewes (Leitner *et al.*, 2004). Individual ewe milk yield loss caused by subclinical mastitis can be as high as 2.6-15% and 3.6–

43.1 % in the case of unilateral and bilateral infection, respectively (Martí-De Olives *et al.*, 2020).

Mastitis alters milk composition in sheep, and it has been reported that infected and uninfected udder halves show significant differences in total solids overall or individual major milk components including protein, fat, and lactose (Burriel, 1997; Leitner *et al.*, 2004). Lactose concentration decreases in mastitic ewes because of tissue damage which interferes with the synthesis of the precursor, glucose, and due to competition with inflammatory cells for available glucose (Leitner *et al.*, 2003a; Malek dos Reis *et al.*, 2013). Moreover, the osmotic regulation role of lactose accentuates the decrease in concentration of mastitic milk. As a result, lactose concentration has been considered a potential indicator of mastitis (Martí-De Olives *et al.*, 2020). Changes in milk fat and protein levels vary depending on the volume of milk loss or the dilution effect during mastitis (Pulina *et al.*, 2007). The protein percentage can also decrease but can sometimes increase because of increased permeability of the blood during mastitis. In contrast, the casein content does not vary in most cases (Leitner *et al.*, 2012).

In non-dairy ewes, studies on the impact of palpable udder defects on milk yield are very limited despite the potentially serious effects on lamb growth and survival to weaning and the frequent subsequent culling of affected ewes (Grant *et al.*, 2016; Griffiths *et al.*, 2019a). Diffusely hard udders and udders that contain lumps have been associated with a decrease in body weight gain in pre-weaned lambs (Griffiths *et al.*, 2019b) and are also a reported reason for the culling of ewes, as it is assumed they have decreased milk production (Grant *et al.*, 2016; Peterson *et al.*, 2017). At slaughter, Smith *et al.* (2015) collected the udders of ewes with chronic mastitis and reported that some udders with abscesses within them contained no milk in the gland.

Moreover, milk samples collected from udders with defects including nodules, lumps, diffuse hardness, and abscesses were reported to contain clots and flakes, serous and purulent fluid and blood-stained secretions (Saratsis *et al.*, 1998). A condition described as "hard udder", which occurs at or shortly after parturition, has been reported to result in udders that appear to be full of milk, but fail to express milk after lambing (Bruce *et al.*, 2013; Skyrme, 1970).

2.7 Mammary Gland Defence and Infection

2.7.1 Overview of the mammary gland defence system

The most common portal of entry of pathogens to the mammary gland is via the ascending papillary duct route; however, in some cases pathogens can also enter following systemic infection (e.g. Maedi visna virus infection of the mammary gland) (Foster, 2017). Generally, the host's mammary immunity, the pathogen, the environment, and interactions among these factors are the major determining factors of mammary disease development (Schlafer & Foster, 2016; Sordillo & Streicher, 2002). The mammary immune system, as part of the ewe's body, has multidimensional mechanisms to prevent or control infection and disease development. Both innate and adaptive immune systems are involved and coordinate together in mammary gland defence (Sordillo & Streicher, 2002), but the innate system is more important (Foster, 2017; Schlafer & Foster, 2016; Uthaisangsook *et al.*, 2002) as the adaptive system is slow in response to novel threats (Stelwagen *et al.*, 2009).

Innate immunity comprises physical, soluble and cellular factors (Foster, 2017). Physical anatomic structures are the barrier and first line of defence for mammary infection. The sphincter muscle of the teat maintains tight closure of the papillary duct while the stratified squamous epithelium of the teat duct secretes keratin which plugs the duct to prevent bacterial penetration between milking/suckling and dry period and has an antibacterial effect (Paulrud, 2005; Sordillo & Streicher, 2002). If bacteria pass through the physical barriers of the teat end, they then encounter non-specific defence by cells and soluble factors of the mammary immune system (Sordillo & Streicher, 2002).

Lactoferrin, lysozyme, complement, cytokines, chemokines, and growth factors are humoral (non-cellular) factors found in secretions of leukocytes like neutrophils and macrophages. These non-cellular factors are bacteriostatic components that work independently and in concert with Ig and cellular factors (Sordillo *et al.*, 1997). Lactoferrin is an iron-binding protein which withholds iron from bacteria by preventing the production of the bacterial enzyme, dismutase. This bacterial enzyme dysfunction inactivates superoxide radicals which may enhance killing by phagocytes (Smith & Oliver, 1981). Complement is a collection of serum and milk proteins that performs in association with a specific antibody to cause lysis of invading bacteria (Barrio *et al.*, 2003). In addition, these factors induce the release of pro-inflammatory cytokines that attract neutrophils for phagocytosis (Carlsson *et al.*, 1989; Ezzat Alnakip *et al.*, 2014; Schlafer & Foster, 2016).

Mammary immune responses are also mediated by innate immune cells comprising neutrophils, macrophages, natural killer cells and dendritic cells (Rainard & Riollet, 2006). In the normal mammary gland these immune cells provide surveillance for mastitis-causing pathogens and are involved in restructuring during involution. Following bacteria entry into the mammary gland, the immune cells participate in phagocytosis and initiation of inflammation (Barber *et al.*, 1999; Paape *et al.*, 2000). Neutrophils are the predominant cell types found at the site of infection and in the mammary secretion during the early stage of bacterial inflammation (Sordillo & Streicher, 2002). Macrophages are the predominant leukocyte in non-infected mammary glands and are involved in non-specific innate immunity. Macrophages also contribute to the specific response through antigen presentation and processing to lymphocytes (Bradley & Green, 2005). In addition, macrophages participate in phagocytosis and are associated with the detection of invading pathogen and initiate inflammation; however, they are less active in phagocytosis than neutrophils. Moreover, cytotoxic natural killer cells are critical to the intracellular pathogens (Sordillo, 2005; Sordillo & Streicher, 2002) and kill both gram-positive and gram negative bacteria (Shafer-Weaver & Sordillo, 1996).

If bacteria overcome the ewe's innate immunity and are not eliminated, the body elicits specific acquired immune responses (Uthaisangsook *et al.*, 2002). Igs are glycoproteins that form a major part of the acquired immune response and play a significant role in specific immune response via detecting specific pathogens and their selective elimination. There are five classes of Ig: IgG, IgM, IgE, IgD and IgA. The concentration of each Ig varies based on lactation cycle and inflammatory conditions (Stelwagen *et al.*, 2009) .

2.7.2 Intramammary infection

Intramammary infection (IMI) in sheep can be caused by a wide range of pathogens such as bacteria, mycoplasmas, virus (small ruminant lentivirus), algae and fungi, although bacteria are the major intramammary pathogens (Ariznabarreta *et al.*, 2002; Gonzalo *et al.*, 2002; Spuria *et al.*, 2017). Staphylococcus (*S. aureus* and other staphylococcus spp.), Mannheimia spp., Streptococcus spp., Enterobacteriaceae, Pseudomonas aeruginosa, Corynebacteria and other bacteria can produce IMI in small ruminants such as sheep (Contreras *et al.*, 2007; Gelasakis *et al.*, 2015; Peterson *et al.*, 2017). Generally, the most commonly detected IMI in sheep is Staphylococcus. In dairy sheep, coagulase negative staphylococcus (CNS) is the main pathogen isolated from clinically heathy ewes with subclinical infection whereas, *S. aureus* has been the principal cause of clinical mastitis (Bergonier *et al.*, 2003; Contreras *et al.*, 2007; Dore *et al.*, 2016). On the other hand, in non-dairy sheep studies, the majority of clinical mastitis has been associated with *M. haemolytica* or *S. aureus* (Arsenault *et al.*, 2008; Koop *et al.*, 2010; Mavrogianni *et al.*, 2007b; Omaleki *et al.*, 2010) while CNS species are the most prevalent in sub-clinical mammary infection (Persson *et al.*, 2017; Spuria *et al.*, 2017). The majority of clinical cases of mastitis are seen in the first week of lactation (Mørk *et al.*, 2007). Mastitis caused by *S. aureus* is more common in ewes rearing twins or triplets compared with those rearing singletons (Arsenault *et al.*, 2008; Koop *et al.*, 2010)

Several CNS species have been isolated from sheep milk, most commonly *S. epidermidis* (Onni *et al.*, 2010) but also *S. chromogenes*, *S. simulans* and *S. xylosus* are frequently isolated and can cause persistent subclinical infection (Fthenakis & Jones, 1990a; Gelasakis *et al.*, 2015). The distribution of CNS species in non-dairy sheep varies among studies (Persson *et al.*, 2017) which could be due to management system differences (Contreras *et al.*, 2007). IMI infection with CNS, irrespective of species, causes an increase in SCC and changes in the composition of milk (Burriel, 1997).

Mannheimia species, *M. haemolytica, M. glucosida and M. ruminalis*, have been isolated from cases of acute mastitis (Omaleki *et al.*, 2010; Omaleki *et al.*, 2011) with *M. haemolytica* identified as the most common cause of mastitis in flocks of non-dairy sheep. *M. haemolytica* has been isolated to a comparable or even greater level than *S. aureus* in dual purpose sheep with clinical mastitis (Arsenault *et al.*, 2008; Koop *et al.*, 2010; Omaleki *et al.*, 2010; Persson *et al.*, 2017). The frequency of Streptococci, Enterobacteria, Corynebacteria and Pseudomonas species is low, but these are still important pathogens (Fernández-Garayzábal *et al.*, 1998; Lafi *et al.*, 1998; Mørk *et al.*, 2007; Smith *et al.*, 2015).

Limited studies have been published regarding the isolation of bacteria from palpable udder defects in sheep. However, some studies isolated bacteria from various mammary abnormalities/secretions such as lumps and diffuse "hard udder" or chronic mastitis during the non-lactating period, and reported staphylococci (*S. aureus* and coagulase-negative isolates) as the most frequently isolated bacteria in ewes (Onnasch *et al.*, 2002; Peterson *et al.*, 2017; Saratsis *et al.*, 1998). Smith *et al.* (2015) also reported that *S. aureus* was the most frequently detected bacteria from udder samples with lumps/abscesses from non-dairy sheep collected at an abattoir. The same authors reported that in 40% of cases, bacterial polyinfection was present when swab samples were collected from mammary abscesses. This is greater than the level of polyinfection identified in milk samples and the difference may be due to the nature of closed abscesses which remain relatively stable, enhancing accumulation of bacteria in one area (Cheng *et al.*, 2011; Cheng *et al.*, 2010). In ewes with palpable udder defects, the association between bacterial occurrence and progression of udder defects has not been studied, although repeated udder examinations have shown varying percentages (number) of ewes with defective udders across a year or two years (Grant *et al.*, 2016; Griffiths *et al.*, 2019a; Griffiths *et al.*, 2019b). Persistency of bacterial species over time is unknown; however, *M. haemolytica* infections were stable during three-week time interval samplings during lactation whereas the stability of staphylococcus spp. was moderate (van den Crommenacker-Konings *et al.*, 2021).

2.7.3 Milk somatic cell count

Milk somatic cells are a mixture of body-derived immunologic and milk-secreting cells that are normally present at low levels in milk (Alhussien & Dang, 2018). Milk somatic cell count (SCC) is an estimation of somatic cells in the milk and it has been used as a marker for udder health and milk quality in ruminants worldwide (Sharma *et al.*, 2011); however, sheep milk, unlike in cows and goats, does not have widely accepted reference ranges (Riggio & Portolano, 2015). Generally, sheep milk has higher SCC than cows and goats (Maisi *et al.*, 1987; Riggio & Portolano, 2015). It has been recommended that milk SCC in dairy ewes should be below 500 $\times 10^3$ cells ml^{-1} (Souza *et al.*, 2012; Zafalon *et al.*, 2018) although healthy ewe udders have been found with very high SCC (Riggio & Portolano, 2015; Riggio *et al.*, 2010). Udder halves having IMI after lambing and at weaning in non-dairy ewes have had SCC ranging from 297,000–2,097,000 cells/ml and 174,000–3,929,000 cells/ml, respectively (Persson et al., 2017). The effect of non-infectious factors such as age, parity, breed, flock, year and lactation stage are reported to be minimal in comparison to infectious factors (Bonelli *et al.*, 2013; Gonzalo *et al.*, 2002; Kern *et al.*, 2013; Othmane *et al.*, 2002; Paape *et al.*, 2007).

Physiological variations in lactation stage affect SCC or proportion of somatic cells (Cuccuru *et al.*, 1997; Morgante *et al.*, 1996; Winnicka *et al.*, 1999). Lower SCC in early lactation has been reported and it is thought this may be due to minimal contamination from the lamb's mouth at that stage (Arsenault *et al.*, 2008; Gougoulis *et al.*, 2008) whereas there is a decline in SCC during the end of lactation which is associated with mammary gland involution (Tatarczuch *et al.*, 2002; Tatarczuch *et al.*, 2000). In uninfected sheep milk, the proportion of mammary epithelial cells to inflammatory cells throughout lactation varies in different studies (Bonelli *et al.*, 2013; Leitner *et al.*, 2003a; Rainard & Riollet, 2006) which could be due to variation in the methods of identification and definition of non-infected glands and cell types by different authors (Leitner *et al.*, 2012; Mehne *et al.*, 2010; Rainard & Riollet, 2006).

Milk SCC and bacterial culture are reported to be dependable diagnostic methods for identifying mammary infection in non-dairy (Fthenakis & Jones, 1990a) and dairy breeds in sheep (Gonzalo *et al.*, 1994; Leitner *et al.*, 2004). IMI is positively associated with SCC in one or both halves of the udder in non-dairy sheep infected with different bacterial species including CNS, Mannheimia spp., *S. aureus*, Enterococcus spp. or Streptococcus species (Arsenault *et al.*, 2008; Moroni *et al.*, 2007; Persson *et al.*, 2017; Zafalon *et al.*, 2018). However, there has not been a consensus in SCC cut-off in sheep which might be related to the occurrence of bacterial pathogens as the SCC depends on the bacterial species isolated (Zafalon *et al.*, 2016).

Staphylococcus aureus, the principal cause of both clinical and subclinical mastitis in dairy ewes, has been associated with the highest SCC among bacterial causative agents (Arsenault *et al.*, 2008; Bergonier & Berthelot, 2003; Bergonier *et al.*, 2003; Moroni *et al.*, 2007). CNS species have been associated with an SCC range of 4.94 to 6.27 log cells/ml and reported to be the most prevalent cause of subclinical infection (Gonzalo, 2017; Persson *et al.*, 2017; Souza *et al.*, 2012). Most of the milk SCC studies in sheep have been performed in dairy flocks. Chronically infected mammary glands, which are characterized by udder asymmetry, sclerosis and mammary abscesses, have higher than normal values of SCC in both dairy and non-dairy sheep (Bergonier & Berthelot, 2003), but there is still a scarcity of literature available in non-dairy sheep particularly in association with the dynamic progressive nature of udder defects in ewes.

2.8 Udder Defects in Ewes: Examination, Occurrence, Pathogenesis, Gross Lesions and Histopathology

2.8.1 Udder and teat defects examination and scoring in ewes

Internationally, several researchers have recommended systematic udder examination at the beginning and end of lactation and before mating for the next season both in dairy and non-dairy ewes (Bergonier & Berthelot, 2003; Blagitz *et al.*, 2014; Fthenakis *et al.*, 2012; Kirk *et al.*, 1980; Saratsis *et al.*, 1998; Watson *et al.*, 1990). According to Smith *et al.* (2015), farmers in the UK examine ewes' udders at weaning or premating for the purpose of culling or selecting ewes for the next breeding season. Likewise in New Zealand, few reports have mentioned the relevance of a systematic and close udder examination in ewes to detect mastitis and palpable udder detects in non-dairy ewes (Bruce *et al.*, 2013; Griffiths *et al.*, 2019a; Peterson *et al.*, 2017). Furthermore, a survey on farm-management tool used by New Zealand sheep farmers reported that more than 75% of the farmers practiced udder examination as part of routine farm management at least once a year (Corner-Thomas *et al.*, 2016). Udder examination can be undertaken when ewes are in the upright standing position in a race (Grant *et al.*, 2016) or in the sitting position by turning each ewe on its rump (similar to the shearing position) restrained by an assistant (Hayman *et al.*, 1955).

In dairy sheep, udder and teat linear scoring systems have been mainly used for udder conformation assessment in association with mastitis or machine milkability (Casu *et al.*, 2006; Casu *et al.*, 2010), weight gain in lambs (Huntley *et al.*, 2012) and traumatic teat lesions (Cooper *et al.*, 2013). In other studies udder defects have been classified as subclinical, acute or chronic based on clinical findings (Fragkou *et al.*, 2014; Grant *et al.*, 2016). However, some authors have attempted to standardize scoring systems into different classes. Quinlivan (1968a) classified udder lesions into four classes with classes 1 and 2 for chronic lesions; class 3 describing an acute form; and class 4 representing per-acute clinical mastitis. In addition, one study developed an udder and teats palpation and inspection scoring system which constituted palpation of the udder to describe consistency along with inspection of udder and teat volume, symmetry and presence of injury or lesion (Blagitz *et al.*, 2014). A teat lesion scoring system for traumatic and non-traumatic lesions has been proposed by Cooper *et al.* (2013). This scoring system has seven lesion categories that range from small tooth mark lesions to large areas of skin damage. Recently, in New Zealand, udder and teat palpation scoring systems were developed in consultation with experts and farmers, and by reviewing previously available scoring systems (Griffiths *et al.*, 2019a) as shown in Table 2.3. This scoring system uses scales of 1 to 5 for teat lesions and 1 to 7 for the udder palpation (table below).

Table 2.3 Udder and teat defects palpation scoring system in non-dairy ewes.

Trait	Levels (scores)	Description	Analysis levels (scores)
Udder palpation (Sitting position)	7	Diffuse hard consistency of udder	Hard
	6	Firm consistency of udder with small nodule(s) (lumps) > 2cm in size	Lump
	5	Firm consistency of udder with small nodule(s) (lumps) < 2cm in size	Lump
	4	Soft consistency of udder with small nodule(s) (lumps) > 2cm in size	Lump
	3	Soft consistency of udder with small nodule(s) (lumps) < 2cm in size	Lump
	2	Diffuse firm consistency of udder	Normal
	1	Diffuse soft consistency of udder	Normal
Teat Palpation (Sitting position)	5	Teat obstruction ('blind teat')	Abnormal
	4	Dense, vertical cord in center of teat	Abnormal
	3	Hard consistency	Abnormal
	2	Thickened teat end	Abnormal
	1	Soft consistency	Normal

Source: Griffiths *et al.* (2019a)

2.8.2 Occurrence of various udder defects in ewes

Defective udders that are judged unfit for the next breeding season have been described using different terminologies including "impaired udders", "imperfect udder", "unsound udder", "faulty udder", "udder with abnormality" and "defective udder" (Griffiths *et al.*, 2019a; Hayman *et al.*, 1955; Quinlivan, 1968b; Saratsis *et al.*, 1998). Defective udders have also been termed "dry ewe mastitis" as the timing of udder examination is typically during the non-lactating period (Quinlivan, 1968b). Udders have been considered as defective when detected with any abnormality during careful and close examination (inspection and palpation) of the udder halves and teats (Blagitz *et al.*, 2014; Hayman *et al.*, 1955; Saratsis *et al.*, 1998). Defects can range from mechanical to non-mechanical gross injuries and acute mastitis to clinically detectable IMM associated with chronic mastitis (Madel, 1981). Several mammary gland abnormalities have been described including abnormal mammary secretion, inflammation, nodules or IMM, diffuse hardness fibrosis, teat occlusion and teat canal cord formation, different sized masses (abscesses) and ruptured abscesses on the udder (Cooper *et al.*, 2013; Grant *et al.*, 2016; Griffiths *et al.*, 2019a; Quinlivan, 1968a; Saratsis *et al.*, 1998; Smith *et al.*, 2015).

Lamb growth rates and lamb survival to weaning are dependent on the udder health (Hayman *et al.*, 1955). Udder defects including mastitis in lactation have been negatively associated with daily lamb weight gain (Arsenault *et al.*, 2008; Fthenakis & Jones, 1990b; Grant *et al.*, 2016; Huntley *et al.*, 2012; Moroni *et al.*, 2007). In ewes, acute mastitis at premating, diffuse hardness of an udder at docking or with lump (which vary in size and consistency) at weaning increase the odds of lamb loss (Dwyer *et al.*, 2016; Forrest *et al.*, 2007; Griffiths *et al.*, 2019a; Hayman *et al.*, 1955).

Acute mastitis can cause both local and systemic signs in ewes. The mammary gland becomes painful, swollen and hot which may result in partial or complete damage of mammary gland function (Fthenakis & Jones, 1990b; Smith *et al.*, 2015). Abnormal mammary secretion such as clots, flakes, purulent, serous and blood stained fluids are also commonly reported clinical signs (Mørk *et al.*, 2007). Systemic signs may include anorexia, lameness, anxiety, restlessness, changes in feeding behaviour and pain in affected ewes (Foster, 2017; Fthenakis & Jones, 1990a). The flow-on effects of acute mastitis can be sudden death, loss of udder function, chronic mastitis with detectable masses in the mammary gland, raised SCC or recurrent infection (Bergonier & Berthelot, 2003; Marogna *et al.*, 2010; Menzies & Ramanoon, 2001; Watson & Buswell, 1984).

Traumatic and non-traumatic teat lesions have been reported as common findings of udder examinations (Cooper *et al.*, 2013; Huntley *et al.*, 2012). Teat lesions have been associated with IMI that may result in development of acute or chronic mastitis (Cooper *et al.*, 2013; Fragkou *et al.*, 2007; Mavrogianni *et al.*, 2006). In the early weeks of lactation, traumatic teat lesions, potentially caused by lambs' teeth are more prevalent because lambs depend on ewes' milk only during this period (Cooper *et al.*, 2013). In addition, higher litter size per ewe is associated with increased frequency of suckling (Waage & Vatn, 2008), while total suckling time is positively associated with risk of teat bite and associated lesions (Teke & Akdag, 2011). On the other hand, the incidence of non-traumatic teat lesions such as proliferative skin lesions, warts, pustules or combinations of these, increase gradually after lambing (Huntley *et al.*, 2012) with greater prevalence reported between weeks 7-10 after lambing (Cooper *et al.*, 2013).

Intramammary masses (IMM) can be defined as any palpable or detectable mass of different consistency from the rest of the gland tissue (Grant *et al.*, 2016) which is usually associated with chronic mastitis (Bergonier *et al.*, 2003). Most of the IMM palpated vary in size and consistency of lumps and diffuse hardness, and are associated with abnormal secretions or abscesses (Peterson *et al.*, 2017; Saratsis *et al.*, 1998). In most cases, IMM in non-dairy ewes are abscesses (Smith *et al.*, 2015). Based on palpation characteristics, palpable masses in the udder can be broadly categorised into lumps with different size of nodules or diffuse hard consistency of udders (Griffiths *et al.*, 2019b). In dairy ewes such udder defects can be identified during routine milking allowing early detection. In contrast, non-dairy ewes do not receive such regular and close attention and also as the defect develops slowly there is high possibility of missing an early diagnosis or the lesion remaining undetected during pregnancy and lactation (Fragkou *et al.*, 2014). According to Grant *et al.* (2016), IMM has been associated with acute mastitis and traumatic teat lesions, underfeeding energy in pregnancy and IMM in the previous lactation.

The incidence rate of acute mastitis in non-dairy ewes has been reported to be from 0 to 6.6% in Quebec, Canada (Arsenault *et al.*, 2008), 0 to 5% in England and Ireland (Cooper *et al.*, 2016; Grant *et al.*, 2016; Onnasch *et al.*, 2002) and 3 to 5% in Australia (Barber, 2015). Prevalence rates ranging from less than 1% or up to 5% have also been reported in New Zealand (Clark, 1980; Griffiths *et al.*, 2019a; Quinlivan, 1968a, 1968b). The incidence risk of IMI or clinical mastitis increases during the immediate post-partum period or initial stages of lactation (Bergonier *et al.*, 2003; Mavrogianni *et al.*, 2014). Risk factors such as age, litter size, breed, management system, mastitis history and body condition score (BCS) have been associated with acute mastitis in non-dairy ewes (Arsenault *et al.*, 2008; Koop *et al.*, 2010; Mørk *et al.*, 2007; Waage & Vatn, 2008).

There are few studies investigating IMM in non-dairy ewes. Chronic mastitis characterized by IMM has been reported to affect 2.8% of ewes in Ireland (Onnasch *et al.*, 2002), while in the UK, Grant *et al.* (2016) reported 4.7% of ewes had chronic mastitis during pregnancy and 10.9% during lactation. Meanwhile, in Greece, Saratsis *et al.* (1998) reported 5.1% cumulative incidence of udder abnormalities during the dry-period in ewes including all abnormalities in the udder (abnormal secretions, nodules, lumps, diffusely hard udders and cysts) and teat lesions. Recently, Griffiths *et al.* (2019a) found 5.0-7.4% of ewes had abnormal udder palpation scores across four inspection times (pre-mating, pre-lambing, docking and weaning) over a year. Variation in occurrence rates could be due to changes in the maturation cycle of the IMM (abscess) (Cheng *et al.*, 2011; Smith *et al.*, 2015). Studies of repeated udder examination of ewes suggest udder defects have a dynamic nature. A New Zealand study with four observations within one year (Griffiths *et al.*, 2019a) and a UK study with four observations over two years during pregnancy and lactation and Grant *et al.* (2016) showed changes in the number (percentage) of ewes having udder defects at subsequent examinations.

Palpable udder defects or abscesses should be differentiated from milk cysts which occur occasionally (Watkins & Jones, 2007). Milk cysts are also referred as cranial midline lump (CML) or subcutaneous lump based on their location and palpation appearance (Griffiths *et al.*, 2019a; Ridler *et al.*, 2021). These cysts contain a bacterial negative milk-like secretion (Saratsis *et al.*, 1998). Milk cysts were located near the intra-mammary groove and either cranially or caudally (Griffiths *et al.*, 2019a; Ridler *et al.*, 2021; Saratsis *et al.*, 1998). This condition has been reported in goats in association with 'weeping teats' and it occurs during or shortly after lactation as pressure around the mammary gland becomes higher (Matthews, 2016). Milk cysts are self-resolving if pressure on the area can be avoided (Gazzola *et al.*, 2021).

2.8.3 Mechanism of palpable udder defects development, persistence, and recurrence

Intramammary masses (IMM) are palpable udder defects that develop after chronic IMI; however, not all IMI cause IMM (Smith *et al.*, 2015). Abscesses are the most common palpable udder defects located either in the glandular or cistern part of the mammary gland (Madel, 1981; Smith *et al.*, 2015). Multiple micro-abscesses (3-5 mm) distributed throughout the parenchyma of the affected gland give a nodular appearance (Madel, 1981). Clinical examination of ewes with palpable udder defects can show externally visible pus or abscesses in or on the udder (Peterson *et al.*, 2017).

Abscesses, defined as inflammatory lesions releasing purulent material, are standard responses for many different biological, chemical or physical insults to host tissues (Kobayashi *et al.*, 2015). There are two contradictory hypotheses about the function of abscess formation. According to Krishna and Miller (2012), abscess formation is used by the host to contain and ultimately eliminate the pathogen; in contrast, some studies suggested that abscess formation is a pathogen-driven process against host responses to the infection and ease bacterial replication (Cheng *et al.*, 2011; Mansour *et al.*, 2016). Moreover, the immunopathogenesis of tissue response to infection during formation of an abscess has not been fully elucidated (Tzianabos *et al.*, 2000). However, abscess formation involves several characteristics from both invading bacterial pathogen and the host cells (Kobayashi *et al.*, 2015).

The pyogenic abscess begins as a localized host acute inflammatory response to bacterial infection (Krishna & Miller, 2012). Polymorphonuclear neutrophils (PMNs) are the most important cellular defence against invading bacteria and are a major component of abscesses (De Souza *et al.*, 2012). The host secretes pro-inflammatory and chemotactic factors that promote recruitment and attract PMNs to the site of infection (Rainard & Riollet, 2006). Phagocytosis is then initiated to remove invading microorganisms, a sequence which is promoted by opsonization of bacteria with antibody and complement (Kobayashi *et al.*, 2015). The centre of the abscess contains an acute inflammatory exudate typically composed of many viable and necrotic PMNs, tissue debris, fibrin, and both live and dead bacteria (Kumar *et al.*, 2005) (Figure 2.4). Subsequent to leukocyte phagocytosis, bactericidal mechanisms take place with the production of reactive oxygen species or enrichment of antimicrobial peptides, proteins, and degradative enzymes in the phagosome (Quinn & Gauss, 2004). Although host cells produce antimicrobial agents with the purpose of protecting the body and killing the bacteria, the nonspecific nature of these agents may cause damage to host cells and contribute to the production of abscess (Quinn & Gauss, 2004).

Bacterial pathogens, mainly *S. aureus*, produce virulence factors that evade host innate and adaptive immunity (Vautor *et al.*, 2009) which protect the bacteria from the bactericidal activity of PMNs or directly modify neutrophil recruitment and phagocytosis function (Rainard & Riollet, 2003). Virulence factors can also cause direct lysis of neutrophils and result in formation of the fibrin capsule as a barrier (Durr *et al.*, 2006). Furthermore, the bacterial coagulases and von Willebrand factor-binding protein promote fibrin production and formation of coagulation at the site of infection (Cheng *et al.*, 2010). These deposits form a pseudocapsule, which functions as a barrier to protect the bacteria from the immune cells, so they can replicate without interference (Cheng *et al.*, 2011; Smith *et al.*, 2015).

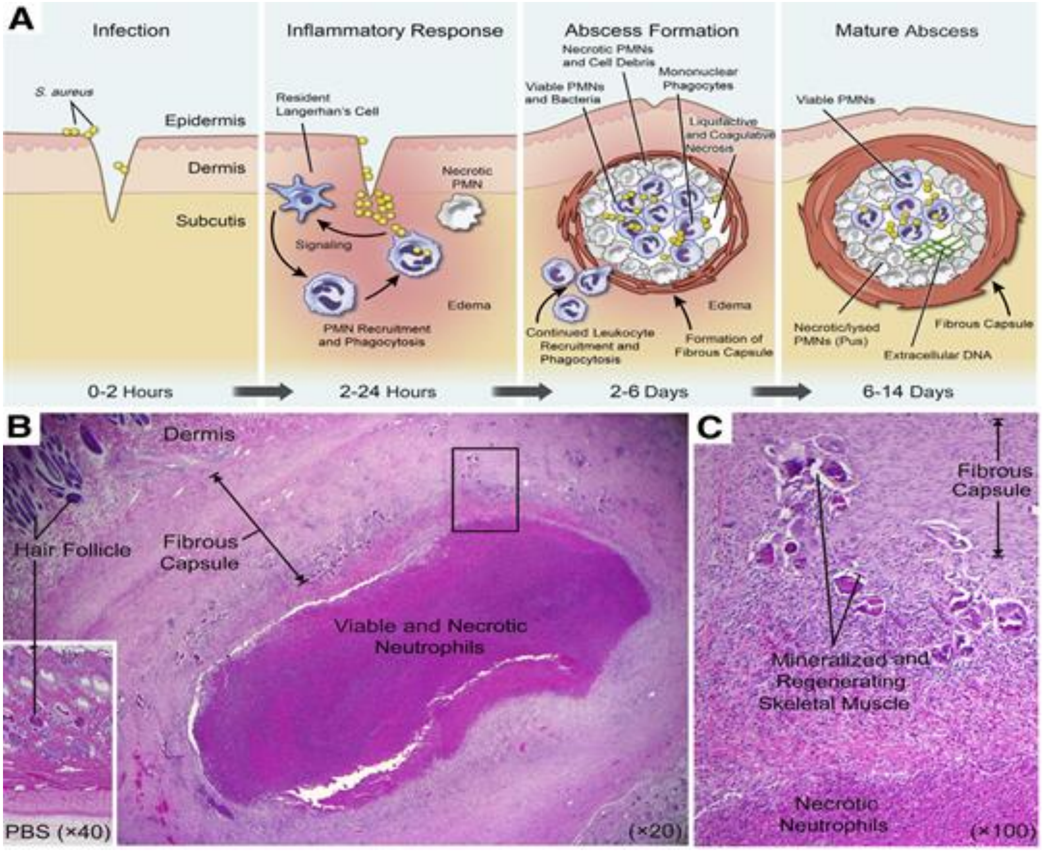

Figure 2.4 Staphylococcus aureus skin abscesses. A: Formation of a *S. aureus* skin abscess. B: Representative histopathological section of a typical rabbit skin abscess at day 14 after infection. C: Increased magnification of the boxed area shown in B (Kobayashi *et al.*, 2015).

2.8.4 Persistence and recurrence of palpable udder defects

As part of the abscess maturation cycle, the bacterial multiplication and abscess formation continues for weeks and eventually ruptures (Kobayashi *et al.*, 2015; Lowy, 1998). Abscess rupture has been widely considered a physiological feature of healing and tissue repair (Cheng *et al.*, 2009); however, the rupture spreads bacteria that reform and repeat the abscess in other sites (Cheng *et al.*, 2011; Smith *et al.*, 2015). In non-dairy ewes with IMM, masses are not consistently detected during subsequent udder examinations (Grant *et al.*, 2016; Griffiths *et al.*, 2019a) which is probably due to the dynamics of maturation, rupture and spread of bacterial and reformation of abscess.

According to Grant *et al.* (2016), if ewes are detected once with IMM, the defect is 3 to 5 times most likely to recur in the future. Therefore, ewes with IMM can be a risk factor for future mammary infection. In humans, one study showed that the occurrence of primary abscess is higher in non-lactating than in lactating women. In addition, recurrence of primary mammary abscess was recorded only in non-lactating women (>50%) (Bharat *et al.*, 2009).

2.8.5 Gross and histologic lesions of udder defects in ewes

Pathological examination of mammary gland lesions is a useful diagnostic procedure for diagnosis of udder defects (Arteche-Villasol *et al.*, 2022). Effective host defences control entrance, multiplication, and tissue invasion of bacterial infection (Sordillo & Streicher, 2002). However, once bacteria pass the physical barriers and overcome the host's immunity, they can cause mild to severe or acute to chronic lesions (Foster, 2017). Bacterial invasion and multiplication in mammary epithelium may contribute to epithelial cell death (Akers & Nickerson, 2011; Zhao & Lacasse, 2008). In addition, toxins produced by some bacteria can damage parenchymal tissue of the gland (Cifrian *et al.*, 1996; Zhao & Lacasse, 2008).

Grossly, the mastitic udder half usually appears slightly larger in size than the contralateral non-defective (el-Etreby & Abdel-Hamid, 1970). Well-developed lesions of mastitic udders in ewes were usually observed 2 to 3 days after presentation of clinical signs (Watkins & Jones, 2007). With advancement of the defect, reddening and lobular mottled appearance of the cut surface were reported in the affected udder halves. Further, degeneration and necrosis of epithelial cells, and plugging of ducts and cistern with clots of milk and fibrin could be evident (El-Masannat *et al.*, 1991).

The common mammary tissue reactions to infection are inflammation, epithelial cell hyperplasia, squamous metaplasia, and epithelial necrosis, oedema, granulation and scarring (Foster, 2017), changes which can generally be observed on routine histology. Mammary inflammation increases milk somatic cells (Burriel, 1998). These reactions to the infection are essential to combat infections agents; however, they can result in accumulation of exudate that may block the normal milk secretions and cause secondary lesions (Foster, 2017).

Several pathogens, mainly bacteria, have been isolated from the mammary gland of ewes (Bergonier *et al.*, 2003), while different pathogens are variably associated with mild to severe, or transient to persistent, forms of infection. Pathogenesis, course and consequences of mammary infection of ewes are similar to that of bovines (Schlafer & Foster, 2016). The lesions can be either severe (necrotizing), suppurative or granulomatous (Foster, 2017). The severe forms of the disease (necrotizing or gangrenous) typically occur shortly postpartum and lead to high death rates, whereas the mild form is non-fatal with a variety of grossly detected lesions (Schlafer & Foster, 2016).

The severe necrotizing (gangrenous) form is usually associated with systemic effects such as fever, anorexia, leukopenia, hyperfibrinogenemia, and hypocalcemia. Gangrenous mastitis is typically caused by gram-negative bacteria and some necrotizing gram-positive bacteria like *S. aureus*. This type of infection is life-threatening because of both the effect of endotoxins secreted by the bacteria, and glandular damage that leads to reduced immunity and makes affected individuals prone to secondary infection (Foster, 2017). In gangrenous mastitis the affected half of the udder, usually only one side, becomes enlarged, necrotic and blue-black, often with a clear line of demarcation between affected (necrotic or gangrenous) and apparently normal tissue. There are watery mammary secretions with some flakes in them (Schlafer & Foster, 2016). Necrosis and severe vascular leakage of mammary tissue can be seen as dry and friable areas of necrosis and sequestration of mammary tissue bordered by hyperemia and hemorrhage. Oedema of the mammary gland is also obvious and results in swelling of the mammary gland with watery milk secretion containing fibrin. In the non-necrotizing severe form, although there is ductular and glandular tissue necrosis, there is no necrotic sequestrum formation and no systemic effects (Foster, 2017). If the ewe survives the acute form with systemic effects, the mammary gland typically develops abscessation within weeks (Cheng *et al.*, 2011; Schlafer & Foster, 2016). Then, after maturation of the abscess it often ruptures and forms fistulae, and progressively most of the affected gland sloughs (Schlafer & Foster, 2016).

Chronic suppurative cases are usually caused by pus-forming gram-positive bacteria and commonly occur in the non-lactating period. Suppurative exudate or abscesses accumulate in small ducts, and may heal with scar formation in the ducts and lobules, whereas in larger ducts (lactiferous and papillary ducts) exudate typically forms large abscesses remodeled by granulation tissue (Schlafer & Foster, 2016). In the early stages, the affected part of the gland shows serous to purulent secretions and epithelial lining of the ducts may appear normal or red granular with distinct lobulations due to papillary duct swelling and involution of nearby glands. The epithelial ducts, in both udders with abscessation, become hyperplastic and more squamous, resulting in thickening and narrowing of the duct walls followed by periductal fibrosis and lymphocyte and plasma cell accumulation within fibrous septa (Schlafer & Foster, 2016). These lesions are mainly restricted to large ducts in the distal portion of the mammary gland (Foster, 2017; Schlafer & Foster, 2016). At a later stage, fibrosis and thickening of walls of larger ducts, and protrusion of rounded polypoid projections into the lumen can be seen (Schlafer & Foster, 2016). Particularly in hard udders, mammary atrophy, galactophoritis and fibrosis can be seen (Bruce *et al.*, 2013).

2.8.5.1 Gross and histological lesions associated with Staphylococcus and Mannhemia haemolytica

Differences in toxin production and invasiveness among Staphylococcus species (*S. aureus* and CNS) and difference in host response result in variation in histologic features. Acute *S. aureus* infection has been characterized by abundant serous exudate, neutrophil infiltration in acinar and ducts, minimal vascular changes, and necrosis and desquamation in different lobules (el-Etreby & Abdel-Hamid, 1970). Epithelial hyperplasia and squamous metaplasia of ducts, and lymphoid follicles in the lactiferous sinus and ducts, death and exfoliation of epithelial cells, peri-ductal fibrosis and atrophy of glandular cavity occurs, which may obstruct the milk flow (Chen *et al.*, 2018; Schlafer & Foster, 2016; Sordillo *et al.*, 1989). In addition, it can be characterized by central necrotic foci surrounded by neutrophils and macrophages; reticulo-endothelial cells and capsule while the adjacent granular tissues may appear normal (el-Etreby & Abdel-Hamid, 1970).

In CNS species infection, variation in pathogenicity among CNS species is associated with different clinical status and histological features (Fthenakis & Jones, 1990a). Macroscopically, the udder shows congested tissue and connective tissue proliferation and some tiny firm white nodules (Burriel, 1998). Histologically, early-stage infection shows varying degrees of neutrophil infiltration (Fthenakis & Jones, 1990a), fat globules (amylacea), and cellular debris in the alveolar lumina and inter-alveolar spaces. Within 2-3 weeks, mild to extensive epithelial involvement occurs as cisternal epithelium and subepithelium become edematous. In addition, proliferation of connective tissue, formation of fibrotic tissue, and mononuclear tissue infiltration have been described with more chronic lesions (Burriel, 1998; Fthenakis & Jones, 1990a; Stabenfeldt & Spencer, 1966).

Mannheimia haemolytica can cause gangrenous mastitis with a bluish demarcated area that eventually swells and sloughing of skin. In the initial stage of *M. haemolytica* infection, the mammary secretion is watery, but gradually it becomes yellowish with mucus or, in some ewes, results in red-yellowish milk discoloration (Al-Ani *et al.*, 1997). The parenchymal lesions include subcutaneous oedema, the presence of sanguineous fluid from parenchymal sections, and enlargement and oedema of the supramammary lymph nodes (Mavrogianni *et al.*, 2006). Histological findings of massive neutrophilic infiltration, necrosis, oedema and vascular changes are characteristically seen (Al-Ani *et al.*, 1997; Mavrogianni *et al.*, 2006).

2.9 Summary of Literature Review and Identified Knowledge Gaps

Lamb production is the key driver of sheep production in New Zealand. Pre-weaning lamb survival and growth rate are highly dependent on ewe's udder health. Udder defects can be defined as udder abnormalities detected by palpation with different consistency and size such as lumps in different locations of the gland or diffuse hardness of the tissue. They have an important impact on udder health and may reduce milk yield and alter milk composition and increase SCC. Lambs reared by ewes with udder defects and mastitis have been associated with lower survival to weaning and poor growth rates.

Udder assessment, achieved by visual inspection and palpation, has been a vital tool for farmers and veterinarians for assessing udders to make culling decisions in ewes. In dairy ewes, udder defects can be detected more readily because ewes are checked during regular milking; however, udder defects in non-dairy ewes are usually not identified until at weaning or when animals are examined for ewe selection for the next breeding season. However, a consistent, easy-to-use udder scoring system that is associated with production traits is lacking for non-dairy ewes.

Several bacterial species, principally *M. haemolytica* and Staphylococcus species, have been associated with IMI in non-dairy ewes. However, in ewes very few studies have been done on isolation of bacteria from diffusely hard udders or udders with lumps of various size and consistency. Moreover, there is disparity among studies about association of bacterial pathogens isolated and various udder defects. SCC is positively correlated to IMI, but there has not been consensus on appropriate SCC cut-off values and no detailed information exists on the relationship between SCC and IMM in non-dairy ewes. There is generally a paucity of literature on gross pathology, histology and bacterial studies in non-dairy ewes. In some studies, repeated udder examinations have implied that the udder defects could change over time, based on fluctuations of percentage of ewes with defects, but to the author's knowledge, no previous studies have attempted to assess the changes of udder defects over time or describe their effect on milk yield and composition.

Chapter 3: General Methodology and Thesis Design

This chapter describes general methodology of all the studies undertaken as part of this PhD thesis and outlines how individual aspects of each study/experiment are allocated to the various thesis chapters. Chapter-specific methods, on the other hand, are detailed in their respective chapters. There is overlap between individual studies; therefore, the thesis is structured such that each chapter focuses on a specific common component(s) across the various studies/experiments of udder defects (e.g., pathology, and bacteriology) rather than having a chapter for each specific study/experiment.

Additionally, this chapter includes case definitions for some key terminologies. When exploring the literature on udder defects in ewes, it became apparent that differing terminologies have been used previously to describe similar or nearly the same concepts. Hence, definition of important terminologies sub-section of the chapter (see section 3.9) is used to prevent misunderstanding regarding terminologies used in this manuscript. The four studies/experiments that were undertaken as part of this PhD thesis are outlined in Table 3.1.

Table 3.1 The three studies that were undertaken as part of this PhD and thesis chapters

Study / Experiment Title		Thesis Chapters	Thesis Chapters Tittle
Study A	A longitudinal study of changes in ewe palpable udder defects over time	Chapter 5	Assessment of changes in udder half defects over time in non-dairy breed (Romney) ewes
		Chapter 6	Isolation of aerobic bacterial species associated with palpable udder defects in non-dairy breed (Romney) ewes
		Chapter 7	Gross pathology and histological changes of palpable udder defects in non-dairy breed (Romney) ewes
Study B	A cross-sectional study of palpable udder defects in ewes from a commercial farm	Chapter 6	Isolation of aerobic bacterial species associated with palpable udder defects in non-dairy breed (Romney) ewes
		Chapter 7	Gross pathology and histological changes of palpable udder defects in non-dairy breed (Romney) ewes
Study C	Longitudinal milking study over 6 weeks of lactation on ewes with udder defects	Chapter 4	Effect of palpable udder defects on milk yield, somatic cell count, and milk composition in non-dairy ewes
		Chapter 5	Assessment of changes in udder half defects over time in mixed-age non-dairy breed (Romney) ewes
		Chapter 6	Isolation of aerobic bacterial species associated with palpable udder defects in non-dairy breed (Romney) ewes
		Chapter 7	Gross pathology and histological changes of palpable udder defects in non-dairy breed (Romney) ewes

3.1 Study A: Longitudinal Study on Changes in Ewe Udder Defects Over Time

This study/experiment was based on data gathered over a three-year longitudinal period from 2016 to 2018 at Massey University's Riverside farm, Wairarapa, New Zealand. The study utilized a total of 1200 mixed-age Romney ewes at the time of enrolment.

3.1.1 Study animals

The study comprised the entire cohort of 2013 and 2014 born ewe replacements on Massey University's Riverside farm, Wairarapa, New Zealand. All ewes had lambed at least once prior to joining the study. Ewes were ear-tagged with visual (VID; Allflex, Palmerston North, New Zealand) and electronic (EID; Allflex, Palmerston North, New Zealand) identification.

The ewes were farmed exclusively outdoors, grazing under standard commercial New Zealand conditions on pasture. The predominant pastures were ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*). From weaning until pre-lambing, ewes were rotationally grazed in large mobs with paddock shifts every 2-5 days. At pre-mating, approximately 10 days before the expected start of lambing, ewes were allocated into different paddocks for lambing at a rate of approximately 7 to 12 ewes per hectare, based on pregnancy rank. They remained set-stocked until weaning. Individual feeding decisions were made at the discretion of the farm manager and pasture measurements were not taken.

Rams were introduced to the ewes for breeding at a ratio of 1:150 for two estrus cycles (34-days) each year. Transabdominal ultrasonography was undertaken to diagnose pregnancy and determine the number of fetuses by a commercial practitioner during mid-pregnancy. Ewes lambed during the August to October period. Over the lambing period, ewes were monitored twice daily to match lambs to their dams. Lambs were given an electronic identification tag and relevant information on new-born lambs was recorded: dam identification, date of birth, sex, birth rank, and birth weight.

3.1.2 Assessment and measurements of ewes

Ewes were enrolled in the study in January 2016 and were assessed four times a year for three consecutive years at pre-mating, pre-lambing, docking (3-8 weeks after lambing), and at weaning. These time points are key management events when farmers traditionally closely observe individual ewes. On these occasions, the udders of ewes were palpated and scored using a standardized udder scoring system (se section 3.6). Body condition scoring (BCS) and live weight measurement of each ewe was also assessed (se section 3.4 and 3.5) on these days.

3.1.3 Further investigation of individual ewes

At the end of the three-year study, milk samples were collected from selected ewes (n=101) at weaning (5th December 2018) to identify aerobic bacterial pathogens (detail in Chapter 6). Ewes with cranial midline lumps (CML) (n=7) were transported to Massey University and humanely euthanised. Post-mortem udder examination was undertaken for gross examination samples collected for histological examination (Chapter 7).

3.2 Study B: Cross-sectional Study of Udder Defects in Ewes from a Commercial Farm

Ewes were sourced from a single commercial farm from North Canterbury in the South Island, New Zealand. The farmer recognized a higher-than-usual proportion of ewes with udder defects during the 2018 season and identified 80 ewes with udder defects during lactation. Post-weaning, the farmer and attending veterinarian palpated and scored udders of these 80 ewes using the scoring system described by Griffiths *et al.* (2019a). They identified 74 ewes with palpable udder defects. In March 2019, the ewes were purchased, and all ewes deemed as fit for transport by the attending veterinarian were transported via a commercial transport agency to Massey University's Tuapaka farm, 15 km north-east of Palmerston North City (40°20′ S, 175°43′ E).

The ewes remained at the Tuapaka farm for a week under standard commercial pasture grazing conditions. During this time, the udder of each ewe was scored using the system described by Griffiths *et al.* (2019a) (detail below). Ewes were then slaughtered either via a local abattoir (n=70) or, if deemed unfit for slaughter, were humanely euthanised at Massey University (n=4). In both cases, the whole udder was collected (detail below) for bacterial isolation (Chapter 5), and gross and histological examination (Chapter 7).

3.3 Study C: Longitudinal Milking study on Ewes with Udder defects

3.3.1 Ewe selection

Eighty ewes aged 5-6 years were obtained from Massey University's Riverside farm, located 11 km north to north-west of Masterton (40°50′ S, 175°37′ E), Wairarapa, New Zealand, based on udder traits evaluated during the previous three years in Study A. Ewe selection was based either on the presence of one or more udder defects (hard and/or lump, Griffiths *et al.* (2019a) on selection day or a history of udder defects identified during at least three out of 12 udder examinations over the previous three years (Study A), with a focus on ewes with defects during examinations nearer to the selection date. Twenty 'normal' ewes were selected based on an absence of udder defects on selection day and no recorded defects during 12 udder examinations over the previous three years. Selection of the ewes took place on weaning day (5th

December 2018), at which time the udder of all ewes were scored. The ewes were transported to Massey University's Keeble farm (5 km southeast of Palmerston North, New Zealand, 40°24' S and 175°36' E) in January 2019.

3.3.2 Animal management

From the time of arrival at Keeble farm, the ewes were managed as a single flock under commercial grazing conditions. For breeding, ewes were progesterone-synchronized by inserting a controlled release intravaginal device (Eazi-Breed CIDR®, Zoetis New Zealand) on 13 March 2019 for a period of 12 days. At removal of the CIDRs six crayon-harnessed rams were introduced. The crayon colour was changed four days later (March 29th) and rams were removed on 15th April. The colour pattern on the rump of the ewes was utilized to identify individual ewe's breeding period. Pregnancy diagnosis was undertaken twice in mid-pregnancy (June 4th and July 25th) using trans-abdominal ultrasonography undertaken by a commercial operator to confirm pregnancy and fetal number. Single and twin-bearing ewes were retained while non-pregnant and triplet-bearing ewes were removed (n=21). Additionally, 11 ewes were culled due to routine farm practice.

In pregnancy, ewes were managed under commercial feeding conditions to meet pregnancy requirements. Forty-eight ewes were retained for lambing and during the lambing period, a twice-daily lambing beat was undertaken (at approximately 8AM and 4PM daily). New-born lambs were weighed and electronically tagged, their sex was determined, and they were matched to their dams. The lambing period occurred from 13th of August to 9th of September 2019. On milking days (see below) lambs were separated from their dams for six hours between morning and afternoon milkings, during which time each lamb was bottle-fed 300g of ewe's milk (Peterson *et al.*, 1997). Lambs were weighed on each milking day and at weaning. When any ewe produced less than 1200g/day of milk or abandoned their lamb/s, the lamb/s was/were removed from the ewe and fostered. These ewes were excluded (n=18) from the remainder of the milking study but were included for the study of assessment of changes in udder half defect over time (Chapter 5). Thirty ewes lactated throughout the entire six-week lactation period and were milked at each event at weekly intervals to measure milk yield, SCC, and milk composition (Chapter 4), and samples were collected for bacterial isolation (Chapter 6). All 48 ewes had their udders examined and scored six times during lactation at weekly intervals. The ewes were culled or euthanised, and the udders were collected for examination by gross pathology and histopathology (Chapter 7).

3.4 Body Condition Scoring (BCS)

In all studies, BCS was assessed by experienced staff by palpating the lumbar region feeling the vertical (spine) and horizontal processes along the loin area for sharpness or roundness. The body condition scoring system has 5 points (1-5, where: 1= thin and 5 =obese) assessed in 0.5 intervals (Jefferies, 1961; Kenyon *et al.*, 2014).

3.5 Body Weight Measurement

Ewes were weighed using static digital weighing scales (model XR5000, Tru-Test Group, Auckland, New Zealand) to the nearest 0.2 kg. Young lambs in the milking study were weighed to the nearest gram by using a table-top weighing scale (Sartorius, Germany) by putting them into a bucket.

3.6 Udder Scoring System

Ewe udders were assessed with ewes restrained by experienced sheep handlers in a sitting position. Once restrained, the udders were visually inspected and then palpated according to Griffiths *et al.* (2019a). This procedure took approximately 3-5 minutes per ewe. Summary of the udder half scores is indicated in Table 2.3 (Chapter 2). For analysis of the data, the 7-category udder scoring system was condensed into three categories: Score 7 was categorised as ‘hard’, scores 3-6 as ‘lump’, and scores 1 and 2 as ‘normal’.

3.7 Post-Mortem Udder Collection from Ewes

In all three studies, the whole udder was collected immediately after slaughter/euthanasia. Routine humane slaughtering procedure for the abattoir was implemented, where the use of intracranial captive bolt was followed by immediate exsanguination. These were then packed in an ice bath for transport back to Massey University. Each whole udder was washed thoroughly with tap water and 70% alcohol and wiped by sterile teat whips (Zoetis New Zealand Limited) before sample collection. For ewes that were humanely euthanised at Massey University, the same udder collection and preparation was undertaken, but the udders were transported to Massey University and examined within one hour of collection. For ewes humanely euthanised on-farm, the same udder collection and preparation was undertaken, but udders were transported to Massey University and examined within three hours of collection.

3.8 Animal Ethics Approval

All procedures involving the use of live animals were undertaken in accordance with animal welfare standards and approved by the Massey University Animal Ethics Committee. The studies and animal ethics application numbers are provided (Table 3.2).

Table 3.2 Studies undertaken as part of this PhD and Massey University Animal Ethics Committee approvals with application numbers

Study number	Study title	Ethics application number
Study A	Longitudinal study of changes in ewe udder defects over time	MUAEC 15/105
Study B	Cross-sectional study of udder defects in ewes from a commercial farm	MUAEC 15/105
Study C	Longitudinal milking study over 6 weeks of lactation on ewes with udder defects	MUAEC 18/99

3.9 Definition of Important Terminologies

A range of terminologies to describe ewe udders have been used in the literature. To avoid ambiguity the following relevant terminologies to this thesis are defined below and have been used consistently throughout the thesis:

Udder defects

Any abnormality of the udder that can be observed or palpated. Mammary gland abnormalities can range from mechanical to non-mechanical gross injuries and acute mastitis to clinically detectable intramammary masses (IMM) including abnormal mammary secretions, inflammation, nodules or IMM, diffuse hardness, fibrosis, teat occlusion and teat canal cord formation, different sized masses (abscesses) and ruptured abscesses on the udder (Cooper *et al.*, 2013; Grant *et al.*, 2016; Griffiths *et al.*, 2019a; Quinlivan, 1968b; Saratsis *et al.*, 1998; Smith *et al.*, 2015). Similar terminologies used by other author/s: Faulty udder/imperfect udder (Hayman *et al.*, 1955); Udder abnormality/Udder lesion (Madel, 1981; Saratsis *et al.*, 1998).

Palpable udder defects

Diffuse hard consistency of an udder half or any palpable or detectable mass of different consistency from the rest of the gland tissue. Similar terminologies used by other author/s: Intramammary mass (IMM) (Grant *et al.*, 2016). Note: Palpable udder defects are usually associated with chronic IMI (Madel, 1981; Smith *et al.*, 2015); however, not all IMI cause such defects.

Mastitis

An inflammatory change of the mammary gland which, along with physical, chemical, and microbiological changes, is characterized by an increase in somatic cells, especially leukocytes, in the milk and by pathological changes in the mammary tissue (Menzies & Ramanoon, 2001) .

Clinical mastitis (Acute)

Mammary inflammation with obvious clinical signs such as pain, heat, swelling, redness, and macroscopic changes in the milk.

Sub-clinical mastitis

Mastitis associated with no macroscopic signs of inflammation, but milk tests show an increase in somatic cell counts and alterations in the chemical proportions of the milk.

Chronic mastitis

Udder inflammation with abnormal sub-clinical or clinical changes that continues over a long period. It may progressively develop to udder atrophy, sclerosis, asymmetry, mammary abscesses or scar tissue and a simultaneous reduction in milk yield.

Dairy ewes

Selectively bred sheep breeds that specialize in high milk production and are characterized by longer lactation length.

Non-dairy ewes

Breeds of sheep primarily produced for purposes other than milk production such as meat or wool. They produce significantly lower milk yields than the dairy breeds. Similar terminologies used by other author/s: Dual purpose breed; Suckler ewes/ Meat breeds/Wool breeds (Cooper *et al.*, 2016)

Udder

Two mammary glands in sheep which is considered as a single mass (Dyce *et al.*, 2010) suspended and supported to the body wall by medial elastic and non-elastic lateral suspensory ligaments. The ligaments are connected to the abdominal cavity via the inguinal canal that allow passage of blood vessels, nerves, and lymphatics.

Udder half

One of the two mammary glands of the udder in ovine species which is totally independent of the other having a separate secretory unit, duct system, blood supply, nerve supply, suspensory apparatus, and teat (Fails & Magee, 2018).

Udder half milk yield

Milk production expressed from an udder half, either the right- or left-hand side

Contralateral gland/ udder half

The opposite or another gland/udder half of the same ewe

Whole udder milk yield

Combined milk yield of two udder halves of a ewe. Similar terminologies used by other author/s: Individual/ewe /combined udder half milk yield (Leitner *et al.*, 2004; Marti De Olives *et al.*, 2013)

Chapter 4: Effect of Palpable Udder Defects on Milk Yield, Somatic Cell Count, and Milk Composition in Non-Dairy Ewes

Full chapter Published as:

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Note that this chapter is the copy of the published paper and part of the materials and methods is repeated from the general methodology (Chapter 3).

4.1 Abstract

AIM: In non-dairy ewes, udder defects hinder the survival and weight gain of their pre-weaned lambs. The objectives of this study were to determine the effects of palpable udder defects on milk yield, somatic cell count, and milk composition in non-dairy Romney ewes.

MATERIALS AND METHODS: Ewes with a history of udder defects or normal udders were selected for the study. Of a total of 48 ewes that lambed, 30 ewes reared at least one lamb, and were milked six times, once weekly, for the first six weeks of lactation. Udder halves were palpated and scored at each milking event. Multivariate linear mixed models examined the impacts of udder defects on udder-half and whole-udder milk yield, SCC, and milk composition (protein, fat, lactose, total solids, and solids non-fat).

REESULTS: Across the six examinations, 24.7% of the total 352 udder-half examinations were observed to be defective. Udder halves that were defective at least once produced on average 57.9% less ($p < 0.05$) milk than normal udder halves, while normal udder halves with a contralateral defective half yielded 33.5% more ($p < 0.05$)

milk than normal udder halves. Successive occurrence of both hard and lump udder defect categories in an udder-half, udder defect detection early in lactation, and a high frequency of udder defect detection were all associated with udder-half milk yield loss ($p < 0.05$). At the whole-udder level, no differences in milk yield ($p > 0.05$) were observed between those with one udder-half defective and both normal udder-halves. However, udders in which one udder half was categorised as hard but progressed to lump and remained as lump until 42 days of lactation produced less ($p < 0.05$) milk compared with normal udders. With the exception of solids non-fat, there were no significant associations ($p > 0.05$) between milk composition parameters and udder defect.

CONCLUSION: Overall, these findings emphasise the importance of udder health in non-dairy ewes and the potential effect of udder defects on their lambs.

Key words: Hard udder, Lactation, Milk yield, Udder health, Mammary

4.2 Introduction

Various udder defects including mastitis have been reported in both dairy and non-dairy ewes (Hayman *et al.*, 1955; Saratsis *et al.*, 1998). Clinical presentations of these defects include signs of inflammation, abnormal secretion, abscesses, lumps, nodules, diffuse hardness, and cysts (Hayman *et al.*, 1955; Quinlivan, 1968a; Saratsis *et al.*, 1998). Udder defects cause mild to serious damage to the mammary gland in ewes (Fthenakis & Jones, 1990c; Madel, 1981; Mavrogianni *et al.*, 2005). This damage can hinder the synthetic capacity of epithelial cells and/or increase the permeability of blood components into the milk (Burriel, 1997; Harmon, 1994). The occurrence and impact of these defects varies greatly depending on many factors, such as the individual animal, causative agent, severity of infection, management factors, production systems, genetics/breed, udder morphology, production level, and whether both or single udder-halves are involved (Gelasakis *et al.*, 2015; Gonzalo *et al.*, 2002; Marti De Olives *et al.*, 2013). Globally, the prevalence of udder defects in non-dairy ewes ranges from 0 to 10.9% (Arsenault *et al.*, 2008; Barber, 2015; Cooper *et al.*, 2016; Grant *et al.*, 2016; Onnasch *et al.*, 2002; Saratsis *et al.*, 1998). The prevalence of udder defects in New Zealand is up to 7.5% (Griffiths *et al.*, 2019a; Peterson *et al.*, 2017; Quinlivan, 1968a; Ridler *et al.*, 2021; Skyrme, 1970).

Udder defects in dairy ewes can be detected more frequently compared with non-dairy ewes because farmers check the udder during regular daily milking events (Fragkou *et al.*, 2014). In non-dairy ewes, udder defects are usually only identified at weaning or prior to mating, when farmers are selecting ewes for the next breeding season (Saratsis *et al.*, 1998). However, a condition described as "hard udder" has been observed during early lactation (Bruce *et al.*, 2013), while the prevalence of clinical mastitis has been reported to be higher shortly after lambing (Mørk *et al.*, 2007), which is a time when most farmers would not be checking the udders of non-dairy breed ewes in outdoor lambing systems.

Clinical and sub-clinical mastitis have a significantly negative impact on milk yield in ewes (Fthenakis & Jones, 1990c; Gonzalo *et al.*, 2004; Martí-De Olives *et al.*, 2020; Mørk *et al.*, 2007; Smith *et al.*, 2015). In dairy ewes, studies on the effect of mastitis on milk yield and composition have been undertaken for the purposes of quantifying milk losses and assessing milk quality for cheese production (Burriel, 1997; Leitner *et al.*, 2003). In contrast, in non-dairy ewes, the focus of studies investigating mastitis (Cooper *et al.*, 2016; Huntley *et al.*, 2012; Martí-De Olives *et al.*, 2020) or palpable udder defects (Grant *et al.*, 2016; Griffiths *et al.*, 2019a; Hayman *et al.*, 1955) have been to investigate growth rates and survival of pre-weaned lambs. Mastitis decreases milk yield and alters milk composition, including protein, fat, and lactose (Burriel, 1997; Griffiths *et al.*, 2019a; Leitner *et al.*, 2004). Lactose concentration decreases in mastitic (subclinical and clinical) ewes because of tissue damage, which interferes with its synthesis (Leitner *et al.*, 2003) and osmotic regulation role, which accentuates the decrease (Martí-De Olives *et al.*, 2020). Changes in milk fat and protein levels vary depending on the volume of milk loss or the dilution effect during mastitis (Leitner *et al.*, 2012; Pulina *et al.*, 2007).

An individual ewe whole-udder assessment approach has been used to diagnose mastitis, quantify milk yield loss, and assess milk composition for both dairy and non-dairy ewes (Leitner *et al.*, 2003; Madel, 1981; Saratsis *et al.*, 1999). However, due to the anatomic, functional, and infection independency of the two udder-halves, and the compensatory effects in both unilateral and bilateral infection, assessment of the whole-udder has been associated with huge individual variation (Gonzalo *et al.*, 2005; Gonzalo *et al.*, 2004; Marti De Olives *et al.*, 2013). Hence, determining milk losses due to mammary infection is better studied by assessment of individual udder-halves (Martí-De Olives *et al.*, 2020).

In non-dairy ewes, there are a limited number of studies on the impact of udder defects on milk yield despite the potential serious consequences to lamb growth and survival to weaning and subsequent culling of affected ewes (Grant *et al.*, 2016; Griffiths *et al.*, 2019a; Hayman *et al.*, 1955). “Hard udder”, a diffuse hard consistency of the udder occurring at or shortly after parturition, has been reported to result in udders that appear to be full of milk but fail to express milk after lambing (Bruce *et al.*, 2013; Skyrme, 1970). Hard udders have been associated with teat cord (hard core) formation, which may prevent milk removal (Peterson *et al.*, 2017). Ewes whose mammary glands contain lumps have been associated with decreased body weight gain in pre-weaned lambs (Griffiths *et al.*, 2019b). The presence of mammary glands with lumps is also a reported reason for culling of ewes, as it is assumed they have decreased milk production (Grant *et al.*, 2016; Peterson *et al.*, 2017) and reduced overall productivity (Flay *et al.*, 2020). Moreover, milk samples collected from ewe udders with defects including nodules, lumps, diffuse hardness, and abscesses were reported to contain clots and flakes, serous and purulent fluid, and blood-stained secretions (Saratsis *et al.*, 1998).

To the authors' knowledge, no previous studies have assessed the effect of palpable udder defects on milk yield and composition in either dairy or non-dairy ewes. Therefore, the objectives of this manuscript were to quantify the effects of palpable udder defects on udder-half and whole-udder milk yield, somatic cell count, and milk composition in mixed-age non-dairy breed (Romney) ewes. It was hypothesized that udders with palpable defects would show decreased milk yield and altered milk composition compared with normal udders.

4.3 Materials and Methods

4.3.1 Ewe selection

Eighty commercial ewes aged five to six years were selected from Massey University's Riverside farm, Wairarapa, New Zealand, based on udder and teat traits evaluated during the previous three years (2016, 2017, and 2018) and at the time of selection (Griffiths *et al.*, 2019a). In total, 60 ewes with defects or a history of defects and 20 with consistently normal udders were selected. These ewes were transported to Massey University's Keeble farm (Palmerston North, New Zealand) in January 2019.

4.3.2 Animal management

From the time of arrival at Keeble farm, the ewes were managed as a single flock under commercial grazing conditions. For breeding, ewes were progesterone-synchronised by inserting a controlled release intravaginal device (Eazi-Breed CIDR®, Zoetis New Zealand) on March 13th, 2019, for a period of 12 days. Pregnancy diagnosis was undertaken twice in mid-pregnancy (June 4th and July 25th) using trans-abdominal ultrasonography undertaken by a commercial operator to confirm pregnancy and fetus numbers. Single and twin-bearing ewes were retained while non-pregnant and triplet-bearing ewes were culled (n = 21).

On July 30th, 2019, all ewes were weighed, body condition scored, and moved to a single paddock closer to the milking parlour with a pasture requirement according to commercial conditions for late pregnant ewes (pre-lambing). Forty-eight ewes were retained for lambing and the lambing period occurred between August 13th and September 9th, 2019. On milking days (see below), lambs were separated from their dams for six hours between morning and afternoon milkings, during which time each lamb was bottle-fed 300 g of ewe's milk (Peterson *et al.*, 1997). If a ewe produced less than 1200 g/day of milk or abandoned their lamb/s, the lamb/s was/were removed from the ewe and fostered. These ewes, and ewes for whom all their lambs died (18), were excluded from the milking study.

4.3.3 Milking and milk yield calculation

Each ewe was milked six times during lactation at seven-day intervals, beginning 5-8 days after lambing. There were two milking groups: ewes that lambed on Sunday-Tuesday were milked on Mondays while those that lambed on Thursday-Saturday were milked on Fridays. Ewes that lambed on Wednesday (August 21st, 2019) were milked once on Wednesday and then joined the Friday milking group to balance group sizes. Each milking group contained ewes with a history of both normal udders and udder defects. The milking procedure was as follows. Briefly, milking was achieved by injecting oxytocin (oxytocin-EA (Ethical Agents Ltd, Auckland) (1 IU) diluted with 0.9 mL of physiological saline into the jugular vein 0.5–1 minute before milking commenced (Peterson *et al.*, 1997).

At each milking (morning (approximately starting at 9 am) and afternoon (approximately starting at 3 pm)), ewes were machine-milked for approximately two minutes after which any remaining milk in the mammary gland was expressed by hand. In the morning, the time of milking was recorded individually for each ewe. The ewes, without their lambs, were then put back onto pasture until the afternoon milking. In the afternoon, milk from each udder-half was collected into a separate container and the weight of milk (g) collected and time were recorded. To estimate a 'daily' milk yield, the weight of milk collected from the afternoon milking and the inter-milking interval time were used to calculate a 'daily' (24 h) milk yield from each udder-half. The 'daily' whole-udder milk yield was calculated as the sum of milk yield from two udder halves for each ewe (van der Linden *et al.*, 2011).

4.3.4 Milk sampling and laboratory analysis

The milk collected was gently mixed by inverting the collection container and 10 and 50 ml of milk were removed into separate sampling vials for SCC and milk composition analysis, respectively. SCC was assessed 2–4 h post-collection on the same day (see section 4.3.5). Milk samples collected for composition analysis were stored in a refrigerator (+4 °C) overnight and analysed the next day.

4.3.5 Somatic cell count (SCC) determination

Somatic cell count analysis of milk from each udder-half was performed using a DeLaval cell counter (DCC) (DeLaval Int. AB, Tumba, Sweden) according to the method of Gonzalo *et al.* (2004). Milk was extracted from the sample vial into the DCC cassette, which contained a small amount of fluorescent stain and Triton X-100, and provided cell counts per ml within approximately 1 minute. The DCC captured a digital picture of each somatic cell's nucleus, which was then stained in the cassette with a DNA-specific fluorescent reagent enabling each cell's nuclei to be counted. A small number of samples with clots or blood (signs consistent with clinical mastitis) could not be analysed as the presence of clots meant they were unable to be read by the machine; these were excluded from the analysis.

4.3.6 Milk component analysis

Analysis of milk components including fat, protein, lactose, total solids, and NFS were determined using a Milkoscan FT1 (Foss Electric, Hillerød, Denmark). The stored milk samples were placed in a water bath (40º C) before the analysis and carefully inverted to homogenize the fat. The Milkoscan was set to allow triplicate analyses from the single sample and provided these three results along with the mean and standard deviation. Sheep milk calibration was developed using Fourier transform infrared spectroscopy (FTIRS) through a Milkoscan FT1 machine and the details of the Milkoscan calibrations are summarized in Appendix I.

4.3.7 Udder scoring

Udder scoring of the ewes was undertaken using the methods described by Griffiths *et al.* (2019a) each week for the first six weeks of lactation (on the same day as milking). All ewes were udder-scored at these times, regardless of whether they reared a lamb and were milked.

4.3.8 Data management

For analysis of the data, the 7-category udder scoring system as described by Griffiths *et al.* (2019a), was condensed into three categories: Score 7 was categorised as 'hard', scores 3–6 as 'lump', and scores 1 and 2 as 'normal' (Griffiths *et al.*, 2019a). Udders or udder halves categorised as 'hard' or 'lump' were considered defective. During the six examinations in lactation, it was observed that most udder-half defects changed category over time (e.g., hard changed to lump). To account for these changes and potential effects on the trait measured, additional sub-categories were developed (Table 4.1).

Combined over the six once-weekly milk collections from the 30 ewes, the dataset for udder-half consisted of 352 daily milk yields. Four ewes missed the last milking. When udder-half observations with no milk were excluded, the dataset included 262 values. This was further reduced to 255 values for SCC and 243 values for milk composition analyses. Fewer samples were available for SCC and milk composition due to no expression of milk or an inadequate volume of milk to conduct the appropriate tests. The total number for the analysis of whole-udder milk yield consisted of 176 whole-udder observations (i.e., half of 352 daily yields; however, only 123 individual udder-half observations expressed milk from both udder halves). For the analysis between the overall defect type and whole-udder milk yield, six observations from five udders with bilateral udder defects were excluded (n = 170).

4.3.9 Statistical analysis

All data were entered to MS-Excel (2016) and analysed using the statistical package R version 3.6.0 (R Core Team, 2020). Analyses were undertaken both at the udder-half and whole-udder level.

4.3.9.1 Udder-half Data Analysis

Udder-half milk yield, milk natural log-SCC, and milk composition (fat, crude protein, lactose, total solids, and SNF) were the dependent variables considered. Independent variables considered included udder-half defect occurrence, number of lambs born, number of lambs reared, udder-half (left/right), pre-lambing body condition score (BCS), pre-lambing body weight, udder defect history (over the previous three years), age of ewe, and days in milk. SCC was considered as a predictor variable of milk yield and composition only in those udder halves that expressed milk.

The association of udder-half milk yield was also analysed with various udder-half defect parameters (Table 4.1). The association of some udder-half defect variables (weekly defect occurrence and weekly and overall udder-half defect type) with SCC and milk composition were not undertaken due to the low numbers of defective udder halves from which milk could be expressed and tested for SCC and composition.

4.3.9.2 Whole-udder Data Analysis

The association between whole-udder milk yield and udder defect parameters is summarized in Table 4.1. The same independent variables were considered as those for the udder-half analysis except for udder-half (left/right).

4.3.9.3 Multivariate Model Development and Diagnostics

Multivariate linear mixed models with ewe identification number as a random effect and AR1 correlation structure were used to analyse the impact of udder-half or whole-udder defect occurrence, types, frequency, and first detection time, on milk yield, natural log-SCC, and milk composition. For this purpose, “nlme” package in R was used. A stepwise variable selection based on significance level ($p < 0.1$) was implemented. Interaction of days in lactation with udder defects and its quadratic relationship was tested during the models’ development. The confounding effect of variables was progressively assessed during steps of model development by monitoring for a substantial increase in beta coefficient and/or decrease in standard errors.

Histograms and QQ plots were used for assessing the normality of the data. Homoscedasticity and linearity were checked using fitted vs. predicted values plot, and the variance inflation factor (VIF) was used to determine the multicollinearity among independent variables. R^2 was used to assess the goodness fit for the models. Outlier observations were removed based on Cook’s distance and improvement of R^2. Least squares mean (LSM ± SEM) of dependent variables were calculated by adjusting for significant fixed-effect variables

Table 4.1 Description of udder-half and whole-udder analysis types (*n* = 12) and categories used to investigate the effects of palpable udder defects on milk parameters in mixed-age non-dairy breed (Romney) ewes, which were examined six times at weekly intervals, starting at day 7 of lactation.

Analysis Type and Number	Categories for Analysis	Description of Categories	Parameters Analysed
Weekly udder-half defect occurrence a (1)	Defective Normal (defective) Normal (normal)	Defective: udder-half categorised as hard or lump Normal (defective): normal udder-half but with hard or lump on the contralateral side Normal (normal): normal udder-half with a normal udder-half on the contralateral side	Udder-half milk yield
Overall udder-half defect occurrence (across all six examinations) (2)	Defective Normal (defective) Normal (normal)	Defective: udder-half categorised as hard or lump on at least one of the six occasions Normal (defective): normal udder-half contralateral side to overall defective Normal (normal): normal udder-half with a normal udder-half on the contralateral side in all six occasions	Udder-half milk yield, SCC, milk composition
Udder-half defect type at single point in time (weekly) (3)	Hard Lump Normal (defective) Normal (normal)	Hard: hardness in udder-half Lump: lump in udder-half Normal (defective): normal udder-half but with hard or lump on the contralateral side Normal (normal): normal udder-half with a normal udder-half on the contralateral side	Udder-half milk yield
Udder-half defect type change (overall) (4)	Hard-normal Lump-normal Hard-lump Hard-lump-normal Normal (defective) Normal (normal)	Hard-normal: udder-half was hard but subsequently changed to normal and remained normal Lump-normal: udder-half was lump but subsequently changed to normal and remained normal Hard-lump: udder-half was hard but subsequently changed to lump and remained as lump Hard-lump-normal: udder-half was hard but subsequently changed to lump and then changed to normal and remained as normal Normal (defective): normal udder-half contralateral side to overall defective Normal (normal): normal udder-half with a normal udder-half on the contralateral side in all six occasions	Udder-half milk yield
Udder-half defect frequency (5)	High Low Normal (defective) Normal (normal)	High: hard or lump identified on at least 3 examinations Low: hard or lump identified on 1 or 2 examinations Normal (defective): normal udder-half but with hard or lump on the contralateral side Normal (normal): normal udder-half with a normal udder-half on the contralateral side	Udder-half milk yield
Udder-half defect first time detection (6)	Day 7 Day 14 Normal (defective) Normal (normal)	Day 7: hard or lump first detected at first udder examination (Day 7) Day 14: hard or lump first detected at second or subsequent udder examinations (Day 14 or greater) Normal (defective): normal udder-half but with hard or lump on the contralateral side Normal (normal): normal udder-half with a normal udder-half on the contralateral side	Udder-half milk yield
Weekly whole-udder defect occurrence [a] (7)	Defective Normal (both)	Defective: One or both udder halves categorised as hard or lump Normal (both): both udder halves normal in all six occasions	Whole-udder milk yield

Table 4.1. Continued

Whole-udder defect across all six examinations (overall) (8)	Defective Normal (both)	Defective: One or both udder halves categorised as hard or lump on at least one of the six occasions Normal (both): both udder halves normal in all six occasions	Whole-udder milk yield
Whole-udder defect type at each examination (weekly) (9)	Hard Lump Normal (both)	Hard: One or both udder halves categorised as hard or 'lump' on one udder-half and 'hard' on the other half Lump: One or both udder halves categorised as lump Normal (both): both udder halves normal	Whole-udder milk yield
Unilateral udder defect type change (overall) (10)[b]	Hard-normal Hard-lump Hard-lump-normal Lump-normal Normal (both)	Hard-normal: one udder-half was hard but subsequently changed to normal and remained normal Hard-lump: one udder-half was hard but subsequently changed to lump and remained as lump Hard-lump-normal: udder-half was hard but subsequently changed to lump and then changed to normal and remained as normal Lump-normal: udder-half was lump but subsequently changed to normal and remained normal Normal (both): both udder halves normal in all six occasions	Whole-udder milk yield
Whole-udder defect frequency [c] (11)	High Low Normal (both)	High: hard or lump identified on at least 3 examinations Low: hard or lump identified on 1 or 2 examinations Normal (both): both udder halves normal	Whole-udder milk yield
Whole-udder defect first time detection (12)	Day 7 Day 14 Normal (both)	Day 7: One or both udder halves categorised as hard or lump first detected at first udder examination (Day 7) Day 14: One or both udder halves categorised as hard or lump first detected at second or subsequent udder examinations (Day 14 or greater) Normal (both): both udder halves normal	Whole-udder milk yield

Note: [a] Individual udder-half or whole-udder defects could change in their defect category over time. [b] Only unilateral udder defects were included (n = 170) to show change of defect over time. [c] Bilateral udder defect from a ewe on one observation was counted as a single occurrence.

4.4 Results

4.4.1 The occurrence of udder-half defects (weekly and overall)

From the total of 48 ewes that lambed, 18 ewes were excluded from the milking study in the first week of lactation because they had lost all their lamb/s. Based on udder palpation seven days after lambing, of these 18 ewes, 39% were categorised as hard in both udder halves, 33% were categorised as hard in one udder-half, and 28% were categorised as lump in one udder-half. Of the 30 ewes that were milked, 14 were single bearing and 16 were twin-bearing, with 9 twin-bearing ewes rearing both lambs.

During the udder palpation assessments, it was observed that most ewes' udder-half defect category changed over time. Figure 4.1 summarises the occurrence of weekly (A) and overall (B) udder-half defects during the six-week period. The maximum number of udder-half defects was recorded on day 21 (week 3, 35% of 60 udder halves) and the minimum on day 42 (week 6, 5% of 52 udder halves, Figure 4.1). Across all six examinations, 87 udder halves (24.7% of the 352 udder-half observations) were defective. Note that four ewes (eight udder halves) were not milked on day 42.

The highest number of udder halves in the category 'hard' occurred at day seven (28% of the 60 udder halves, Figure 4.1A) but decreased to 3.8% by day 42 (week six). A total of 35% of the 60 udder halves were categorised as 'hard' at least once during the six-weeks. In contrast, udder halves in the category 'lump' were lowest at day seven (week one, 1.7% of the 60 udder halves) but had increased to 16.7% by day 14 (week two) and remained at similar levels to day 42 (week six). A total of 35% (of the 60 udder halves) were categorised 'lump' at least once over the six weeks. Combined over all six weeks, 50% of 60 udder halves were defective at least once (Figure 4.1B).

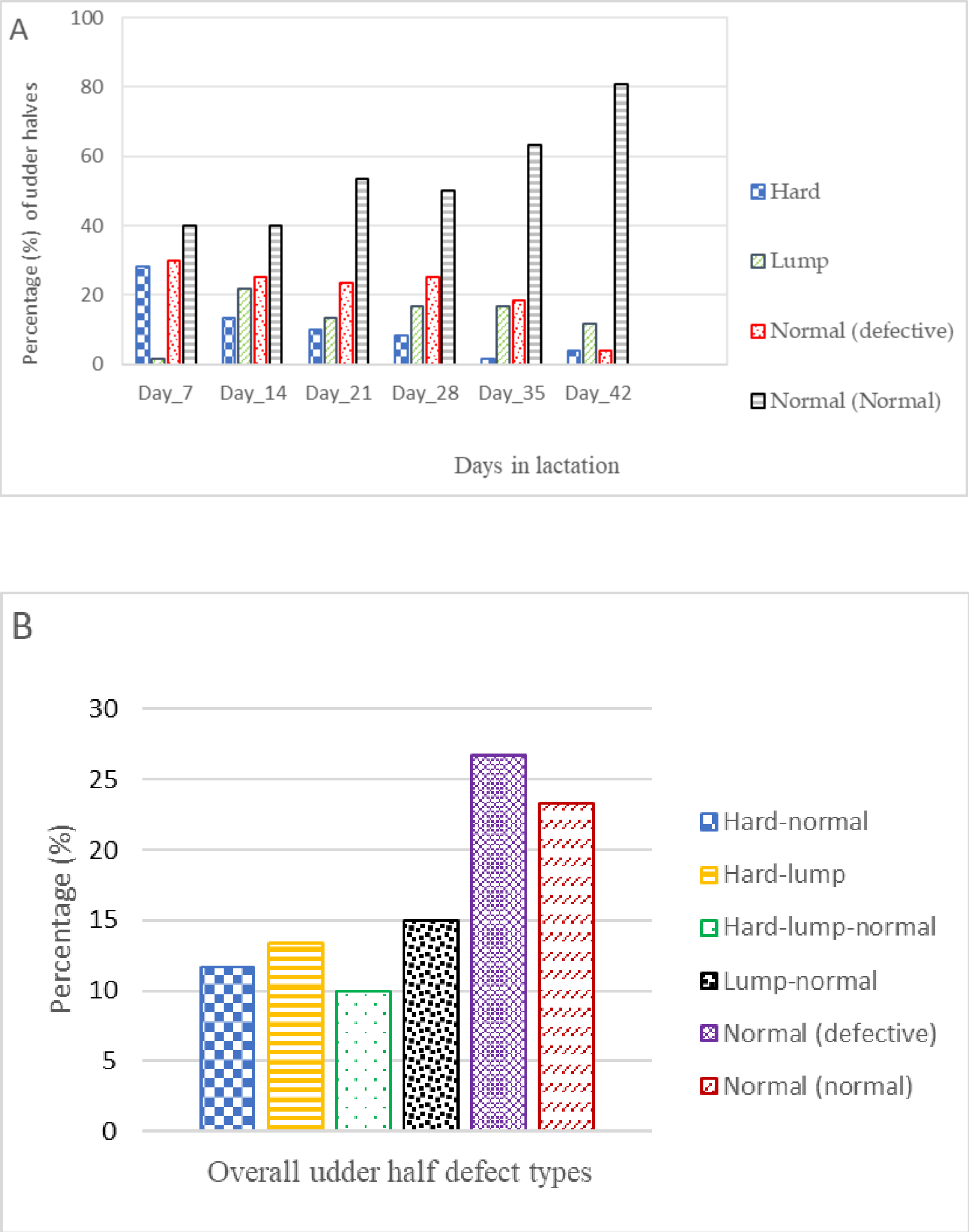


Figure 4.1 The percentage (%) of udder-half defect occurrence (*n* = 60, i.e., 30 ewes in total), by category in non-dairy ewes whose udder was scored once weekly for the first six-weeks of lactation. A: percentage of weekly udder-half defect types; B: Percentage of overall (i.e., classification over the entire six measurements) udder-half defect types; normal udder halves (both in weekly and overall) were sub-categorised into normal (normal) and normal (defective) to account for possible compensatory effects of a normal udder-half when the contralateral udder-half was defective. Normal (normal) refers to a normal udder-half from a ewe in which both udder halves were normal. Normal (defective) refers to a normal udder-half from a ewe where the contra-lateral udder-half was defective. The naming of the overall (progressive) defect categories describes the change in the defect category over time (e.g., hard-lump refers to an udder-half that was first categorised as 'hard' then subsequently was categorised as 'lump' and remained 'lump' until day 42. Note: Four ewes did not have an udder examination on day 42.

Most of the udder-half defects (83.3% of the 30 defective udder halves) were detected in the first two weeks (14 days) post-lambing. Five udder halves (16.7% of the 30 udder halves) were identified as defective only once, while the rest were observed to be defective on two or more occasions.

During the six weeks, many of the udder-half defects changed in category over time. A third of the 21 udder halves initially categorised as 'hard' changed to normal and the rest changed to 'lump' (Figure 4.1B). Of those that changed to 'lump', more than half remained as 'lump' while the others changed to normal.

4.4.2 Udder-half milk expression

Based on weekly milking status, 18/60 (30%) of the udder halves included in the study expressed no milk at least once during the six-week period. At day 7, 12/60 (20% of the udder halves) expressed no milk and this increased to 16/60 (27%) on day 35 (i.e., week five). Of these 18 udder halves that expressed no milk, 55.5% expressed no milk on all six occasions, 16.6% on five, 22.2% on four, and 0. 5% on just one occasion.

Over the entire 42-day (six-week) milking period, data were available for 352 udder halves of which 90 had no milk expression. Of these 90 udder halves, 28% were in the category 'hard', 38% were 'lump', while 34% were 'normal'. In contrast, when an individual half udder was categorised based on the entire six-week (42-day) period (category 2 in Table 4.1), the 30 normal (normal) and normal (defective) udder halves expressed milk at every milking. Of the 30 udder halves that expressed no milk, 23% were categorised as hard-normal 27% hard-lump, 20% hard-lump-normal, and 7% lump-normal.

4.4.3 Relationship between udder-half defect occurrence and udder-half milk yield

Normal (normal) udder halves produced more ($p < 0.01$) milk than defective udder halves based on both weekly and overall udder-half defect analysis (Table 4.2 and Figure 4.2). Further, normal (defective) udder halves produced more ($p < 0.01$) milk than normal (normal) udder halves. Overall, defective udder halves (defective at least once during the six-weeks) produced 57.9% less milk than normal (normal) udder halves. In contrast, normal (defective) udder halves produced 33.5% more milk than normal (normal) udder halves.

Using the multivariate model, weekly udder-half defect occurrence and the number of lambs each ewe was rearing (rearing rank) were predictors ($p < 0.05$) of udder-half milk yield ($R^2 = 0.366$, Table 4.3), where weekly udder-half defect occurrence explained 36.6% of the variation in milk yield. The overall udder-half defect explained 39% ($p < 0.05$) of the milk yield at the half udder level (Table 4.3).

Further analysis was undertaken to assess the relationship between udder-half defect occurrence and udder-half milk yield, using only those udder halves where milk was actually expressed ($n = 262$, of the total 352 potential udder-half collections over the six-week period). This analysis identified differences ($p < 0.05$) among weekly and overall udder-half defect categories; however, overall, the differences between the normal and defective categories were less than those identified in the analysis including all 352 udder halves as outlined earlier (Table 4.2 and Table 4.3).

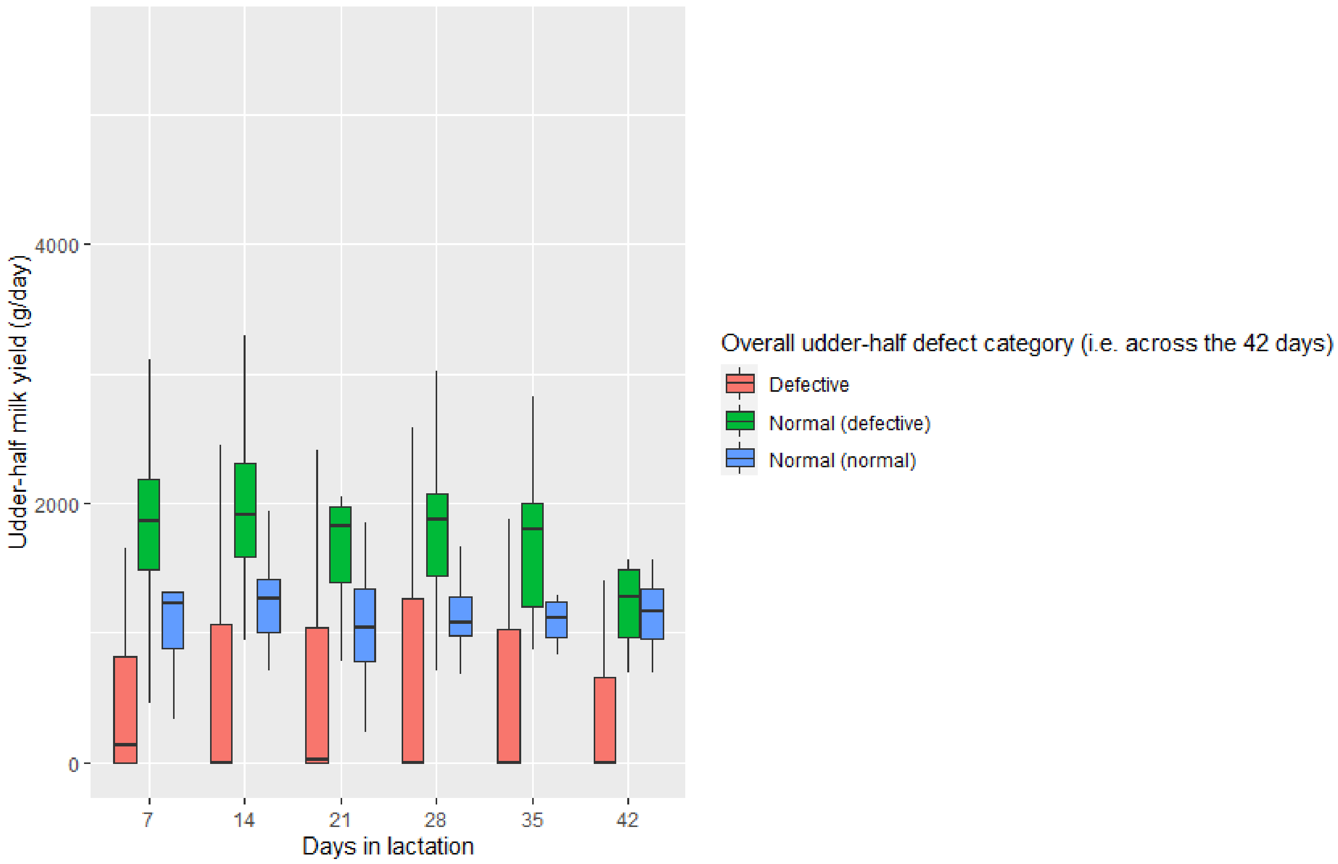


Figure 4.2 Udder-half milk yield (median and interquartile range) based on overall udder-half defect (i.e., across the 42 days/six-week period) categories in non-dairy Romney ewes (n = 30) milked once weekly during the first six weeks of lactation. Udder halves that were categorised as 'hard' or 'lump' on at least one of the six occasions were considered Defective. Normal (defective): was used to describe a normal udder-half where the contralateral udder-half was defective. Normal (normal): was used to describe a normal udder-half where the contralateral udder-half was also normal.

Table 4.2 The effect of weekly[4] and overall[5] udder-half defect occurrence on udder-half milk yield (n = 352), somatic cell count (SCC) (n = 255), and milk composition (n = 343) (LSM + SEM) in non-dairy breed (Romney) ewes examined and milked once weekly during the first six weeks of lactation.

	Udder-half Defect Category					
Dependent Variables	Normal (normal)1		Defective2		Normal (defective)3	
		SEM	LSM	SEM	LSM	SEM
Udder-half milk yield	LSM					
Weekly defect4 (g/d)	1118[b]	54.1	320[a]	83.7	1785[c]	90.4
Overall defect5 (g/d)	1204[b]	76.8	507[a]	53	1811[c]	73.4
SCC (1000)	647	86.5	680	82.6	633	74.9
Milk composition						
Fat (%)	7.57	0.23	7.92	0.25	7.26	0.22
Crude protein (%)	4.95	0.04	4.83	0.04	4.89	0.04
Lactose (%)	7.57	0.23	7.92	0.25	7.26	0.22
Total solids (%)	18.3	0.25	18.7	0.29	18.5	0.26
SNF (%)	11.3[b]	0.07	11.0[a]	0.08	11.3[b]	0.07

[1] Normal (normal): a normal udder-half where the contralateral udder-half was also normal. [2] Defective: udder-half categorised as hard or lump either in one or both udder halves. [3] Normal (defective): a normal udder-half where the contralateral udder-half was defective. 4 Weekly defect: udder-half defect category at each examination. 5 Overall defect: udder-half category for all six examinations (identified as defective by being 'hard' or 'lump' at least once during the six examinations). For weekly udder defect, individual udder-half could be identified in a different defect category each week whereas, the overall udder defect reflects a single overall udder-half category over the six-week (42 days) period. log-SCC: Natural log of somatic cell count. LSM: least square means. Different superscripts [a,b,c] within a row indicates significant differences (p < 0.05).

4.4.4 Relationship between udder-half defect occurrence and somatic cell count (SCC)

The log-SCC was not affected (p >0.05) by overall udder-half defect occurrence (Table 4.2). Among the independent variables included for udder-half log-SCC analysis, the best fit for the multivariate linear mixed model included birth rank and udder defect history as predictors ($p < 0.05$, $R^2 = 0.46$, Table 4.3), where ewes that gave birth to twins and those with a previous history of udder defects had higher log-SCC than those that gave birth to a single lamb and had no history of udder defects.

4.4.5 Relationship between udder-half defect occurrence and milk composition

There was no association between overall udder-half defect occurrence and milk fat, crude protein, total solids, and lactose ($p > 0.05$, Table 4.2). However, defective udder halves had lower ($p < 0.05$) SNF than the normal (normal) udder halves, whereas no difference was observed between normal (defective) and normal (normal) udder halves ($p > 0.05$).

Days in lactation and SCC were significantly correlated with milk fat, lactose, and SNF ($p < 0.05$, Table 4.3). As days in lactation progressed, fat and total solids increased, but SNF decreased ($p < 0.05$). Fat percentage increased with SCC whereas lactose and SNF were negatively associated ($p < 0.05$).

4.4.6 Relationship between udder-half defect type and udder-half milk yield

Udder halves categorised as hard or lump at each examination had lower milk yield ($p < 0.05$) compared with normal (normal) udder halves (Table 4.4). There were no udder-half milk yield differences ($p > 0.05$) in the overall udder-half defect category (hard-normal) compared with normal (normal) udder halves. However, udder halves that were categorised as hard and then progressed to lump and either remained as lump (hard-lump) or progressed to normal (hard-lump-normal) had a lower milk yield than normal (normal) udder halves (Table 4.4). Additionally, milk yield in udder halves identified as lump that changed to normal (lump-normal) was lower ($p < 0.05$) than normal (normal) udder halves (Table4). For the multivariate analysis of udder-half weekly milk yield, there was a significant interaction ($p < 0.05$) between days in lactation and udder halves categorised as hard (Table 4.4). This was due to an increase in the udder-half milk yield in the later days of lactation for udder halves categorised as hard ($p < 0.05$). This is likely explained by a decreased occurrence of udder halves categorised as hard as the days in lactation advanced, as the category changed to lump or normal (Figure 4.1). Hard-lump and hard-lump-normal udder-half categories had lower ($p < 0.05$) udder-half milk yield compared with normal (normal) udders (Table 4.4).

Table 4.3 Multivariate regression coefficient (Estimate ±SEM) of udder-half log-SCC (*n* = 255) and milk composition (fat, crude protein, lactose, total solids, and SNF) (*n* = 243) based on overall[4] udder-half defect from 30 non-dairy breed (Romney) ewes examined and milked weekly during the first six weeks of lactation.

Parameters	Intercept		Defective[1]		Normal (defective)[2]		Days in lactation		SCC (10^6)		Birth rank		Rearing rank		Udder defect history		R^2
	Est.	SE	Est.	SE	Est.	SE	Est.	SE	Est.	SE	Est.	SE	Est.	SE	Est.	SE	
Milk yield																	
Weekly	744.50	153.70	-739.40	98.90	755.6	103.7							224.1	103.9			0.366
Overall	1195.25	129.19	-762.73	153.78	674.07	168.13											0.386
Milk composition																	
Fat (%)	5. 97	0.32					0.04	0.01	0.71	0.19							0.67
Protein (%)	4.83	0.05							0.1	0.03							0.49
Lactose (%)	5.58	0.04							-0.19	0.04							0.32
Total solids (%)	17.05	0.34					0.37	0.05									0.64
SNF (%)	11.74	0.16	-0.30	0.18	0.07	0.20	-0.01	0.00	-0.26	0.06							0.30
Log-SCC	4.84	0.35									0.59	0.20			0.46	0.19	0.46

Normal (normal): a normal udder-half where the contralateral udder-half was also normal. Normal (normal) was used as a reference in all cases. [1]Defective: an udder-half categorised as hard or lump either in one or both udder halves. [2]Normal (defective): a normal udder-half but with hard or lump on the contralateral side. [3] Weekly defect: udder-half defect category at each examination. [4] Overall defect: udder-half category for all 6 examinations (identified as defective by being 'hard' or 'lump' at least once during the six examinations). For weekly udder defect, individual udder-half could be identified in a different defect category each week whereas, the overall udder defect reflects a single overall udder-half category over the six-week (42 days) period. Log-SCC: Natural log of somatic cell count. All intercepts and estimates were significantly different ($p < 0.05$), except where blank values are shown. Est.: indicates estimate of multivariate coefficient.

Table 4.4 Multivariate analysis coefficient (Estimate ± SEM) of weekly udder-half milk yield (g/d) based on either weekly[7] or overall[8] udder-half defect types from 352 individual udder-half observations from 30 non-dairy breed (Romney) ewes examined and milked weekly during the first six weeks of lactation.

Udder-half defect parameters	Udder-half milk yield (g/d)				P-value
	Weekly defect		Overall defect		
	Estimate	SEM	Estimate	SEM	
Intercept	1255.49	151.37	1197.73	135.05	<0.001
Hard	-924.50	245.97			<0.001
Lump	-362.50	279.55			NS
Days in lactation	-71.17	32.86			0.03
Hard X days in lactation	229.74	75.85			<0.001
Lump X days in lactation	-2.82	67.20			NS
Normal (defective) X days in lactation	-152.43	60.07			0.01
Normal(defective)[1]	943.35	215.62	647.77	170.15	<0.001
Hard-normal[2]			-231.77	216.39	NS
Hard-Lump[3]			-1303.20	195.39	<0.001
Hard-lump-normal[4]			-1189.52	209.03	<0.001
Lump-normal[5]			-356.22	192.04	NS
R^2		0.28		0.53	

Normal (normal): normal udder-half with a normal udder-half on the contralateral side. Normal (normal) was used as a reference in all cases. [1]Normal (defective): normal udder-half but with hard or lump on the contralateral side. [2] Hard-normal: udder-half was hard but subsequently changed to normal and remained normal. [3] Hard-lump: udder-half was hard but subsequently changed to lump and remained as lump. [4] Hard-lump-normal: udder-half was hard but subsequently changed to lump and then changed to normal and remained as normal. [5] Lump-normal: udder-half was lump but subsequently changed to normal and remained normal. [6] Weekly defect: udder-half defect category at each examination. [7] Overall defect: udder-half category for all six examinations (identified as defective by being 'hard' or 'lump' at least once during the six examinations). For weekly udder defect, individual udder-half could be identified in a different defect category each week whereas, the overall udder defect reflects a single overall udder-half category over the six-week (42 days) period. All intercepts and estimates were significantly different ($p < 0.05$), except where blank values are shown.

Table 4.5 Multivariate analysis (estimate ±SE) of weekly udder-half milk yield (g/d) based on udder-half defect detection frequency and first defect detection time from 352 udder-half observations from 30 non-dairy breed (Romney) ewes examined and milked weekly during the first six weeks of lactation.

Parameters	Udder-half milk yield (g/d)		R^2
	Estimate	SE	
Effect of udder-half defect frequency			
Intercept (g/d)	1246.19	116.69	0.46
High (3-6)[1]	-1081.73	149.02	
Low (1-2)[2]	-266.16 [NS]	156.67	
Normal (defective)[3]	510.15	152.88	
Effect of udder-half defect first detection time			
Intercept	1279.23	115.67	0.29
Day 7[4]	-841.39	143.35	
Day 14 and above[5]	-585.27	161.63	
Normal (defective)[3]	526.49	163.68	

Normal (normal): normal udder-half with a normal udder-half on the contralateral side. Normal (normal) was used as a reference in all cases. [1] High: hard or lump identified on at least three examinations. [2] Low: hard or lump identified on one or two examinations. [3] Normal (defective): normal udder-half but with hard or lump on the contralateral side. [4] Day 7: hard or lump first detected at first udder examination at end of week one (Day seven). [5] Day 14: hard or lump first detected at second or subsequent udder examinations (Day 14 or greater). LSM: least square means. All estimates were significant ($p < 0.05$), except with NS.

4.4.7 Relationship between udder-half defect frequency and udder-half milk yield

Udder halves with a high udder defect frequency (defects identified on three to six occasions) produced less milk ($p < 0.05$, 88.5% less) than normal (normal) udder halves (Table 4.5). However, no differences ($p > 0.05$) were observed between udder halves with low defect frequency (one to two occasions) and normal (normal) udders. The best fit multivariate model for udder-half milk yield consisted only of udder-half defect frequency, which explained 46% ($p < 0.005$) of the variation (Table 4.5).

4.4.8 Relationship between first defect detection time and udder-half milk yield

Udder halves detected with a defect for the first time on day seven (end of week one) had lower ($P < 0.05$) milk yield than normal (normal) udder halves, but there was no difference ($p > 0.05$) in milk yield between udder halves detected with a defect on day 14 (end of week two) or later and normal (normal) udder halves (Table 4.5). The best fit multivariate model for udder-half milk yield consisted only udder-half defect first detection time, which explained 29% of the variation (Table 4.5).

4.4.9 The occurrence of whole-udder defects

Figure 4.3 shows the occurrence of weekly whole-udder defects during the first six weeks of lactation. A total of 23 whole-udders from the 30 ewes (77%) were defective at least once during the six once-weekly examinations. Most whole-udder defects were unilateral (i.e., defect in one udder-half only), except in five ewes, in which a bilateral defect was observed on one or two occasions.

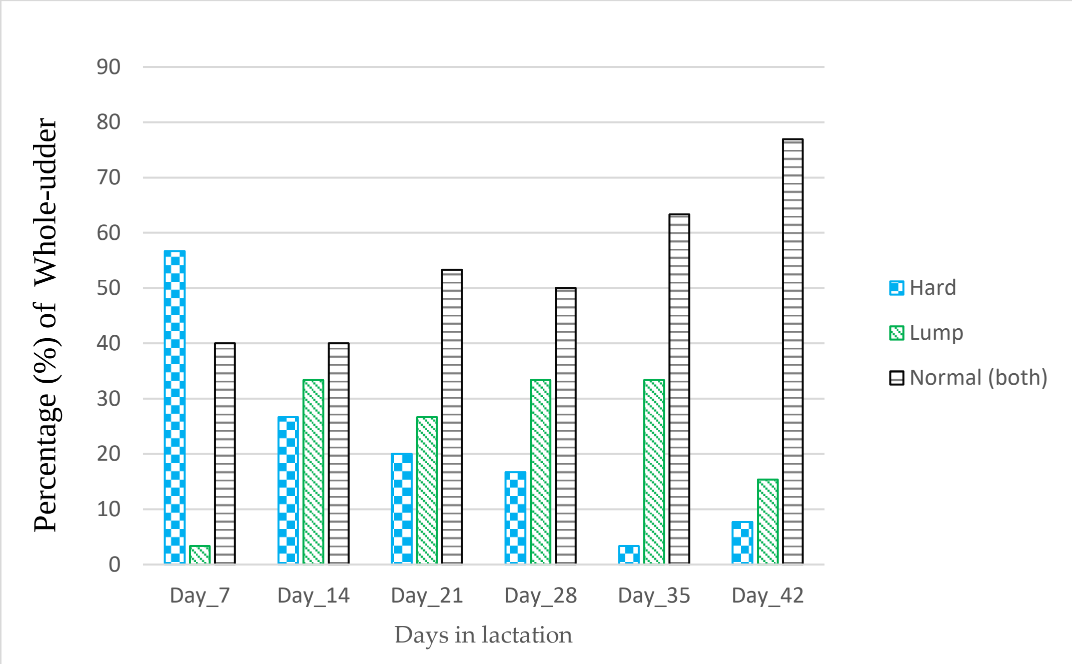


Figure 4.3 The percentage (%) of weekly whole-udder defect types in 30 non-dairy Romney breed ewes udder scored once weekly during the first six weeks of lactation. The naming of the overall (progressive) whole-udder defect types describes the change in defect category over time: Hard: was used to describe occurrence of 'hard' in one udder-half and 'normal' or 'lump' on the other udder halves; Lump: was used to describe occurrence of 'lump' either in one or both udder halves; Normal-both: both udder halves normal; For weekly whole-udder defect, individual udder-half could be identified in a different defect category each week over the six-week (42 days) period. Note four ewes were not udder scored on day 42 (end of week six).

4.4.10 Relationship between whole-udder defect occurrence and whole-udder milk yield

There was no association ($p > 0.05$) between weekly or overall udder-half defect occurrence and whole-udder milk yield. The best fit regression model had rearing rank as a predictor with an intercept of 1532.8 g/d (± SE 255.70) and a rearing rank coefficient of 410.79 (± SE 185.8). As such, twin-rearing ewes displayed higher ($p < 0.05$) whole-udder milk yields compared with single-rearing ewes. Similarly, there was no difference ($p > 0.05$) in whole-udder milk yield between normal (both) and defective whole-udder categories from those udders ($n = 123$) which expressed milk from both udder halves.

4.4.11 Relationship between defect type and whole-udder milk yield

Weekly whole-udder defect type had no effect ($P > 0.05$) on whole-udder milk yield (Table 4.6); however, the whole-udders categorised as hard-lump produced less ($p < 0.05$, 20.7% less) milk compared with normal (both) whole-udders. The overall whole-udder defect and rearing rank were predictors ($p < 0.05$) for the best fit multivariate model ($R^2 = 0.29$) for whole-udder milk yield (Table 4.6).

Table 4.6 Multivariate analysis coefficient (estimate + SEM) of weekly whole-udder milk yield (g/d) based on either weekly[1] or overall[2] udder-half defect types from 170 individual whole-udder observations from 30 non-dairy breed (Romney) ewes examined and milked weekly during the first six weeks of lactation.

Dependent variables	Category	Milk yield (g/d)		R^2
		Estimate	SEM	
Weekly whole-udder defect type[1]				
Intercept		1532.8	255.7	0.26
Rearing rank		410.8	185.8	
Overall whole-udder defect type[2]				
Intercept		1761.2	317.1	
Overall whole-udder defect type	Hard-normal[3]	-232.1[NS]	238.3	0.29
	Hard-lump[4]	-562.4	239.0	
	Hard-lump-normal[5]	213.2 [NS]	236.7	
	Lump-normal[6]	-270.4 [NS]	240.5	
Rearing rank		357.0	176.6	

[1]Weekly whole-udder defect type: udder defect type at each examination. [2] Overall whole-udder defect type: unilateral defect type change across all six examinations. [3] Hard-normal: udder-half was hard but subsequently changed to normal and remained normal. [4] Hard-lump: udder-half was hard but subsequently changed to lump and remained as lump. [5] Hard-lump-normal: udder-half was hard but subsequently changed to lump and then changed to normal and remained as normal. [6] Lump-normal: udder-half was lump but subsequently changed to normal and remained normal. Normal: both udder halves normal (Reference). Normal (both) was used as a reference in whole-udder analysis. Note: Four ewes were not milked on day 42 (end of week six). NS: Not significant ($p < 0.05$).

4.4.12 Relationship between whole-udder milk yield and whole-udder defect frequency

There were no differences (p > 0.05) in whole-udder milk yield between whole-udder defect frequency categories (high: defect identified on three to six occasions, or low: one to two occasions) compared with normal (both) udders (data not shown).

4.4.13 Relationship between whole-udder milk yield and whole-udder defect first defect detection time

Whole-udder milk yield was not different ($p > 0.05$) between udders where both halves were normal compared with udders where one or both udder halves were found to be defective at either day 7 or day 14 and later (data not shown).

4.5 Discussion

This study was undertaken to assess the effect of palpable udder defects on milk yield and milk composition in non-dairy breed (Romney) ewes. Udder defects and milk parameters were analysed both at the udder-half and whole-udder level. Udder defects are relatively common in ewes in New Zealand, with previous studies undertaken between weaning and mating reporting a 2.3–7% prevalence (Peterson *et al.*, 2017; Quinlivan, 1968a; Ridler *et al.*, 2021). Recently, Griffiths *et al.* (2019a) studied palpable udder defects in Romney ewes at pre-mating, pre-lambing, docking (approximately four weeks post-lambing), and weaning and reported an occurrence of udder defects of 6.0, 5.0, 7.5, and 7.4%, respectively. The occurrence of palpable udder defects in early lactation is not well understood, but it has been suggested to be higher than at other times (Peterson *et al.*, 2017). Internationally, studies on udder defects in non-dairy ewes have shown comparable occurrence rates to those reported in New Zealand and have impacted mammary health and lamb survival and growth (Arsenault *et al.*, 2008; Barber, 2015; Cooper *et al.*, 2016; Grant *et al.*, 2016; Onnasch *et al.*, 2002). Despite this, there appears to be no available reports on the effects of palpable udder defects on milk yield and milk composition in ewes.

The present study investigated udder defects in a unique population of ewes that had a history of either known udder defects or no defects in the previous three years. From the total 48 ewes that lambed in the present study, 41 ewes (23 of 30 milked ewes and all 18 of the non-milked ewes) had an udder defect in one udder-half on at least one occasion, during their six once-weekly examinations. Thirty eight percent of the ewes that lambed in this study lost all their lambs and a further 12.5% lost one of their twins. These loss rates are higher than the averages reported in single or twin lambs in New Zealand (Dalton *et al.*, 1980; Griffiths *et al.*, 2019a; Morris & Kenyon, 2014). In contrast, in the present study, for ewes where both udder halves were categorised as normal throughout the study, all lambs survived. This finding agrees with the potential undesirable impact of udder defects on lamb survival reported previously (Grant *et al.*, 2016; Griffiths *et al.*, 2019a; Hayman *et al.*, 1955) and emphasises the importance of udder health and inspection of udders. Ewes that lost all their lambs in the present study either did not express, or only produced very small amounts of, milk from one or both udder halves. Additionally, more than half of the 30 ewes enrolled in the milking study did not express milk from one of the udder halves at least once during the six milkings. Total failure to express milk was seen mainly in udder halves categorised as hard at the time of milking or previously. Similarly, a condition described as hard udder has been reported to interfere with colostrum consumption or cause complete absence of milk, resulting in higher loss rates of lambs and increased culling of ewes (Bruce *et al.*, 2013; Skyrme, 1970).

Diffuse hardness of the mammary gland ('hard') and lumps of various size and consistency ('lump') were the only categories of palpable udder defects presented in this study. Repeated weekly udder examinations over the six-week period revealed that the palpable udder defects changed over time from either defective to normal or from one defective category to another. Overall, it was observed that the occurrence of udder defects decreased as lactation advanced, particularly in relation to 'hard'. The higher occurrence of udder defects observed in the early lactation period could be associated with higher milk production during this time, which may lead to a reduction in immunological competence (Bergonier & Berthelot, 2003) or a change in the severity of infection (Quinlivan, 1968b). A change from hard to normal palpation scores in the weeks following parturition has been previously reported by others (Bruce *et al.*, 2013; Peterson *et al.*, 2017; Skyrme, 1970). However, in some cases, this change took several weeks. This agrees with Grant *et al.* (2016), who reported that some palpable udder defects persisted during lactation and that these may increase the risk of recurrence in the following lactation. This may explain the high rates of udder defects reported in this study, as many of these ewes had a history of udder defects in the previous year/s, with ewes selected specifically because they had a history of palpable udder defects. It might also partially explain the occurrence of a higher percentage of palpable udder defects during lactation than in pregnancy (Grant *et al.*, 2016; Griffiths *et al.*, 2019a).

In this study, which included ewes with a history of udder defects, there was a high occurrence of udder halves categorised as hard at day 7, but which subsequently changed to lump or normal within 2-6 weeks post-lambing. Traditionally, farmers in New Zealand examine the udders of ewes at weaning and/or pre-mating. A recent longitudinal cross-sectional study by Griffiths *et al.* (2019a) assessed ewes' udders during late pregnancy, mid-lactation, weaning, and pre-mating. However, New Zealand-based studies examining udders in early lactation are limited and these included only a small number of ewes and the only focus was a condition described as hard udder (Bruce *et al.*, 2013; Skyrme, 1970). Further investigation of udder defects in early lactation could provide further valuable information to explain the pathological process and bacterial flora associated with this defect and the process of progression over time.

Udder halves with a defect observed at least once in six-weekly examinations produced 57.9% (range 27.5 to 99.8%) less milk than udder halves categorised as normal over the entire period. Further, normal udder halves yielded at least twice as much milk compared with udder halves initially categorised as hard, but which changed to lump or to lump and then normal over the six-week period (i.e., hard-lump or hard-lump-normal). The loss of milk yield in these udder halves was much higher than those categorised as either hard or lump alone. Additionally, udder halves with a higher udder-half defect detection frequency (i.e., defect detected on more than three out of six examinations) produced up to 88.5% less milk than the normal udders, while udder halves detected with a defect in the first week of lactation produced 65.1% less milk than a normal udder-half. Based on these results, it is apparent that sequential occurrence of both hard and lump (e.g., hard-lump) early in the lactation period, and/or persistency of defects over time, resulted in a greater loss of udder-half milk yield. There are no other studies available that report the effect of palpable udder defects on milk yield. However, the occurrence of sub-clinical mastitis in early lactation has been associated with a significant loss of milk yield in ewes (Leitner *et al.*, 2004; Marti De Olives *et al.*, 2013; Pulina *et al.*, 1993).

In the present study, normal udder halves contralateral to a defective udder half (i.e., described as normal (defective)) produced higher milk yield than normal udder halves with a contralateral normal (i.e., described as normal (normal)). This is most likely due to a compensatory increase in milk production by the normal udder halves in response to the decrease by the contralateral defective. As a result, whole-udder milk yield presented no difference between defective and normal udders, except only in the case of whole-udders detected with hard that changed to lump and persisted (hard–lump). Marti De Olives *et al.* (2013) quantified a 15-17% loss of whole-udder milk yield in udders detected with unilateral sub-clinical mastitis compared with normal udders. Hence, while there is a level of compensation by the normal udder-half, it may not be enough to compensate fully for reduced production in the impaired udder, which would impact on total lactation yield and lamb growth and survival as was demonstrated in a large field study (Griffiths *et al.*, 2019a). In ewes with an udder-half defect or no milk expression from one udder-half, twin lambs would likely be most affected due to competition to access adequate milk yield compared with singletons. Even in a fully functional udder, the milk production of twin-rearing ewes is not twice that of a ewe rearing a singleton (Peterson *et al.*, 2006). This situation would likely exacerbate the effect of udder defects on twin lamb survival and weight gain, even if there was no difference in whole-udder milk yield between ewes with normal or defective whole-udders.

Udder halves that expressed very little or no milk during a milking event were excluded from somatic cell count (SCC) and milk composition analysis due to insufficient samples for laboratory testing. Hence, inference of SCC and milk composition parameters should be made in consideration of these limitations. The SCC cut-off point to indicate mammary infection in ewes has been reported to range from 300,000 to 1,000,000 (Murphy *et al.*, 2018), indicating a lack of consensus (Zafalon *et al.*, 2016). The present study found milk SCC was not different between defective and normal categories, as some individual udder halves in both categories were observed to have high SCC (i.e., more than one million). High SCC in the normal udders in the present study could be due to subclinical mastitis, while udder halves with lump (assumed to be abscesses) may change in bacterial phenotypic diversity in the mammary gland (Cheng *et al.*, 2011; Smith *et al.*, 2015), which may result in variation in SCC.

In the current study, only milk solids non-fat (SNF) had any association with udder-half defects while there were no associations with crude protein, lactose, and total solids. There appears to be no available literature on the effects of palpable udder defects on milk composition in ewes. In a review, Martí-De Olives *et al.* (2020) reported that the concentration of lactose decreases, and whey protein increases with subclinical mastitis while other milk components have been inconsistently influenced (decrease, increase, or no difference) by subclinical mastitis in ewes. Changes in fat and protein (casein) percentages depend on the extent of milk yield loss, due to the dilution or concentration effect. In this study, defective udder halves produced less milk than normal udder halves and the resulting concentration effect on milk composition may explain the lack of variation between defective and normal udder halves (Burriel, 1997). In addition, the total crude protein depends on the casein:protein ratio, which usually decreases as a result of infection (Leitner *et al.*, 2011; Marti De Olives *et al.*, 2013; Rovai *et al.*, 2014). However, whey proteins increase during mammary inflammation or damage to the blood milk-barrier, which is likely to leak from blood to milk (Leitner *et al.*, 2011; Marti De Olives *et al.*, 2013). Moreover, variation in milk composition due to mastitis depends on the type of infectious agents (Burriel, 1997; Malek dos Reis *et al.*, 2013). Generally, previous studies have summarized that quantitative milk production is a better indicator of early lamb growth than milk components; however, milk composition may explain a reasonable proportion (Danso *et al.*, 2016; Geenty, 1979).

In conclusion, the results of this study showed a high occurrence of palpable udder defects in early lactation in non-dairy Romney ewes with a previous history of udder defects. Further, palpable udder defects were associated with both udder-half and whole-udder changes in milk yield, in which the degree of milk loss depended on the type and persistence of the udder defects. Therefore, these findings indicate the importance of udder health in non-dairy ewes and the potential effect on their offspring. However, milk SCC and composition parameters, except SNF, presented no association with palpable udder defects. A high proportion of udder halves were hard seven days after lambing and expressed little to no milk but then changed to lump or normal over the following five weeks. The cause and pathogenesis of this defect is worthy of further consideration.

Chapter 5: Assessment of Changes in Udder Half Defects Over Time in Non-Dairy Ewes

5.1 Abstract

AIM: To assess the changes in udder half defect status (hard, lump or normal) over time and to predict the risk of future udder half defect occurrence.

METHODS: A total of 1039 non-dairy breed (Romney) ewes were enrolled in two studies. In the first study, udder halves of 991ewes were assessed utilizing a standardized udder palpation method and scored four times a year, for two successive years (pre-mating, pre-lambing, docking, and weaning). The second study assessed the udder halves at pre-mating, and at six weekly intervals in the first six weeks of lactation in 46 ewes that had defective and normal udder halves. Udder half defect change over time was visualized via lasagna plots, and multinomial logistic regression was used to predict the risk or probability of udder half defect occurrence.

RESULTS: In the first study, the highest occurrence of udder halves categorised as hard was observed at either pre-mating or docking. Udder halves categorised as lump had their highest occurrence at either docking or weaning. Udder halves detected with a defect (hard or lump) at pre-mating were more likely (RRR: 6.8 to 1444) to be defective (hard or lump) at future examinations (pre-lambing, docking, or weaning) within the same year or pre-mating the following year, compared with udder halves categorised as normal. In the second study, change of udder half defect type over the first six weeks of lactation was variable. However, it was observed that the udder half defects, particularly udder halves categorised as hard, decreased during lactation. Failure to express milk in udder halves in early lactation was associated with a higher occurrence and persistency of udder half defects.

CONCLUSION: In conclusion, the occurrence of diffuse hardness or lumps in an udder half changed over time, and the risk of future occurrence of a defect was higher in udder halves previously categorised as either hard or lump. Hence, it is recommended that farmers identify and cull ewes with ewes with udder halves categorised as hard and lump.

5.2 Introduction

Poor udder health can have serious impacts on the welfare of suckling lambs and reduce the longevity of ewes within the flock (Farrell *et al.*, 2019; Stafford, 2013). In non-dairy ewes, defective udders are associated with a lower lamb survival and decreased lamb live weight gain during the pre-weaning period (Griffiths *et al.*, 2019a; Griffiths *et al.*, 2019b). Udder defects are abnormalities of the udder, the causes of which can range from non-mechanical injury to infectious agents (Hayman *et al.*, 1955; Madel, 1981). Several terminologies have been used to describe defective udders such as “impaired udders”, “imperfect udder”, “unsound udder”, “faulty udder”, “udder with abnormality” and “defective udder” (Griffiths *et al.*, 2019a; Hayman *et al.*, 1955; Quinlivan, 1968b; Saratsis *et al.*, 1998). Udder defects include abnormal secretion, inflammation, nodules or mammary masses, diffuse hardness, cysts, fibrosis, teat occlusion and teat canal cord formation, lumps and ruptured abscesses on the udder (Cooper *et al.*, 2013; Gelasakis *et al.*, 2015; Grant *et al.*, 2016; Griffiths *et al.*, 2019a; Mørk *et al.*, 2007; Quinlivan, 1968a; Saratsis *et al.*, 1998; Smith *et al.*, 2015).

Clinically, ewe udder defects can be detected by physical palpation and on occasion visually (Blagitz *et al.*, 2014; Saratsis *et al.*, 1998). Standardized udder assessment is a useful tool to identify defective udders of ewes to provide timely selective treatment or make culling decisions (Bergonier & Berthelot, 2003; Grant *et al.*, 2016). Udder defect assessment in non-dairy ewes usually takes place either at weaning or prior to mating when farmers are selecting ewes for the next breeding season (Saratsis *et al.*, 1998). More than 75% of New Zealand farmers reportedly assess their ewes' udders at least once a year, which is assumed for the purpose of making culling decisions (Corner-Thomas *et al.*, 2016). Ridler *et al.* (2021) recommended that ewe udders are examined at least four to six weeks post-weaning because issues not identified at weaning can be more easily identified.

Palpable udder defects such as hardness within the udder and various sized lumps have been reported during pregnancy, lactation, and/or the dry period in ewes (Grant *et al.*, 2016) . Hard udder has been described as diffuse hard consistency of the udder upon palpation (Griffiths *et al.*, 2019a; Saratsis *et al.*, 1998). Udder lumps (abscesses) have been described by several authors and have been reported to show phenotypic diversity in size, consistency, and location upon clinical and post-mortem examination of udders (Griffiths *et al.*, 2019a; Ridler *et al.*, 2021; Saratsis *et al.*, 1998; Smith *et al.*, 2015; Yusuf *et al.*, 2018).

Repeated udder examinations of the same flock of ewes within a season in New Zealand (Griffiths *et al.*, 2019a) and across two seasons in the United Kingdom (Grant *et al.*, 2016) both showed phenotypic diversity and a varying percentage of udder defects on consecutive examinations. Ridler *et al.* (2021) also reported change in udder defect across two visits, where the first visit was on the day of weaning followed by the second visit four to six weeks' later. In that study, from those ewes with hard udders in the first visit, they changed to normal, lump or remained hard while there was a small number of normal udder halves at the first visit that were found to have defects at the second visit. Bruce *et al.* (2013) reported that "hard udder" typically occurred shortly after lambing and then appeared to regress, with involution of the gland occurring within 3-4 weeks of initial lesion detection. In addition, Peterson *et al.* (2017) reported hard udders identified at palpation three weeks post-weaning had changed to normal palpation scores several months after weaning. Smith *et al.* (2015) hypothesized that the phenotypic diversity of palpable udder lumps (abscesses) could be due to the maturation cycle of the abscesses. Combined these studies imply that udder defects can change over time. Further, Grant *et al.* (2016) reported that ewes that were identified with palpable udder defects were three to five times more likely to have udder defects identified in the future.

Understanding the dynamic nature of palpable udder defects over time could provide important information regarding when higher rates occur and the best possible time for intervention. Additionally, such studies could provide reliable information regarding the appropriate culling of affected ewes, by predicting the likelihood of recurrence within the same or the following year/season. Therefore, the objectives of this chapter were: to assess the changes in udder half defect status over time, both over two full years and during a single lactation; and to predict risk of future udder half defects occurrence.

5.3 Materials and Methods

This Chapter utilized data from two studies. Study A was undertaken under commercial conditions and utilised longitudinal data gathered over a two-year period from 2017 to 2018 at Massey University's Riverside farm, Wairarapa, New Zealand (Chapter 3). Study C used data from 48 ewes with a history of either udder defects or normal udders who were examined and milked once a week for the first six weeks of lactation in 2019 (Chapter 4).

5.3.1 Study A: Ewe selection and management

The study comprised the entire cohort of 2013 (n=590) and 2014-born (n=391) replacement ewes at Massey University Riverside farm (n=981 on February17th, 2017 at pre-mating). All ewes had lambed at least once prior to joining the study. Ewes were ear tagged with visual (VID; Allflex, Palmerston North, New Zealand) and electronic (EID; Allflex, Palmerston North, New Zealand) identification tags. The ewes were managed as outlined in Chapter 3. Ewes were culled based on routine farm practices such as feet and teeth; however, ewes were not culled based on udder health. Non-pregnant ewes were also culled in each year. In addition, throughout the study, ewes that required euthanasia on welfare grounds were euthanised.

5.3.2 Study C: Ewe selection and management

In December 2018, 80 ewes aged 5-6 years were obtained from Massey University's Riverside farm, Wairarapa, New Zealand, based on udder traits evaluated during Study A. Ewe selection was based either on the presence of one or more udder defects (hard and/or lump) (Griffiths *et al.*, 2019a) on selection day, or a prior history of udder defects, with a focus on ewes with defects during examinations nearer to the selection date. An additional group of 20 'normal' ewes were selected based on an absence of udder defects on selection day and no recorded defects during the 12 udder examinations in the previous three years. Selection of the ewes took place at weaning day, at which time the udder and teats of all ewes were scored and the results were recorded. The ewes were transported to Massey University's Keeble farm (Palmerston North, New Zealand) in January 2019 and managed as outlined in Chapter 3. Forty-eight ewes were retained for lambing and during the lambing period, a twice-daily lambing beat was undertaken (at approximately 8AM and 4PM daily). New-born lambs were weighed and electronically tagged, their sex was determined, and they were matched to their dams. The lambing period occurred from 13th of August to 9th of September 2019.

5.4 Body Condition Scoring (BCS) and Live Weight

Body condition scoring and live weight measurement of each ewe was undertaken using the methods outlined in Chapter 3, on the same days that ewes' udders were examined.

5.5 Udder Scoring

Ewe udders were assessed by trained and experienced assessors with ewes restrained by experienced sheep handlers in a sitting position. Udder halves were scored from 1-7 using the system described by Griffiths *et al.* (2019a) (Chapter 3); however the scores were then collapsed down to three categories: normal (scores 1 and 2); hard (score 7); and lump (scores 3-6).

In Study A, udders were assessed four times per year for two consecutive years (2017 – 2018) at pre-mating (February), pre-lambing (September), docking (3-8 weeks after lambing, November), and weaning (December). These time points were selected as they represent key management events when farmers traditionally interact with individual ewes. Four trained operators undertook the udder palpations in Study A. In Study C, udders were assessed at pre-mating and six times weekly in the first six weeks of lactation beginning from 4-10 days after parturition. In Study C a single operator undertook all udder palpations to avoid potential inter-operator variation.

5.6 Data Analysis

In Study A all 981 ewes (1962 udder halves) in 2017, 769 ewes (1538 udder halves) in 2018 and 704 ewes (1408 udder halves) in both years were included in the analysis. While in Study C, 48 ewes (92 udder halves) were assessed weekly in the first six weeks of lactation. However, only udder halves with complete data for all scoring events within a plot were considered for the lasagna plots.

5.6.1 Descriptive analysis - Lasagna plots

To analyse the dynamic nature of udder defects the lasagna plot method (Jones *et al.*, 2014; Swihart *et al.*, 2010) was identified as the most suitable option. Lasagna plots allow visualization of the changes in defect category (hard, lump, or normal) of udder halves over time (for the different udder scoring events). Within the plots, each bar shows a different udder scoring event (i.e. time) while the different colours within each bar represent the defect categories. The data in the table at the top of the plot designates the percentage of each udder half defect category at each event, which corresponds to the percentage of each colour at each event. Change in defect category over time of each udder half can be tracked by following longitudinal transitions across the udder scoring events of stacked bars. A change in the colour of each udder half category at a different time event represents a change in udder-half category. The lasagna plots were created using SAS Statistical software Version 9.4 (SAS Institute Inc., Cary, NC) utilizing the methodology of Jones *et al.* (2014). Three lasagna plots were created using udder half data from Study A: i) four udder examination events in 2017 comprising 1860 udder halves (930 ewes), ii) four udder examination events in 2018 comprising 1428 udder halves (714 ewes), and iii) all eight udder examination events across 2017 and 2018 comprising 1408 udder halves (704 ewes).

Four lasagna plots were created for Study C, i) pre-mating and six udder examination events during their single lactation which comprised data from udder halves from which milk was expressed (43 udder halves), ii) pre-mating and six udder examination events during lactation which comprised data from udder halves from which milk was not expressed (37 udder halves), iii) pre-mating and six udder examination events during their single lactation which comprised data from udder halves from which had previous history of udder half defect (58 udder halves), iv) pre-mating and six udder examination events during lactation which comprised data from udder halves from which milk had no previous history of udder half defect (22 udder halves). Udder halves that did not express milk were defined as those udder halves from which no milk was expressed from at least four of the six milking events and expressed less than 100g/day in the rest, otherwise an udder half was considered as expressed milk.

5.6.2 Multinomial logistic regression

Study A. A series of multinomial logistic regression models were developed utilising data from 1962 udder halves in 2017 and 1562 udder halves in 2018 to predict the relative risk ratio of udder half defect status (hard, lump, or normal) at pre-lambing, docking and weaning for the 2017 year and the 2018 year, based on the udder half defect status at pre-mating of each respective year being analysed. Two models were fitted from data of the 1426 udder halves that were measured across both years to predict the relative risk ratio of the udder half defect status at pre-mating in 2018 based on pre-mating and weaning data in 2017. Birth rank (singleton [1] or twin [2]), rearing rank (no lambs reared [0], reared a single lamb [1] or reared twin lambs [2]), udder half (right/left), age of ewes (2013 or 2014 year of birth) and ewe BCS and bodyweight at premating were included as covariates. For all the models developed, the probability of each udder half defect category at each udder scoring event was extracted.

Study C. Several multinomial logistic regression models were fitted to assess weekly transitional probability using data from 48 ewes during the first six weeks of lactation, conditionally on the preceding udder palpation event (e.g. Day 7 udder defect status was used to predict Day 14 udder defect status, Day 14 for Day 21, and so forth for the six examinations during lactation). Birth rank (singleton [1] or twin [2]), rearing rank (no lambs reared [0], reared a single lamb [1] or reared twin lambs [2]), udder half (right/left) and udder half milk expression status (yes/no) were included as covariates. A separate analysis, using the same model structure, was undertaken to predict the probability of each udder half defect category in the six examination events during lactation (Day 7 to Day 42) based on pre-mating udder half defect status of the ewes at mating in 2019.

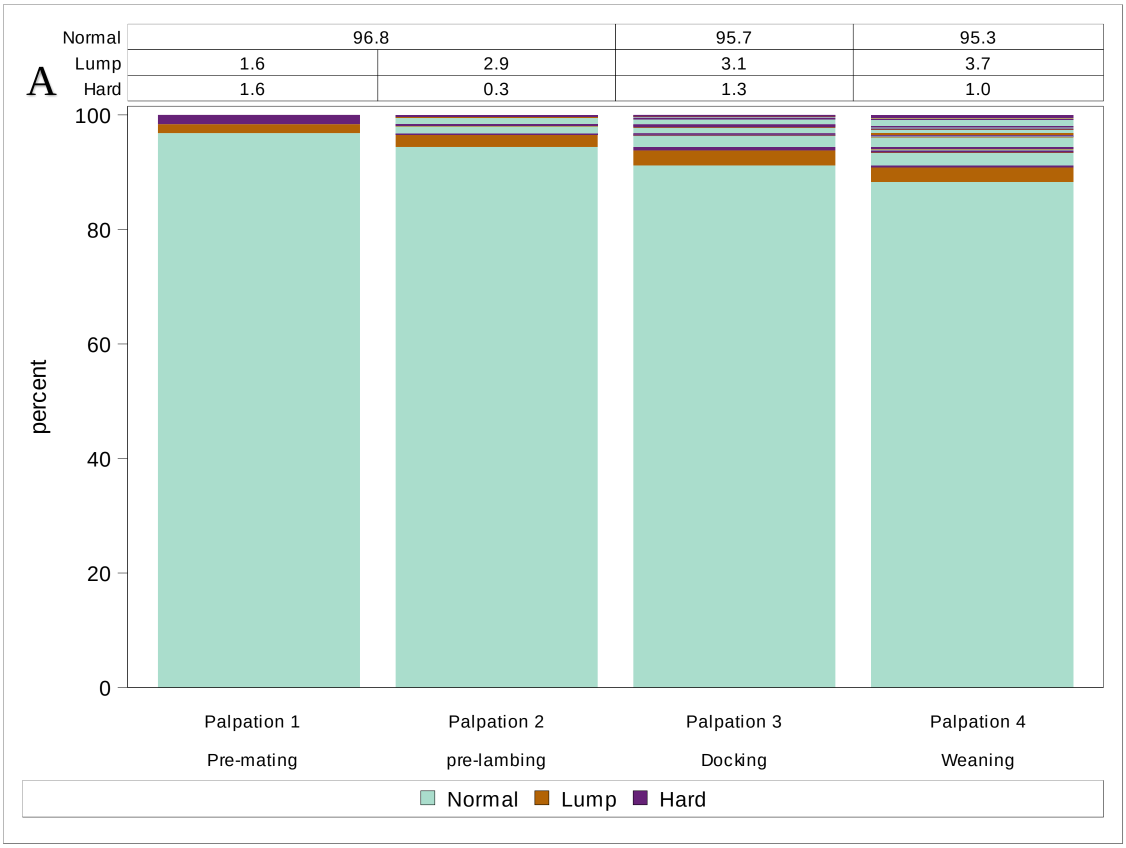
All analyses were performed using a multinomial logistic regression approach with *'multinom'* function from *'nnet'* package in R statistical software version 3.6.3 (R Core Team, 2020). Interactions between variables were tested. The model development process was based on the backward selection of the independent variables mentioned. Goodness of fit of the models was tested for significance by likelihood ratio test (also called model chi square). Variance inflation factor was applied to assess the multicollinearity while the goodness of fit was assessed by McFadden's pseudoR^2.

5.7 Results

5.7.1 Study A: Lasagna plots of udder half defects in 2017 and 2018

Overall, greater than 94.8% of udder halves were normal at each individual udder examination event, although new udder half defects were detected at each additional examination event from those udder halves previously categorised as normal (Figure 5.1). Amongst udder halves that had defects (hard or lump) or those that developed defects, the lasagna plots demonstrate that defects either remained the same over time, changed to normal or changed to another defective category i.e., all possible changes occurred although only a relatively small proportion of ewes had or developed defects. Only six (0.3%) udder halves in 2017 and 16 (1.0%) udder halves in 2018 stayed defective (remain in the same category or changed to another defective category) at all udder examination events within each year. However, none of the udder halves remained defective in all eight udder examinations throughout both years.

In both years, the percentage of udder halves categorised as hard was higher at the pre-mating examination compared with pre-lambing (late-pregnancy). The percentage increased again at docking before slightly decreasing at weaning (Figure 5.1A, B). Udder halves categorised as lump were more commonly identified during lactation (i.e. identified at docking and weaning) compared to during the non-lactation periods (i.e. identified at pre-mating and pre-lambing; Figure 5.1A, B). Comparison of the same examination periods across years showed that the percentage of udder halves that were defective was higher at pre-mating (0.4%) and docking (0.9%) in 2018 compared to 2017 (Figure 5.1C). From eight udder examination events over the two year period, the highest percentage of udder half defects was observed at docking 2018.


A
Normal 96.8 95.7 95.3
Lump 1.6 2.9 3.1 3.7
Hard 1.6 0.3 1.3 1.0
percent
100
80
60
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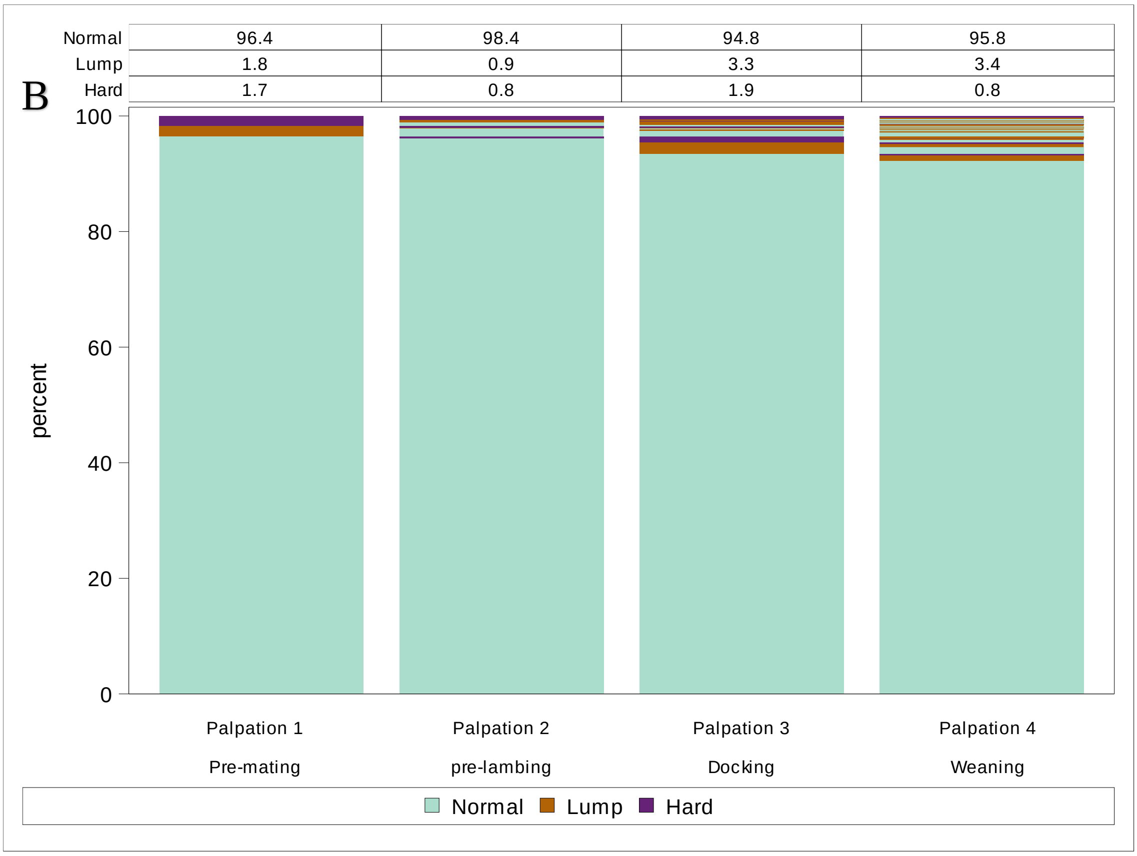
Palpation 1
Pre-mating
Palpation 2
pre-lambing
Palpation 3
Docking
Palpation 4
Weaning
Normal
Lump
Hard


B
Normal 96.4 98.4 94.8 95.8
Lump 1.8 0.9 3.3 3.4
Hard 1.7 0.8 1.9 0.8
percent
100
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Palpation 1
Pre-mating
Palpation 2
pre-lambing
Palpation 3
Docking
Palpation 4
Weaning
Normal
Lump
Hard

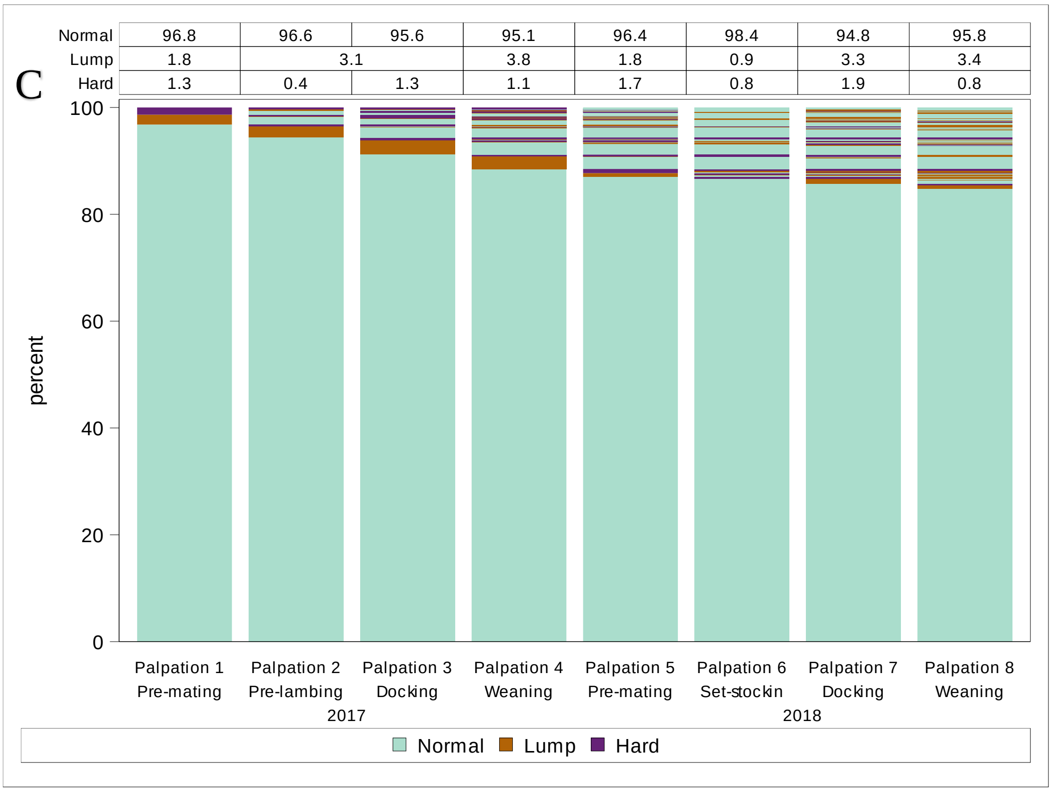


Figure 5.1 Lasagna plot of palpable udder half defects (hard, lump and normal) from non-dairy Romney ewes identified during udder examination events occurring at key management times (pre-mating, pre-lambing, docking, and weaning) from 1860 udder halves (930 ewes) in year 2017 (A); 1428 udder halves (714 ewes) in year 2018 (B); and 1408 udder halves (704 ewes) from 2017 and 2018 combined (C). Note: Within each plot, each bar shows a different udder half scoring event (i.e. time) while the different colours within each bar represent the defect categories. The data in the table at the top of the plot designates the percentage (%) of each udder half defect category at each event, which corresponds to the percentage of each colour at each event. Change in defect category over time of each udder half can be tracked by following longitudinal transitions across the udder scoring events of stacked bars.

5.7.2 Study A: Relative risk ratios of udder half defects at pre-lambing, docking and weaning in 2017 and 2018 based on pre-mating status

If an udder half was categorised as hard at pre-mating, it was more likely to be categorised as hard at pre-lambing, docking, and weaning in both 2017 (Table 5.1) and 2018 (Table 5.2) compared to an udder half that was categorised as normal at pre-mating. The relative risk ratios (RRR) ranged from 14 to 1465 ($p<0.05$), when other variables in the model were held constant. If an udder half was hard at pre-mating (within each respective year), the relative risk of it being categorised as lump at the following pre-lambing in 2017 and pre-lambing, docking and weaning in 2018 was higher (RRR range 14 to1465 times) compared to an udder half that was categorised as normal at pre-mating while keeping all other variables constant.

Udder halves categorised as lump at pre-mating (within each year) were more likely ($p<0.05$) to be categorised as hard at pre-lambing, docking, and at weaning in 2017 and at pre-lambing and docking in 2018, compared with udder halves that had been categorised as normal at pre-mating, when other variables were held constant in the model (RRR range 6 to 67, Tables 5.1 and 5.2). In addition, udder halves categorised as lump at pre-mating were more likely to be categorised as lump at pre-lambing, docking, and weaning in both 2017 and 2018, compared to udder halves that had been categorised as normal at pre-mating, when other variables held constant in the model (RRR range from 6 to 333, Tables 5.1 and 5.2).

Among the covariates considered for models examining udder half defects across key management times, rearing rank was the only covariate that significantly ($p<0.05$) influenced udder half defects and only at docking in both years. A rearing rank of zero (no lambs suckling) was associated with a higher relative risk of an udder half being categorised as lump at docking in 2017 and both hard or lump at docking in 2018, compared with the reference rank of one lamb suckling. In both 2017 and 2018, ewes that reared twins were less likely to have udder halves categorised as hard (OR=0.2, $p<0.05$) compared to the reference of ewes that reared single lambs (Table 5.1 and 5.2).

Table 5.1 Relative risk ratio (RRR ± standard error) of udder half defect category (normal, hard or lump) at pre-lambing, docking, and weaning based on pre-mating udder half defect category from 1962 udder halves (from 981 non-dairy Romney ewes) in 2017.

Predictor variables	Category	Udder half defect (2017)	
		Hard	Lump
		Pre-lambing	
Pre-mating udder half defect	Normal (Reference)	-	-
	Hard	52.9 (0.9)	13.8 (0.4)
	Lump	26.5 (1.2)	6.9 (0.6)
Constant		0.002 (0.6)	0.002 (0.1)
		Docking	
Pre-mating udder half defect	Normal (Reference)	-	-
	Hard	14.1 (0.6)	1.01^{NS} (1.0)
	Lump	42.0 (0.55)	5.5 (0.65)
Rearing rank	Single (Reference)	-	-
	No lambs	1.8^{NS} (0.6)	3.6 (0.5)
	Twins	0.2 (0.65)	1.7^{NS} (0.3)
	Triplets	3.0 ^{NS} (0.8)	1.9 ^{NS} (0.8)
Constant		0.01 (0.3)	0.02 (0.3)
		Weaning	
Pre-mating udder half defect	Normal (Reference)	-	-
	Hard	43.3 (0.6)	3.3^{NS} (0.6)
	Lump	26.0 (0.7)	6.7 (0.5)
Constant		0.01 (0.3)	0.04 (0.1)

Note: All relative risk ratios were significant ($p<0.05$), except those designated as ^{NS} (not significant, $p>0.05$).

Table 5.2 Relative risk ratio (RRR ± standard error) of udder half defect category (normal, hard or lump) at pre-lambing, docking, and weaning based on pre-mating udder half defect category from 1538 udder halves (from 769 non-dairy Romney ewes) in 2018.

Predictor variables		Udder half defect (2018)	
		Hard	Lump
	Pre-lambing		
Pre-mating udder half defect	Normal (Reference)	-	-
	Hard	1465 (1.1)	1462 (1.0)
	Lump	66.6 (1.4)	333 (1.1)
Constant		0.001 (1.00)	0.001 (0.9)
	Docking		
Pre-mating udder half defect	Normal (Reference)	-	-
	Hard	292.5 (0.7)	169.3 (0.6)
	Lump	6.2 (0.8)	16.7 (0.5)
Rearing rank	Single (Reference)		
	No lambs	8.3 (0.8)	7.4 (0.5)
	Twins	0.2 (0.84)	0.7^{NS} (0.5)
	Triplets	0.9^{NS} (1.2)	1.3^{NS} (0.9)
Constant		0.009	0.01 (0.4)
	Weaning		
Pre-mating udder half defect	Normal (Reference)	-	-
	Hard	84.7 (0.7)	76.0 (0.5)
	Lump	9.4 (1.1)	20.8 (0.5)
Constant		0.006 (0.3)	0.02 (0.19)

Note: All relative risk ratios (RRR) were significant ($p<0.05$), except those designated as NS (not significant, $p>0.05$)

5.7.3 Study A: Predicted probabilities of udder half defects at pre-lambing, docking and weaning in 2017 and 2018 based on pre-mating status

For udder halves categorised as normal at pre-mating, the vast majority remained normal at pre-lambing, docking and at weaning across both years (predicted probability (Pp) of 0.96-0.99, Table 5.3). In both years, the probability was highest at pre-lambing followed by docking and then weaning. For udder halves that were categorised as normal at pre-mating, the Pp that they would subsequently change to hard or lump was very low in both years (i.e. less than 0.036, Table 5.3).

For those udder halves that were categorised as hard at pre-mating, the Pp of remaining hard or changing to hard was variable between years and time points; however, it was higher in 2018 than 2017. In contrast, the Pp of these udder halves being categorised as normal ranged from 0.73 to 0.83 in 2017 but was much lower in 2018 (Table 5.3).

For those udder halves that were categorised as lump at pre-mating, the Pp of changing to normal was lowest at docking in both 2017 and 2018 (Table 5.3). The Pp of udder halves categorised as lump changing to hard was higher in 2017 compared to 2018, but the highest probability was observed at docking in both years. The Pp of udder halves categorised as lump remaining as lump ranged from 0.069 to 0.296.

Table 5.3 Predicted probability (Pp) of udder half defect occurrence in non-dairy Romney ewes at pre-lambing, docking, and weaning based on pre-mating udder half defect category from 1962 udder halves (981 ewes) in 2017 and 1538 udder halves (769 ewes) in 2018.

Year	Pre-mating udder half defect category	Change of udder half defect category	Pre-lambing	Docking	Weaning
2017	Normal	Normal to Normal	0.987	0.968	0.959
		Normal to Hard	0.002	0.012	0.005
		Normal to Lump	0.024	0.020	0.036
	Hard	Hard to Normal	0.821	0.834	0.727
		Hard to Hard	0.036	0.149	0.182
		Hard to Lump	0.143	0.017	0.091
	Lump	Lump to Normal	0. 797	0.608	0.714
		Lump to Hard	0.060	0.323	0.107
		Lump to Lump	0.143	0.069	0.179
2018	Normal	Normal to Normal	0.999	0.969	0.973
		Normal to Hard	0.001*	0.010	0.001*
		Normal to Lump	0.001*	0.020	0.021
	Hard	Hard to Normal	0.333	0.160	0.320
		Hard to Hard	0.333	0.440	0.160
		Hard to Lump	0.333	0.400	0.520
	Lump	Lump to Normal	0.786	0.630	0.703
		Lump to Hard	0.036	0.074	0.001*
		Lump to Lump	0.178	0.296	0.296

Note: * indicates probability of 0.001 or less. Predicted probability (Pp) of 0 indicates impossibility of the defect category to occur and 1 indicates certainty.

5.7.4 Study A: Relative risk ratio of udder half defects at pre-mating 2018 based on weaning and pre-mating 2017

If an udder half was categorised as defective (hard or lump) at pre-mating in 2017, the RRR of it still being categorised as defective (hard or lump) at pre-mating in 2018 was higher (RRR range 6.6 to 15.4, $p<0.05$) compared with udder halves categorised as normal at pre-mating in 2017 (Table 5.4). Keeping all other covariates constant, for udder halves categorised as hard at weaning in 2017, the RRR of being categorised as hard or as lump at pre-mating in 2018 was 141 and 53 times higher ($p<0.05$) respectively, compared with an udder half that was categorised as normal at weaning in 2017. If an udder half was categorised as lump at weaning in 2017, the RRR of it being categorised as hard or lump at pre-mating 2018 was 6 and 16 times higher (P<0.05) respectively, compared with an udder half that was categorised as normal at weaning in 2017 (Table 5.4).

Table 5.4 Predicted relative risk ratio (RRR±SE) of 2018 pre-mating udder half defect occurrence based on 2017 pre-mating and weaning udder half defect status from 1408 udder halves (from 704 non-dairy Romney ewes)

Predictor variables	Categories	Pre-mating udder half defect (2018)	
		Hard	Lump
2017 Pre-mating udder half defect	Normal (Reference)		
	Hard	15.4 (0.6)	7.6 (0.8)
	Lump	6.6 (0.8)	13.4 (0.6)
Pre-mating BCS		0.3 (0.4)	0.4 (0.4)
Ewe age (Year born)	2013 (Reference)		
	2014	1.3^{NS} (0.4)	4.7 (0.6)
Constant		0.4^{NS} (1.3)	0.1^{NS} (1.4)
2017 Weaning udder half defect	Normal (Reference)		
	Hard	141.2 (0.62)	53 (0.8)
	Lump	5.9 (0.5)	15.7 (0.5)
Constant		0.011 (0.2)	0.011 (0.2)

Note: All Relative Risk Ratios were significant (p<0.05), except those designated as NS (not significant)

5.7.5 Study C: Lasagna plots of udder half defects at pre-mating and day 7 to 42 of lactation in udder halves with or without a previous history of udder defect

From a total of 22 udder halves that had no history of udder half defects in the previous two years (2017 to 2018), only 9 (41%) udder halves were found to be normal throughout all examinations. In contrast, in udder halves that had a history of udder half defects, 19% exhibited no udder half defects throughout all udder examinations. Udder half defects appeared to be highly variable with multiple changes over time, at pre-mating and during the first six weeks of lactation (Figure 5.2), particularly for those udder halves that had a previous history of defect (Figure 5.2B). The percentage of udder half defects, and their persistence, appeared to be higher in udder halves that had a previous history of defect (Figure 5.2B) compared with those that had not (Figure 5.2A).

From a total of 22 udder halves that had no defect history, only one udder half (4.5%) was categorised as lump on Day 7 while the rest were categorised as normal (Figure 5.2A). The number of udder halves that had no defect history and were categorised as hard was highest on Day 14. All udder halves categorised as hard on Day 14 had changed to normal by Day 21 and this persisted until Day 42; however, two (9%) udder halves changed to lump on Day 35. Udder halves categorised as hard were seen only on Day 14. In udder halves with no defect history, only one new defect was observed at Day 35 and no new defect was observed in the following weeks.

From a total of 62 udder halves that had a previous history of defect, the percentage that were categorised as normal over six weeks of lactation appeared to be lower (45-71%, Figure 5.2B) compared with those that had no defect history (50-95%, Figure 4.2B). Fifty percent of udder halves that had a previous defect history were categorised as hard at Day 7 and over subsequent weeks, these udder halves changed category, such that no udder half remained consistently hard at all examinations. As the days in lactation increased, the percentage of udder halves categorised as hard decreased substantially. The percentage of udder halves that had a previous history of defect and were subsequently categorised as lump, was higher at Day 14 compared with Day 7. At subsequent examinations the percentage categorised as lump fluctuated over the weeks.

5.7.6 Study C: Lasagna plots of udder half defects at pre-mating and day 7 to 42 of lactation in udder halves that did and didn't express milk

Milk expression status (yes/no) had a significant ($p<0.05$) effect on udder half defect. Compared to normal udder halves, those udder halves that didn't express milk had higher RRR of being hard (OR=7.9-35.4) on days 14 and 21 and higher RRR of being lump (OR=6-26, $P<0.05$) on all six occasions during lactation compared to the normal udder halves. Except for one udder half, all udder halves that had no history of defect expressed milk whereas only 35% of udder halves that had a previous history of udder half defect expressed milk. Plots were made for udder halves that did express milk (Figure 4.3A) and those that didn't (Figure 5.3 B).

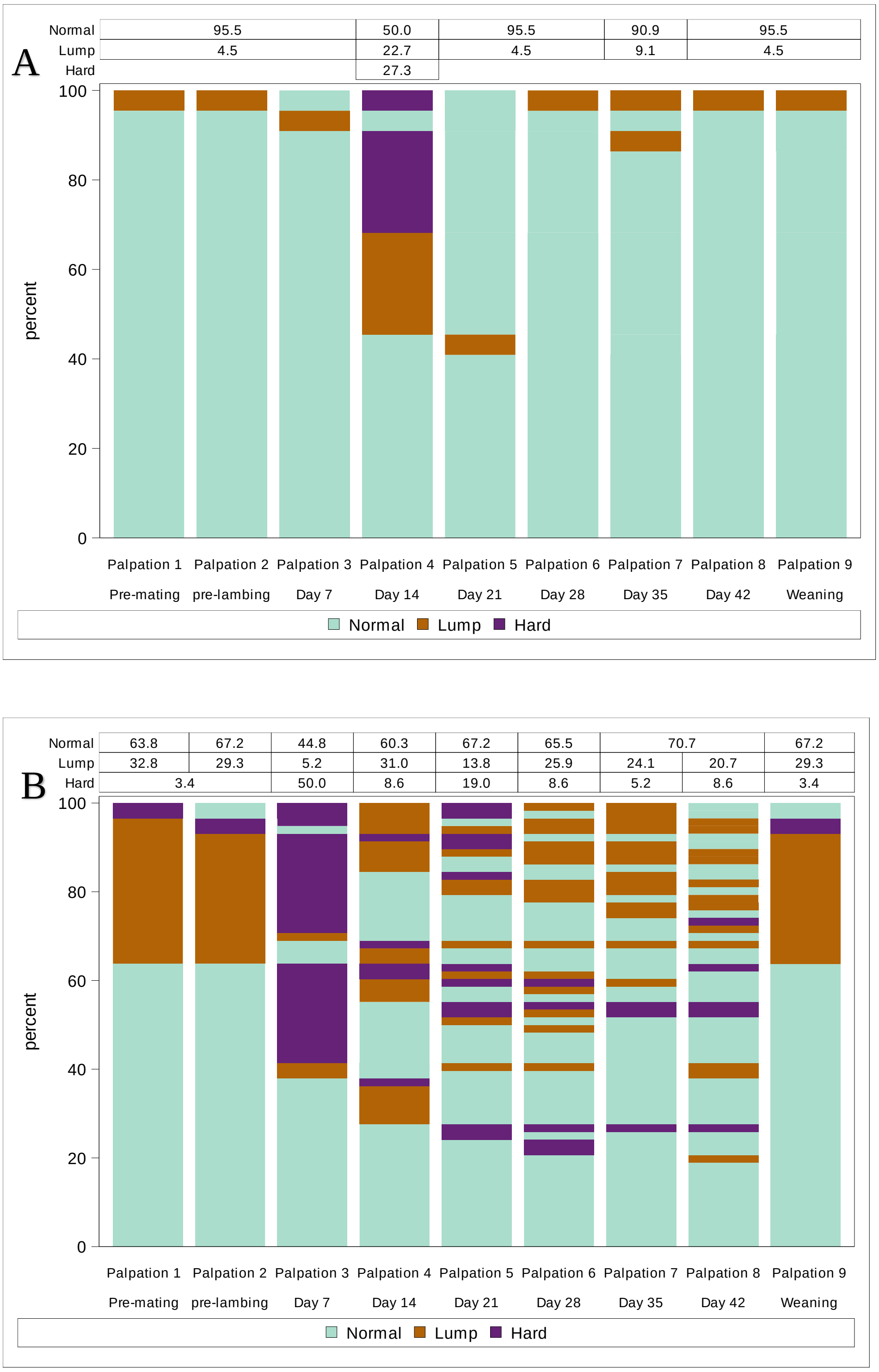
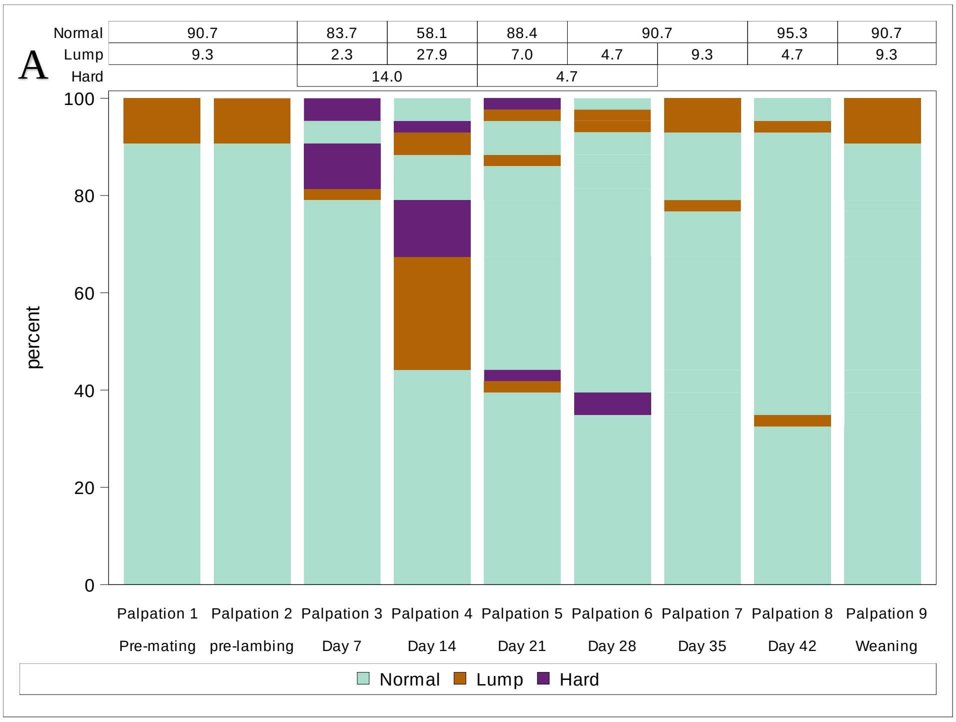


Figure 5.2 Lasagna plot of palpable udder half defects (hard, lump, and normal) in the first six weeks of lactation in non-dairy Romney ewes (Study C). Udder examination was undertaken at weekly intervals from 22 udder halves that had no history of defect in the previous two years (2017 and 2018); (A) and from 58 udder halves that had a history of defects (B). Note: Within each plot, each bar shows a different udder scoring event (i.e. time) while the different colours within each bar represent the defect categories. The data in the table at the top of the plot designates the percentage of each udder half defect category at each event, which corresponds to the percentage of each colour at each event. Change in defect category over time of each udder half can be tracked by following longitudinal transitions across the udder scoring events of stacked bars. Six ewes that missed the udder examination on Day 42 were excluded from the plot.



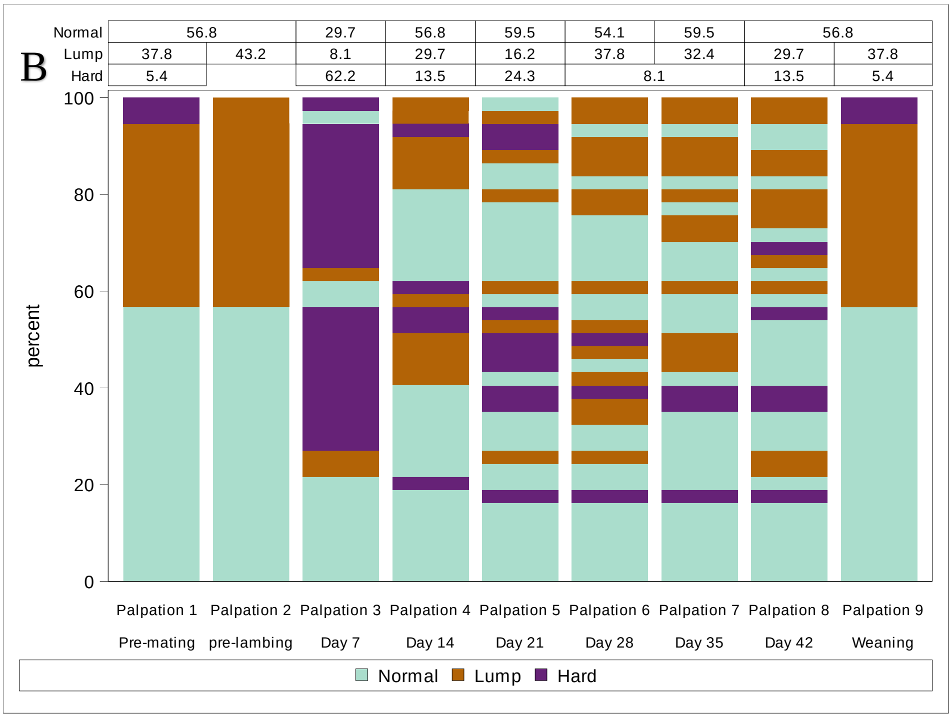


Figure 5.3 Lasagna plot of palpable udder half defects (hard, lump, and normal) in the first six weeks of lactation in non-dairy Romney ewes (Study C). Udder examination was undertaken at weekly intervals from 43 udder halves that did express milk (A) and from 37 udder halves that did not express milk (B). Note: Within each plot, each bar shows a different udder scoring event (i.e. time) while the different colours within each bar represent the defect categories. The data in the table at the top of the plot designates the percentage of each udder half defect category at each event, which corresponds to the percentage of each colour at each event. Change in defect category over time of each udder half can be tracked by following longitudinal transitions across the udder scoring events of stacked bars. Note that an udder half that did not express milk refers to an udder half which did not express milk at all in at least four occasions and expressed less than 100 g/day on the other two occasions.

5.7.7 Study C: Weekly transitional probability of udder half defects from day 7 to 42 of lactation

In the first six weeks post-lambing, weekly udder half defect category transitions (from day 7 to 14, from day 14 to 21 and so on) identified that all possible transitions across categories occurred (i.e., normal to normal; normal to hard or lump; hard or lump to normal; hard or lump to hard or lump, Figure 5.3a). The probability of weekly transition of udder halves categorised as normal remaining normal, increased as days in lactation advanced from Day 7 to 42. In contrast, the transitional probability of a normal udder half changing to an udder half categorised as either hard or lump declined (Figure 5.4A).

The weekly transitional probability of udder halves categorised as hard changing to normal was high (>0.8) during the first three weeks (Day 7 to 21) of lactation and remained steady until it declined from Day 28 (Figure 5.4B). The weekly transitional probability of an udder half categorised as hard that remained hard, increased as lactation advanced from Day 7 to 42. Whereas the probability of transition from hard to lump slightly decreased. The weekly transitional probability of udder halves categorised as lump transitioning to normal declined from day 7 to Day 35 but, then started to rise at Day 42 (Figure 5.4C). In contrast, the weekly transitional probability of udder halves categorised as lump remaining as lump increased from Day 7 to day 35 and then declined at day 42. The probability of an udder half categorised as lump transitioning to hard was variable among the udder scoring days, but it was generally low (<0.09).

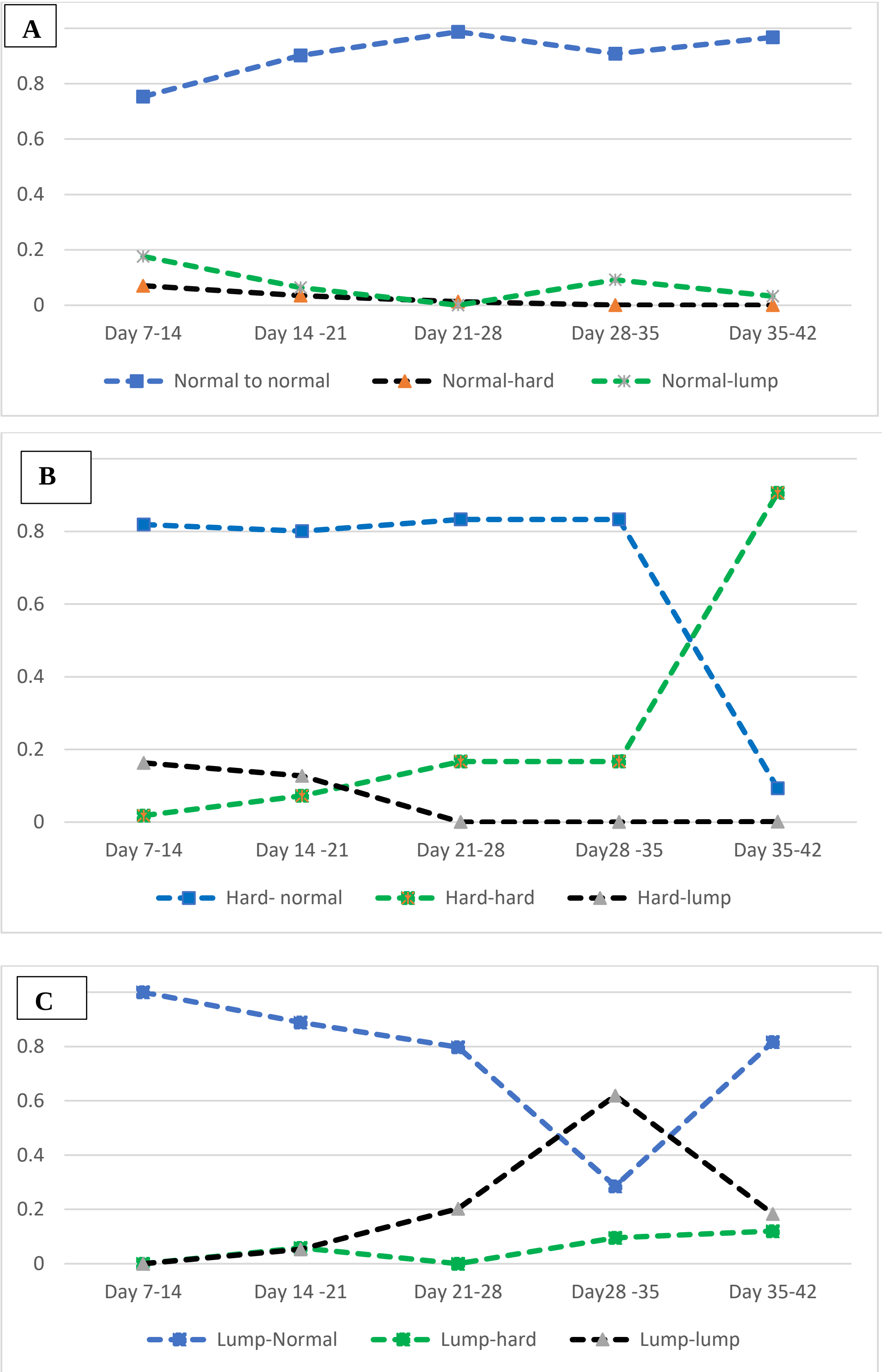


Figure 5.4 Line graph of transitional probability of udder half defects in the first six weeks of lactation from 92 udder halves (from 46 non-dairy Romney ewes) in 2019 (Study C). A) Transitional probability of udder half defect from preceding normal state; B) Transitional probability of udder half defect from preceding hard state; C) Transitional probability of udder half defect from preceding hard state. Probabilities were predicted conditionally on the preceding udder palpation event (e.g. Day 7 udder defect status was used to predict Day 14 udder defect status, Day 14 for Day 21, and so forth for the six examinations during lactation).

5.7.8 Study C: Predicted probability (Pp) of udder half defect from Day 7 to 42 in lactation based on pre-mating udder half defect

The predicted probability (Pp) of udder half defect in the first six weeks of lactation (Day 7 to Day 42) based on pre-mating udder half defect status is summarized in Table 5.5. For udder halves that were normal at pre-mating, the Pp of an udder half remaining normal was high (Pp range 0.674-0.986) at all six events. For those that were categorised as normal at pre-mating, the Pp of being categorised as hard was highest at Day 7, before it dropped close to zero for the next four weeks and then rose again on Day 42. Whereas the Pp of an udder half being categorised as lump was low at Day 7, peaked at Day 14 and then declined.

For udder halves that were categorised as hard at pre-mating, the Pp of being normal on Day 7 was very high, then declined. While the Pp of being categorised as hard declined over lactation. For those that were categorised as hard at pre-mating, the Pp of being categorised as lump was very low on Day 7 and was then higher in the subsequent weeks.

For udder halves categorised as lump at pre-mating, the Pp of being normal increased with days in lactation, until Day 28, before declining in the following two weeks. For those udder halves that were categorised as lump at pre-mating, the Pp of being categorised as hard was very high on Day 7 and then decreased from Day 14 while the Pp of being categorised as lump was variable over time but was highest on Day 42.

All three udder halves categorised as hard at pre-mating expressed no milk during the first 42 days in lactation, but due to low numbers this was not significant ($p<0.05$). Udder halves categorised as lump at pre-mating were four times more likely ($p<0.05$) not to express milk, compared to udder halves categorised as normal at pre-mating.

Table 5.5 Predicted probability (Pp) of udder half defects change over time from Days 7 – 42 of lactation based on pre-mating udder half defect category in 92 udder halves (from 46 non-dairy Romney ewes).

Udder half defect category		Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Pre-mating	Lactation						
Normal	Normal	0.864	0.674	0.904	0.986	0.961	0.883
	Hard	0.113	0. 107	0.037	0.001*	0.001*	0.067
	Lump	0.023	0.220	0.060	0.014	0.039	0.050
Hard[ǂ]	Normal	0.907	0.001*	0.674	0.297	0.001*	0.001*
	Hard	0.093	0.001*	0.001*	0.001*	0.001*	0.001*
	Lump	0.001*	0.999	0.326	0.999	0.999	0.999
Lump	Normal	0.574	0.708	0.804	0.923	0.616	0.500
	Hard	0.396	0.090	0.021	0.001*	0.001*	0.056
	Lump	0.030	0.201	0.175	0.077	0.384	0.444

Note: Predicted probability of each udder half defect category in the six examinations during lactation (Day 7 to Day 42) was undertaken based on pre-mating udder half defect status of the ewes. [ǂ] Only three udder halves were categized as hard at pre-mating. * indicates probability of 0.001 or less. Predicted probability (Pp) of 0 indicates impossibility of the defect category to occur and 1 indicates certainty.

5.6 Discussion

The objectives of the study were to assess the changes in udder half defects over time and determine risk of future occurrence of udder half defects in ewes. For this purpose, across two studies, a standardized udder assessment method was implemented at pre-mating, pre-lambing, docking and weaning for two years or weekly six times in the first six weeks in lactation. The assessment was undertaken at udder half level as each mammary gland is anatomically and/or physiologically independent. Lasagna plots were used to present the data as it enabled easy visualisation of changes in udder half category over time.

In Study A, which evaluated udder halves at eight time points over two years, most udder halves were categorised as normal while only a low percentage of udder halves were categorised as hard or lump, matching previous studies (Griffiths *et al.*, 2019a; Peterson *et al.*, 2017; Ridler *et al.*, 2021) when time specific evaluations have been undertaken. However, the relatively large number of ewes included in the study still meant that meaningful analysis could be undertaken. Most udder halves categorised as having defects (either hard or lump) changed in category over time, while a small percentage of new defective udder halves (i.e. from udder halves previously categorised as normal) were observed at each time point. This approach demonstrated the dynamic nature of udder half defects and helps explain why repeated examinations with the same flock, has previously resulted in different percentages of udder defects (Grant *et al.*, 2016; Griffiths *et al.*, 2019a).

The proportion of udder halves categorised as hard was higher at docking (Study A) or the first couple of weeks in lactation (Study C). A higher occurrence of hard udder or other udder defects shortly after lambing (Barbagianni *et al.*, 2017; Bruce *et al.*, 2013; Mavrogianni *et al.*, 2014; Skyrme, 1970) could be due to the peri-parturient relaxation of immunity which makes the udder susceptible to infection (Coop & Kyriazakis, 1999; Ezzat Alnakip *et al.*, 2014; Mizuno *et al.*, 2012). The occurrence of udder halves categorised as hard was also higher at pre-mating. The post-lactation involution period has been associated with mammary gland susceptibility, increased risk of infection or advancement of subclinical infection to clinical because of compromised mammary defences in this period (Fthenakis & Jones, 1990b; Petridis *et al.*, 2012). It can also be related to the occurrence of post-weaning chronic mastitis (Quinlivan, 1968b).

Udder halves categorised as lump were more commonly identified during lactation (i.e. identified at docking and weaning) compared to during the non-lactating period (i.e. identified at pre-mating and pre-lambing). This agrees with a Grant *et al.* (2016) who reported higher palpable udder defect occurrence in lactation compared to pregnancy. Lumps or intramammary abscesses usually occur following an intramammary infection (IMI) (Smith *et al.*, 2015), which, in the case of this study, is likely to be first observed after early post-lambing infection or complication. Higher occurrence of lumps at weaning might be associated with physiological changes in early cessation of lactation and initiation of involution that compromise mammary gland immunity, particularly in those that didn't express milk (Petridis & Fthenakis, 2014; Petridis *et al.*, 2012).

Utilizing an objective udder assessment method and making rational culling decisions is important to improve the health and welfare of pre-weaned lambs (Blagitz *et al.*, 2014; Griffiths *et al.*, 2019b). This can optimally be achieved by predicting the occurrence of udder defects in the next breeding season based on udder examination prior to mating at the time of ewes' selection. Pre-mating udder examination has been suggested to be an appropriate time to identify ewes that are likely to be unsuitable for retaining in the breeding flock (Griffiths *et al.*, 2019a) as ewes with udder halves categorised as hard or lump at pre-mating were associated with lower lamb survival and body weight gain in pre-weaned lambs (Flay *et al.*, 2020; Griffiths *et al.*, 2019a; Griffiths *et al.*, 2019b). In this study, an udder half categorised as hard or lump at pre-mating was more likely to remain as hard or change to lump compared with the normal udder halves in at least one of the key management times in the same year (pre-lambing, docking, and weaning). The findings of this study support the practice of farmers culling ewes identified with defects at weaning or premating (Corner-Thomas *et al.*, 2016; Grant *et al.*, 2016).

Comparison of the same examination periods across years (Study A) showed that the percentage of udder halves that were defective at pre-mating and docking was higher in 2018 than in 2017. Similarly, palpable udder defects during a previous lactation was associated with increased risk of udder defects in the current lactation (Grant *et al.*, 2016). This is likely a function of time; in this study ewes with udder defects were not culled and so as more ewes developed udder defects over time the percentage of identified defects would have increased. Further, given the high reappearance rates in Study C and impact on lamb performance, the results of these studies suggest that ewes with hardness or lump/s within one or both udder halves should be culled regardless of the apparent severity of the defect at the time of examination.

In ewes with a previous history of udder half defects (Study C), the presence of udder half defects during the first six weeks of lactation appeared to be high and highly variable with numerous defect type changes. The high occurrence in early lactation could be due to high production stress stimulating compromises in mammary defence systems following lambing (Coop & Kyriazakis, 1999; Ezzat Alnakip *et al.*, 2014; Mizuno *et al.*, 2012; Sordillo, 2005). During lactation, the occurrence of udder half defect was higher and more consistent over time in those udder halves that did not express milk in comparison with udder halves from which milk was expressed. Smith *et al.* (2015) reported that palpable udder defects are associated with abnormal secretions or abscesses in the mammary gland while abscesses take a couple of weeks to mature before spontaneously resolving or bursting (Cheng *et al.*, 2011; Kobayashi *et al.*, 2015). This could explain the high occurrence and persistency of defects over time in those udder halves that didn't express milk. Others have reported that udder defects can also induce early involution in those udder halves which may lead to further compromise of the mammary immunity (Petridis *et al.*, 2012; Wilde *et al.*, 1997). On the other hand, mammary emptying in those udder halves that do express milk might be helpful to prevent further health complications (Gelasakis *et al.*, 2015).

The overall frequency of defective udder halves decreased as the days in lactation increased. Udder halves categorised as hard declined substantially, as an udder half categorised as hard was more likely (80%) to change to normal in the first five weeks of lactation. This result agrees with previous reports of higher occurrence of "hard udder" shortly after lambing but within two to three weeks the udder appeared normal (Bruce *et al.*, 2013; Skyrme, 1970). Although the number of udder halves categorised as hard decreased significantly over time, if an udder half was still hard on Day 35, the probability of remaining hard was very high (>90%). This could be due to considerably decreased milk production or initiation of early involution which compromises the mammary immunity as in the case of normal involution during drying off (Petridis *et al.*, 2012). An udder half categorised as lump was more likely to change to normal in the first five weeks of lactation, but the probability declined as the days in lactation increased. The probability of an udder half categorised as lump to remain lump increased for the first four weeks in lactation and started to decrease on Day 35 after. This might be associated with development maturation of an abscess which bursts a couple of weeks later (Cheng *et al.*, 2011; Kobayashi *et al.*, 2015). In the first six weeks of lactation, the probability of new defect development (normal to hard or lump) was very low, and it decreased as the days in lactation increased.

Udder palpation scoring is an easy and practical method of udder assessment in ewes. Nevertheless, the technique could be relatively subjective depending on the physiological state of the udder (i.e., lactating or involuted) and the operators skill. To minimize potential variation or misclassifications, in Study A, the same four trained operators undertook the udder palpations while in Study C the same person undertook all udder palpations. However, the outcomes may have been missed, mis-classified or over-interpreted, and therefore, interpretation these results should be done with caution.

In conclusion, udder half defect status changed over key management times such as pre-mating, pre-lambing, docking and weaning in a year, and also weekly in early lactation. The highest occurrence of udder halves categorised as hard was observed during the non-lactating period and early lactation (pre-mating or docking) whereas in udder halves categorised as lump, the highest occurrence was during lactation (docking and weaning). Udder half defects categorised as hard or lump at pre-mating were more likely to be defective (hard or lump) at future examinations at key management times in the year or pre-mating of the following year. Udder halves that did not express milk in early lactation had a higher occurrence and persistency of udder half defects compared with those that did express milk. Hence, decisions on culling or keeping ewes based on udder palpation should be made with consideration of the dynamic pattern of udder half defect changes over time and their persistency, and status of the contralateral udder half and ultimate effect on whole udder milk production.

Chapter 6: Isolation of Aerobic Bacterial Species Associated with Palpable Udder Defects in Non-Dairy Ewes

6.1 Abstract

AIMS: Firstly, to identify associations between udder half defect (hard or lump) and bacteria isolated from milk or mammary tissue swabs; secondly, to compare with samples from normal udder halves at different physiological time points; and finally, to compare bacterial species isolated via milk and swabs of mammary tissue from within the same udder halves.

METHODS: Samples were aseptically collected from each udder half of 223 non-dairy breed (Romney) ewes from three different studies. The first two studies utilized cross-sectional sampling, either milk samples at weaning (Study A, n=101) or mammary tissue swab samples at premating (Study B, n=74). In the third study (Study C, n=48), milk samples were collected weekly for the first six weeks of lactation, at weaning and three weeks post-weaning and mammary tissue swab samples were collected post-weaning, after slaughter. Conventional bacterial culture and Matrix-assisted laser desorption/ionization time-of flight mass spectrometry was used for bacterial identification. Association between udder half defect, bacterial positivity, and time, and the agreement between milk and swab samples from the same udder halves were quantitatively analysed.

RESULTS: *Mannheimia haemolytica* and *Streptococcus uberis* were the dominantly identified bacterial species from defective udder halves, either hard or lump, whereas CNS, mostly *Staphylococcus simulans* and *Staphylococcus chromogens*, were more frequently isolated from normal udder halves. *Staphylococcus aureus* was predominantly identified from defective udder halves during lactation while it was

isolated from both defective and normal udder halves in similar proportions both at weaning and pre-mating. Persistency of bacterial species over time was variable depending on the species involved although less frequently identified species showed less stability over time. *Mannheimia haemolytica, Staphylococcus xylosus* and *Streptococcus pluranimalium* had the greatest persistence over time while *Staphylococcus aureus* was observed with both very short and long persistency. A very high agreement (91.5%) of bacterial species identified was observed between the mammary tissue swab and udder half milk samples during post-weaning.

CONCLUSION: In summary, palpable udder half defects were associated with bacterial positivity and the persistency of the bacteria over time was dependent on the species involved. Hence, culling ewes with palpable udder half defects that had more stable bacterial species over time could contribute to reducing the recurrence of palpable defects or mastitis.

6.2 Introduction

Udder defects have been associated with reduced milk production and changes in milk composition in both dairy and non-dairy ewes (Fthenakis & Jones, 1990a; Saratsis *et al.*, 1999; Yusuf *et al.*, 2018). Further, low survival rates (Griffiths *et al.*, 2019a) and reduced live weight gains (Griffiths *et al.*, 2019b) have been observed in pre-weaned lambs reared by ewes with udder defects. These defects can be associated with mild to clinically noticeable changes in milk production and the udder structure (Arsenault *et al.*, 2008). A range of bacterial species have been isolated from either milk or in abscesses from defective udders, with Staphylococci (*S. aureus* and coagulase-negative isolates) and *Mannheimia haemolytica* (*M. haemolytica*) the most frequently reported isolates (Arsenault *et al.*, 2008; Peterson *et al.*, 2017; Ridler *et al.*, 2021; Smith *et al.*, 2015; van den Crommenacker-Konings *et al.*, 2021). These studies also reported isolation of other relatively less frequently isolated bacterial species such as Streptococci, Enterobacteria, Corynebacterium and Pseudomonas. Over the past five decades in New Zealand, only a small number of studies have been undertaken investigating bacterial species isolated from defective udders and teats have reported isolation of these and related bacterial species from clinically mastitic milk (Ekdahl, 1972; Peterson *et al.*, 2017; Quinlivan, 1968b; Ridler *et al.*, 2021). These studies were undertaken either shortly after lambing or during the post-weaning period and in most cases involved taking milk samples at a single point in time from ewes whose history of udder defects was unknown.

Upon palpation of an udder, any detectable mass of different consistency from the rest of the gland tissue or diffuse change in consistency of the whole mammary gland can be considered as a palpable udder defect (Griffiths *et al.*, 2019a). A diffuse hard consistency of the whole mammary gland is a unilateral or bilateral udder defect with no to a small amount of milk expression from the gland (Peterson *et al.*, 2017; Skyrme, 1970). This defect type can be observed during both lactation and the dry or the pregnancy period (Grant *et al.*, 2016; Griffiths *et al.*, 2019a; Saratsis *et al.*, 1998). Recently, Ridler *et al.* (2021) reported at least seven different bacterial species from milk samples collected from udder halves categorised as hard, one week after weaning in non-dairy ewes in New Zealand. *Staphylococcus aureus* (*S. aureus*) was identified as the dominant bacterial species in that study, although 19.4% (7/36) of the udder halves categorised as hard yielded no bacteria.

Udder lumps have been described as intramammary masses (IMM) that can vary in size, consistency, and location (Griffiths *et al.*, 2019a; Saratsis *et al.*, 1998). Smith *et al.* (2015) who collected mammary gland samples with lumps (abscesses) reported that *S. aureus* was the most frequently detected bacterial species. Ridler *et al.* (2021)

reported various species of bacteria isolated from milk samples from udder halves that contained lumps, including Staphylococcus, Streptococcus and Mannheimia. Distinct subcutaneous lumps immediately cranial or sometimes caudal to the udder have been reported in some ewes at weaning or shortly thereafter (Ridler *et al.*, 2021). Such lumps were described by Saratsis *et al.* (1998) as bacterial negative cysts filled with milk-like fluid.

Previous studies have shown changes in the percentage of palpable udder defects in repeated examinations within the same flock, indicating the dynamic nature of palpable udder defects (Grant *et al.*, 2016; Griffiths *et al.*, 2019a). Further, Ridler *et al.* (2021) reported changes in udder defect status (defective to normal, defective to other defect category (e.g. hard to lump) or normal to defective) across two visits four to six weeks apart following weaning. However, there has been no research evaluating bacterial isolation in defective udder halves over time.

Combined, the data indicate that while there has been research on bacterial isolation from udders with palpable udder defects in non-dairy ewes, most were undertaken during the short period between weaning and mating. Moreover, the association between bacterial isolation and udder half defect category or changes in defect category over time has not been studied. Therefore the objectives of the current studies were to firstly, identify aerobic bacteria species isolated from milk and mammary tissue swab samples collected from normal udder halves and those with palpable defects at different physiological times (pre-mating, early lactation, weaning and post-weaning); secondly, identify associations between udder half defect and bacterial isolation; thirdly, assess changes in bacterial isolation from the same udder halves over time; and lastly, compare bacterial isolation from udder halves sampled via milk samples compared with swabs of mammary tissue.

6.3 Materials and methods

This chapter comprises data collected during three separate studies previously described as studies A, B and C in the General Methodology (Chapter 3).

6.3.1 Animal selection and management

Study A: Ewes in this study were part of a three-year longitudinal study from 2016 to 2018 at Massey University's Riverside farm, Wairarapa, New Zealand. The ewes were managed under standard commercial New Zealand conditions on pasture. A total of 101 Romney ewes aged four to five years were selected based on: 1. their udder half defect history (33 ewes); 2. their udder half defect status at weaning on December 5th, 2018 (48 ewes); 3. history of no udder half defects in the previous three years and with no udder half defect at weaning on December 5th, 2018 (20 ewes). At weaning on December 5th, 2018, milk samples were collected from each udder half for bacterial identification from all 101 ewes. Additionally, seven ewes with cranial midline lump/s were humanely euthanised at Massey University and udder samples were collected (detail below). A total of eight swab samples were collected from the cranial midline lump/s (one each from seven ewes and two from one ewe) as described below.

Study B: Seventy-four Romney ewes that had been culled by the owner due to udder defects were obtained from a single commercial farm in North Canterbury, South Island, New Zealand and were transported to Massey University's Tuapaka farm, Palmerston North in May 10th, 2019. The ewes remained at Tuapaka farm for a week under standard commercial pasture grazing conditions. During this time the udder and teats of each ewe were scored using the system described by Griffiths *et al.* (2019a). Ewes were then slaughtered either at a local abattoir (n=70) or, if deemed unfit for transportation to slaughter, were humanely euthanised at Massey University (n=4). In all cases, the whole udder was collected, transported to Massey University and a mammary gland tissue swab was collected from each udder half within three to six hours (see below for further detail).

Study C: Forty-six ewes joined this study from a three-year (2016 to 2018) longitudinal study at Massey University's Riverside farm, Wairarapa, New Zealand based on udder defect history and udder defect status on the selection day. This study comprised lactating ewes with both normal and defective udder halves. Milk samples were collected from each udder half six times during lactation (on approximately days 7, 14, 21, 28, 35 and 42 of lactation), at weaning (November 29^{th}, 2019) and three weeks after weaning (December 18^{th}, 2019). Two ewes were culled at day 42 due to routine farm management and four ewes missed day 42 milk sample collection. Udder halves were scored (Chapter 3) just before sample collection on all sampling days. On December 21^{st}, 2019, all ewes were slaughtered through a commercial abattoir and udders were collected for mammary tissue swab sampling (see section 6.3.4 below).

6.3.2 Udder scoring

Each udder half was scored according to Griffiths *et al.* (2019a) and categorised into hard, lump, or normal (Chapter 3, 3, Section 3.6).

6.3.3 Post-mortem udder collection

Immediately post-slaughter (Studies B and C) the whole udder was removed, transported to Massey University, and washed and thoroughly cleaned (Chapter 3, Section 3.7).

6.3.4 Mammary swab sample collection

A swab sample was collected from each udder half (gland) using a sterilized blade to incise the udder skin at the base of teat and at the gland cistern, to expose the whole gland cistern. Several dissections were made using new sterilized blades to check the presence of lumps in the different parts of the udder (cranial or caudal to gland cistern). In each udder half, the gland cistern and/or contents of defects found were swabbed using a transport swab (Fort Richard Laboratories, Auckland). The ewes' identification number, site of collection, nature of the contents and number of samples were recorded for each udder half independently. For the seven ewes with cranial midline lump/s (CML), an incision was made into the lump and swab samples were collected. The swab samples were cultured immediately or kept for a maximum of 12 hours in the refrigerator (4° C) until culturing.

6.3.5 Milk sampling

An aseptic milk sampling procedure was conducted to collect milk samples from each udder half (Studies A and C). Before sampling, teats were thoroughly cleaned with 70% ethanol and wiped with commercial teat wipes (Zoetis Mediwipes, Zoetis New Zealand limited). The first three milk squirts were discarded and then 3-4 ml of milk was collected from each udder half into sterile tubes for bacteriological testing. Milk samples were cultured (see section 6.3.6 below) in less than one hour after collection or kept for a maximum of 12 hours in the refrigerator (4° C) until culturing.

6.3.6 Bacterial culture and identification

Milk and swab samples were processed at the Massey University School of Veterinary Science bacteriology laboratory. The media used was Colombia blood agar with 5% sheep blood, which was incubated aerobically at 37°C for 48 hours. Swabs were streaked directly onto the plate. Milk samples (10µL) were put on a plate using micropipettes and streaked with a sterilized inoculation loop. Cultures with three or more morphologically distinct colonies on a single sample plate were considered positive and morphological characteristics were assessed (Adkins *et al.*, 2017).

Cultures with three or more different colony types without dominant colony type were considered contaminated. For all the isolates, single distinct colonies were picked further for sub-culture, Gram stain and catalase and coagulase testing. Purified isolates were then stored at −80°C until tested using Matrix-assisted laser desorption/ionization time-of flight (MALDI-ToF) mass spectrometry to identify isolates.

6.3.7 Matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry identification

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry was used to identify isolates (Alatoom *et al.*, 2011). All the bacterial isolates were analysed using the direct transfer method, by directly smearing bacterial colony to a target plate (Bruker MALDI Biotyper) at SC Bio Limited, Auckland and Massey University, Palmerston North, Palmerston North, New Zealand. An extended direct method with addition of 1µL of 70% formic acid or ethanol extraction method was applied for isolates that didn't identify by the direct method. MALDI-TOF results were interpreted based on three categories: high confidence identification (range 2.0-3.0), low confidence identification (range 1.70-1.99) and unidentified (less than 1.70) (Almuzara *et al.*, 2016). Unidentified isolates either at genus or species level were described based on their Gram staining and morphological characteristics.

6.3.8 Statistical analysis

Chi-square/Fisher's test of independency was used to assess associations between udder half defect and bacterial positivity or bacterial species at weaning (Study A), pre-mating (study B) and lactation (day 7, 14, 21, 28, 35 and 42), weaning and post-weaning (Study C). Udder halves with no milk expression and contaminated culture samples from Study A were excluded from the statistical data analysis. However, exclusion of these udder halves did not change defective to normal proportion difference ($p>0.05$). The strength of these associations were measured by Cramer's V Coefficient (McHugh, 2013). The Coefficient ranges from 0 (no association at all) to 1 (perfect association), where high (>0.5), moderate (0.3-0.5), low (0.1-0.3) and little (0-0.1) indicate the range in between.

Multinomial logistic regression was applied to assess the association of time and bacterial positivity using “nnet” package in R statistical software (R Core Team, 2020). Lasagna plot was used to visualize longitudinal bacterial positivity (positive/negative or no milk expression) in an individual udder half across six times in the first six weeks of lactation, at weaning and three weeks post-weaning. In addition, descriptive frequency was used to summarize the persistency of each bacterial species identified in an individual udder half over eight repeated occasions (six times in the first six weeks of lactation, at weaning and three weeks post-weaning). The frequencies were described in three categories: occurrence of only once in an individual udder half, two to three times in an individual udder half or more than four in an individual udder half in those udder halves that expressed milk at least seven times.

The agreement between milk and swab samples from the same udder halves (Study C) was analysed using Cohen’s Kappa (Landis & Koch, 1977). The strength of the Kappa agreement is described as: poor (<0.00), slight (0.00-0.20), fair (0.21-0.40), moderate (0.41-0.60), substantial (0.61-0.80) and almost perfect (0.81-1.00).

6.4 Results

6.4.1 Study A: Bacterial species identified from ewes' milk samples collected at weaning and their association with udder defects

In study A, of the 202 udder halves (from 101 ewes) where attempts were made to collect milk samples, 36 udder halves didn't express milk (Table 6.1). No milk expression was seen from 5/11 (45%), 15/46 (33%) and 16/145 (11%) of udder halves categorised as hard, lump and normal, respectively. The bacterial culture of those that did express milk showed that 41 udder halves were positive for bacteria, 68 had no growth of bacteria, and 57 were contaminated (more than 3 bacterial colony types). Contamination was seen in samples from all udder half defect categories (hard, lump and normal). All culture positive udder halves grew a single colony type, except one udder half that grew two colony types identified as *E. coli* and *M. haemolytica*. Bacterial species identified were CNS species, *M. haemolytica*, *S. aureus*, *Streptococcus uberis* (*S. uberis*) and others (Table 6.1).

Chi-square test of independence between bacterial positivity (positive/negative) and udder half defect (hard, lump, or normal) was significantly ($p<0.05$) associated, but the strength of the association was low (Cramer's V=0.26, Table 6.2). In udder halves that expressed milk and with no contamination of bacterial culture, bacteria were isolated from 3/3 (100%) udder halves categorised as hard and 19/25 (76%) udder halves categorised as lump (Table 6.2). In contrast, 62 out of 81 (76.5%) of normal udder halves yielded no bacteria. *M. haemolytica* and Streptococcus species were predominantly isolated from defective udder halves, whereas the majority of CNS were isolated from normal udder halves. *S. aureus* was isolated from both normal and udder halves categorised as lump. Association between udder half defect and bacterial species was observed, but the strength was weak (P=0.039, Cramer's V= 0.20, Table

6.2). Of the eight swab samples collected from seven ewes with cranial midline lumps (CMLs), no bacteria were isolated from any.

6.4.2 Study B: Bacterial species identified from mammary swab samples collected during pre-mating and their association with udder defects

From a total of 148 udder half (from 74 ewes) mammary swab samples cultured from commercial ewes prior to mating, 91 (61.5%) were culture negative (Table 6.1). Eleven udder halves grew more than two dissimilar colony types and were categorised as contaminated. The remaining 46 udder halves were identified as bacterial culture positive with a single colony type, except for three udder halves that yielded two colony types (Table 6.1). *S. aureus, M. haemolytica,* and *S. uberis* were the dominant bacterial species identified and were isolated from udder halves categorised as hard, lump, and normal (Table 6.1).

The association between udder half defect and bacterial positivity (positive or negative) was significant ($p<0.05$), although the strength of the association was weak (Cramer's V = 0.24; Table 6.3). Eleven of 14 (78.6%) udder halves categorised as hard were bacterial culture positive, one third of the udder halves categorised as lump were culture positive whereas most (69.3%) udder halves categorised normal were culture negative (Table 6.3). However, the analysis of association between udder half defect and bacterial species was not significant ($P>0.05$).

As in the case of Study A*, M. haemolytica* and Streptococcus species were the major bacterial species isolated from defective udder halves (Table 6.1). *S. aureus* and *Staphylococcus simulans* (*S. simulans*) were isolated in comparable numbers from both normal and defective udder halves. *Trueperella pyogenes* (*T. pyogenes*) was isolated in lower frequency and only from defective udder halves. A weak association

($p<0.05$) was seen between udder half defect and bacterial species involved ($P<0.05$, Cramer's V = 0.24; Table 6.3).

Table 6.1 Frequency of bacterial species identified from milk samples collected at weaning (Study A, n = 101 ewes) and swab samples collected at pre-mating (Study B; n=74 ewes) from udder halves categorised as hard, lump and normal in non-dairy breed (Romney) ewes.

Bacterial species	Study A (Weaning, 2018)				Study B (Pre-mating, 2019)			
	Hard	Lump	Normal	Study A Total	Hard	Lump	Normal	Study B Total
Staphylococcus aureus	0	3	3	6	2	1	3	6
Staphylococcus caprae	0	0	2	2				
Staphylococcus chromogenes	0	1	3	4	0	0	1	1
Staphylococcus simulans	1	2	3	6	0	3	3	6
Staphylococcus devriesei	0	0	1	1				
Staphylococcus warnerii	0	0	3	3				
CNS (species unidentified)	0	1	0	1	0	1	0	1
Streptococcus oralis	0	1	0	1				
Streptococcus pluranimalium	0	0	1	1				
Streptococcus uberis	0	3	1	4	1	3	1	5
Streptococcus salivarius					0	1	0	1
Streptococcus suis					0	2		2
Streptococcus (species unidentified)	0	3	0	3	0	0	1	1
Mannheimia haemolytica	1	5	1	7	4	0	2	6
Arcanobacterium pluranimalium	1	0	0	1	1	0	0	1
Trueperella pyogenes					2	1	0	3
Corynebacterium pseudotuberculosis					0	1	1	2
Helcococcus ovis					0	1	0	1
Gram-positive bacilli					0	0	2	2
Mixed infection								
Escherichia coli & *M. haemolytica*	0	0	1	1	1	2	0	3
Staphylococcus aureus & *Helcococcus ovis*					0	1	0	1
S. parasanguinis &*Neisseria flavescens*					0	0	1	1
S. uberis & Helcococcus ovis					0	1	0	1
S. uberis & Streptococcus suis					0	1	0	1
Streptococcus spp & Staphylococcus spp					0	0	1	1
Contamination *	3	6	48	57	0	5	6	11
No bacterial growth	0	6	62	68	3	34	54	91
No milk sample expressed	5	15	16	36				
TOTAL	11	46	145	202	14	58	76	148

* Bacterial cultures with three or more dissimilar colony types. CNS: coagulase negative staphylococcus. Note: Culture of samples from most udder halves presented a single colony type, but one udder half from Study A and three udder halves from Study B each yielded two distinct types of colonies.

Table 6.2 Association between udder half defects (hard, lump or normal) and bacteria identified from 109 udder half milk samples collected at weaning (Study A, December 5th, 2018) from 101 non-dairy breed (Romney) ewes.

Parameters	Udder half defects				X^2 (p-value)	Cramer's V
	Hard	Lump	Normal	Total		
Bacterial positivity						
Positive	3	19	19	41	<0.05	0.26
Negative	0	6	62	68		
Bacterial species						
M. haemolytica	1	5	1	7	<0.05	0.23
S. aureus	0	3	3	6		
Streptococcus	0	7	2	9		
CNS	1	4	12	17		
Others	1	0	2	3		

CNS: coagulase negative staphylococcus. Note: Thirty-six udder halves expressed no milk and 57 udder halves with three or more dissimilar colony types (contaminated) were excluded from the analysis.

Table 6.3 Association between udder half defect (hard, lump or normal) and bacteria identified from swabs of mammary tissue from 74 non-dairy breed (Romney) ewes at pre-mating (Study B, May 10th, 2019).

	Udder half defect category					
Parameters	Hard	Lump	Normal	Total	X^2 (p-value)	Cramer's V
Bacterial positivity						
Positive	11	20	15	46	<0.05	0.24
Negative	3	34	54	91		
Bacterial species						
M. haemolytica	4		2	6	>0.05	0.25
S. aureus	2	1	3	6		
Streptococcus	1	7	1	9		
CNS		4	4	8		
Mixed infection		4	2	6		
Gram-positive bacilli	4	4	3	11		

CNS: coagulase negative staphylococcus. Note: Thirty six udder halves expressed no milk and 57 udder halves with three or more dissimilar colony types (contaminated) were excluded from the analysis.

6.4.3 Study C: Bacterial species identified from milk samples collected from ewes during lactation, weaning and post-weaning at Keeble Massey University farm

Udder half milk expression status and bacterial positivity varied throughout six occasions during lactation, at weaning and at post-weaning in 96 udder halves (48 ewes; Table 6.4, Figure 6.1). The number of udder halves that did not express milk varied from 39 on day 7 to 44 at post-weaning. Udder halves categorised as hard, lump, and normal were observed on all eight occasions except at weaning when no udder halves were categorised as hard (Table 6.4). The frequency of udder halves that were bacterial culture positive became less frequent as time went on: it was highest at day 7 of lactation and lowest at weaning (Table 6.4). From 752 potential udder half milk sampling occurrences, on 339 occasions no sample was able to be obtained. Of the 413 milk samples that were collected, five were contaminated, 270 grew no bacteria while at least 19 different bacterial species were isolated from the 138 samples that grew bacteria (Table 6.5). The most commonly isolated bacteria were coagulase negative staphylococcus (CNS) species, in particular *S. simulans, S. chromogenes, S. xylosus, and S. haemolyticus. M. haemolytica* and *Streptococcus species* recorded a reasonably high frequency of occurrence while the rest of the bacterial species identified were found at low frequency (Table 6.5).

Multinomial logistic regression analysis of association between udder half bacterial positivity and time (day 7 of lactation through to post-weaning) was only significant ($p<0.05$) at weaning compared to day 7. The relative risk ratio (±SE) of an udder half being bacterial culture positive at weaning was 0.39 (0.45) times compared with day 7 of lactation (the reference).

Table 6.4 Bacterial positivity of milk samples collected from udder halves categorised as hard, lump or normal from 46 non-dairy breed (Romney) ewes, during the first six weeks of lactation (day 7, 14, 21, 28, 35, 42), at weaning and three weeks post-weaning (Study C).

		Udder half defect category									Total Pos	Total Neg	Total NS	Udder halves
		Hard			Lump			Normal						
Timing	Category	Pos	Neg	NS	Pos	Neg	NS	Pos	Neg	NS				
Lactation	Day 7	3	4	28	2	1	3	19	28	8	24	33	39	96
	Day 14	7	5	8	6	7	15	8	20	20	21	32	43	96
	Day 21	3	1	9	0	1	14	20	28	20	23	30	43	96
	Day 28	3	1	5	2	2	17	16	29	21	21	32	43	96
	Day 35	2	1	4	1	2	14	15	32	25	18	35	43	96
	Day 42	2	1	4	1	1	14	9	33	23	12	35	41	88
Weaning		0	0	0	1	2	9	10	36	34	11	38	43	92
Post-weaning		0	0	1	2	3	8	11	32	35	13	35	44	92
Total		20	13	59	15	19	94	108	23 8	186	143	270	339	752

Pos.: Bacterial culture positive; Neg.: Bacterial culture negative; NS: No sample collected due to no milk expression from the udder half. Note: No data was collected from four ewes on day 42 and two ewes at weaning and three weeks post-weaning.

Table 6.5 Bacterial species identified from milk samples collected from non-dairy breed (Romney) ewes in the first six weeks in lactation (day 7, 14, 21, 28, 35, 42), at weaning and three weeks post-weaning (Study C).

Bacterial spp	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Weaning	Post-weaning	Total
Aerococcus viridans				1					1
Corynebacterium lipophiloflavum	1	2	1						4
Escherichia coli			1						1
Kocuria carniphila				1		1			2
Mannheimia haemolytica	3	1			1	1	1	1	8

Staphylococcus aureus	1	2	1			1			5
Staphylococcus auricularis	2	1	1		1	1			6
Staphylococcus capitis				2				1	3
Staphylococcus chromogenes		2	3	2	3	1		2	13
Staphylococcus haemolyticus	1	1	1	1	1	1	3		9
Staphylococcus petrasii			1			1			2
Staphylococcus simulans	5	5	5	5	5	2	3	4	34
Staphylococcus warneri			1	1	1		1	1	5
Staphylococcus xylosus	2	2	2	2	2		1	1	12
Streptococcus pluranimalium	1	1	1	1	1	1		1	7
Streptococcus uberis	1	2	2	4	2	1	1	1	14
Staphylococcus auricularis & *Streptococcus parasanguinis*							1		1
Aerococcus viridans &*Staphylococcus simulans*	1								1
Corynebacterium spp		1							1
Psychrobacter spp			1						1
Gram-positive bacilli	1		1					1	3
CNS-unidentified		1	1	1	1	1			5
Contaminated	5								5
No bacterial growth	33	32	30	32	35	35	38	35	270
No Sample	39	43	43	43	43	41	43	44	339
Total	96	96	96	96	96	88	92	92	752

CNS: Coagulase negative staphylococcus

6.4.4 Study C: Udder half bacterial positivity and species identified over time during lactation, weaning and post-weaning at Keeble Massey University farm

Figure 6.1 describes the repeated culture result from each udder half over the eight milk sampling occasions. The plot demonstrates that udder halves that were bacterial negative on day 7 of lactation almost always remained negative throughout lactation, at weaning and at post-weaning. In the one udder half which did subsequently present a culture positive result, this only occurred at one sampling time. In contrast, almost all udder halves that were bacterial culture positive at day 7 either remained positive through to and including post-weaning or subsequently no milk sample was able to be expressed (Figure 6.1). The majority of udder halves that didn't express milk during early lactation continued to not express milk until post-weaning; for those that did express some milk it was generally bacterial positive (Figure 6.1).

To investigate persistency of bacterial species over time, individual udder halves from which specific bacterial species were isolated were classified into three categories based on how many times they were culture positive on the eight sampling occasions: single, two to three or more than four occasions. Of the total bacterial species identified, more than six species occurred on only a single occasion in an individual udder half. *Staphylococcus aureus, S. uberis,* and CNS species such as *S. similans, S. chromomogens, S. auricularis, haemolyticus,* and *S. warneri* were observed on both one occasion as well as on more than two occasions in an individual udder half. However, *M. haemolytica, S. xylosus* and *S. pluranimalium* were only isolated from an individual udder half on more than four occasions, apparently indicating persistence of these organisms over time (Table 6.6). Table 6.6 includes those udder halves that expressed milk on at least six occasions (n=71). *M. haemolytica* (2), *S. chromogenes* (2), *S. aureus* (1), *S. uberis* (1) *S. simulans* (1) and no growth (2) were detected in those udder halves that expressed milk on only one or two occasions (n=9).

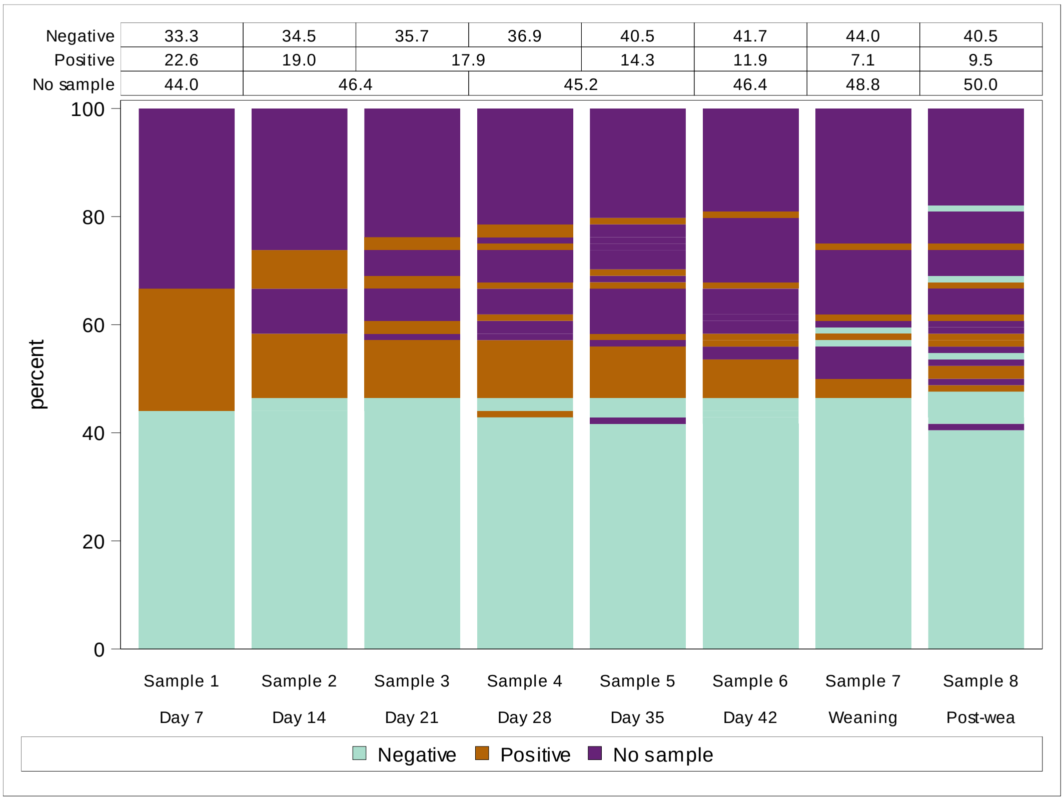


Figure 6.1 Lasagna plot of bacterial culture positivity (negative, positive or no sample) from eight repeated milk samples collected from each udder half over the first six weeks in lactation (Day 7, 14, 21, 28, 35, 42), at weaning and three weeks post-weaning in 40 non-dairy breed (Romney) ewes (Study C) Note: Each bar shows a different udder half milk collection (i.e. time) while the different colours within each bar represent the bacterial culture positivity (positive, negative or no sample). The data in the table at the top of the plot designates the percentage of each bacterial culture positivity at each event, which corresponds to the percentage of each colour at each event. Change in bacterial positivity over time of each udder half can be tracked by following longitudinal transitions across the udder scoring events of stacked bars.

Table 6.6 Frequency of isolation of bacterial species from milk samples collected from individual udder halves on eight occasions during the first six weeks in lactation (Day 7, 14, 21, 28, 35, 42), at weaning and three weeks post-weaning, from 40 non-dairy breed (Romney) ewes (Study C).

Bacterial species	Number of individual udder halves		
	Single occasion	Two or three occasions	Four or more occasions
A. viridans	1		
C. lipophiloflavum	4		
E. coli	1		
K. carniphila	2		
S. petrasii	2		
Psychrobacter spp.	1		
A. viridans and S. simulans	1		
S. *auricularis* and *S. parasanguinis*	1		
Contaminated	4		
Gram-positive bacilli	1		
S. aureus	1	1	
S. capitis		1	
CNS-unidentified	3	1	
S. simulans	2		6
S. warneri	1		1
S. haemolyticus	1		1
S. auricularis	1		1
S. chromogenes	2	1	1
S. uberis	1	1	2
M. haemolytica			1
S. xylosus			2
S. pluranimalium			1

Note that this table only includes data from udder halves where milk samples were collected on six or more occasions.

6.4.5 Study C: Association of udder half defect and bacterial isolation over lactation, weaning and pre-mating

The analysis of association between udder half defect (hard/lump/normal) and bacterial culture positivity (positive/negative) at each time point (Day 7, 14, 21, 28, 35 and 42 of lactation, weaning and post-weaning) was not significant ($p>0.05$). However, *M. haemolytica, Streptococcus uberis* and *Staphylococcus aureus* were more frequently isolated (descriptively) from defective udder halves (hard/lump) whereas CNS were more frequently identified from normal udder halves (Table 6.7).

Table 6.7 Frequency of bacterial species identified from udder halves categorised as hard, lump or normal from 46 non-dairy breed (Romney) ewes, during the first six weeks of lactation, at weaning and three weeks post-weaning (Study C).

Bacterial species	Udder half defect category			
	Hard	Lump	Normal	Total
Staphylococcus aureus	3	1	1	5
Staphylococcus capitis			3	3
Staphylococcus chromogenes			13	13
Staphylococcus haemolyticus			9	9
Staphylococcus petrasii			2	2
Staphylococcus simulans	4	3	27	34
Staphylococcus warneri	1		4	5
Staphylococcus xylosus	1	1	10	12
Staphylococcus auricularis	1		5	6
Streptococcus pluranimalium		2	5	7
Streptococcus uberis	6	1	7	14
Mannheimia haemolytica	2	5	1	8
Aerococcus viridans			1	1
Corynebacterium lipophiloflavum	1		3	4
Escherichia coli			1	1
Kocuria carniphila			2	2
Corynebacterium spp	1			1
Aerococcus viridans and Staphylococcus simulans			1	1
Staphylococcus auricularis and Streptococcus parasanguinis			1	1
Psychrobacter spp			1	1
CNS (unidentified)			5	5
Gram-positive bacilli		1	2	3
Contaminated*		1	4	5
No bacterial growth	13	19	238	270
No milk expressed	59	94	186	339
Total	92	128	532	752

* Bacterial cultures with three or more dissimilar colony types.

6.4.6 Study C: Bacterial species identified from udder half mammary tissue swab samples collected post-weaning and their association with udder half defects

Swab samples were taken from 92 udder halves (from 46 ewes) at slaughter three weeks after weaning and only 17 (18%) showed bacterial growth. Four of 16 udder halves categorised as lump (25%) and 13 of 78 (16.7%) udder halves categorised as normal grew bacteria. There was only one udder half categorised as hard at this time.

Fifteen samples grew a single species of bacteria while two udder halves grew two species of bacteria. The bacterial species identified were *Staphylococcus aureus* (2), *S. simulans* (4), *Streptococcus uberis* (2), *Streptococcus pluranimalium* (1), *Acinetobacter indicus* (1), *Trueperella pyogenes* (1), *M. haemolytica* (1), *S. epidermidis* (1), *Staphylococcus xylosus* (1), *Acinetobacter schindlerim* (1) and unidentified gram-positive rods (2). The association between udder half defect and bacterial positivity was not significant (p>0.05).

From a total of 43 udder halves (nine ewes with both halves and 25 single udder halves) that didn't express milk on at least six occasions, a culture positive result was observed from seven udder halves from swab samples post-weaning. No significant (p>0.05) association was observed between udder half defect and bacterial positivity. Swab samples from normal udder halves grew *Staphylococcus aureus, S. epidermidis, Streptococcus mitis,* and *Staphylococcus simulans* while *Trueperella pyogens and Streptococcus uberis* were isolated from lumps.

6.4.7 Study C: Comparison of bacterial isolation from ewes' udder half milk and swab samples collected at post-weaning

From a total of 92 udder halves from which milk sampling was attempted three weeks post-weaning, 46 (50%) didn't express milk. Thus, the agreement analysis of bacterial isolation from milk and mammary tissue swabs could only be undertaken for the 46 udder halves from which both types of samples were collected. All udder halves that had culture positive milk samples also had culture positive swab samples. However, four udder halves with bacterial culture positive milk samples were bacterial culture negative from the swab samples. These bacterial species identified from four milk samples were S. *simulans, S.warneri,* and *S. capitis*. Udder half swab and milk sampling methods showed a substantial strength of agreement with a k-coefficient of 0.724 ($p<0.001$).

6.5 Discussion

To identify bacterial species and assess their association with udder half defect, milk and/or mammary tissue swab samples were collected at pre-mating, in lactation, at weaning and/or post-weaning from three different populations of non-dairy breed (Romney) ewes. Overall, more than 30 bacterial species of 10 genera were identified during this study. The type and range of bacteria isolated were similar among populations of sheep and time-points of sampling (lactation, weaning, post-weaning and pre-mating). CNS (mainly *S. simulans* and *S. chromogens*), *M. haemolytica, Staphylococcus aureus* and *Streptococcus uberis* were the most frequently isolated bacterial species. Previous studies have similarly reported *Staphylococcus aureus*, *M. haemolytica* and CNS as the most frequently isolated bacterial species in non-dairy ewes in New Zealand (Koop *et al.*, 2010; Peterson *et al.*, 2017; Quinlivan, 1968b) and internationally (Arsenault *et al.*, 2008; Mavrogianni & Fthenakis, 2007; Omaleki *et al.*, 2010).

Overall, a significant but weak association between udder half defect and bacterial culture positivity was observed at weaning and premating whereas no association was found during lactation. Across these three studies, bacteria were isolated from 50-78% of udder halves categorised as hard. Similarly, Ridler *et al.* (2021) isolated bacteria from 28/36 (78%) udder halves categorised as hard. Other authors have also isolated various bacterial species from defective udder halves including those udder halves categorised as hard (Peterson *et al.*, 2017; Saratsis *et al.*, 1998). Bacteria were isolated from 33-61% of udder halves categorised as lump across the three studies. Likewise, in a study undertaken at weaning, 30% of udder halves categorised as lump isolated bacteria (Ridler *et al.*, 2021). Smith *et al.* (2015) reported identification of several bacterial species from lumps (abscesses), but some failed to grow bacteria. No bacteria were isolated from 69-85% of normal udder halves in the current three studies which is comparable to the 75% previously reported by Ridler *et al.* (2021). The bacteria isolated from normal udder halves (15-31%) could be due to subclinical mastitis or opportunistic normal skin or environmental microbial flora such as CNS (Spuria *et al.*, 2017). Marogna *et al.* (2010) analysed the correlation between bacterial positivity and clinical findings such as visible abscesses, nodules and milk abnormalities and reported that ewes with such clinical findings were 105 to 317% more likely to be bacterial positive compared to samples from udders/milk with no abnormalities.

Combined these current studies showed that a considerable percentage of udder halves categorised as either hard or lump isolated no bacteria while a small proportion of normal udder halves did isolate bacteria. This variation in the association between udder half defect and bacterial positivity might be due to differences in bacterial species pathogenicity (i.e. infection with some species may be more likely to result in palpable defects) or persistency over time with the dynamic nature of the defects (van den Crommenacker-Konings *et al.*, 2021). In many cases the defects were likely to have been chronic and, while they are most likely to have been initiated by bacterial infection the bacteria may have been cleared from the mammary gland over time. It is possible the negative culture results could also be due to low accuracy of traditional bacterial culture (Zadoks *et al.*, 2014). Overall, the association between palpable udder defects and bacterial culture positivity indicates the effect of aerobic bacteria; however, the weakness of the association could also be suggestive of involvement of pathogens other than aerobic bacteria such as anaerobic bacteria, rickettsial organisms, viruses or sterile cysts (Greeff & du Preez, 1985; Minguijón *et al.*, 2015).

Mycoplasma species have been reported to cause contagious agalactia in dairy sheep and goats overseas (Migliore *et al.*, 2021), but this condition has not been reported in sheep in New Zealand, nor have any of the four Mycoplasma species implicated in this condition been isolated from New Zealand sheep. In the present study Mycoplasma organisms were not tested for.

Among the commonly identified bacterial species in the present studies, *M. haemolytica* and *Streptococcus uberis* were predominantly isolated from defective udder halves (hard or lump) during lactation, weaning, post-weaning and pre-mating. Similarly, in previous studies *M. haemolytica* and *Streptococcus uberis* were isolated from hard udder halves or udder halves with lump in the post-weaning period (Ridler *et al.*, 2021; Smith *et al.*, 2015). These bacterial species have also been described as an important and highly frequent bacterial species isolated from clinically mastitic milk in non-dairy breed ewes (Arsenault *et al.*, 2008; Barber *et al.*, 2006; Omaleki *et al.*, 2011; Omaleki *et al.*, 2016). Watkins and Jones (1992) suggested that *M. haemolytica* was equally important or more significant than *S. aureus,* the latter of which is the most identified bacteria from udders of dairy ewes (Gelasakis *et al.*, 2015), possibly due to transmission of the bacteria via lamb suckling. *M. haemolytica* resulted in higher SCC response than *S. aureus* which showed the pathogenicity of the pathogen in non-dairy ewes (Persson *et al.*, 2017). Experimental infection of ewe udders wtih *M. haemolytica* has been associated with small nodules with thin walled abscesses (Watkins & Jones, 1992) and serious pathological changes with little or no milk expression in later stages of lactation (El-Masannat *et al.*, 1991). *Streptococcus uberis* is a common pathogen found in milk of both dairy cows and dairy sheep and is known to form mammary gland abscesses in sheep (Rosa *et al.*, 2019; Smith *et al.*, 2015). The isolation of this bacterium from udder halves with palpable udder defects in the present study may have been due to the chronicity of the defects which provided extended time to develop large sized abscess.

Staphylococcus aureus has been reported to be the most commonly identified bacterial species in both dairy and non-dairy ewes with subclinical mastitis (Koop *et al.*, 2010; Mavrogianni & Fthenakis, 2007), acute clinical mastitis or chronic mastitic udders with lump or abscesses (Smith *et al.*, 2015), and defective udders with lumps, diffuse hardness, nodules or open abscess (Saratsis *et al.*, 1998). In the present study, *S. aureus* was isolated in comparable numbers from defective (lump or hard) and normal udder halves at weaning, pre-mating and isolated pre-dominantly from defective udder halves during lactation. Ridler *et al.* (2021) and Smith *et al.* (2015) isolated the most *S. aureus* from udder halves categorised as hard or lump during the dry period. *S. aureus* have been reported to be less frequent in sub-clinical infection (Arsenault *et al.*, 2008). Nevertheless, the comparable number *S. aureus* isolation from udder halves with or without clinically identifiable or palpable udder half defects during the dry period in the present study might be because some of the udder halves categorised as normal in the present studies had a previous history of udder defects.

It has been reported that CNS species were the most prevalent bacteria isolated in clinically normal or sub-clinical mammary infection (Gelasakis *et al.*, 2015; Persson *et al.*, 2017; Spuria *et al.*, 2017). In the present study, CNS were predominantly isolated from normal udder halves with no palpable defects, which agrees with (Ridler *et al.*, 2021). However, CNS were also isolated from udder halves categorised as either hard or lump which could suggest potential pathogenicity of some CNS species (such as *S. simulans* and *S. chromogens*) to be associated with clinically noticeable changes, as it was observed in experimental infection with *S. simulans* in non-dairy ewes (Fthenakis & Jones, 1990a). CNS species have been isolated from up to 20% of clinical mastitis cases in cows and has been referred to as an emerging mastitis pathogens (Persson Waller *et al.*, 2011; Pyörälä & Taponen, 2009). *Trueperella pyogenes* was only isolated from swab samples collected at pre-mating and post-weaning from udder halves categorised as hard or lump, but not during lactation or milk samples at weaning. Similarly, this bacterium has been associated with dry period mastitis (Saratsis *et al.*, 1998).

Individual udder halves that were bacterial culture negative at the start of lactation almost always remained negative in repeated milk samples across the first six weeks of lactation, weaning and post-weaning. However, udder halves that were bacterial culture positive in the first week of lactation almost always either persisted with positivity or resulted in no expression of milk at later stages. *M. haemolytica, S. xylosus* and *S. pluranimalium* were persistently observed over four or more weeks in an individual udder half. *S. aureus* and other CNS species were either present for a short period or remained stable for four or more weeks. The other less frequently identified bacterial species mostly occurred for a very short period. This finding agrees with a previous study which reported *M. haemolytica* being stable in its presence over a three week period in lactation, but *S. aureus* and CNS were only moderately stable (van den Crommenacker-Konings *et al.*, 2021). While udder half defects change over time (Chapter 4: e.g. defective udder halves changed to normal as days in lactation advanced), persistence of these commonly identified bacterial species in an udder half may indicate stability of these bacterial species even after a defective udder half changed to normal. This could cause future defect recurrence in these udder halves or be a potential source of infection for other ewes in the flock.

As expected, those udder halves that did not express milk at sample collection time in the early weeks of lactation had less chance of expressing milk at later stages of lactation or at post-weaning. However, in those udder halves that did express small amounts of milk at later stages, the samples were bacterial positive. In these cases, *M. haemolytica S. chromogenes*, *S. uberis S. aureus*, and *S. simulans* were identified which may imply the persistency or severity of these species. In those udder halves that did not express milk at all during lactation, 83% were bacterial culture negative in post-mortem swab samples, collected three weeks post-weaning. It is possible that these udder halves were bacterial negative throughout lactation, but it is more likely that they were positive at some time in lactation and then resolved by three weeks post-weaning. In chronic mammary inflammation, the mammary immunity can clear or reduce bacterial load to below limits of detection by bacterial culture at post-weaning (Kuehn *et al.*, 2013) and therefore be reported as culture negative. In those udder halves that did not express milk, therefore, assessing the bacterial status while the udder was detected with either diffusely hard or lump during lactation, before changing into normal or other defect category may provide satisfactory explanation.

Bacteria identified from mammary swab and milk samples collected in individual ewes post-weaning were almost always (91.3%) in agreement. The only difference occurred in four udder halves that had swab negative cultures, but the milk sample was positive. The agreement between milk and tissue swab bacterial species identified was higher compared to a previous report by Smith *et al.* (2015). This might be because most of the lumps in this study were in the gland cistern area and were not capsulated or closed, therefore if bacteria were present in the lump, they were also present in milk. Alternatively, the three CNS species found only in the milk could be opportunists from the teat skin or environment or possibly transfered to milk by suckling from the mouth of the lamb (Dahlberg *et al.*, 2020; Fragkou *et al.*, 2007; Mavrogianni *et al.*, 2007a; Spiegel *et al.*, 2022). Nearly all the swab samples collected from palpable udder defects (abscess) in the current studies showed one distinct colony type on culture. Overseas authors have reported that mammary abscesses are often polymicrobial (Brook, 2002; Smith *et al.*, 2015). It is possible that not only the presence of abscesses but also the location and degree of capsulation of abscesses may play a role in the bacterial diversity of the abscesses and/or agreement between mammary abscess swab and milk samples. Overall, the very high agreement of bacterial species isolated from milk and mammary tissue swabs indicates that identifying bacterial species in udder halves by milk sampling can be a reliable and practical method.

In the current studies, three or more dissimilar colony types with no dominant colony type were considered contaminated (Adkins *et al.*, 2017). Only a small number of contaminated samples were identified in Study B and Study C, which is comparable to previously reported findings (Mørk *et al.*, 2007; Moroni *et al.*, 2007; Ridler *et al.*, 2021). However, Study A recorded a higher-than-expected contamination percentage in milk samples which may be due to several factors. At the time of sample collection (weaning) some ewes expressed very little milk and therefore, discarding the first few squirts of milk from the teat before sample collection was not practically applicable, particularly in cases where mammary secretion was thick and exudative. In addition, due to persistent rain the ewes were wet and had soil contamination on the udder/teats on the day of sample collection, which likely increased the contamination of the teats and hence the milk samples despite efforts to collect samples in an aseptic manner. In this study, udder halves with no milk expression during lactation and those with contaminated bacterial findings were excluded from the statistical data analysis, but exclusion of these udder halves did not change defective (hard and lump) to normal udder half proportion.

In the current studies, commonly identified pathogenic bacterial species were associated with udder halves categorised as hard or lump, confirming the most likely cause of these palpable udder half defects. Bacterial species such as *M. haemolytica* were stable over time, which could result in future recurrence of udder defects or act as a source of infection for other ewes. However, in the present study the number of bacterial species identified to assess persistency over time in an individual udder half was not large enough to undertake statistical analysis; hence the descriptive analysis should be interpreted with cation. To more accurately describe the bacterial species' change over time, a higher number of isolates would be needed for each bacterial species, which would require a greater number of ewes than the current study.

In conclusion, common bacterial species that were isolated from udder halves categorised as either lump or hard were *M. haemolytica* and *Streptococcus uberis* whereas CNS were more commonly isolated from normal udder halves. *S. aureus* was isolated from both defective and normal udder halves in comparable numbers with combined high frequency at pre-mating and weaning but predominantly from defective udder halves during lactation. Udder half milk bacterial positivity was stable in repeated assessment across lactation, weaning and post-weaning; however, the stability over time varied among the species involved. *M. haemolytica S. xylosus* and *S. pluranimalium* were persistent while Staphylococcal species including *S. aureus* stability were variable in their persistency. Mammary tissue swab and udder half milk methods of bacteria sampling presented a very high agreement of bacterial positivity and species identified at post-weaning. Hence, identifying ewes with bacterial species predominantly identified from udder halves categorised as hard or lump and which appear more stable over time could contribute to making culling decisions and minimize future recurrence of palpable defects or mastitis.

Chapter 7: Gross Pathology and Histological Changes of Palpable Udder Defects in Non-Dairy Ewes

7.1 Abstract

AIM: To describe gross and histological lesions of palpably defective (hard or lumps) udder halves of non-dairy (Romney) ewes at weaning, three weeks post-weaning, and at pre-mating.

METHODS: Udder halves were palpated from a total of 127 mixed-age non-dairy breed (Romney) ewes across three studies, and the whole udder was collected after slaughter/euthanasia. Post-mortem, each udder half was externally examined and systematically dissected for internal gross examination. At least one sample was collected from each udder half for histology, and a semi-quantitative histological grading system was applied to assess mammary inflammation and tissue damage. Data analysis included descriptive statistics, chi-square test of independence, and multinomial logistic regression.

RESULTS: Mammary cranial midline lumps were typically broadly spherical subcutaneous masses, histologically comprising variably sized multifocal cysts containing fluid consistent with normal milk. Udder halves categorised as hard were diffusely enlarged and typically contained a single large abscess within the gland on gross dissection. Histologically, udder halves categorised as hard consistently contained one or more pyogranulomas (92.8% (12/13) in Study B and 100% (1/1) in Study C) within glandular tissue. In contrast, udder halves categorised as lump showed marked variation in both gross pathology and histology, ranging from normal appearance to severe chronic inflammation with a purulent exudate. Udder halves categorised as defective (hard or lump) were respectively five times and 145 times

more likely ($p<0.05$) to have a moderate or severe histological inflammatory grade, compared to udder halves categorised as normal. In contrast to defective udder halves, most (82.2% (60/73) in Study B and 77.1% (54/70) in Study C) of the udder halves categorised as normal on palpation were grossly and histologically normal or showed only minimal to mild histological inflammation.

CONCLUSION: These findings suggest cranial midline lumps are not typically associated with significant inflammation or structural tissue change and may not cause permanent damage to the mammary gland. Therefore, these ewes with cranial midline lumps should not be culled. However, udder half hardness is consistently associated with typically chronic and more extensive inflammation, suggesting ewes with palpable hard udder halves should be culled to avoid likely future impacts on milk production. Histological inflammation within udder halves categorised as lump was much more variable in both extent and chronicity, and consequently, the impact on milk production of udder halves with lumps is less certain. Further studies are needed to better determine the impact of udder halves categorised as lump on milk producton.

7.2 Introduction

Palpable udder defects constitute any abnormal mammary tissue consistency that can be detected by palpation. In non-dairy ewes, diffuse hardness of the udder half and one or more lumps of various sizes within the udder half parenchyma are the two most commonly reported palpable udder defects in non-dairy ewes (Griffiths *et al.*, 2019a; Ridler *et al.*, 2021). High lamb mortality and reduced growth rates have been reported in lambs born and reared by ewes with defective udders (Griffiths *et al.*, 2019a; Griffiths *et al.*, 2019b). Udder halves categorised as either hard or lump have been associated with either low milk yield or expression of no milk (Zeleke *et al.*, 2021).

In udder halves categorised as hard, the whole udder half has been described as having diffusely firm or hard consistency without overt signs of inflammation or pain upon palpation (Griffiths *et al.*, 2019a; Saratsis *et al.*, 1998). Bruce *et al.* (2013) described very hard udder halves in ewes in early lactation, a condition they termed “hard udder”, and characterized this as chronic mastitis with non-suppurative to multifocal abscessation and severe galactophoritis. However, gross and histological changes in udder halves categorised as hard are not well-described in the published literature. In contrast, udder lumps are typically small to large intramammary nodules with variably firm or soft consistency within the udder halves (Blagitz *et al.*, 2014; Griffiths *et al.*, 2019a).

Incision and gross examination of udders with one or more lumps typically reveal a single or multiple abscesses of various sizes in different locations within the gland (Peterson *et al.*, 2017; Smith *et al.*, 2015). Udder lumps are variably associated with open abscesses or healed scars/scabs of ruptured abscesses. In addition, udder halves with lumps often expressed or contained abnormal mammary gland secretions including clots and flakes, serous fluid and purulent, blood-tinted or frank hemorrhagic secretion (Saratsis *et al.*, 1998; Smith *et al.*, 2015; van der Molen *et al.*, 1985; Watkins & Jones, 2007). Occasionally, discrete masses in the cranial midline of the udder (cranial midline lumps or “CMLs”) have also been reported as a palpable udder defect (Ridler *et al.*, 2021). Unlike lumps located in the core of the mammary gland, CMLs are not reported to be associated with failure of lambs to survive or reduced growth rate from birth to weaning (Griffiths *et al.*, 2019b).

According to the available literature, the occurrence of udder defects in ewes in New Zealand ranges from approximately 2-7.5% (Griffiths *et al.*, 2019a; Peterson *et al.*, 2017). Survey results indicate that approximately 75% of farmers in New Zealand examine the udders of their non-dairy ewes (Corner-Thomas *et al.*, 2016), with findings assumed to contribute to decisions about animal culling or retention. Use of a standardized udder defect palpation (and inspection) scoring system and its systematic application at weaning or pre-mating has been suggested as useful to assess to identify ewes that are likely to be unsuitable for retaining in the breeding flock (Blagitz *et al.*, 2014; Griffiths *et al.*, 2019a). However, understanding the pathology of palpable udder defects may also provide additional pertinent information and contribute to more rational and evidence-based culling decisions of ewes with udder defects. However, detailed descriptions of the pathology of the various udder half defects are lacking. Therefore, the objective of this chapter was to describe, and where possible, further characterise gross and histological lesions of palpably defective (hard or lumps) udder halves of non-dairy breed (Romney) ewes at weaning, three weeks post-weaning and at pre-mating.

7.3 Materials and Methods

For this chapter, udder and udder lesion samples were collected from mixed age non-dairy breed (Romney) ewes culled or euthanised from one of three different studies. These studies are described in detail as Studies A, B and C in the General Methodology chapter (Chapter 3) and are re-summarized below.

Study A was based on longitudinal udder defect data gathered in non-dairy breed (Romney) ewes over a three-year period from 2016 to 2018 at Massey University's Riverside farm, Wairarapa, New Zealand. Manual udder assessment was performed at pre-mating, pre-lambing, docking and weaning in each year. At the end of the three-year study period, seven ewes with CMLs were identified on the day of weaning (December 5th, 2018) from 752 udders (1504 udder halves) and samples from these seven ewes were used for the pathology studies in this chapter. These ewes with CMLs were humanely euthanised immediately prior to sampling.

Study B was based on 74 ewes with palpable udder defects from a farm reporting higher than usual numbers of udder half defects post-weaning in North Canterbury, in the South Island of New Zealand. All ewes deemed fit for transport by the attending veterinarian were transported via a commercial transport agency to Massey University's Tuapaka farm, Palmerston North (March 2019). One week after arrival, these ewes were slaughtered through a commercial abattoir (n=70) or euthanised at Massey University (n=4).

Study C was a one-year longitudinal study that comprised 48 mixed age non-dairy breed (Romney) ewes. These ewes were selected from a commercial Massey University (Riverside) farm. At the time of selection, ewes with known udder defect history, both defective (hard or lump) and normal, were included in the study. Forty-six of these ewes were culled and slaughtered through a local commercial abattoir at the end of the study approximately three weeks post-weaning (December 21st, 2019). Tissue samples were collected from 92 udder halves (46 ewes).

7.3.1 Udder scoring

Each udder half was scored according to Griffiths *et al.* (2019a) and categorised as hard, lump, or normal (Chapter 3, Section 3.6).

7.3.2 Post-mortem udder collection

As detailed in Chapter 3 (section 3.7), in all three studies, the whole udder was collected immediately after routine humane slaughtering/euthanasia, with gross examination undertaken and tissues collected for histology within six hours of euthanasia.

7.3.3 Gross udder examination

External and internal gross examination was performed separately for each udder-half. Udder halves were inspected externally and palpated thoroughly for any abnormality including hardness, lumps, nodules, blind teats, fistulas, scabs, abscesses, or evidence of injury. Milk expression was checked by squeezing the teats prior to dissection.

Following external gross examination, an incision with a scalpel at the base of the teat (gland cistern) was made to confirm the presence or type of any secretion in the gland cistern. The incision was then extended longitudinally to expose the teat cistern and canal ventrally and parenchyma of each udder-half dorsally by dividing the udder-half roughly into two halves. Multiple parallel parasagittal incisions were made to assess the presence of any lesion within the core of the parenchyma (Madel, 1981; Smith *et al.*, 2015). If present, the location of any defect(s) were recorded as being in one of three main parts of the gland: the area surrounding the gland cistern with the teat at the centre (zone 1); the caudal and cranial core of the mammary tissue (zone 2) or the cranial or caudal extension outside the core mammary tissue from the parenchyma (zone 3) as shown in Figure 7.1. All relevant information and any lesions present were recorded using the same format (Appendix II).

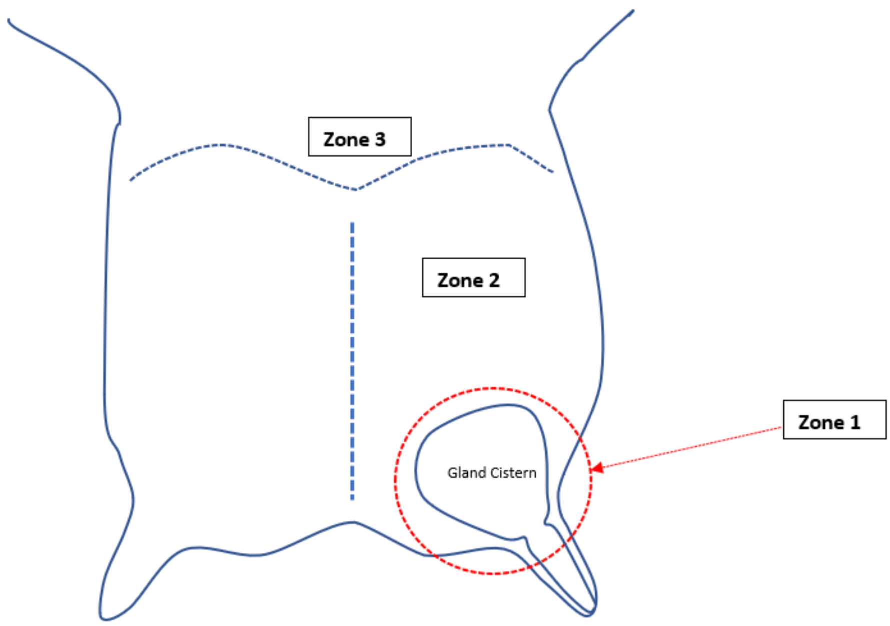


Figure 7.1 Diagrammatic illustration of an udder showing three palpable udder defect occurrence and tissue sample collection zones in non-dairy breed (Romney) ewes (viewed from the rear/front). Zone 1: an area with the gland cistern at the centre and immediate surrounding mammary tissue; zone 2: the caudal or cranial core of the mammary gland tissue; and zone 3: the dorso-cranial or dorso-caudal region outside the core mammary tissue.

7.3.4 Histological examination and grading

Post-mortem, representative tissue samples were collected from all udder halves. For a palpably normal udder half, a single sample was collected from the mammary parenchyma adjacent to the gland cistern (zone 1). For an udder half with a defect located in zone 1, one or two samples were collected from the same site as the normal udder halves in zone 1. For an udder half with a defect located in zone 2 or 3, one or two samples were collected from the site of the defect in zone 2 or 3 in addition to a single sample from zone 1. Each sample was trimmed to approximately 6-8mm thickness and fixed in 10% buffered formalin for at least 24 hours, then routinely processed and embedded into paraffin blocks. Haematoxylin and eosin (H&E) stains were performed on 4µm sections of tissue cut from the paraffin blocks.

Following sub-gross assessment of each tissue section to identify areas of interest or inconsistency, a minimum of ten high power fields (400x magnification) which included glandular tissue (if present on the section) were assessed and graded using a semi-quantitative (0-4) histological inflammation grading system based on and modified from previous studies (Breyne *et al.*, 2017; Camperio *et al.*, 2017; Gans, 2020). Briefly, sections were evaluated for the presence and extent of a range of histological features previously assessed in mammary gland lesions as consistent with the presence of inflammation or tissue damage, including the presence of inflammatory infiltrate (interstitial or alveolar/ductal), fibrosis or granulation tissue, lymphocytic foci, alveolar/ductal dilation, epithelial cell degeneration or necrosis, loss of tissue architecture and alveolar atrophy. An overall or average grade from 0-4 for each section was given (Table 7.1), where grade 0 indicated that inflammation was essentially absent and tissue architecture was maintained; grade 1 indicated the presence of minimal focal or multifocal inflammation with maintenance of tissue architecture; grade 2 indicated mild inflammation consistently or multifocally present with maintenance of tissue architecture; grade 3 indicated moderate inflammation consistently or multifocally and prominently present with possible loss of tissue architecture and/or necrosis in places; grade 4 indicated severe and extensive inflammation consistently and diffusely or overwhelmingly present with necrosis or effacement of tissue architecture in multiple areas.

In addition to the inflammation grade of 0-4, in tissue sections where inflammation was present (i.e. grade 1 or higher), the predominant type of inflammation was recorded as a suffix of either acute ("-A") (mainly neutrophilic and /or fibrinous inflammation), chronic ("-C") (mainly macrophages, lymphocytes/plasma cells and/or fibrosis) or mixed ("-M") (a combination of both acute and chronic findings).

Overall, the grade result for each sample was summarized by combining both the grade (0-4) system and the predominant inflammation type (-A, -C or -M). For example, grade 2-C represented findings of mild chronic inflammatory changes within the examined tissue section(s) including mainly macrophages, lymphocytes/plasma cells and/or fibrosis.

Table 7.1 The semi-quantitative histological inflammation grading system of the mammary gland applied in the assessment of palpable udder defects in the non-dairy breed (Romney) ewes.

Inflammatory grade	General grade descriptor of inflammation	Approximate percentage of section affected by lesion	Mammary tissue histology description
0	Absent	0%	None of the histologic features (necrosis, neutrophils, lymphocytes, or bacteria) observed in the examined 400x field or observed but considered part of normal structures/tissue (e.g. neutrophils present but part of blood). Normal tissue architecture maintained.
1	Minimal	1-5%	One or more of the histologic features focally to multifocally present in very low numbers/amounts in the examined 400x field; tissue architecture maintained and no necrosis.
2	Mild	5-20%	One or more of the histologic features consistently or multifocally present in low numbers/amounts in the examined 400x field; tissue architecture maintained and no necrosis.
3	Moderate	20-60%	One or more of the histologic features consistently or multifocally present and prominently present in the examined 400x field; tissue architecture variably maintained or damaged/lost in occasional places with necrosis present.
4	Severe	>60%	One or more of the histologic features consistently, extensively, diffusely or overwhelmingly present in the examined 400x field; tissue architecture is damaged/lost in multiple areas and necrosis is extensively present.

7.3.5 Data analysis

As the number of udder halves categorised as either hard or lump was low, these categories were combined for statistical analysis. Hence, udder halves were recategorised as either defective or normal for quantitative data analysis, while histological inflammatory grades were categorised into five grades: grade 0 (no inflammation), grade 1 (minimal), grade 2 (mild), grade 3 (moderate) and grade 4 (severe) regardless of chronicity. For instance, a histological grade of 2 (mild) could include acute, chronic or mixed mild inflammation. Descriptive statistics were used to describe gross pathological and histological features and the results tabulated for all three studies. Further, analysis was undertaken to quantify the association between udder defect category and histological lesions in Studies B and C. Chi-square test of independency was used to assess associations between udder half defect and the histological grade at pre-mating (Study B) and three weeks post-weaning (Study C). Further, multinomial logistic regression was applied to assess the association of udder half defect (defective vs normal) and udder half histological grade (none, minimal, mild, moderate or severe) using “nnet” package in R statistical software (R Core Team, 2020).

7.4 Results

A total of 127 udders across the three studies (A (n=7), B (n=74) and C (n=46)) were collected and grossly examined and samples taken for histology from all udder halves. Gross examination were made and samples for histology were collected from each udder half separately. Twenty-eight samples were collected for histology from the seven ewes from Study A at weaning. One hundred and sixty-one samples were collected from 142 udder halves from Study B at pre-mating, while 93 samples for histology were collected from 83 udder halves from Study C three weeks post-weaning.

7.4.1 Study A: Cranial midline lumps (CMLs)

Of the 752 ewe udders assessed at weaning in Study A, seven ewes presented with either one or two CMLs on palpation.

7.4.1.1 Gross pathology

Both udder halves of all seven ewes with CMLs in Study A were normal in both zones 1 and 2 on external gross appearance and on incision apart from the presence of one to three easily palpable and broadly spherical masses (CMLs) located superficially beneath the skin within the subcutaneous tissue in zone 3 of the cranial midline of the udder (Figure 7.2). These CMLs ranged in diameter from 2cm to 10cm and were palpably turgid and slightly firmer than the adjacent unaffected udder tissue. On external examination, CMLs initially appeared separate from the adjacent mammary gland. However, based on palpation and observation after removing the udder skin, a clear demarcation between the periphery of the CML and the rest of udder tissue was not evident. On incision, all CMLs contained variable amounts of opaque cream-coloured fluid with the consistency and appearance of normal milk.

7.4.1.2 Histology

In six of the seven ewes from Study A, histology from both the right and left halves of the udder parenchyma showed typical features of normal lactating mammary gland, with lobules mainly comprising acini lined by cuboidal to flattened epithelium and variable numbers of discrete clear cytoplasmic vacuoles (lipid droplets). Most acini contained pale eosinophilic fluid admixed with clear vacuoles (consistent with normal milk; proteinaceous fluid containing lipid droplets; Figures 7.3A and B). Moderate numbers of acini contained dense amorphous basophilic and concentrically lamellated or ringed structures up to 100μm diameter (corpora amylacea). Interlobular fibrous and adipose tissue was variably present but generally sparse.

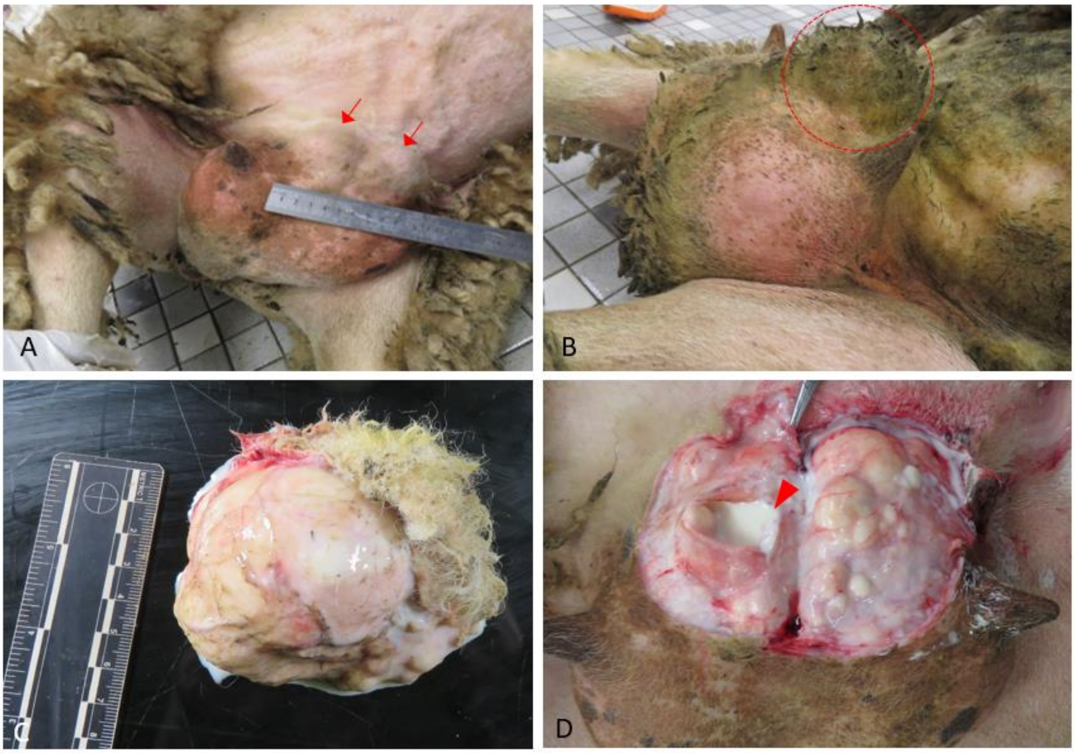


Figure 7.2 Study A: Typical gross pathology of mammary cranial midline lumps (CMLs). A and B: Intact CML (red arrows and within red circle) with ewe is sitting on rump. C: Large intact CML (approximately 8cm diameter) after excision. D: CML after skin and lump have been incised and exposed showing multiple cream-coloured nodules within the CML; one nodule has been incised (red arrowhead) and contains cream-coloured fluid consistent with normal milk.

In one of the 7 ewes (#1710), sections of both the right and left udder halves showed evidence of moderate chronic-active mastitis in both left and right udder halves (Figures 7.3C and D). In these udder halves, multifocal small to medium-sized aggregates of lymphocytes and neutrophils admixed with lower numbers of macrophages within both interlobular fibrous tissue as well as individual acini were present in both udder halves. Inflammatory cells occasionally effaced acini and ducts, and intact glandular epithelial cells often appeared degenerate.

Sections of CMLs from 6 of the 7 ewes comprised multifocal cysts of variable and often large size lined by a single layer of cuboidal to flattened epithelium that were generally either empty (likely due to content displacement during processing) or containing homogenous eosinophilic fluid (consistent with high protein fluid such as milk; Figures 7.4A-D). These CMLs were considered histologically consistent with dilation(s) of mammary ducts or acini forming cysts, and were located within normal udder tissue with adjacent normal acini variably containing secretion.

In one ewe (#1710), the CML was histologically different to CMLs in all other (6) ewes. In this animal, the wall of the CML comprised a thick fibrous capsule surrounding large numbers of neutrophils admixed with fewer macrophages; fibrin, karyorrhectic cellular debris and clear acicular clefts within central areas of the CML were also observed. This CML was considered histologically consistent with a chronic abscess.

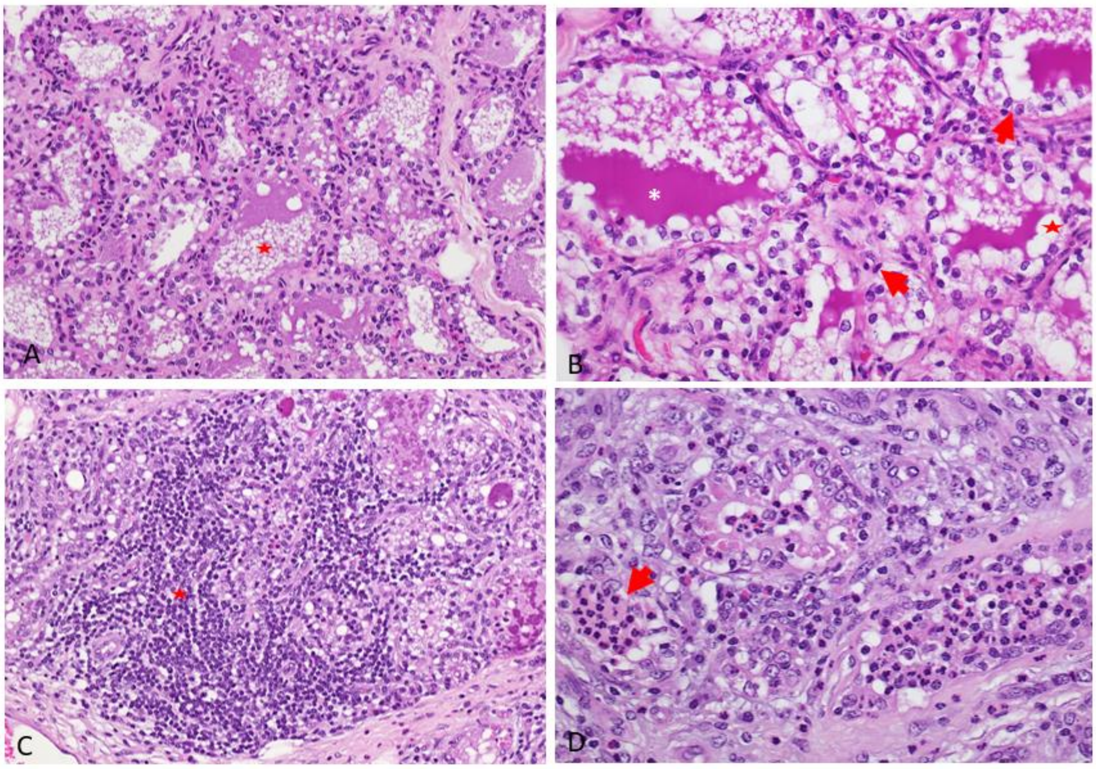


Figure 7.3 Study A: Histology of mammary gland tissue collected from zone 1 in udders with CMLs. Haematoxylin and eosin staining. A (200x) and B (400x): Typical findings in ewes with CMLs showing parenchymal mammary gland tissue with lobules comprising large numbers of acini lined by cuboidal to flattened epithelium (red arrows) and containing eosinophilic fluid (white asterisk) and clear cytoplasmic vacuoles (red stars); C (200x) and D (400x): Atypical findings in one ewe with CML, showing parenchymal mammary gland tissue containing lymphocytes (red star) and neutrophils (red arrow) admixed with lower numbers of macrophages within individual acini and extending into interlobular tissue (C (200x) and D (400x)).

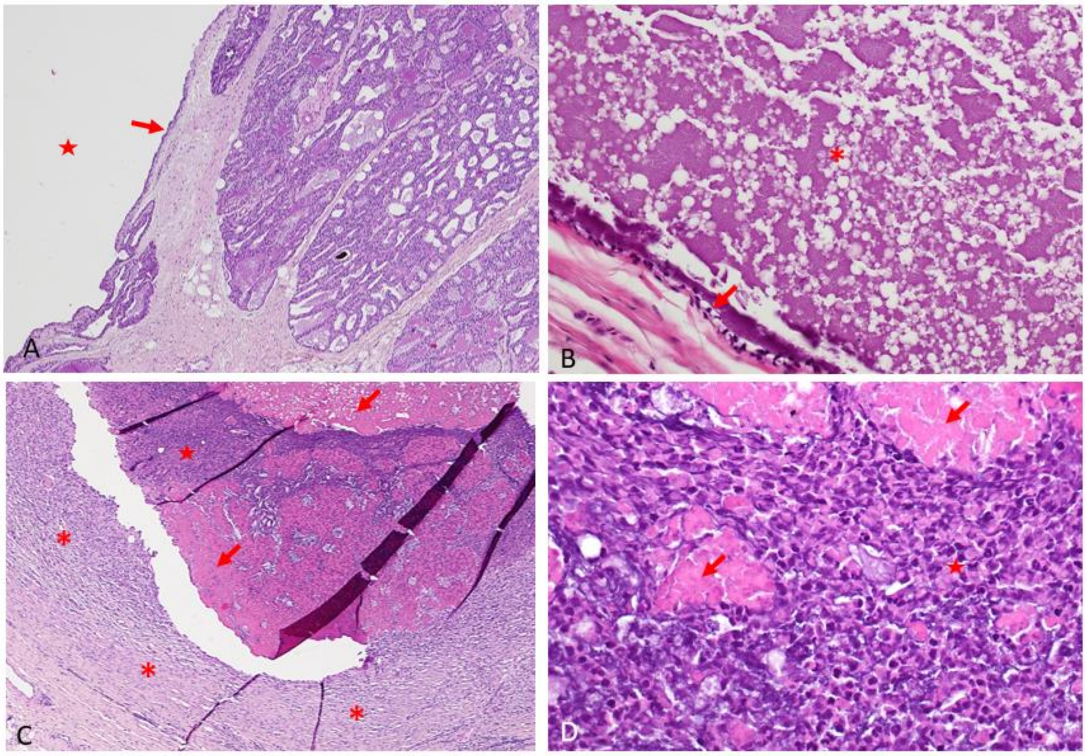


Figure 7.4 Study A: Histology of mammary CMLs collected from zone 3 in ewes with CMLs. Haematoxylin and eosin staining. A (40x) and B (40x): Typical findings in 6/7 ewes with CMLs showing dilated mammary cyst with either empty content (red star) likely due to displacement during processing or eosinophilic fluid containing many large clear (likely lipid) vacuoles (red star). Cysts are lined by a single layer of cuboidal to flattened epithelial cells (red arrows) containing homogenous eosinophilic fluid with many lipid vacuoles (red asterisk). C (40x) and D (400x): Atypical findings in one ewe with CML showing abscess formation comprising a thick fibrous capsule (red asterisks) surrounding a central region with myriad neutrophils (red stars) admixed with fewer macrophages, fibrin (red arrows) and karryorhectic cellular debris. Folding of the tissue section apparent in Figure 7.4C is an artefact of slide preparation.

7.4.2 Study B: Udder halves categorised as hard, lump and normal

In Study B, from the total of 148 udder halves (74 ewes) assessed, 14 (9.5%) were categorised as hard, 60 (40.5%) as lump, and 74 (50%) as normal. Gross pathological characterization of udder halves categorised as hard, lump, or normal is presented in Table 7.2.

7.4.2.1 Gross pathology

Gross pathology: udder halves categorised as hard

The 14 udder halves categorised as hard appeared normal in shape and slightly enlarged in size compared to their contralateral normal udder halves on gross examination (Figure 7.5A). No external signs of redness/inflammation were observed in these udder halves. The consistency of the whole udder half was firm, and ewes did not exhibit signs of pain on udder palpation. All udder halves categorised as hard were unilateral, with a palpably normal contralateral udder half. Milk could not be expressed at all from teats on the affected udder half. In udder halves categorised as hard, one n=1 (7.1%) presented with yellow purulent discharge at the skin surface on the external skin of the udder consistent with recent rupture (Table 7.2).

On incision, mammary parenchymal tissues of the hard udder halves were variably soft to firm with occasionally gritty internal consistency. All except one udder half categorised as hard contained a single large (4-10cm diameter) lesion containing variable amounts of thick yellow to greenish purulent material (consistent with suppurative inflammatory exudate) on incision. The lesion occupied the whole parenchymal core including the gland cistern (Figure 7.5B, Table 7.2). Of these lesions, nine (64.3%) were contained within a fibrous capsule and were classified as abscesses (Figures 7.5B).

Table 7.2 Study B, summary of gross pathological findings based on gross appearance of udder halves categorised as hard (n=14), lump (n=60), and normal (n-=74) mixed age non-dairy breed (Romney) commercial ewes from North Canterbury in South Island, New Zealand examined prior to mating.

Gross appearance	Category	Hard	Lump	Normal	Total
Udder defect location	The whole udder half	14			14
	Zone 1		55		55
	Zone 2 (caudal)		4		4
	Zone 2 (cranial)		1		1
	Zone 3 (cranial midline)	-	-	-	-
	No defect			74	74
External udder appearance: Udder open (burst) with yellow purulent discharge or scab/scars	Yes	1	4		5
	No	13	56	74	143
Udder consistency on incision	Firm	8	28	1	37
	Soft	6	32	73	111
Mammary content/ secretions	Thick yellow to greenish purulent fluid	13	32	11	56
	Dark tan bloody fluid		3	3	6
	Clear watery fluid		2	16	18
	Milk like clumps/fluid		6	22	28
	No fluid/content	1	17	22	40

Note: Zone 1 refers to the area surrounding the gland cistern with the teat at the centre; zone 2 represents the caudal and cranial core of the mammary tissue, and zone 3 indicates the cranial or caudal extension outside the core mammary tissue from the parenchyma.

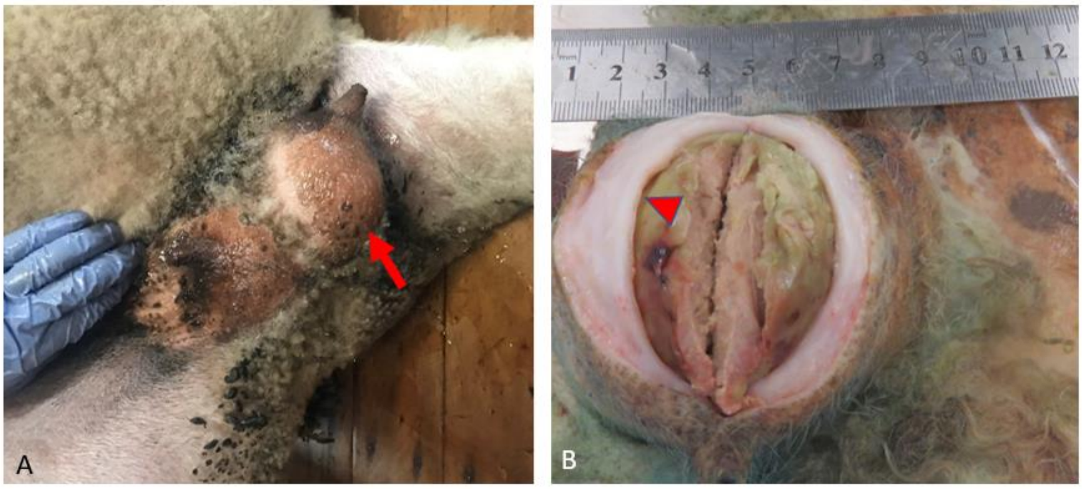


Figure 7.5 Study B. Typical gross findings in udder halves categorised as hard in non-dairy (Romney) ewes. A. Mammary gland from a ewe on sitting on her rump (shearing position) with the left udder half (arrow) categorised as hard appearing normal in shape but enlarged in size. B: Incision of the left mammary gland shown in A, showing large thick yellowish purulent exudate surrounded by a thick fibrous capsule (abscess) (arrowhead) occupying the whole parenchyma core and gland cistern.

Gross pathology: udder halves categorised as lump

Of the 60 udder halves categorised as lump in Study B, 54 (90%) were located in zone 1 while the remaining 10% were located within either the cranial or caudal mammary parenchymal core (zone 2; Table 7.2). Four (6.7%) udder halves categorised as lump showed either a recently ruptured abscess or more chronic scabs/scars on the udder skin (Table 7.2). Approximately half (n=32, 53.3%) of the udder halves categorised as lump contained thick opaque yellow exudate (pus) in gland cisterns on incision, while dark tan bloody fluid was observed in three (5%) udder halves. Clear watery fluid (3.3%) and white milk-like clumps/fluid (10%) were also observed (Table 7.2). After incision of the udder skin, 55 (91.5%) of udder halves categorised as lump contained a single nodule ranging from <1 cm to 4cm in diameter, while five of 60 (8.3%) contained multiple (two to five) nodules. Of the 32 udder halves with nodules containing yellow to greenish purulent discharge, 13 (40.6%) were contained within a fibrous capsule and were consistent with abscesses.

Gross pathology: udder halves categorised as normal

Grossly, all udder halves categorised as normal (n = 74) were of normal size and shape with homogenous soft consistency of the gland on palpation. Seventy-three of these udder halves (98.7%) were soft and elastic on incision (Table 7.2). The remaining one udder half was normal on palpation, but subsequently found to be firm on incision. Upon incision, udder halves categorised as normal contained secretion with variable gross appearance, ranging from white to cream-coloured fluid consistent with milk (n = 22, 29.3%) or no content/secretion (n = 22, 29.3%), while 16 (21.3%) contained clear watery fluid within the gland cistern. Low numbers of palpably normal udder halves contained yellowish purulent thick fluid (n = 11, 18.6%) or dark tan bloody fluid (n = 3, 5.1%) within the gland cistern (Table 7.2).

7.4.2.2 Histology

The histological features and inflammatory grades of udder halves categorised as hard, lump or normal in Study B showed variability in the presence of inflammatory lesions and grades seen. To better describe these inflammatory lesions, the dominant histologic grades, mostly observed within a minimum of ten high power fields, and range of observed grades are summarized and presented in Table 7.3.

Table 7.3 Study B. Histology grading of udder halves categorised as hard, lump and normal in 74 mixed age non-dairy (Romney) ewes from a commercial farm in North Canterbury, South Island, New Zealand prior to mating.

Dominant histological grade	Observed grade range	Frequency of udder halves			
		Hard	Lump	Normal	Total
0	-		-	-	-
1C	0C -2C		4	20	24
2C	1C - 3C	1	23	39	63
2M	2M			1	1
3C	2C - 3C		11	11	22
3M	3M		1	1	2
4C	0, 2C- 4C	8	11	1	20
4M	2M - 4M	4	6		10
Total		13	56	73	142

Note: The histological inflammatory grading scale (0-4, -A, -C and -M) used is as described above in section 7.3.4 and Table 7.1.

Histology: udder halves categorised as hard

All except one (n=12, 92.9%), of the udder halves categorised as hard had a dominant histological inflammatory grade of severe (grade 4) with either chronic or mixed inflammation observed (Table 7.3). A common (12/13, 92.3%) feature of these udder halves was the presence of a single (occasionally multiple) and often large abscess or pyogranuloma within the glandular tissue including the presence of mature granulation tissue and a chronic inflammatory infiltrate (Figure 7.6). Minimal to moderate chronic interstitial inflammation in the rest of the udder half section including within glandular tissue adjacent to the pyogranuloma was typically observed. Both abscesses and pyogranulomas typically comprised a central region of eosinophilic cellular and karyorrhectic debris admixed with viable and degenerate neutrophils (necrosis) and occasionally contained keratin fragments (Figures 7.6A and B), although in some sections at least part of the central core was absent from the section (likely lost during histological processing). The necrotic central region was typically surrounded by extensive mixed but mainly chronic inflammation, typically comprising epithelioid macrophages, lymphocytes (occasionally forming small aggregates) and plasma cells admixed with variable numbers of neutrophils (Figure 7.6 C) and mature granulation tissue or fibrosis more peripherally (Figure 7.6D). Three udder halves (21.4%) categorised as hard contained a significant number of dilated glands and ducts containing homogenous eosinophilic fluid (consistent with high protein fluid such as milk).

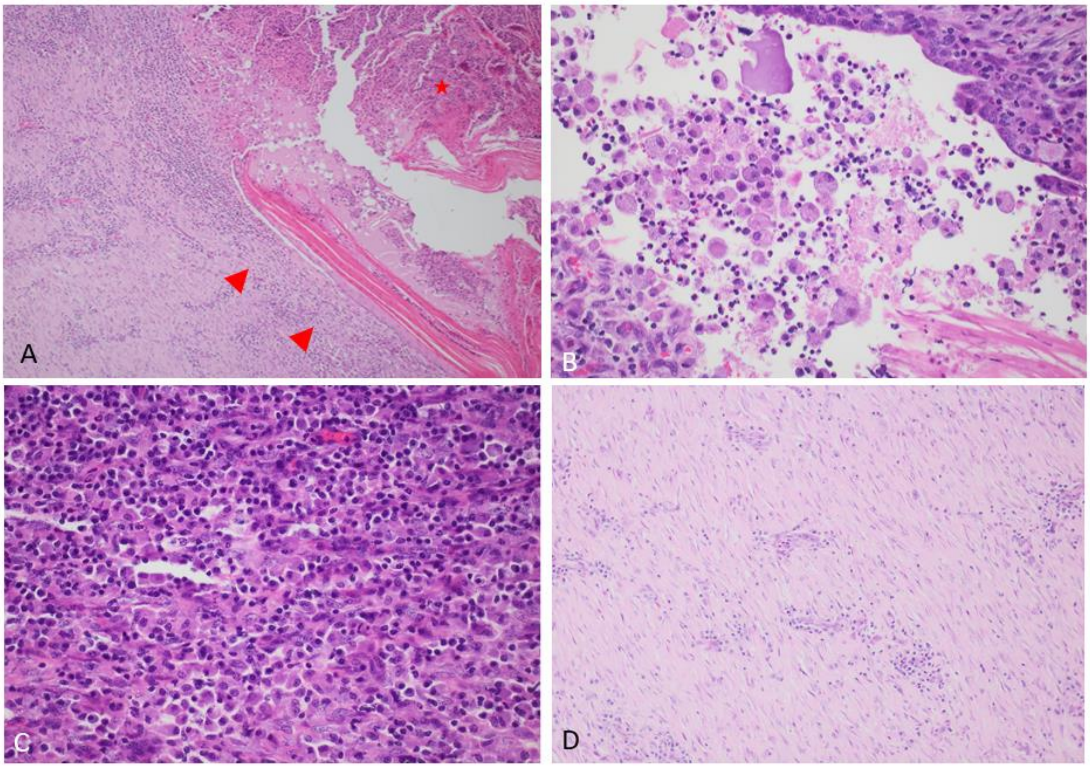


Figure 7.6 Study B: Histology of pyogranuloma in the mammary gland tissue collected from zone 1 in udder halves categorised as hard. Haematoxylin and eosin staining: A. Pyogranuloma with central region comprising eosinophilic cellular and karyorrhectic debris admixed with neutrophils (red star) surrounded by extensive mixed inflammatory cells and fibrosis more peripherally (red arrowheads). Brightly eosinophilic layers of keratin can be seen in the lower right quadrant of the image (40x). B. Central region of pyogranuloma showing eosinophilic cellular and karyorrhectic debris, neutrophils and epithelioid macrophages. Brightly eosinophilic keratin fragments can be seen in the lower right corner of the image (400x). C. Mixed inflammatory exudate surrounding central regions of the pyogranuloma comprising neutrophils, lymphocytes, and plasma cells (400x). D. Periphery of pyogranuloma with mature granulation tissue mainly comprising fibrosis admixed with low numbers of mixed inflammatory cells including lymphocytes and plasma cells (200x).

Histology: udder halves categorised as lump

In contrast to udder halves categorised as hard, the histological grade and findings within udder halves categorised as lump were extremely variable in Study B (Table

7.3). Across all examined samples, dominant histological grades ranged from minimal (score 1; n = 4, 7.1%) to mild (score 2; n = 23, 41.1%, Figure 7.7A) to moderate (score 3; n = 12, 21.4%, Figure 7.7B) to severe (score 4; n = 17, 30.3%, Figure 7.7C, Table 7.3). No udder halves categorised as lump had a dominant histological grade of 0 (no/minimal inflammation). Histological findings of chronic inflammation (-C) were present in the majority (n = 49, 87.5%) of udder halves categorised as lump, while a small number (n = 7, 12.5%) showed more mixed (-M, concurrent acute and chronic) inflammation. From the udder halves categorised as lump which had a severe histological inflammatory grade (n=17), most (n = 13/17, 76.5%) contained one or more pyogranulomas. Of the udder halves categorised as lump that had significant inflammatory changes (i.e. either moderate (grade 3) or severe (grade 4) inflammation, n = 29 in total), most (21/29, 72%) contained grossly visible purulent exudate on dissection, while the remaining (8/29, 28%) contained either non-purulent exudate or no content. However, from the udder halves categorised as a lump which had less extensive histological inflammation (i.e. either minimal (grade 1) or mild (grade 2) inflammation, n = 27 in total), 70% (19/27) contained either non-purulent exudate or no content.

7.4.2.3 Study B: Association of udder half defect status and histology grading

Compared to udder halves categorised as normal, udder halves categorised as defective were five times more likely ($p<0.05$) to have a moderate inflammatory grade on histology and 145 times more likely ($p<0.05$) to have a severe histological inflammatory grade (Table 7.4).

Table 7.4 Study B: Odds ratios (and SEM) of udder halves' histological inflammatory grades in udder halves categorised as defective compared to normal udder halves from 74 mixed age non-dairy (Romney) ewes from a commercial farm in North Canterbury, South Island, New Zealand prior to mating.

	Udder half histological inflammatory grades		
Udder half defect	Mild (Grade 2)	Moderate (Grade 3)	Severe (Grade 4)
Constant	2.00 (0.27)	0.60 (0.36)	0.05 (1.02)
Normal	Reference	Reference	Reference
Defective	3.0 (0.61)^{NS}	5.0 (0.68)	145.1(1.15)

Note: All estimates are significant except ^{NS} which refers to not significant (p>0.05)

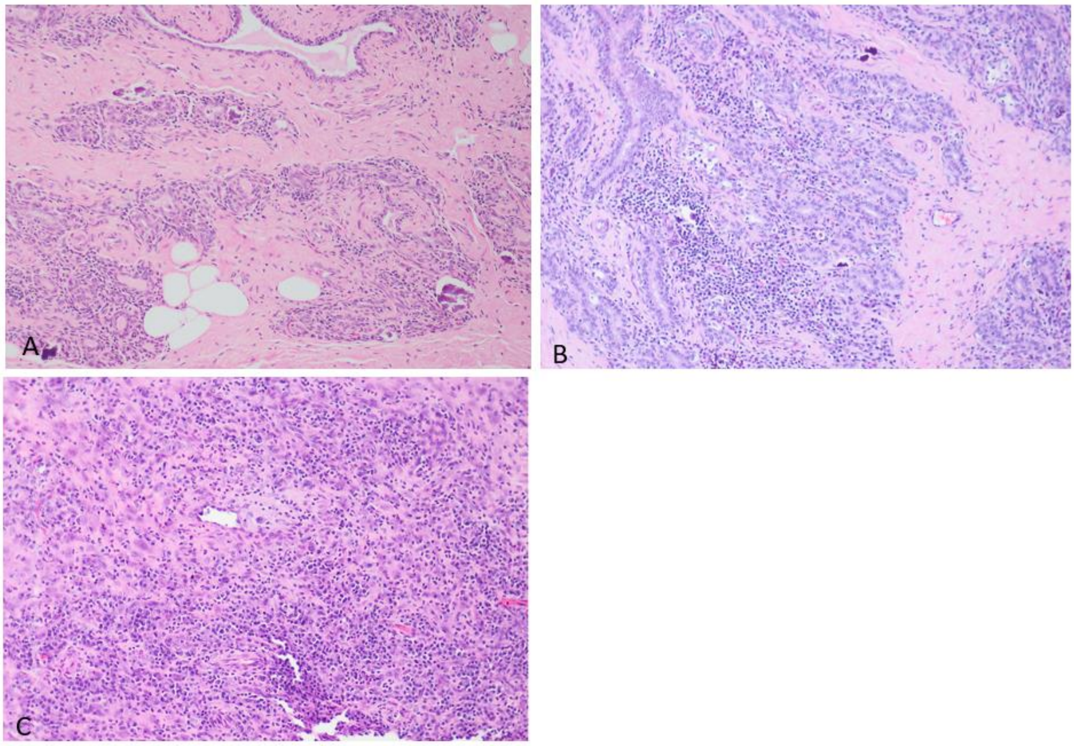


Figure 7.7 Studies B and C: Variable histology grades (mild, moderate and severe inflammatory) in mammary gland tissue collected from zone 1 in udder halves categorised as lump. Haematoxylin and eosin staining (200x): A. Mild mammary parenchymal inflammation (predominantly lymphocytes and plasma cells) and with maintenance of tissue architecture. B. Moderate mammary parenchymal inflammation with partial loss of tissue architecture C. Severe and extensive mammary parenchymal inflammation with effacement of tissue architecture.

Histology: udder halves categorised as normal

In Study B, the variability of histological grades within each udder half categorised as normal was much lower than those udder halves categorised as lump (Table 7.3). The majority (n = 60, 82.2%) of udder halves categorised as normal on palpation had minimal to mild histological inflammatory grades (grades 1 or 2), Table 7.3), and typically showed retention of normal mammary tissue architecture and intact glandular acini that contained secretion. However, low to moderate interstitial lymphocytic infiltration with lower percentages of plasma cells (57/60, 95%) or lymphocytic infiltration admixed with neutrophils (3/60, 5%) was consistently observed microscopically in these palpably normal udder halves. A histological grade of moderate inflammation (grade 3) was observed in 12/73 (16.4%) while only one (1.4%, 1/73) udder half presented a histological grade of severe (Table 7.3).

7.4.3 Study C: Udder halves categorised as hard, lump and normal post-weaning

Of the total 92 udder halves from 46 ewes assessed three weeks post-weaning (pre-slaughter) in Study C, most were categorised as normal (76/92, 82.6%), Table 7.5); only one (1.1%) of the udder half was categorised as hard while 15 (16.3 %) were categorised as lump. All 16 udder halves classified as defective (either hard or lump) had a history of one or more udder defects on palpation sometime in the previous three years. Of the 76 udder halves categorised as normal, 54 (71.1%) had a history of one or more udder defects on palpation within the last four years. The remaining 22 (28.9%) were categorised as normal throughout the previous four years with no history of any udder defect recorded within that period (Table 7.5).

Table 7.5 Study C: Gross pathology of udder halves categorised as hard (n=1), lump (n=15), and normal (n=66) from mixed age non-dairy breed (Romney) ewes.

		Udder defect history				Total
		Defective			Normal	
Udder defect status	Category	Hard	Lump	Normal	Normal	
Udder defect location	Whole udder half	1				1
	Zone 1		14	13	9	29
	Zone 2 (cranial)		1			1
	Zone 3 (midline)					
	No defect			41	13	58
Udder open (ruptured) yellow purulent discharge or scab/scars	Yes	1	2		1	4
	No		1		21	88
Udder consistency on incision	Firm/hard	1	2	4	2	3
	Soft		13	9	7	13
Defect content/ mammary secretions	Abscess/pus		8	12	7	27
	Dark tan bloody fluid			1		1
	Clear watery fluid/clump		1		9	10
	Milk like fluid		5	17	1	23
	No fluid	1	1	24	5	31

7.4.3.1 Gross pathology

Gross pathology: udder halves categorised as hard

Only one of the 92 udder halves in Study C was categorised as hard. This udder half was normal in shape, slightly enlarged in size and had diffuse firm consistency of the whole udder half on palpation with thick scabs on the udder skin. The parenchymal tissue was firm on incision and no fluid or abnormal content was observed in the gland cistern or mammary core.

Gross pathology: udder halves categorised as lump

The vast majority (n = 14, 93.3%) of the 15 udder halves categorised as lump were in zone 1, while the remaining one (6.7%) was located within zone 2 (the cranial mammary core), Table 7.5. Two (13.3%) udder halves categorised as lump had either an open (likely recently ruptured) abscess or an older scab or scar present on the udder skin and were firm/hard on incision (Table 7.5). Palpation of udder halves categorised as lump most commonly revealed a single nodule (86.7%), but less commonly the presence of two or three nodules (13.4%); on incision, nodules ranged from 1cm to 4cm in diameter. Just over half (n=8, 53.3%) of udder halves categorised as lump contained yellowish thick purulent exudate within the gland cistern or mammary core, four (50%) of which were contained within a thick fibrous capsule and considered consistent with abscesses, while white milk-like fluid or clots (n = 5, 33.3%) or clear watery fluid (n = 1, 6.7%) were also observed. One (6.7%) udder half contained no abnormal content or secretion (Table 7.5). Of the udder halves categorised as lump that showed more extensive inflammation microscopically (i.e. both moderate (grade 3) and severe (grade 4), n = 5), 60% (3/5) contained grossly visible thick yellow opaque exudate (consistent with pus) on dissection, while the remaining 40% (2/5) had non-purulent exudate or no content at all. In udder halves categorised as lump which showed less extensive inflammation microscopically (i.e. either minimal (grade 1) or mild (grade 2), n = 7), 57% (4/7) presented non-purulent exudate or no content.

Gross pathology: udder halves categorised as normal

There were 54 udder halves with a previous history of udder defects but categorised as normal at the time of Study C. All were of normal size, shape and consistency on external examination but, on incision, 13 (24.1%) contained abnormal content in the gland cistern (zone 1, Table 7.5). Where present, the abnormal content was most often (n = 12, 92.3%) yellow to greenish thick opaque fluid consistent with pus while one (7.8%) contained dark tan bloody fluid. However, most normal udder halves contained either no content or secretion (n = 24, 44.4%) or white milk-like fluid (n = 17, 30.3%) (Table 7.5). Of those udder halves (n=12) containing pus, three (25%) were encapsulated by a fibrous capsule and classified as abscesses (Table 7.5).

All 22 udder halves with no previous history of udder defects and categorised as normal at the time of Study C were of normal size, shape, and consistency on external examination; however, one udder half presented with a scab on the udder skin and 7 (31.8%) contained yellow to greenish thick opaque fluid consistent with pus in the gland cistern upon incision which was not encapsulated (Table 7.5). On incision of the gland cistern, the remaining udder halves categorised as normal with no history of previous defects contained clear watery fluid (40.9%), no content or secretion (22.7%) or white milk-like fluid (4.5%, Table 7.5).

7.4.3.2 Histology

The histological inflammatory grades of udder halves categorised as hard, lump or normal in Study C are summarized in Table 7.6. with the dominant grades and grade ranges in each udder half defect category. Those categorised as normal were sub-categorised into those with a history of being normal or defective during the previous three years.

Histology: udder halves categorised as hard

Only one udder half with a history of previous udder defect was categorised as hard in Study C and it had a severe histological (grade of 4, chronic inflammation; Table 7.6), with similar appearance to that seen in udder halves categorised as hard in Study B with pyogranuloma(s) (see section 7.4.2.2). Within the adjacent glandular tissue, secretory acini were often collapsed with minimal secretion observed and moderately increased fibrous connective tissue between collapsed lobules.

Histology: udder halves categorised as lump

Udder halves categorised as lump in Study C showed high variability in the dominant histological inflammatory grade, with similar proportions of severe (grade 4, n=4, 33.3%), moderate (grade 3, n=3, 25%) and mild (grade 2, n=4, 33.3%) inflammation seen; minimal inflammation (grade 1, n=1, 8.3%) was observed less frequently (Table 7.6). All udder halves categorised as lump with a dominant histological inflammatory grade of 4 (severe) contained one or more pyogranulomas, with similar features to the descriptions for pyogranulomas in udder halves categorised as hard or lump in Study B above in section 7.4.2.2 and for the udder half categorised as hard in Study C. Typically within the glandular tissue adjacent to pyogranulomas, acini were often small or collapsed with abundant interstitial lymphocytes and lower numbers of plasma cells around glands in the interstitial and connective tissues. Mild to moderate fibrous connective tissue within or between the lobules was also typically observed.

In udder halves categorised as lump that had minimal or mild (grade 1 or 2) microscopic inflammatory changes, mammary architecture appeared normal and homogenous eosinophilic fluid with lipid vacuoles consistent with milk was present within glandular acini. Low numbers of interstitial lymphocytes and plasma cells were present in all 12 udder halves categorised as lump. In addition, neutrophils were variably admixed (n = 7/12, 58.3%) with variable amounts of interstitial connective tissue within or between lobules. In udder halves categorised as lump that had extensive inflammation microscopically (i.e. both moderate (grade 3) and severe (grade 4), n=5), 60% (3/5) had grossly visible purulent exudate on dissection while the remaining 40% contained either non-purulent secretion or no content. In those with less extensive inflammation microscopically (i.e. both minimal (grade 1) or mild (grade 2), n=7), less than half (3/7, 43%) showed grossly visible purulent exudate on dissection.

Histology: udder halves categorised as normal

The vast majority of udder halves with no history of defects and categorised as normal in Study C (19/22, 86.4%) had a low histological inflammatory grade (grade 1-2, minimal to mild inflammation). In these samples, the histological architecture of the mammary gland appeared normal with variable numbers of intact acini containing small amounts of bright pink secretion present within each lobule. Low numbers of interstitial lymphocytes and plasma cells and occasionally neutrophils were variably present around acini in some sections. Only three normal udder halves (13.6%) showed a higher histological inflammatory grade (all grade 3, moderate inflammation), and typically showed normal glandular structure and secretion in alveoli.

The majority (n = 35/48, 72.9%) of udder halves with a history of defect but categorised as normal in Study C had a dominant histological grade of minimal or mild (grades 1-2). Lesions were similar to those described in udder halves categorised as normal in Study C above; however, the percentage of udder halves graded as moderate histological inflammation (grade 3) was higher (n = 12/48, 25%). In addition, unlike those with no previous udder half defect history and categorised as normal in the current udder assessment, a low number of udder halves (n= 2, 4.2%) showed extensive grade 4 (severe inflammation), with histological features similar to those previously described.

Table 7.6 Study C: Udder half histological grading of udder halves categorised as hard, lump and normal in 46 mixed age non-dairy (Romney) ewes three weeks after weaning.

History of udder half defect	Dominant score	Observed scores	Frequency of post-weaning udder half defects			Total
			Hard	Lump	Normal	
Normal	1C	1C –2C			6	6
	1M				1	1
	2C	1C - 2C			11	11
	2M	1M-3M			1	1
	3C	-			1	1
	3M	1M - 4M			2	2
	4C	-				
	4M				-	
	Sub-total				22	22
Defective	1C	0 -2C		1	2	3
	1M	-			3	3
	2C	1C - 3C		3	28	31
	2M	-		1	2	3
	3C	0 - 4C		3	9	12
	3M	2M-3M			3	3
	4C	1C- 4C	1	3	2	6
	4M	2M - 4M		1		1
	Sub-total		1	12	48	61
Total			1	12	70	83

Note: The histological inflammatory grading scale (0-4, -A, -C and -M) used is as described above in section 7.2.1 and Table 7.1.

7.5 Discussion

The studies comprising this chapter describe the gross and microscopic pathology of a large number of palpably normal and defective udders in ewes including both diffusely hard udder halves and udder halves with lumps, with the intention that this information may be used to contribute to more rational and evidence-based culling decisions. Previous New Zealand studies indicate that the occurrence of palpable udder defects in ewes ranges from 2% to 7.5% (Griffiths *et al.*, 2019a; Peterson *et al.*, 2017; Ridler *et al.*, 2021) and show the negative effect of udder defects on milk yield (Zeleke *et al.*, 2021), increased lamb mortality (Griffiths *et al.*, 2019a) and reduced body weight gain in pre-weaned lambs (Griffiths *et al.*, 2019b). However, descriptions of the pathology of these defects are limited in both scope and in the number of animals evaluated (Benites *et al.*, 2002; Serres *et al.*, 2011). Key findings from the studies comprising this chapter include the consistent presence of non-inflammatory cyst(s) in ewes with CMLs (Study A) and of a single large abscess or pyogranuloma with more extensive and usually chronic inflammation in udder halves classified as hard (Studies B and C). However, pathological findings within udder halves classified as lump in both Studies B and C were much less consistent.

All CMLs were discrete masses that were palpably turgid and slightly firmer than the adjacent normal mammary tissue and grossly contained fluid consistent with normal milk when incised. Histologically, all but one CML examined comprised dilation(s) of mammary ducts or acini filled with homogenous fluid consistent with milk and without associated inflammation. The palpable consistency of the CMLs in Study A is consistent with the findings of Griffiths *et al.* (2019a) and Ridler *et al.* (2021) in earlier studies, while the gross appearance of the CML content is similar to that described by Saratsis *et al.* (1998). However, while previous studies reported mid-line lumps located both cranial (Ridler *et al.*, 2021) and caudal (Griffiths *et al.*, 2019a; Ridler *et al.*, 2021; Saratsis *et al.*, 1998) to the udder, in the current Study A CMLs were only seen in the cranial midline of the udder.

Based on the typical histological findings of dilated but otherwise normal ducts and acini, the occurrence of CMLs in Study A could be explained by pressure expansion and accumulation of milk forming a discrete cyst, similar to the development of cystic dilation in "weeping teats" described in goats during or shortly after lactation (Matthews, 2016). The high pressure could be associated with the accumulation of milk in the gland cistern shortly after cessation of suckling when ewes lose their lambs or at the time of weaning (Petridis & Fthenakis, 2014, 2019). The accumulated milk could form a clot until phagocytized by the process of apoptosis (Petridis & Fthenakis, 2019; Tatarczuch *et al.*, 1997), which can block the milk flow and result in it accumulating in other locations outside the gland cistern forming a cyst (Dingwell *et al.*, 2004; Matthews, 2016). Bacteria were not isolated from CMLs in the present study (and see also Chapter 6). Similarly, CMLs in ewes have previously been reported to be negative for the presence of bacteria (Ridler *et al.*, 2021; Saratsis *et al.*, 1998) and self-resolving if pressure on the area can be avoided, as in the case of other mammary cysts (Gazzola *et al.*, 2021). Given these findings and the typical lack of inflammation or intralesional bacteria, once the blockage of milk flow resolves, most CMLs would be expected to resolve and disappear following mammary gland involution and have little effect on long-term milk production. Therefore, ewes with CMLs should not be culled.

Histological evidence of moderate mixed and generally chronic inflammation was observed in a single udder half with a CML in Study A. Inflammation was observed both in the mammary core (zone 1) as well as surrounding the CML. The source of inflammation in this CML could be hypothesized in two ways: firstly, the inflammation in the CML (zone 3) might be due to the extension of initial mastitic milk from the mammary core (zone 1). Secondly, the accumulation of milk in CML may itself have contributed to the development of a bacterial infection by providing a growth medium. The first theory is considered more likely here, as samples collected from zone 1 of both the right and left udder halves of the affected ewe contained inflammation as well. The latter theory appears less likely because previous studies have not cultured bacteria from milk cysts and found no association between bacterial culture and the presence of milk cysts in goats (Gazzola *et al.*, 2021; Saratsis *et al.*, 1998). However, further studies would be needed to confirm this conclusion.

Udder halves categorised as hard were typically normal in shape but moderately enlarged in size, with diffusely firm consistency of the entire udder half both at pre-mating (Study B) and post-weaning (Study C). On incision, all except one udder half categorised as hard contained a single large abscess comprising thick opaque yellowish exudate consistent with pus within a fibrous capsule. These gross findings (diffusely firm consistency on palpation and abscessation on incision) are also consistent with multiple previous studies (Griffiths *et al.*, 2019a; Kumar *et al.*, 2015; Ridler *et al.*, 2021; Saratsis *et al.*, 1998). Watkins and Jones (2007) described similar udder defects to these as having hard consistency on palpation and forming single to multiple encapsulated abscesses, containing purulent material, within the gland or subcutis. Similarly, Smith *et al.* (2015) dissected palpable udder defects and described the lesion as an accumulation of abscesses; however, unlike the current study, the palpated udder defects were not categorised as hard or lump. The udder abscesses reported by Smith *et al.* (2015) ranged from 1 to 7 cm in diameter, and description of the larger abscesses (6 to 7 cm diameter) was similar to the udder halves categorised as hard in the present studies. Previous studies have recovered numerous bacterial species from diffusely hard udder defects and associated bacterial infection as the likely cause of defects (Peterson *et al.*, 2017; Ridler *et al.*, 2021; Smith *et al.*, 2015). In agreement with these previous findings, several bacterial species were also isolated from udder halves categorised as hard in the present studies (see Chapter 6). The diffusely firm consistency of udder halves in the present studies could be due to the build-up of large quantities of highly viscous purulent exudate in the whole mammary gland and more importantly because of the fibrous capsule surrounding the purulent exudate.

Histologically, udder halves categorised as hard typically showed features of severe chronic inflammation including granulation tissue and were more consistent with multifocal pyogranuloma formation that frequently effaced alveoli and ducts within multiple lobules rather than a single abscess as appeared in gross examination. The presence of laminated keratin fragments in some samples may suggest effacement of larger ducts or teats. These histological findings are similar to those of Serres *et al.* (2011) who also described hard udder halves as containing purulent exudate with multifocal pyogranulomatous mammary gland lesions in affected ewes. The predominantly chronic inflammation observed in the present studies was not unexpected, as the time between initial identification of the udder defect to the time of slaughter and udder sample collection in Study B was long (12 weeks) while most ewes in Study C had a history of udder defects in the previous four years. Combining gross and histological findings in diffusely hard udder halves with the recovery of bacteria from 70% and 100% of udder halves categorised as hard in Study B and C, respectively, suggests that the initiating cause may be bacterial infection of the udder half followed by the development and persistence of (chronic) inflammation.

The gross finding of what appeared to be one or more large abscesses occupying most of the mammary core coupled with histologically severe chronic inflammation and parenchymal damage likely explains the absence of, or very low levels of, milk expression from udder halves categorised as hard (Chapter 4) (Zeleke *et al.*, 2021) and is consistent with the previous study by Peterson *et al.* (2017). The histological changes of severe chronic inflammation observed in these udder halves are reportedly more likely to have permanent effects on mammary gland function and milk production from the affected udder half due to the associated loss of secretory parenchyma, duct and acini block, age, and proliferation of granulation tissue within the affected udder half (Benites *et al.*, 2002; Helmboldt *et al.*, 1953). This suggests a negative impact of udder halves categorised as hard on future milk yield and consequentially on survival and body weight gain of pre-weaned lambs. Hence, the present findings provide more support for culling ewes with diffusely hard udder halves.

Udder halves categorised as lump grossly contained one or more discrete nodules within the mammary core, which showed wide variation in size (ranging from less than 1 cm to up to 7 cm in diameter), as well as in the consistency, location within the udder and number of nodules present. Just over half of these udder halves contained thick opaque yellow exudate (consistent with pus) within either the gland cistern (zone 1) or mammary core (zone 2) of the mammary gland. The findings have been previously considered consistent with abscessation (Kumar *et al.*, 2015; Smith *et al.*, 2015); however, in half of the samples categorised as a lump in the present studies, the exudate did not appear to be contained within a fibrous capsule. These findings have some similarities to those in the study by Smith et al. (2015) which described udder lumps as abscesses but observed variation in gross appearance. The same authors suggested the maturation cycle of abscesses as a reason for the diversity of the gross appearance. Abscesses mature by forming a capsule surrounding the purulent exudate, which eventually ruptures and spreads the bacteria into a different location by creating a fistula with a fresh purulent exudate or eventually forming a scar/scab on the external udder skin (Cheng *et al.*, 2010; Kobayashi *et al.*, 2015). This can elicit a repeated cycle of maturation in the new location (Arteche-Villasol *et al.*, 2022; Cheng *et al.*, 2011). This explanation is considered likely to explain some findings in the present studies, as bacteria were isolated from a substantial proportion (from30 to 60% across the three studies) of udder halves categorised as lump, as detailed in Chapter 6.

Just under half (47% in Study B and 49% in Study C) of udder halves categorised as lump did not express or contain purulent exudate within the mammary gland or on the udder skin. This may appear to contradict the description of lumps as abscesses and the hypothesis of the abscess maturation cycle by Smith *et al.* (2015) above. However, the absence of purulent exudate in the gland cistern or parenchymal core of the gland could be explained by leakage via the teat. Since most purulent exudates associated with udder halves categorised as a lump in the present studies were unencapsulated and located in zone 1 (gland cistern), it is reasonable to assume leakage of purulent exudates rather than rupture followed maturation of the abscess. Further, the presence of purulent exudate blocking the teat canal, forming a hard core, in half of the udder halves categorised as a lump but with no grossly visible purulent exudate in the udder (Study B) may also support this assumption, indicating recent intramammary abscessation. Similarly, accumulation of purulent exudate in the teat canal was reported by Peterson *et al.* (2017) and Watkins and Jones (2007), and as a palpable hard core on rolling the teat between the thumb and forefinger. In those udder halves categorised as lump and containing a sterile abscess, it is possible these could be idiopathic granulomatous mastitis, which is characterized by the formation of an abscess, no bacterial isolation, and lobular distribution of granulomatous inflammation, as described in humans (Baslaim *et al.*, 2007).

Histologically, udder halves categorised as lump also showed wide variation in the presence, severity and nature of inflammation, ranging from minimal inflammation with normal tissue architecture (grade 1) to severe inflammation with multiple areas of tissue architecture damage (grade 4) in the current studies. Chronic inflammation was predominant in these udder halves. Approximately half (42% in Study B and 52% in Study C) of the udder halves categorised as lump had more extensive inflammation histologically (moderate or severe). Forty-five percent of the udder halves with lumps in Study B and 60% of those in Study C contained single or multiple pyogranulomas with often extensive granulation tissue. These findings indicate chronic inflammation or long-term damage which has been present for some time (at least several weeks and likely longer), is less likely to resolve, and is more likely to adversely affect future milk production performance (Benites *et al.*, 2002; Helmboldt *et al.*, 1953). However, findings were variable, as approximately half of the udder halves categorised as lump showed less extensive inflammation histologically (minimal or mild) with less damage to the secretory parenchyma of the gland. Although these milder microscopic changes observed may not be detrimental to milk production at the time of udder assessment, they could present a risk for future udder defect occurrence (Grant *et al.*, 2016). Consequently, ewes with udder halves categorised as lump may need to be culled to prevent potential recurrence of udder defects and adverse effect on milk production. However, further studies are needed here.

In udder halves categorised as lump, 72% (Study B) and 100% (Study C) of udder halves with extensive inflammation microscopically had grossly visible purulent exudate while 30% (Study B) and 43% (Study C) of the udder halves with less extensive inflammation microscopically were associated with purulent exudate grossly. This suggests that udder halves categorised as lump and containing grossly observed pus or evidence of abscessation on incision were more likely to be associated with significant histological inflammation, mammary tissue damage and long-term negative impacts on milk production. Hence, identifying those udder halves categorised as a lump which are likely to have a more profound effect on the secretory parenchyma and cause permanent damage to the mammary gland (e.g. those which cause significant tissue effacement and granulation tissue formation) is crucial. While manual udder palpation alone does not appear to allow reliable differentiation between these different types of lumps, other methods such as ultrasonography may provide additional valuable information to do this. This would help to identify and classify contents or secretions in live ewes to complement external examination and help identify ewes with lesions likely to have long-term adverse effects on milk production (Hiepler *et al.*, 2009; Lazaridis *et al.*, 2012). However, further studies are required to confirm this assumption.

All udder halves categorised as normal on palpation were typically normal in size, shape and consistency on external examination (Studies B and C), and typically retained normal mammary parenchymal tissue structure with only minimal to mild inflammation. Nevertheless, 32% (Study B) and 24% (Study C) of palpably normal udder halves contained abnormal content (e.g., purulent exudate or bloody secretion) on dissection, which were not consistent with a normal udder. These findings could be due to limitations in the accuracy of udder palpation to detect certain udder defects such as those located deep within the intramammary tissue (Hiepler *et al.*, 2009) or to differentiate the appearance of abnormal fluids in the gland cistern (Flöck & Winter, 2006). Ewes with udders palpated as normal should not be culled; however, identifying those udder halves palpated as normal but containing abnormal content or with major microscopic changes would be beneficial to making culling decisions. Furthermore, only 13% of udder halves categorised as normal with no history of defect in the previous four years showed extensive inflammatory changes microscopically in Study C; however twice as many (26%) udder halves palpated as normal but with a previous history of udder half defect showed similar inflammatory changes, suggesting a history of udder defect is important in interpretation of udder palpation findings. This is similar to the findings of Grant *et al.* (2016), which reported that previous history of palpable udder defect increased the risk of future recurrence. This shows that those udder halves with a previous history of palpable defects may return to normal, but had a higher risk of retaining abnormal content and significant inflammation with negative impact of milk production compared to palpably normal udder halves with no history of defect.

While the gross pathology and microscopic changes were assessed in individual udder halves in the present studies, knowing the defect status and associated pathological changes in milk yield of the contralateral udder half is also essential to more fully determine the possible effect on whole udder milk production of an individual ewe. In the current studies, none of the ewes had bilateral diffusely hard udder halves, although 21% of the udder halves categorised as hard in Study B had a contralateral udder half categorised as a lump. Both unilateral and bilateral occurrence of lumps were seen in the current studies, but the number of unilateral defect occurrences was higher, which is similar to previous studies (Griffiths *et al.*, 2019a; Ridler *et al.*, 2021).

In addition to the current pathological findings, other factors such as involvement of the contralateral udder half, rearing of the lambs, a potential source for new infection, and practicality of treatment options should be considered.

Variations in gross external appearance (size, consistency, or location), internal gross content (e.g. purulent exudate), and inflammatory histology grade were observed in defective udder halves, particularly in udder halves categorised as a lump. Merging the initial seven-category udder palpation scores into three categories (hard, lump, and normal) can be considered a limitation of the studies. Thus, it is important to assess and analyse specific lump scores independently to further evaluate the effect in detail in future studies.

To the author's knowledge, there is no published histological grading system to assess the histology of mammary gland inflammation in ewes. The semi-quantitative histological grading system applied in the present studies was modified and adopted from previous experimental and other studies on mice and cattle. An inflammatory grading system was used as previous studies suggest that inflammation is associated with many udder defects and may have significant long-term effects on milk production. While the grading system used here allowed easy explanation and clear presentation of large numbers of results and ascertainment of trends across studies and defect groups, it has limitations. First, some mammary gland tissues in the present studies showed marked variation in lesion appearance within a single tissue sample, and the final average or overall histological inflammatory grade of a sample used does not always reflect that variation. To try to reduce inconsistency, a minimum of ten high power fields (400x magnification) were assessed, and both the dominant inflammatory grade and ranges of grades observed in the sample were also reported. Nevertheless, inconsistencies may have not been fully avoided because of uneven or multifocal lesions across several lobules of the gland. Secondly, the grading system did not include non-inflammatory lesions such as duct dilation, corpora amylacea or the presence or absence of fluid within glands, which may have been associated with gross lesions, palpable abnormalities or reduced milk production. Therefore, interpretation of histological grading should be done with caution, and undertaking further studies to more accurately grade, describe and characterize the lesions with variation (e. g. udder halves categorised as lump) would be beneficial.

In conclusion, CMLs are typically cysts dilated with milk-like fluid which are not typically associated with inflammation, and which appear to have a very limited effect on milk secretion of the associated mammary gland. Udder halves categorised as hard were associated with more extensive and severe inflammation and damage to the mammary secretory parenchyma, which likely indicate long-lasting effects on the gland and future milk yield of the affected udder half. The effect of udder halves categorised as lump ranged from minor changes to severe inflammation and damage to the mammary parenchyma. Identifying those lumps which are more likely to cause permanent damage to the mammary gland is crucial, but not possible from palpation and gross appearance alone. Hence, culling decisions should be made by considering pathological changes of these defects and other factors such as udder defect status and milk yield of the contralateral udder half and rearing rank to keep ewes that are more likely to sufficiently feed and rear the pre-weaned lambs.

Chapter 8: General Discussion

8.1 Introduction

In ewes, diffuse hardness of the whole mammary gland or discrete lumps are recognized as common palpable udder defects (Griffiths *et al.*, 2019a; Peterson *et al.*, 2017; Ridler *et al.*, 2021). Udder palpation is a key tool for udder examination in non-dairy ewes (Blagitz *et al.*, 2014; Corner-Thomas *et al.*, 2016; Grant *et al.*, 2016; Saratsis *et al.*, 1998). Palpable udder defects have been detected during pregnancy and lactation, and at weaning, post-weaning and pre-mating (Grant *et al.*, 2016; Griffiths *et al.*, 2019b; Peterson *et al.*, 2017). Griffiths *et al.* (2019a) found that udder defects identified at pre-mating had a negative impact on the growth rate and survival of pre-weaned lambs. Further, effects of udder defects on reduction of ewe longevity and lamb performance have been reported (Flay *et al.*, 2021; Grant *et al.*, 2016; Hayman *et al.*, 1955; Peterson *et al.*, 2017). Abnormal milk secretion including clots and flakes, serous and purulent fluid, and blood secretion have been detected in ewes with diffusely hard udders or udder lumps (Peterson *et al.*, 2017; Saratsis *et al.*, 1998). It has also been well established that milk somatic cell count (SCC) is positively correlated to intramammary infection (IMI) (Gelasakis *et al.*, 2015). However, very few studies have been undertaken to assess the impact of palpable udder defects on milk yield and composition, and milk SCC.

Studies of udder assessment within the same flock over six weeks to two years have shown that palpable udder defects can change over time (Grant *et al.*, 2016; Griffiths *et al.*, 2019a; Ridler *et al.*, 2021). Smith *et al.* (2015) identified phenotypically diverse udder defects in non-dairy ewes ranging in diameter from 1 to 7cm during post-mortem examinations, which is suggestive of different stages or types of udder defects. Grant *et al.* (2016) reported that ewes that were identified with palpable udder defects were three to five times more likely to have udder defects identified in the future. When combined, these findings imply that palpable udder defects may persist and/or change over time but the timing of these changes and possible pathological mechanisms are yet to be fully understood. To make more informed culling decisions, it is important to better understand how udder defects change over time and the of these defects impact milk production.

Numerous bacterial species have been identified from ewes with palpable udder defects post-weaning, with *S. aureus* being the most common (Ridler *et al.*, 2021; Smith *et al.*, 2015). Bacteria isolated from milk samples taken three weeks apart in early lactation have shown that mammary bacterial populations can change depending on the bacterial species present (van den Crommenacker-Konings *et al.*, 2021); however, bacterial identification from defective udders over time has not been studied. Hence, further research is required to identify bacterial species from defective udders over time. Gross pathological and histological studies of an udder can also provide evidence to support the assessment of an udder using palpation, but pathological alterations associated with each palpable udder defect are unknown.

In non-dairy ewes a key driver of production is the weight of lambs weaned per ewe (Flay *et al.*, 2020) and therefore, high lamb survival and pre-weaning growth rates are critical. Culling of ewes with palpable udder defects such as diffuse hardness and lumps has been recommended (Griffiths *et al.*, 2019a; Griffiths *et al.*, 2019b). However, culling ewes also comes at a cost, as for each ewe that is culled, a replacement ewe-lamb either needs to be retained or purchased (Farrell *et al.*, 2019). Therefore, understanding the fundamental dynamics and pathology of udder defects is critical to making more balanced culling decisions. Greater knowledge of udder defects may allow introduction of more timely interventions and would aid evidence-based decisions regarding culling. Hence, the general objectives of this thesis were to assess the relative importance of udder defects in non-dairy ewes with a specific focus on assessing the effects, dynamics, bacteriology and pathology of palpable udder defects and to examine the impact of udder defects on milk production parameters.

8.2 Summary of Experimental Chapters

8.2.1 Chapter 4: Effect of palpable udder defects on milk yield, somatic cell count and milk composition in non-dairy ewes

Romney ewes with and without a previous history of palpable udder defects were selected and milked weekly during the first six weeks of lactation to assess udder-half and whole-udder milk yield, SCC, and milk composition (fat, protein, lactose, total solids, and solids non-fat (SNF)). Defective udder halves had lower milk yield than normal udder halves with a contralateral normal udder half. Further, whole udder milk yield from normal udder halves with a contralateral defective udder half, was higher than whole udders with both normal udder halves. This result suggests a compensatory effect. Successive udder-half defects over time, udder half defects in early lactation, and a high frequency of udder half defects were all associated with lower udder-half milk production. SNF was lower in defective udder halves, and it was the only milk composition parameter associated with udder half defect. In summary, these findings emphasise the impact of palpable udder defects on udder half milk production of ewes and their potential effect on growth and survival of their lambs. Multiple-born (twin or triplet) lambs would likely be most affected due to competition to access adequate milk yield compared to singletons.

8.2.2 Chapter 5: Assessment of changes in udder half defects over time in non-dairy ewes

This chapter comprised two studies. In the first study, ewe udder halves were assessed and scored for two successive years, four times a year at the key management times of pre-mating, pre-lambing, docking, and weaning. The second study assessed ewe udder halves at pre-mating, and six times at weekly intervals in the first 42 days of lactation. The findings of this chapter demonstrated that udder half defects (categorised as hard, lump or normal) changed over time. The highest occurrence of udder halves categorised as hard was observed at either pre-mating or docking. Udder halves categorised as lump had a highest occurrence at either docking or weaning. In addition, udder half defects categorised as either hard or lump at pre-mating were more likely to be defective (hard or lump) at future examinations during that year or at pre-mating the following year. Change in udder half defect status across early lactation showed high variability. Failure to express milk in udder halves in early lactation was associated with a higher occurrence and persistency of udder half defects. In summary, this chapter showed that the occurrence of palpable udder defects changed over time, and that the risk of future defect occurrence was a minimum of seven times higher in udder halves previously categorised as having either a hard or lump defect.

8.2.3 Chapter 6: Isolation of aerobic bacterial species associated with palpable udder defects in non-dairy ewes

Milk or tissue swab samples were aseptically collected from each udder half of Romney ewes across lactation and, at weaning and/or post-weaning. Pure isolates from conventional bacterial culture were identified by Matrix-assisted laser desorption/ionization time-of flight (MALDI-ToF) mass spectrometry. Repeated examinations, up to eight times were undertaken from each udder half to assess the dynamics of bacterial species. *Mannheimia haemolytica* and *Streptococcus uberis* were the most common bacterial species identified from defective (hard or lump) udder halves. *Mannheimia haemolytica, Staphylococcus xylosus and Streptococcus pluranimalium* had the greatest persistence over the lactation, weaning and post-weaning periods. Staphylococcus species had comparatively high isolation frequency, but stability over time was variable. *Staphylococcus aureus* was isolated from both defective and normal udder halves in similar proportions, while CNS were more frequently isolated from normal udder halves. Mammary tissue swab and udder half milk samples for bacterial sampling, presented a very high agreement of bacterial positivity and species identified post-weaning. Overall, results of this study indicated that palpable udder defects were associated with bacterial positivity and the persistency of the bacteria over time was dependent on the species involved.

8.2.4 Chapter 7: Gross pathology and histological changes of palpable udder defects in non-dairy ewes

Gross and histological assessment of cranial midline lump (CML), diffuse udder half hardness, and lumps were undertaken during the non-lactating period (weaning, three weeks post-weaning, and at pre-mating) in non-dairy breed (Romney) ewes. In ewes, detected with CMLs, the mammary tissue appeared normal in both right and left mammary core (zone 1). However, they had multifocal cyst/s of variable sizes containing fluid consistent with normal milk on the cranial midline of the udder (zone 3). The results suggest CMLs may not cause damage to the mammary gland and are assumed to be temporary. Udder halves categorised as hard were described as a single large abscess within the mammary core, occupying the gland cistern and most of the mammary core, on gloss dissection. The typical histological lesion of udder halves categorised as hard was described as occurrence of pyogranuloma with extensive mixed inflammatory cells, effacement of the glandular tissue, and mature granulation tissue. In udder halves categorised as hard, the results are suggestive of long-term damage to the mammary tissue. Gross appearance and histology of udder halves categorised as lump noticeably varied from normal appearance to severe chronic inflammation with a purulent exudate. Hence, further study is required to assess udder halves categorised as lump as the lesions markedly varied in extent and chronicity.

8.3 Summary of Main Findings and Practical Recommendations

Each chapter of this thesis was focused on a specific aspect of palpable udder half defects in non-dairy breed (Romney) ewes. However, presenting a summary of the combined aspects of each defect category (e.g., CML, hard, or lump) is essential to obtain a complete understanding of the causes, pathological changes over time and clinical importance. In the three studies undertaken (Chapters 4-7), all parameters, including udder palpation, were at the individual udder half level but palpable udder defects were observed both unilaterally and bilaterally. Defect status or milk compensation of the contralateral udder half substantially affected the whole udder milk yield. Hence, discussing the combined findings of both udder halves can provide valuable evidence for practical application on commercial farms, to allow for evidence-based intervention or culling decisions. Therefore, several factors should be considered including involvement of udder halves (unilateral or bilateral), milk yield and compensation of the non-defective contralateral udder half and potential rank (i.e. number) of lambs to be reared, when making culling decisions based on an individual ewes udder defect.

Udder half defect status changed over key management times such as pre-mating, pre-lambing, docking and weaning in a year, and also weekly in early lactation. The lowest occurrence of udder halves categorised as both hard and lump was observed at pre-lambing. The highest occurrence of udder halves categorised as hard was observed during the non-lactating period and early lactation (pre-mating or docking) whereas in udder halves categorised as lump, the highest occurrence was during lactation (docking and weaning). In the first six weeks of lactation, the change in udder defects was highly variable; however, overall defect (hard) decreased as lactation advanced.

Udder half defects categorised as hard or lump at pre-mating were more likely to be defective (hard or lump) at future examinations at key management times during that year or pre-mating of the following year. Udder halves found defective at weaning were more likely to be defective (hard or lump) at pre-mating. Additionally, some udder halves that were normal at weaning were subsequently found to be defective at pre-mating. Further, the data suggests that pre-mating is the preferred time to examine udders to identify defects and was also shown by Flay *et al.* (2020) to be the best time to examine udders to predict effects on lamb performance for the coming season.

CMLs are broadly spherical subcutaneous masses extending from the mammary core that are located cranial to the udder (zone 3). In these studies, CMLs were found to contain variable amounts of bacterial negative fluid consistent with normal milk. Histologically, CMLs comprised multifocal cysts of variable sizes lined by a single layer of cuboidal to flattened epithelium and were considered consistent with dilation(s) of mammary ducts or acini. In addition, according to Griffiths *et al.* (2019a), no association was observed between CML in ewes and survival and body weight gain of their pre-weaned lambs. Hence, the current findings suggest that CML are unlikely to cause significant effects on the future mammary health or milk production and therefore ewes with such defects need not be culled.

Udder halves categorised as hard were moderately enlarged, contained purulent exudate in the whole gland and expressed very little to no milk. Milk and swab samples collected from these udder halves showed that between 79 and 100% were bacteria culture positive, and numerous bacterial species were identified during lactation, weaning and at pre-mating. This hardness appeared as single large intramammary mass (IMM) comprising thick opaque yellowish purulent exudate within a fibrous capsule (abscesses) with microscopic changes including severe damage of the secretory parenchyma, interstitial infiltration of inflammatory cells and increased granulation tissue formation. These pathological changes are likely to be indicative of long-term effects on the secretory parenchyma of the gland. Moreover, the analysis of repeated udder palpations within a year and across two seasons confirmed that udder halves categorised as hard were more likely to be defective in the future, within the same year (pre-lambing, docking or weaning) or in the next season (pre-mating), compared to udder halves categorised as normal.

The degree of whole udder milk loss depended on the type and persistence of the udder half defects in unilateral defective (hard) udders. Udder halves categorised as hard which changed to lump and persisted produced less milk than udder halves categorised as normal from ewes where both udder halves were normal. Even though compensation of milk yield was observed at the whole udder level, ewes with a diffusely hard udder half should be culled due permanent damage and the likelihood of defect persistence and/or recurrence.

Udder halves categorised as lump showed high variation in lump size, consistency, number of nodules, and location in the gland on palpation. Further, on incision, the contents varied from abnormal purulent exudate to normal milk-like fluid or no abnormal contents. Similarly, the histology ranged from minimal inflammation with no significant damage to the secretory parenchyma, to severe inflammation associated with likely permanent injury to the secretory alveoli and ducts, which was largely replaced by granulation tissue. Also, a reasonable proportion of udder halves categorised as lump across lactation, weaning, post-weaning and pre-mating were bacteria culture positive. Distinguishing between udder halves categorised as lump likely to result in permanent damage to the secretory parenchyma from those udder half defects causing minor damage would be ideal; however, it couldn't be possible with udder palpation.

Regardless of these observed variations, udder halves categorised as lump had lower udder half milk yield and the occurrence of lumps at pre-mating was associated with increased relative risk of future occurrence within a season or following season. Pre-mating udder palpation score of lump was associated with increased odds of lambs not surviving to weaning (Griffiths *et al.*, 2019a) and reduced growth to weaning of lambs (Griffiths *et al.*, 2019b). Therefore, it can be argued that ewes with udder halves categorised as lump should be culled; however, further studies are needed to more accurately determine the pathological changes, and impact on milk production, and possibilities of treatment options in those udder halves with minor damage.

All udder halves categorised as normal appeared typically normal on palpation. No bacteria were isolated from at least 68% of udder halves categorised as normal across the studies or time points, and at least 82% of those udder halves did not show any gross lesion/content or major histological changes. However, a limited number of normal udder halves had abnormal content (e.g. purulent exudate or bloody secretions) in the gland cistern, and minor to moderate changes on histological examination. This suggests that udder palpation may be insufficient to detect some minor defects or abnormalities.

Overall, the studies in this thesis were conducted in a confined population of non-dairy Romney breed ewes from one commercial farm in the South Island and two flocks from Massey University (Riverside) farm in the North Island. To validate the findings and further improve the practical implications, it would be important to undertake further studies by increasing the number and types (i.e., rearing rank, age, and breed) of ewes that are investigated. Additionally, following ewes over multiple seasons would be beneficial to more accurately determine the change over time across the seasons.

8.4 Limitations and Further Areas of Research

Udder palpation scoring according to Griffiths *et al.* (2019a) was applied in all three of the present studies to assess udder defect status and change over time. It must be recognised that while udder palpation is a practical and efficient method of examining udders, it is a relatively subjective technique and outcomes may differ due to the skills or biases of the operator. In Study A (Chapter 5), four people undertook the udder palpations and while they were trained there was the potential for variation. However, in Study B and C (Chapter 4-7) the same person undertook all udder palpations. The ability to identify udder half defects is also likely to vary depending on the physiological state of the udder (i.e. lactating or involuted). Defects may be more difficult to detect in udder halves in early lactation due to an increase in the size, internal changes and the amount of milk within the udder (Tucker *et al.*, 2009). As demonstrated in Chapter 7, some udder halves that appear normal on palpation may still have abnormal content or histological features.

The udder palpation scoring method comprised seven categories that were then summarized into three udder defect type categories: hard, lump, and normal. Due to relatively low numbers of affected udder halves, the scores that described the number and size of lumps (scores 3-6) were merged into a single ‘lump’ category. This may explain some of the variation in the effects on milk yield, bacterial isolation and pathobiology of udder halves categorised as lump. Further detailed study of udder halves with palpable lumps with scores 3-6 considered independently instead of combining into one category, would be warranted to determine if differences exist. However, this would require a much larger cohort of ewes studied in order to have adequate numbers within each lump score. The occurrence of bilateral udder half defects was much lower than unilateral, both in udder halves categorised as either hard or lump. The effect of bilateral diffuse hardness or lump on whole udder milk yield was not analysed due to the small number of occurrences found. To characterize these impacts with any degree of accuracy, further studies would also require a significant increase in ewe numbers.

The longitudinal study (Chapter 5; Study A) utilized two years data to assess changes in udder defect status both within a year and/or across seasons. Four key management times with different physiological states (dry (non-pregnant), late pregnancy, lactation, and cessation of lactation) were utilized to assess changes within a year. Udder half defects were observed to change within season (2017 and 2018). However, with only one seasonal transition period (i.e. between 2017 and 2018), there may not have been adequate data available to fully examine effects in the transition period. Further, the number of ewes included in the second year (2018) were less than ewes in the first year (2017) due to ewe loss and incomplete data. Further studies should examine the transition period and include a larger population of ewes.

Most of the udder halves categorised as hard or lump observed during early lactation changed to normal as lactation progressed, through to weaning and post-weaning. As a result, gross intramammary and histological changes of these udder half defects may have been missed because of the post-weaning timing of the pathological study. Post-slaughter, measures were taken to collect a representative tissue sample for histology and a semi-quantitative histology grading was applied. However, such applied techniques do not guarantee complete consistency of sampling and grading, as lesions are diffusely multifocal and distributed across various lobules of the mammary gland and therefore there is potential for misrepresentation of the data. To address this, future studies may wish to collect more tissue samples per gland and improve the current grading system.

Milk samples for bacterial identification were collected during lactation, at weaning, post-weaning and in the pre-mating period. No milk samples could be collected from udder halves with palpable defects that expressed no milk. However, post-mortem tissue swab samples were also collected from all udder halves irrespective of whether milk samples could be collected in lactation, at pre-mating or post-weaning. Nevertheless, the change in udder half defect category over time might have also affected the stability of the bacterial species involved, particularly less stable bacterial species such as Staphylococcus. Another potential issue observed was the variation of bacterial culture contamination, (i.e. three or more dissimilar colony types with no dominant colony type), in some samples in all three studies. In Study A (Chapter 5), a higher-than-expected contamination percentage was observed in milk samples. However, in the later studies there were minimal levels of contamination.

8.5 Additional Research Not Included as Part of this Thesis

Two further sections (chapters) entitled "Ultrasonography of palpable udder defects in non-dairy breed (Romney) ewes" and "Hard udder in ewes in early lactation in non-dairy breed (Romney) ewes" were proposed for this thesis, but not included due to insufficient time as a result of the Covid-19 global pandemic and the nationwide lockdowns. This negatively affected data and laboratory analysis. However, the raw data was collected. Following completion of the PhD, these findings will be analysed and communicated. These are briefly outlined below.

Udder ultrasonography has been suggested to complement the external clinical examination (udder palpation) by providing additional understanding of the palpable udder defects' content and mammary internal structural changes (Hiepler *et al.*, 2009). As part of the present studies (Studies B and C), udder ultrasonography was undertaken with the aim of describing mammary internal structural changes and to further assess changes over time in live ewes. Ultrasonography of normal and defective udder halves was conducted on 74 ewes at pre-mating in Study B, while in Study C, five sequentially repeated udder half ultrasonographic scans of 46 ewes was implemented both in pregnancy and during lactation.

In Study C (Chapter 4-7), the highest occurrence of diffuse hardness of udder halves was observed on Day 7 of lactation and decreased as days in lactation advanced, generally changing to either lump or normal. In addition, the udder halves categorised as hard in early lactation in Study C had similar characteristics to those previously reported as a defect called 'hard udder' (Bruce *et al.*, 2013; Skyrme, 1970). This 'hard udder' syndrome was described as a rock-hard palpation of an udder with a sharp dorso-cranial border which occurred shortly after lambing and regressed within three to four weeks (Bruce *et al.*, 2013; Skyrme, 1970). Consequently, with a special focus on early lactation 'hard udder', an additional experiment (Study D) entitled "Hard udder in ewes in early lactation" was carried out in 2020. Eighteen ewes with unilateral or bilateral hard udders identified in the first week of lactation were sequentially (weeks 1, 2, 4 or 6 of lactation) euthanised to assess the pathological changes and dynamics in early lactation. For this purpose, udder palpation scoring, ultrasonography and mammary tissue biopsy were applied when the ewes were alive. Post euthanasia, mammary tissue swabs (for bacterial culture), mammary tissue impression cytology and tissue for histology were collected.

8.6 Conclusion

The goal of this PhD thesis was to explore detailed understanding of palpable udder defects during lactation, weaning and the dry period to make evidence-based culling decisions in non-dairy ewes. CMLs are described as an accumulation of bacterial negative fluid consistent with normal milk and this defect should be differentiated from other abnormal palpable udder defects as affected ewes do not need to be culled. Diffusely hard udder halves and udder halves with lump in the mammary parenchyma appeared as accumulations of purulent exudate (abscess) associated with bacterial infection and mild to severe histological changes. The assessment of palpable udder defects (lump and hard) presented a pattern of change over time. Udder palpation can practically be applied to predict future occurrece based on current pre-mating udder examination. Thus, it is recommended to examine udders at pre-mating and cull those with palpable udder defects, both hard and lump, regardless of the category, size and consistency. Culling of ewes with udder halves categorised as hard should improve overall flock lamb survival and body weight gain to weaning. Further studies should be undertaken in udder halves categorised as lump to more accurately determine the observed variability in pathological changes. The use of a more sensitive methods of udder defect assessment (e.g., udder ultrasonography) are recommended in assessing the content of udder halves categorised as either lump or normal.

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Appendices

Appendix I: Milkoscan FT 1 machine sheep milk calibrations for fat, crude protein, lactose, and total solids.

Component	Intercept	Coefficients	R-Squared	Absolute Accuracy	Relative Accuracy (%)
Fat (N = 51)	0.901	0.902	0.965	0.298	3.87
Crude protein (N = 54)	-0.61	1.11	0.977	0.067	1.134
Lactose (N = 51)	0.840	0.765	0.796	0.10	2.18
Total solids (N = 51)	1.878	0.909	0.986	0.206	1.113
SNF (N = 51)	-1.78	1.14	0.816	0.148	1.37

Note: The absolute accuracy expresses Standard Error of Prediction whereas the relative accuracy states Standard Error relative to the mean of the reference values.

Appendix II: Udder external and internal gross examination (lesion) recoding sheet in non-dairy ewes

Date:	Sample collector:
Animal Id:	Recorder:

Udder half	Right	Left
External udder examination		
External Gross defect	Teat- Absent/Present Udder - Absent/Present Inflammation/nodule/mechanical injury/scar/other ______	Teat - Absent/Present Udder - Absent/Present Inflammation/nodule/mechanical injury/scar/other _______
Udder defect category	Hard/lump	Hard/lump
Open or burst abscess	Absent/Present Fresh/Scab or scar	Absent/Present Fresh/ Scab or scar
Consistency of udder on incision	Soft/Firm or hard	Soft/Firm or hard
Post-incision (internal) gross appearance of udder		
Prescence any lesion or content	Absent/Present	Absent/Present
Lesion or content location	Zone 1 (centering glandular cistern) Zone 2 (Core of mammary tissue): Cranial/caudal Zone 3 (Udder cranial midline of the udder outside the core mammary tissue)	Zone 1 (centering glandular cistern) Zone 2 (Core of mammary tissue): Cranial/caudal Zone 3 (Udder cranial midline of the udder outside the core mammary tissue)
No. of nodules	1/ 2/ 3+	1/ 2/ 3+
Lesion/Mammary content	Colourless watery secretion Milky white secretion Blood tinged red to dark section Yellow to greenish purulent discharge (viscous or watery)	Colourless watery secretion Milky white secretion Blood tinged red to dark section Yellow to greenish purulent discharge (viscous or watery)
Pseudo-capsulation of the purulent discharge	Absent/Present	Absent/Present
Mammary tissue sampling		
Sample collected for histology	Collected/Not collected Zone 1/ Zone 2/ Zone 3	Collected/Not collected Zone A/ Zone B/ Zone C

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