Improved understanding of the transmission of mastitis in ewes and strategies for its control

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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself under the supervision of my supervisors Professor Laura Green and Dr Kevin Purdy, and has not been submitted in any previous application for any degree.

Summary

Mastitis in ewes is a painful disease that negatively impacts sheep health and welfare, and farm productivity, through reduced milk yield and quality, premature culling and in some cases death. The aim of this study was to increase our understanding of mastitis in suckler ewes by investigating transmission and persistence of bacteria, and strategies for its control.

A systematic review was developed and conducted to collate all information from peer-reviewed papers on risk factors for mastitis. The results identified that hygiene and nutrition were the two most frequently mentioned risk factors.

Based on the results from the systematic review, an intervention study was conducted to test the impact of an improved hygiene regime during lambing on an indoor lambing flock of suckler ewes. The impact of an improved hygiene protocol from the time a ewe lambed to leaving housing on the prevalence and occurrence of acute and chronic mastitis was investigated. From a flock of ewes, 737 ewes were examined for the presence or absence of intramammary masses (IMM) on five occasions; pregnancy, lambing, early lactation, late lactation and pre-tupping. At first signs of lambing the ewes were alternately allocated to either a control or intervention treatment group. The intervention ewes received additional hygiene protocols and were managed by the researchers, whereas the control ewes were managed by the farm staff as they normally would. There were no significant associations between improved hygiene regimes and the occurrence or prevalence of chronic and acute mastitis in the flock. However, a significant association was identified between chronic and acute mastitis, with the presence of one heavily influencing the presence of the other.

A subset of 10 study ewes from the flock were sampled to investigate transmission and persistence of bacteria, and any influence of the improved hygiene regime using matrix assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-ToF-MS). Strains of bacteria were identified to persist in ewes and possible transmission events were identified particularly involving the lambs. The community of bacteria isolated from the mouths of the lambs were highly similar to the community of bacteria isolated from the milk of the study and mastitic ewes as well as the nose samples from the study ewes. The role of the environment as a reservoir for bacteria and the threat it may pose to the ewe by facilitating the introduction of bacteria into the udder is also discussed. Reduced presence and proportions of overall isolated species, strains and reoccurring strains were reduced when the ewes were housed individually with their lambs and on bedding that was more frequently changed.

Having identified that IMM and acute mastitis were significantly associated, any associations between the presence versus absence of IMM on the bacterial communities isolated from mastitic and study ewes were explored. There appears to be a complex relationship between IMM and acute mastitis, and IMM and bacterial diversity. The results support a theory that the presence of IMM is associated with an udder microbiota with lower bacterial diversity and that IMM played a role in the development of acute mastitis.

List of abbreviations

AHDB	Agricultural and Horticultural Development Board
AM	Acute mastitis
BCS	Body condition score
BHBA	β-hydroxybutyric acid
BMSCC	Bulk milk somatic cell count
BST	Bovine somatotropin
BTSCC	Bulk tank somatic cell count
CI	Confidence interval
CMT	California mastitis test
CNS	Coagulase-negative staphylococci
Defra	Department for Environment, Food & Rural Affairs
DCT	Dry cow therapy
E. coli	*Escherichia coli*
ID	Identification
IMI	Intramammary infections
IMM	Intramammary masses
MALDI-ToF-MS	Matrix assisted laser desorption/ionisation time of flight mass spectrometry
NEFA	Non-esterified fatty acids
OR	Odds ratio
PCR	Polymerase chain reaction
PFGE	Pulse-field gel electrophoresis
QC	Quality control
QMMS	Quality Milk Management Services
RCT	Randomised control trial
Ref	Reference
rRNA	Ribosomal ribonucleic acid
S. aureus	*Staphylococcus aureus*
SBA	Sheep's blood agar
SCC	Somatic cell count
SCS	Somatic cell score
SPC	Standard plate count
TBC	Total bacterial counts

Chapter 1. General Introduction

1.1 Mastitis and its impact on farm economics and sheep health and welfare

Mastitis is an inflammation of the mammary gland, usually caused by bacterial infection. The introduction and multiplication of the invading microorganisms, generally via the teat, can result in an immune response attracting somatic cells, such as neutrophils, to the area (Harmon, 1994, Kehrli Jr and Shuster, 1994). Mastitis, like other endemic diseases, impacts heavily on farm economics and the health and welfare of affected animals. It has been ranked as one of the most important diseases affecting suckler ewes; sheep bred to suckle its young raised for the lamb market (Davies *et al.*, 2009). Annual losses due to mastitis of up to £2.7 million have been estimated for the Texel breed alone (Conington *et al.*, 2008). With approximately 1.6m Texel ewes (including crossbred ewes) out of a total UK population of approximately 13.5m ewes, the costs to the industry as a whole will be well above this figure. The economic costs of mastitis are due to direct effects, such as death, culling, and administration of medication; and indirect costs from reduced milk yield and quality, that in turn leads to lower lamb growth rates (Huntley *et al.*, 2012, Grant *et al.*, 2016).

Mastitis is a painful disease that negatively effects ewe health and welfare. Restlessness, changes in feeding and flock interaction behaviours, limping due to udder pain, increased vocalisation and decreased behaviours that “call” lambs to suck have been associated with mastitis (Gelasakis *et al.*, 2015; Gougoulis *et al.*, 2008). Hyperalgesia (increased sensitivity to pain) has been detected in ewes with chronic inflammation further supporting that this is a painful condition (Dolan *et al.*, 2000).

1.2 Ewe mammary gland anatomy and physiology

A ewe’s udder is divided into two halves by connective tissue and each half, also known as a mammary gland, has a teat and streak canal to drain the gland (Figure 1.1). Each gland contains millions of alveoli (microscopic sacs lined with epithelial cells) that produce and secrete milk. When the surrounding muscle cells contract, milk is squeezed from the alveoli into the milk ducts. From here the milk passes to the teat cistern, located at the base of the teat, where it collects until it is stimulated (via sucking or milking machinery) to be let down through the teat. The teat end has only one opening and this sphincter can stay dilated for up to two hours after stimulation, potentially providing an entry point for bacteria.

Mammary gland development in sheep does not noticeably occur in the post-parturient (post-lambing) period. A study assessing the mammary gland growth in sheep identified that 20% of growth occurred from birth to pregnancy, 78% during pregnancy and only 2 % during early lactation (Anderson, 1975). Reduced alveolar cell integrity, induced apoptosis and sloughing of cells have been thought to occur in the mammary gland due to mastitis (Akers and Nickerson, 2011). Decreased alveoli will have a negative impact on milk production and yield as less milk will be produced and moved into the gland cistern for release.

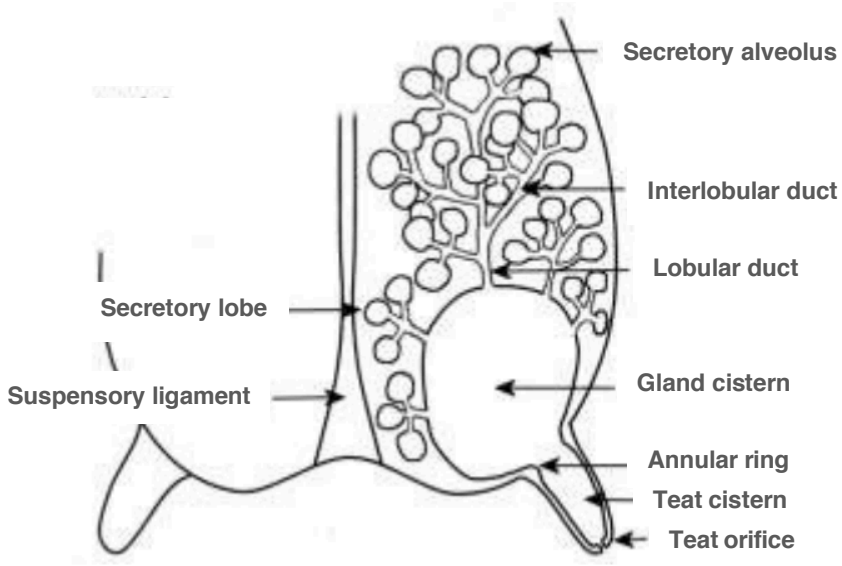

Figure 1.1 Anatomy of the ewe udder
(Sourced from http://www.uoguelph.ca/~pmenzies/Dairy_Sheep/Pdf/I-1_Normal.pdf)

Mastitis-causing pathogens have been isolated from the teats of ewes (Cooper, 2015; Mavrogianni *et al*., 2006). It has been reported that the teat is a main entry point for mastitis causing pathogens such as, *Staphylococcus aureus* and *Mannheimia haemolytica* (Mavrogianni *et al*., 2007) and that damage to the teat, for example, teat lesions, predisposes the ewe to developing mastitis by providing a route for bacteria to enter and speeding up infection (Mavrogianni *et al*., 2006; Fragkou *et al*., 2007). The sphincter of the teat canal is the first line of defence for the udder (Figure 1.2), closing tightly in order to prevent entry and invasion from possible pathogens. Bacteriostatic keratin-secreting cells in the canal also play a defensive role (Sordillo and Streicher, 2002). There is evidence of a protective role of sub-epithelial lymphoid tissue at the border of the teat duct (streak canal) and teat cistern, protecting the mammary gland from, and helping to fight off, any invading bacteria (Fragkou *et al*., 2007). If bacteria do manage to pass the ewe's initial defences then the second line of defence is the immune response. This

response can vary depending on the ewe immune system and immune memory which in turn will influence the infection outcome.

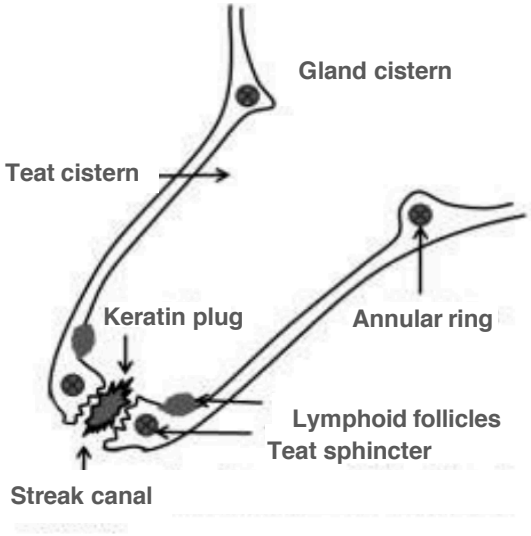

Figure 1.2 Anatomy of the ewe teat
(Sourced from http://www.uoguelph.ca/~pmenzies/Dairy_Sheep/Pdf/I-1_Normal.pdf)

1.3 Presentations of mastitis and incidence rates

Mastitis presents as subclinical disease or clinical disease, and the latter can be characterised as acute (rapid) or chronic (long-lasting).

1.3.1 Subclinical disease

No visible or physical signs of disease, but an infection is present. Although the milk looks normal subclinical infection often leads to reduced milk quality and production, and consequently impacts on lamb health and growth rates (Huntley *et al.*, 2012). Subclinical infections in dairy ewes are more likely to be detected because the ewes' udders are checked each day at milking, with less than 10% incidence rate reported in intensively managed dairy flocks (Albenzio *et al.*, 2002). Subclinical mastitis is generally detected by measuring somatic cell counts, which is explained in further detail in section 1.5.2, as there are no visible signs to indicate disease is present. Subclinical mastitis is likely to be underestimated in suckler ewes because it may not be detected; as much as 50% of a flock might be infected (Grant *et al.*, 2016).

1.3.2 Acute clinical disease

Characterised by visible, physical and potentially systemic signs of disease. The udder may be red, hot and swollen, producing an unusual discharge, watery milk or a pus-like secretion. Manual expression of milk might be difficult or non-productive (Huntley, 2013). This presentation is often painful for the ewe which may cause her to limp to prevent the udder from touching the hind legs causing

more pain. If the infection progresses, the udder can sometimes become cold and discoloured and eventually slough off as the necrotic tissue dies. This is referred to as toxic (gangrenous) mastitis and known colloquially as black bag (Figure 1.3a). It is only caused by specific strains of bacterial species such as *Escherichia coli* and *Staphylococcus aureus* (Bleul *et al.*, 2006; Mørk *et al.*, 2007). Toxic mastitis can progress quickly and lead to death (Vautor *et al.*, 2009). Prevalence of intramammary infections (IMI) in suckler ewes ranges from 0 to 6.8% per flock (Cooper *et al*, 2016; Larsgard and Vaabenoe, 1993; Arsenault *et al.*, 2008). Acute mastitis has been reported in up to 37.1% of ewes in a flock at any one time (Grant *et al.*, 2016). Results from studies identify that acute mastitis peaks in the first 4 weeks of lactation (Mørk *et al.*, 2007; Cooper *et al.*, 2016), which is also confirmed by anecdotal reports from farmers.

1.3.3 Chronic clinical disease

Detected by examining the udder; hard masses or lumps (intramammary masses or IMM) that feel different to the rest of the udder tissue are indicative of chronic mastitis (Figure 1.3b). A previous study has identified these masses as pus-filled abscesses that have probably formed as a result of bacteria infecting the gland, and can be any size or shape (Grant *et al.*, 2016; Smith *et al.*, 2015). It has been hypothesised that because of the life cycle of an abscess, where they grow, burst and then reform (Cheng *et al.*, 2011), the size of the mass is not linked to the severity of the disease (Grant *et al.*, 2016). Prevalence of chronic mastitis can vary from 1.4 to 40% of a flock (Grant *et al.*, 2016). One explanation for this wide range in prevalence could be that some farmers may not use the presence of IMM as a reason to cull ewes.

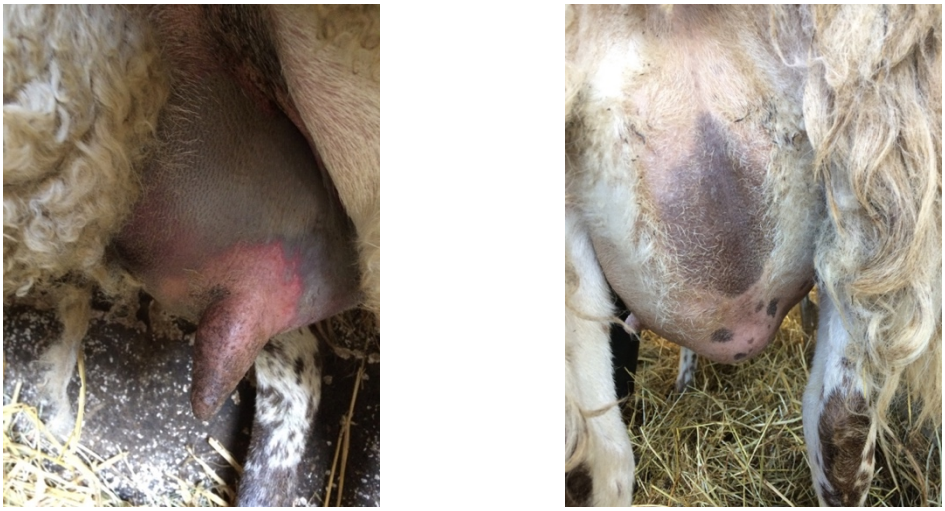

Figure 1.3a & b Ewe with toxic mastitis (left) taken from side orientation. Picture on the right is of a ewe with chronic mastitis, the intramammary mass can be seen visibly

1.4 Causative bacterial species and the ruminant udder microbiome

Over 130 bacterial species have been reported to infect the mammary gland of dairy cows (Watts, 1988). The number may be similar for sheep, however at present, more than 30 species of bacteria have been isolated from sheep milk by culture (Smith *et al.*, 2015). The major species associated with mastitis include *Staphylococcus aureus*, *Mannheimia haemolytica*, *Streptococcus agalactiae*, *Escherichia coli* and coagulase-negative staphylococci (CNS) (Bergonier and Berthelot, 2003, Gelasakis *et al.*, 2015, Smith *et al.*, 2015). These bacterial species have been categorised by their transmission routes. Contagious pathogens may spread from sheep to sheep via the mouths of cross-sucking lambs. For example, *Mannheimia haemolytica* has been isolated from the mouths of lambs and may invade the teat during suckling (Fragkou *et al.*, 2011). Environmental bacteria such as *Escherichia coli* may spread via contaminated bedding into the mammary gland and spread can be exacerbated by damaged teats (Bergonier *et al.*, 2003). However, this may be an oversimplification, species and strains of bacteria have been identified to behave as both environmental and contagious (Bradley and Green, 2001; Sommerhäser *et al.*, 2003), and all of these would have originated from the environment.

Although areas, such as the gastrointestinal tract, have been described as having complex microbial communities (Grigg and Sonnenberg, 2017), there are those who argue that the ruminant mammary gland has been considered sterile (Rainard, 2017). This view has come into question with studies putting forward a theory that the mammary gland is also made up of a microbial community (Quigley *et al.*, 2011; Oikonomou *et al.*, 2012; Monaghan, 2015). For example, the presence of an intramammary microbial community (a 'microbiome'), has been shown to contain up to 23 species per milk sample using molecular techniques (Monaghan, 2015). In addition, species of bacteria that are associated with mastitis have been isolated from milk of clinically healthy individuals (Braem *et al.*, 2012; Oikonomou *et al.*, 2014; Mongahan, 2015), suggesting that these bacterial species can also be part of the normal mammary gland bacterial community. One theory is that fluctuations within this bacterial community cause dysbiosis (imbalance of the microbiota), which could lead to the sheep being susceptible to mastitis by providing favourable conditions for a bacterial species to flourish and dominate (Fragkou *et al.*, 2007; Monaghan, 2015; Kuehn *et al.*, 2013).

1.5 Detection methods for mastitis

1.5.1 Observation of clinical mastitis

The most common method of mastitis detection in meat/suckler sheep is observation for visible signs of disease. Behavioural signs include the ewe hanging back from flockmates, inappetence, lameness due to udder pain, or refusing to suckle their lambs (Fthenakis and Jones, 1990). Consequently, observing lamb growth rates and identifying those that gain weight slower than other lambs could help in detection.

1.5.2 Somatic cell counts

The somatic cell count (SCC) is a measure of the concentration of white blood cells (leukocytes, mainly neutrophils) in milk, and can vary depending on lactation stage, physiological state of the animal or parity (Gonzalo *et al.*, 1994; Kraličková *et al.*, 2012; Jan *et al.*, 2010). The number of white blood cells increases as part of the immune response triggered during an infection. This is the case during mastitis as the immune system responds to pathogens in the udder. In the dairy industry, SCCs are monitored regularly, due to human consumption and concerns for human health, however this is not the case for non-dairy animals.

In dairy cattle, an udder quarter's SCC can range from below 10,000 to over 100,000,000 cells/ml and a SCC >200,000 cells/ml is considered an active infection (Green *et al.*, 2006). The European Union has a SCC threshold for bulk tank milk of 400,000 cells/ml (Commission Directive 89/362/EEC (1) Council Directive 92/46/EEC (2, 3)) anything above this level is deemed unfit for human consumption. Dairy farmers are financially rewarded for low SCC levels, although research in dairy cows with low SCCs has brought into question whether very low SCC levels are also a risk factor for mastitis (Green *et al.*, 2004). To date, there are no accepted threshold values of somatic cell counts for sheep, although values between 600,000 to 800,000 cells/ml have been considered to be indicative of infection (Conington *et al.*, 2008). The use of SCC in the sheep industry is limited, but it is used commonly in research as a proxy to detect infections.

1.5.3 Pen-side detection methods

Accurately testing the SCC of milk has to be carried out in a laboratory, however there are animal-side tests that can provide an indication of SCC level. An example is the California mastitis test (CMT). This uses a reagent added to the milk that disrupts cell membranes and reacts with the cell's DNA to form a gel. The reaction is scored from 0-3, score increases as the viscosity of the gel increases; a score of 2 or 3 is considered as positive for mastitis. An evaluation of CMT as a method to detect subclinical mastitis identified that CMT had a

correlation with SCC of 0.82, suggesting that CMT could be considered a good diagnostic tool (González-Rodríguez and Cármenes, 1996). A similar test is the Whiteside test, where the nucleic acids of the leucocytes form a gel. As the gel becomes more viscous the more somatic cells are present indicating infection (Fthenakis, 1994). However, CMT is more commonly used.

1.6 Methods to detect and identify bacteria

1.6.1 Bacterial culture

Bacterial culture is commonly used to identify the causative agents of mastitis (Smith *et al.*, 2015) and remains the primary diagnostic method for identifying bacteria present in sheep milk (Rovai *et al.*, 2014, Smith *et al.*, 2011). Studies have reported that the diversity of bacteria in mastitic milk is lower than in healthy individuals in humans, cows and sheep (Jiménez *et al.*, 2015; Kuehn *et al.*, 2013; Monaghan, 2015). Traditional mastitis bacteriology identified up to three bacterial species per milk sample, and samples that contained more than three species were considered contaminated. However, the view that the mammary gland is not a sterile environment challenges the idea that more than three bacterial species per milk sample must mean the sample is contaminated (Monaghan, 2015; Rainard, 2017). In contrast, culture negative mastitic samples have also been reported which, after investigation with a non-culture dependent method, were identified to have large bacterial diversity (Kuehn *et al.*, 2013). The authors of that study suggest that this reflects that even low abundance of bacterial species or changes in the microbiota can illicit an infection that is below culture detection levels.

Differentiation of cultured bacterial species is often carried out by biochemical tests or Gram staining. There are limitations to culture work, incubation often requires at least 48 hours and subsequent additional identification tests also require time and labour. However, it is a relatively cost-effective method of bacterial identification.

1.6.2 Culture-independent methods

There are various culture-independent methods that have been employed to differentiate between bacterial species in milk. Polymerase chain reaction (PCR) methods have been developed to identify up to seven mastitis causing species of bacteria in bovine milk using 16S rRNA (Meiri-Bendek *et al.*, 2002; Riffon *et al.*, 2001; Lee *et al.*, 2008). Coagulase negative staphylococci have also been identified from sheep milk directly using PCR (Onni *et al.*, 2010) and from cultured isolates which also included the identification of other mastitis causing species (Marogna *et al.*, 2010). These methods are faster but more expensive than culture-

based methods and their development is still focussed more on cow-based models than sheep.

Sequencing has also been implemented to investigate bacterial communities in both bovine and ovine milk (Monaghan, 2015; Kuehn *et al.*, 2013; Oikonomou *et al.*, 2012) and has identified the presence of mastitis causing bacteria in culture negative milk samples. However, techniques such as pyrosequencing will also detect dead organisms and are very sensitive to contamination (Oikonomou *et al.*, 2012).

Pulse-field gel electrophoresis (PFGE) has been used to identify isolates from sheep samples (milk and nasal) to strain level (Marogna *et al.*, 2010; Pilipčincová *et al.*, 2010) and is often used with PCR. Identifying to strain level is important to provide evidence of transmission or habitation of bacteria. However, this procedure is very labour intensive and expensive.

1.6.3 Matrix-assisted laser desorption/ionisation time of flight mass spectrometry

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-ToF-MS) is a soft ionisation mass spectrometry technique that uses a matrix to ionise a sample with a laser beam. Desorption and ionisation of the sample by the laser beam generates protonated ions which have distinct signals based on their mass-to-charge ratio. These can be compared to a known database to identify the bacteria isolates at species and strain levels. This detection method is cost effective, more accurate and faster than conventional molecular and immunological based detection methods (Singhal *et al.*, 2015).

The use of MALDI-ToF-MS as a rapid high-throughput system to identify bacterial species has been validated by many studies, using numerous bacterial species isolated from different sources (Barreiro *et al.*, 2010; Dubois *et al.*, 2010; Hathout *et al.*, 1999; Neville *et al.*, 2011; Singhal *et al.*, 2015; van Veen *et al.*, 2010). Recently more studies have been supporting this protein fingerprint technique's ability to differentiate not only to species level, but subspecies and strain level (Archer *et al.*, 2017; Böhme *et al.*, 2012; Du *et al.*, 2002; Rupf *et al.*, 2005). It has been suggested as methods move to more strain, than species identification, that the discriminatory power of the method must improve as members of the same species will have very similar mass spectra fingerprints (Sandrin *et al.*, 2012). Another aspect to consider is that the differences in spectra between species, subspecies and strain vary between bacteria, some being considered as nearly indistinguishable (Sandrin *et al.*, 2012). This indicates the need for only using high quality spectra that are reproducible.

1.7 Risk factors for mastitis

Mastitis is a multifactorial disease, so as such, many factors can influence its occurrence. A range of risk factors for mastitis have been identified and these can be categorised into internal (animal) and external (management) factors.

1.7.1 Internal risk factors

Internal factors include ewe parity, litter size and stage of lactation (Albenzio *et al.*, 2002; Antunac *et al.*, 2002; Waage and Vatn, 2008) with an elevated risk of mastitis as parity and litter size increase and at the beginning of lactation. Teat and udder conformation have also been identified to be important; pendulous udders, thick, forward facing or vertically pointing teats all being associated with mastitis in suckler ewes (Casu *et al.*, 2010, Huntley *et al.*, 2012). In addition, ewes with chapped teats or lesions (which may also be a result of contagious ecthyma (orf virus)) have been recognised as a risk for mastitis (Burriel, 1997). A body condition of <2 has also been associated with the development of mastitis (Arsenault *et al.*, 2008; Karagiannis *et al.*, 2014). Having a history of mastitis (both chronic and acute) greatly increases the chances of a ewe developing mastitis again (Grant *et al.*, 2016; Waage and Vatn, 2008). Although one study reported that flocks following recovery from Mycoplasma agalactiae were associated with less cases of mastitis, the authors hypothesise that it may be that the animals in that case had become immune to that organism, which is also known to cause mastitis (Al-Momani *et al.*, 2008). Breed differences have been reported (Larsgard and Vaabenoe, 1993), suggesting some genetic effects regarding mastitis risk.

The health status of the ewe will also make them more or less susceptible to infections. If the immune system is already weakened or fighting off another infection then this may make it easier for mastitis causing pathogens to colonise, dominate and develop into an infection. For example, a study investigating trematode infections identified that infected ewes were predisposed to mastitis, hypothesising an impact from the trematode infection on the local cellular defences leaving the ewe susceptible to infection (Mavrogianni *et al.*, 2014). Mastitis is also linked to a viral disease, Maedi-visna, as clinical mastitis is a symptom (Arsenault *et al.*, 2003). Ewes exposed to stress have been associated with a compromised immune system, increased somatic cell counts and cases of mastitis (Caroprese *et al.*, 2010; Sevi *et al.*, 2001b).

1.7.2 External risk factors

Significant external risk factors include the environment such as housing hygiene; poor ambient conditions, increased humidity, dirty bedding and equipment all increasing the risk of mastitis (Albenzio *et al.*, 2002; Sevi *et al.*, 2002; Sevi *et al.*, 2003a; Alexopoulos *et al.*, 2011). As stocking density increases (Caroprese, 2008)

the effects of housing hygiene on increased mastitis levels can be exacerbated if not managed appropriately. Separating diseased animals from healthy has been suggested to decrease the chances of ewe-ewe transmission so therefore further animals developing mastitis (Grant *et al.*, 2016).

Management systems, such as keeping ewes on cultivated pasture rather than mountain pasture, not providing shelter, lambing indoors and not allowing access outdoors to dairy ewes have also been associated with an increased risk of mastitis (Larsgard and Vaabenoe, 1993; Gregory, 1995; Cooper *et al.*, 2016; Casamassima *et al.*, 2001). Studies have reported that increased flock size increases mastitis risk (Zwiefel *et al.*, 2005; D'Amico and Donnelly, 2010). However, another study did not find any association (Carloni *et al.*, 2015) or observed a decrease in milk bacterial count, so reduced risk of infection, as the number of animals milked increased (Alexopoulos *et al.*, 2011). This could reflect different management strategies employed as flocks increase in size, for example implementing better milking practices (Alexopoulos *et al.*, 2011) and vice versa rather than a direct effect from flock size. Recording ewes that have sub-clinical and clinical mastitis has been identified to be negatively associated with somatic cell counts (Molina *et al.*, 2010), perhaps being aware of the ewe's history and possible risk as an infection to the rest of the flock allows more control over the disease.

For dairy ewes the milking technique and hygiene status of any equipment used, has an impact on the occurrence of mastitis. Inappropriate vacuum levels, pulsation rates or inefficient cluster release being among these factors that have been suggested to predispose ewes to developing mastitis, although significant results regarding these factors have not always been identified (Gelasakis *et al.*, 2015; Sinapis *et al.*, 2006; Peris *et al.*, 2003a, Peris *et al.*, 2003b). Studies have found no significant association between drying off techniques (progressive or abrupt) and mastitis levels (Petridis *et al.*, 2013). However, one study identified that 47% of cases developed in the first 3 weeks of cessation of lactation in a flock of dairy ewes (Saratis *et al.*, 1998). Anecdotally meat sheep farmers in England report that high levels of mastitis at weaning, detection bias may need to be considered here as farmers are more likely to examine the ewes at weaning than at other times of lactation.

Recent work has suggested a link between dietary protein and energy fed during pregnancy, and lactation, and the occurrence of mastitis. Feeding insufficient levels of protein in pregnancy was associated with a four-fold increased risk of acute mastitis in lactation. In addition, underfeeding of energy in pregnancy and during lactation increased the risk of intramammary masses six-fold and two-fold respectively (Grant *et al.*, 2016). These findings support an earlier study that

identified an association between an increased risk of clinical and subclinical mastitis post-partum and pregnancy toxaemia brought on by insufficient provision of energy during pregnancy (Barbagianni *et al.*, 2015).

Seasons also appear to have an effect on mastitis. Higher milk somatic cell counts, so increased chances of an infection being present in the udder, were identified in ewes that lambed in the winter compared to ewes that lambed in the autumn. This may be attributed to a rise in ambient temperatures and housing conditions may be less hygienic during the summer months, increasing the chances of bacterial survival (Sevi *et al.*, 2004). Colder, wetter weather has also been associated with prolonging the survival of *Mannheimia haemolytica* in the environment of sheep (Burriel, 1997). Regional differences have also been commented on (Arsenault *et al.*, 2008), which may reflect differences in control measures between regions or different microclimates.

It has been demonstrated that *Mannheimia haemolytica* survives and flourishes in the nasopharynx of sheep (Omaleki *et al.*, 2015) and on the tonsils of lambs (Fragkou *et al.*, 2011). There is also evidence of lambs transmitting *Mannheimia haemolytica* to teats of the ewe via sucking (Fragkou *et al.*, 2011; Gougoulis *et al.*, 2007), however strain typing was not used so some caution should be used interpreting the results. The nasal cavity of sheep has also been suggested as a reservoir of *Staphyloccocus aureus* (Vautor *et al.*, 2005; Mørk *et al.*, 2012) and results using strain typing has suggested that transmission occurs between dam and lambs (Mørk *et al.*, 2012).

Staphylococcus species are part of the commensal flora on the skin of sheep and human hands (Kloos and Musselwhite, 1975; Grice *et al.*, 2008). Increased amounts of bacteria, including *Staphylococcus* and *Streptococcus* species have been isolated at greater loads from hand milked ewes than machine milked (Menzies and Ramanoon, 2001, de Garnica *et al.*, 2013). Authors hypothesised this to be a consequence of the milker's hands aiding the transmission of bacteria from ewe to ewe and possibly from the environment or themselves (Gelasakis *et al.*, 2015).

Cleanliness of equipment used with sheep is important, milking equipment in particular for dairy sheep (Zweifel *et al.*, 2005). Many farmers use pre - and/or post - milking teat disinfection in an attempt to decrease the chance of bacteria being transferred from the milking equipment into the udder, although this is not common place everywhere (Molina *et al.*, 2010). Al-Momani *et al.* (2008) identified an association between improper cleaning of milking equipment and a significant increase in levels of *Mycoplasma agalactiae* in the flock. Suggesting that the milking equipment is a transmission risk.

1.8 Treatment and prevention of mastitis

The speed in detection and treatment of mastitis in sheep has been identified as important as the development of disease and subsequent damage to the udder by mastitis can be rapid. (Fragkou *et al.*, 2014). Once clinical or subclinical infections are identified, prompt treatment using antibiotics is necessary, as it is a bacterial infection. Prompt and appropriate treatment will also aid to decrease the chances of the infection spreading further through the flock (Gelasakis *et al.*, 2015). However, withdrawal periods for dairy sheep that have been treated has to be considered. Separation of infected ewes and their lambs from the flock and culling infected ewes may also help to decrease spread of disease (Grant *et al.*, 2015). The use of non-steroidal anti-inflammatory drugs as pain management for sheep is not frequently used (Lizarraga and Chambers, 2011), however these drugs have been demonstrated to decrease pain and distress, therefore increasing health and welfare, and may speed up recovery from disease (Gelasakis *et al.*, 2015; McKellar, 2006).

Since 2008, in the dairy cow industry, a national mastitis control plan has been available. Using the information gathered from a detailed survey of the farm, milk bacteriology and somatic cell counts, a personalised plan for each individual farm is developed depending on their own identified issues. Significant decreases in the proportion of infected cows on the farms with a plan have been reported (Green *et al.*, 2007). However, no such plan is in place for sheep. Although no effective control method has yet been developed a number of risk factors have been identified, as described in more detail earlier in section 1.7. Using the knowledge of these risk factors, a change in practice or the implementation of preventative measures can help reduce the occurrence of mastitis.

Dry cow therapy (DCT) may be used on many dairy cow farms as the dry (non-lactating) period has been recognised as a risk for infections (Green *et al.*, 2002). DCT involves a long acting antibiotic being administered via the teat end, to aid in preventing new and existing infections. It has been reported to be effective in reducing somatic cell counts and thereby intramammary infections in sheep as well (Olechnowicz and Jaśkowski, 2012; Gonzalo *et al.*, 2005). However, this practice is doubtful to become common practice in the sheep industry and the dairy cow industry is also reducing the use of this and favouring individual over flock treatment, particularly in view of the current antimicrobial climate. The use of teat dips pre- and/or post milking may also be used in the dairy sheep industry to try and prevent new infections and transmission of bacteria between ewes (Molina *et al.*, 2010).

1.9 Methods for studying mastitis

There are various epidemiological and laboratory methods employed by researchers to study mastitis. Below I will discuss the methods used in this PhD project.

1.9.1 Systematic reviews

Unlike traditional literature reviews, systematic reviews use a pre-defined search criterion to answer a specific question. The systematic methods used in the search reduce bias in selection and identification of included studies and makes it a robust and repeatable approach (Sargeant and O'Connor, 2014). The aim of the search is to identify as many relevant studies as possible. The studies that are selected by the search are subjected to critical review and assessed to establish if they fit the pre-defined criterion that is needed to address the specific research question being asked (Sargeant *et al.*, 2006). These criteria could include a certain size of study sample, the study being carried out in a certain geographic location or the study being published within defined time parameters. The criteria may also include exclusion parameters, such as no studies to be included that do not mention the species of animal the review is aimed at addressing. The relevant data is then extracted from the remaining papers that have passed the review stage. This extraction allows the information to be summarised which makes for easier comparisons (Sargeant *et al.*, 2006). Depending on the question of the review the information that is extracted from the studies may be quantitative or qualitative.

Selecting papers from the search results, appraising the studies and extracting the results is more powerful if at least two authors/investigators working independently complete this. Inter-rater agreement can be measured between the investigators for confidence of limited bias occurring and increases accuracy of data extraction (Meade and Richardson, 1997; Sargeant *et al.*, 2006). Some systematic reviews that have quantitative data to summarise use meta-analyses. This is a statistical method to combine results from different studies which can increase the power and accuracy of the estimates of treatment effects and conclusions from the studies (Mulrow, 1994, Sargeant *et al.*, 2006).

Although historically systematic reviews have been used in healthcare, they are being used more and more in the veterinary sciences and the usefulness of them has also been identified in the agri-food sector (Campbell *et al.*, 1998; Sargeant *et al.*, 2006).

1.9.2 Randomised controlled trials

A randomised controlled trial (RCT) is an example of an intervention research method. Intervention studies have been described as being more conclusive than observational studies and less susceptible to confounding (Coggon, 1997; Kristensen, 2005). Kristensen (2005) describes an RCT by four features. The first feature being randomisation of participants into the intervention or control group. This can be done in different ways but the fundamental principle is that the participant is only by chance in one or the other group. The second and third feature is the presence of a control group and that the intervention is only exposed to the intervention group. The control group can then be used to identify any effect of the intervention. The fourth feature is the design where at least one measurement is taken before and after the intervention in both groups, again so the effect of the intervention can be established.

However, there can be a problem obtaining consent for intervention studies, particularly for human studies. If possible, blinding the participants and researchers to which group is receiving the treatment is preferred to prevent any bias.

1.9.3 Milk sampling and longitudinal studies to investigate mastitis

Milk samples have been used in numerous previous studies to investigate mastitis in ewes (Sevi *et al.*, 2003b; Fthenakis, 1995; Mørk *et al.*, 2007; Smith *et al.*, 2015). To investigate communities and possible transmission events, swabs from sheep nasal cavities, the mouths of lambs and ewe teats have also been collected and analysed (Omaleki *et al.*, 2015; Fragkou *et al.*, 2011; Cooper, 2015) as have hand swabs in human studies (Rusin *et al.*, 2002). Taking samples using swabs is a relatively non-invasive, pain free method and are easily stored and cultured from.

Taking repeated samples over time provides a more complete overview of what has changed and provides an opportunity to see effects of any intervention.

1.10 Summary and conclusions

Mastitis is a multifactorial disease that impacts heavily on sheep health and welfare and farm productivity and economics. Prevalence and incidence differ between the presentations of mastitis with the disease ranking as one of the most important in the sheep industry, with costs predicted to exceed £120M/annum. Disease incidence peaks near the beginning of lactation and housing has been identified to increase the risk of mastitis. Although various risk factors have been identified, an effective control mechanism has yet to be established. Over 30 species of bacteria have been associated with mastitis, however within flock transmission routes or pathways are yet to be explored and described. More information regarding transmission and possible prevention strategies during housing may help to decrease the occurrence of mastitis in sheep.

1.11 Thesis aims

The aim of this thesis was to improve our understanding of mastitis in suckler ewes and to explore effective control mechanisms and transmission routes of mastitis. To address this the following objectives were executed:

1. Conducted a systematic literature review of risk factors for mastitis (Chapter 2)
2. Carried out an intervention study on one flock in England to test the impact of raising hygiene in the perinatal period on occurrence of mastitis (Chapter 3)
3. Investigated the effect of additional hygiene protocols during indoor lambing on the transmission and persistence of bacteria within ewes and between ewes and lambs at strain level (Chapter 4)
4. Explored the relationship between intramammary masses and bacterial communities in one flock (Chapter 5).

Chapter 2. Risk factors for mastitis in sheep, a systematic review: Nutrition and hygiene

2.1 Introduction

Mastitis is one of the most important diseases to affect sheep (Davies *et al.*, 2009), and a significant financial burden on the sheep industry. Therefore, an important issue regarding sheep health and welfare and farm productivity. To improve understanding of the risk factors for mastitis a systematic review was performed on current peer-reviewed published research.

Systematic reviews follow a structured process and, as defined by the Cochrane Collaboration, collates all available evidence to answer a specific question using specified criteria (Green *et al.*, 2011). An explicit, systematic method is used to reduce bias in order to produce robust and reliable findings from which conclusions can be drawn (Sargeant *et al.*, 2006). This method has been used in human healthcare since the 1990s and is being used more and more in veterinary medicine and the agri-food industries (Lonch *et al.*, 2015; Mederos *et al.*, 2012; Fogarty *et al.*, 2018; Clark and Magalhães, 2018).

2.2 Aim

The aim was to collate all the information on risk factors for mastitis in sheep by conducting a systematic review. The ultimate aim was to identify risk factors for mastitis in ewes that could be investigated for causality in an intervention study.

2.3 Method

The method used is an adaption of that used by the Cochrane organisation to suit a veterinary framework (Sargeant *et al.*, 2006, Green *et al.*, 2011) resulting in a 10-stage process (Figure 2.1).

2.3.1 Formulation of the question

For all systematic reviews the first step involves formulating a specific question that the review aims to answer. For this review the question was: “What are the risk factors for mastitis in sheep?”.

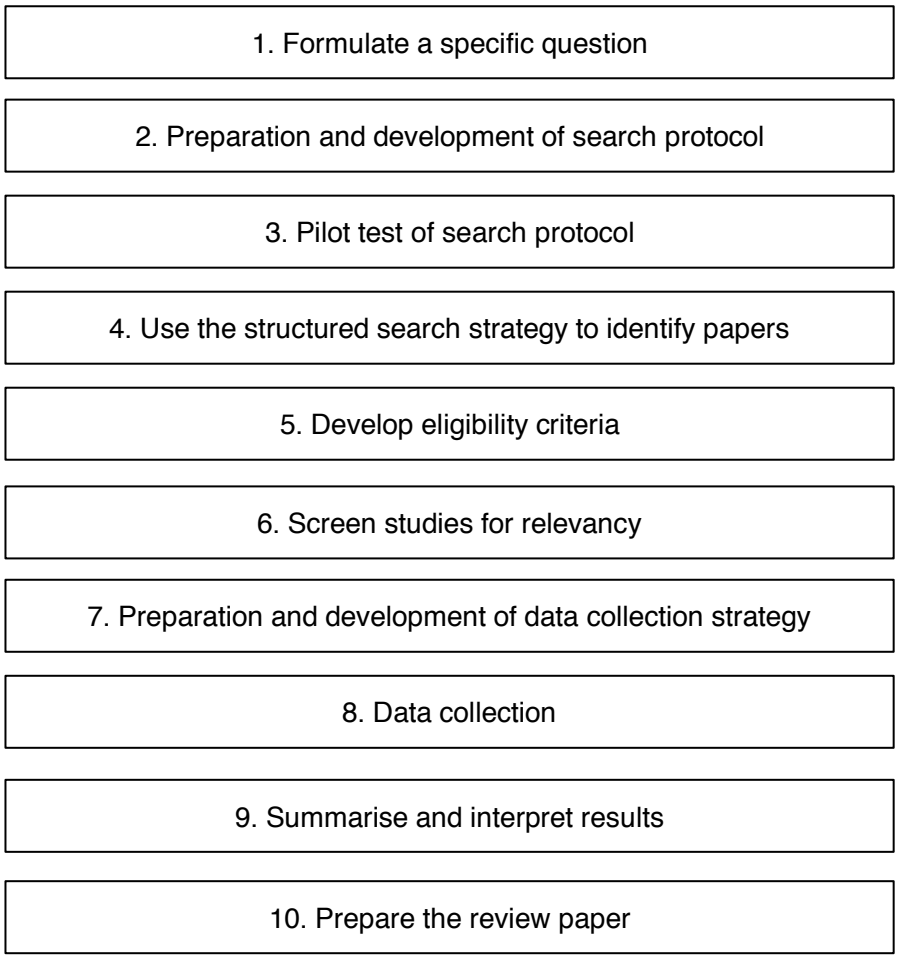

Figure 2.1 10-stage process for the systematic review (Adapted from Sargeant *et al.*, 2006)

2.3.2 Search protocol development

The search strategy aims to capture the maximum number of relevant papers using a transparent, structured and repeatable approach (Sargeant *et al.*, 2006). To increase the retrieval and number of relevant papers and reduce bias, a number of bibliographic databases were used. With the help of an experienced librarian, frequently used and established scientific databases were identified for use. These databases were Scopus (www.scopus.com), Web of Science (apps.webofknowledge.com), Proquest (www.proquest.com) and Science Direct (www.sciencedirect.com), all of which have been used previously in agricultural systematic reviews (Lonch *et al.*, 2015; Clark *et al.*, 2018; Fogarty *et al.*, 2018). CAB abstracts / CABI (www.cabdirect.org) has also been recommended and used for veterinary literature reviews (Grindlay *et al.*, 2012; Mederos *et al.*, 2012). The usefulness of CABI for this review was tested against Scopus. Criteria questions were used to assess the usefulness of papers (abstracts only) captured

by CABI database and Scopus database. The criteria included: is the correct species mentioned, is mastitis (intramammary masses included) mentioned, is there a risk factor mentioned, are somatic cell count (SCC) risk factors mentioned, and are bacterial populations risk factors mentioned? All the criteria must be met for the paper to be included. The results (section 2.4.1) identified that CABI was not necessary to use for the purpose of this review therefore only the other four databases were retained for use.

2.3.2.1 Pilot test

Initially a list of key words and phrases linked to/associated with sheep and mastitis was produced to aid in the development of an optimum search term. A series of trial searches were run in each database. This involved removing and adding words to the search term in a structured manner to identify which words were the most influential and useful in the final search term. Sentinel papers - papers that should be captured by the search as they were known to contain the information required - were used to test the suitability of the searches. In total, 6 such papers were selected (Appendix 1). Different Boolean Operators (these aid with the logical combination of variables using AND, *, OR etc. in the search browser) were used to ensure that all spellings and plurals were captured in the results.

Ultimately the following search term was deemed the most suitable as it maximised the number of relevant results, and minimised the number of irrelevant studies:

(ALL (mastitis OR imi OR "intramammary infection*") AND TITLE-ABS-KEY (sheep OR ewe* OR ovine OR "suckler ewe*") AND ALL ("risk factor" OR scc OR "somatic cell count" OR management) AND NOT TITLE-ABS-KEY (antimicrobial OR "genetic analysis" OR "diagnostic testing" OR molecular OR "acute*phase" OR bola OR "bovine lymphocyte antigen" OR *estrus OR lactoferrin OR antibiotic*).

The filter of TITLE-ABS-KEY (title, abstract, key words) was used for the species search term as this reduced the amount of irrelevant papers in the search results. Once the trial searches had been completed the final search was carried out in the four bibliographic databases and the resulting papers exported into Endnote (EndNote X7.5.3).

2.3.3 Screening for eligibility

Duplicates were removed automatically in Endnote and then a manual check was performed to remove identical papers with different formats missed by Endnote. The abstracts of the remaining papers were screened for relevancy. A list of

criteria was generated to enable accuracy and repeatability of this process, these criteria were used to select the final papers for inclusion in the review. Only peer reviewed documents written in English were included. Papers were removed if they did not contain or mention any of the following; mastitis, intramammary masses, somatic cell count, bacterial count plus any risk factor related to impacting on these. Papers that investigated comparison of treatments for protection were also excluded. Papers identified by the search alerts were also subjected to these criteria.

2.3.4 Information extraction and recording

Data on risk factors and research approaches were collated into an Excel (Microsoft Excel for Mac 2011) spreadsheets. Risk factors for mastitis were identified from the publications and related topics were grouped together. The key facts about the paper research methods and results were also recorded.

2.4 Results

2.4.1 Assessment of CABI inclusion

The search in Scopus and CABI resulted in 2,245 papers. There were more unique papers relevant to the review question in Scopus than CABI. A total of 14 papers were found in CABI alone that were eligible, these papers were from journals of unknown reputability and so it was concluded that CABI was not a necessary database to use.

2.4.2 Summary of search term findings

A total of 1,521 papers were identified by the searches. There were 605 duplicates and 916 different papers (Table 2.1). After eligibility screening 126 papers remained.

Table 2.1 Number of papers at each stage with bibliographical database

Database	Sentinel papers	Imported (+ alert addition)	Automatic duplicates	Manual duplicates	Final number
Scopus[1]	6 (100%)	548 (551)			
WoK[2]	5 (83%)	269			
Sci Direct[3]	6 (100%)	401			
Proquest[4]	4 (67%)	300			
Combined	6 (100%)	1518 (1521)	468	137	916
Selected					126

[1] Scopus (www.scopus.com), [2] Web of Knowledge (https://webofknowledge.com/), [3] Science Direct (www.sciencedirect.com), [4] Proquest (www.proquest.com) - Science and technology

Of the 126 papers, 19 (15%) were literature reviews, 75 (60%) were dairy sheep focused, 21 (16%) suckler focused, and 30 (24%) studied both dairy and suckler sheep. There has been an increasing number of publications on mastitis in both dairy and suckler sheep over the past 25 years (Figure 2.2). Suckler sheep, in particular, increasing in number over the last 5 years.

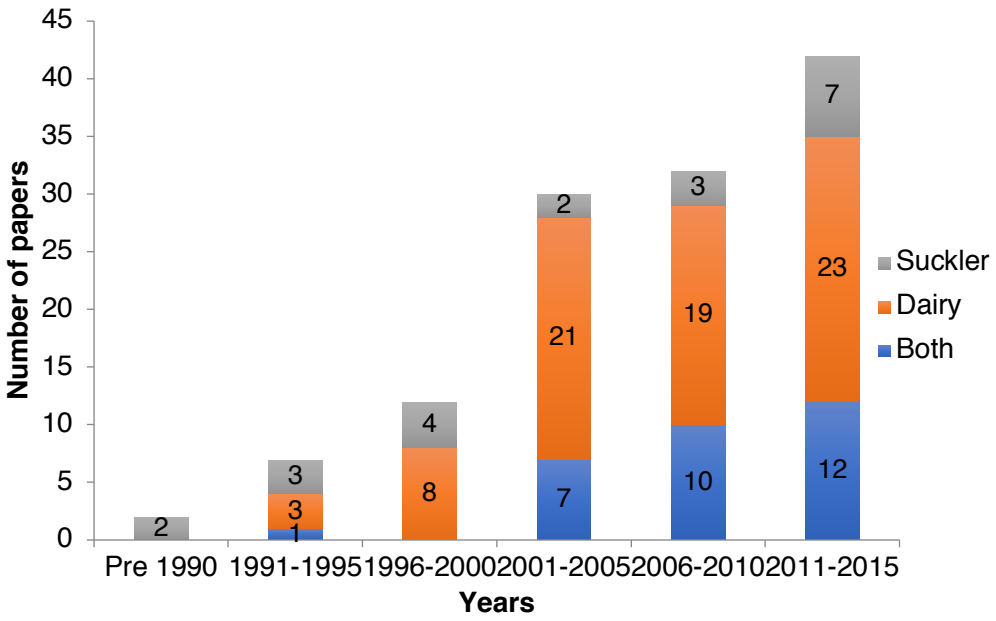

Figure 2.2 Number of papers per year group and by industry

The research publications came from 25 countries, though almost 50% of them were from Greece, Italy or Spain.

The top five risk factors for mastitis in sheep by frequency were hygiene, nutrition, parity, stage of lactation and drying off procedures respectively (Figure 2.3).

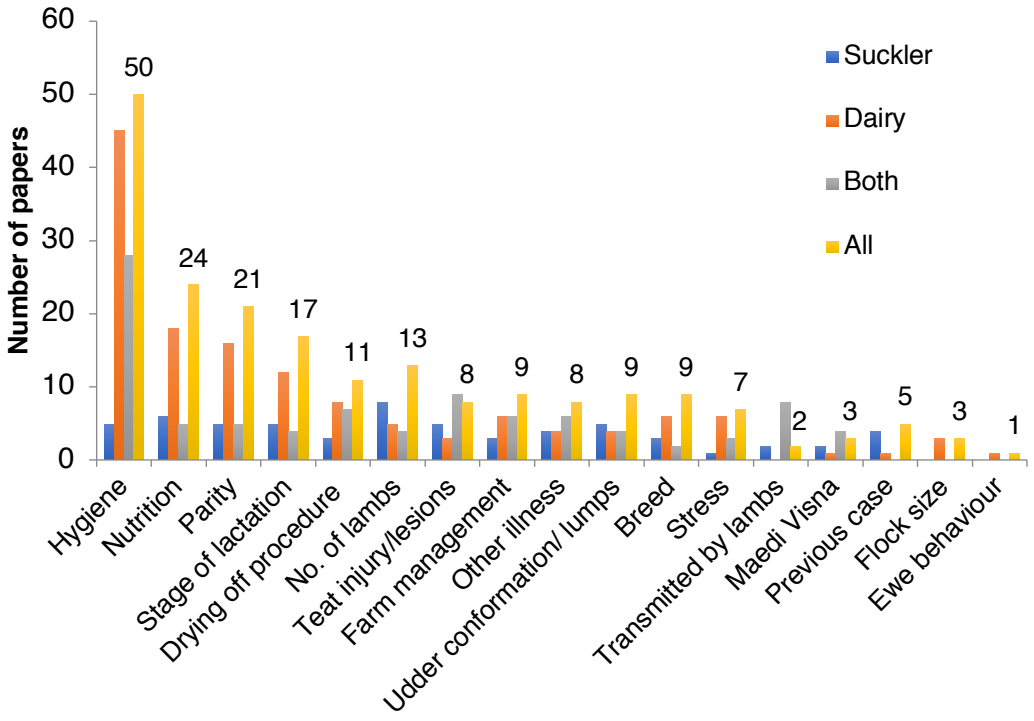

Figure 2.3. Frequency of risk factor identified by the papers. Numbers represent frequency for all

2.5 Summary of extracted information concerning the top two risk factors for mastitis by frequency

The overall aim of this systematic review was to identify risk factors for mastitis that could be tested in an intervention study. Risk factors were prioritised by frequency rather than relative risk because we wanted to identify practices that were common to all farms. If a practice is carried out by a large number of famers, then any benefit found by amending this practice would affect a larger proportion of the population than a practice that was only executed by a few. As nutrition and hygiene were mentioned in over half of the 126 selected papers, it was decided to focus on these two major risk factors in more detail. The information, results and conclusions extracted from the 74 papers that discussed the impacts of either hygiene or nutrition on mastitis, were synthesised qualitatively and discussed below.

2.5.1 Nutrition related risk factors

Correct and adequate nutrition is important in maintaining health and promoting optimal growth. The effects of nutrition on diseases of small ruminants has been investigated and accepted for some years (Caroprese *et al.*, 2015) but its impact on mastitis has only recently been reported. However, it has some popularity as a research topic shown by the fact that it is the second most frequent topic to be

discussed in the final selection of review papers. Nutrition can cover many different aspects; a breakdown on the subtopics and papers concerning these is detailed in Appendix 2 and discussed in more detail below.

2.5.1.1 Body condition score

One practical and non-invasive method of assessing nutritional status is to determine body condition score (BCS). To assess the ewe's body condition the lumbar region is palpated, placing a hand over and around the backbone and transverse processes behind the last rib to feel the amount of fat cover and muscle mass. The score ranges from 1-5, 1 being very thin and 5 very fat, with 0.5 increments, and is defined depending on how prominent and sharp the spinous and transverse processes feel. The more prominent and sharper these feel, the lower the score will be (Defra PB1875).

A low body condition score in pregnancy or lactation has been associated with increased levels of mastitis. Scores below 2.5 have been identified as a significant risk factor for increased somatic cell count (SCC) (Huntley *et al.*, 2012), positive California Mastitis Test (CMT) result (Arsenault *et al.*, 2008) and increased clinical mastitis (Marogna *et al.*, 2010, Karagiannis *et al.*, 2014). However, not all studies identified a significant association between BCS and acute or chronic mastitis incidence and prevalence (Marogna *et al.*, 2010, Grant *et al.*, 2016). The time points of when BCS is measured could be of importance, if there are not enough data points taken this may mask any affects or associations present. Therefore, assessing the ewes BCS once in pregnancy and/or once in lactation as in Grant *et al.* (2016) and Marogna *et al.* (2010) may not be a sufficient number of assessments to identify any associations between BCS and chronic and acute mastitis, which may explain the absence of significant results in these studies. In addition, Marogna *et al.* 2010, only focussed on the relationship between body condition scores over 3 and chronic mastitis incidence. This would have prevented any associations with other body condition scores being identified. Other authors that have investigated pre-lambing BCS on health disorders after lambing found a significant association a BCS of 3.5+ and increased risk of developing a health disorder, of which clinical mastitis was one (Karagiannis *et al.*, 2014).

In summary there is agreement between studies that BCS does have an association with mastitis, both low (<2.5) and high (>3.0). This provides evidence for nutritional influence on disease status.

2.5.1.2 Energy status and needs of the ewe

Negative energy balance can be assessed by measuring β-hydroxybutyric acid (BHBA) and non-esterified fatty acids (NEFA) blood concentrations. Increased

NEFA and BHBA levels indicate (respectively) that free fatty acids are being released from adipose tissue to be used as fuel and that the body is burning fat reserves such as liver glycogen due to insufficient availability of glucose or carbohydrate energy (Karagiannis *et al.*, 2014). High BHBA has been associated with liver damage and parasite infection during pregnancy (Mavrogianni *et al.*, 2014, Caroprese *et al.*, 2015), lactation (Gelasakis *et al.*, 2015) and a decreased energy containing diet (Sevi *et al.*, 1998, Bouvier-Muller *et al.*, 2016). A decrease in BCS has been associated with raised BHBA and NEFA levels (Bouvier-Muller *et al.*, 2016).

Several authors have used BHBA and NEFA levels to assess negative energy balance and its association with mastitis (Karagiannis *et al.*, 2014, Mavrogianni *et al.*, 2014, Barbagianni *et al.*, 2015, Caroprese *et al.*, 2015, Fthenakis *et al.*, 2015, Gelasakis *et al.*, 2015, Bouvier-Muller *et al.*, 2016). Raised BHBA (>1.0mmol/L^{-1}) and NEFA (>0.4mmol/L) levels before parturition have been associated with an increased risk of clinical mastitis (Karagiannis *et al.*, 2014, Mavrogianni *et al.*, 2014, Barbagianni *et al.*, 2015), subclinical mastitis (Barbagianni *et al.*, 2015), increased SCC (Mavrogianni *et al.*, 2014) and positive CMT (Mavrogianni et al., 2014). High BHBA and NEFA levels in lactation have also been associated with increased somatic cell score (SCS) (Bouvier-Muller *et al.*, 2016) and increased incidence of clinical mastitis (Gelasakis *et al.*, 2015).

High BHBA levels, negative energy balance, causes ketosis which is known as pregnancy toxaemia (Barbagianni *et al.*, 2015), this disease only occurs during the last month of pregnancy as the demand on the ewe for energy from the foetus increases and therefore the liver mobilises fat stores to meet this demand. Authors suggest that increased levels of BHBA in pregnancy impedes multiple facets of the immune response such as leukocyte and neutrophil activity (Barbagianni *et al.*, 2015) and the phagocytic behaviour of leukocytes (Mavrogianni *et al.*, 2014, Fthenakis *et al.*, 2015), because less energy is available for the immune system, as it is being diverted to places of need like respiring tissues (Karagiannis *et al.*, 2014). This would consequently increase susceptibility to infectious diseases by weakening the body's defences and predisposing ewes for the development of mastitis (Barbagianni *et al.*, 2015).

Measuring both BHBA and NEFA levels to assess negative energy balance rather than just one is advised as it has been seen to raise one, not always both levels (Bouvier-Muller *et al.*, 2016) and fatty acid release from adipose tissue normally occurs before ketone bodies increase.

Other studies that have looked at the effects of energy on mastitis incidence and prevalence, but have not used BHBA or NEFA levels as indicators, also identified

significant associations. Insufficient energy fed in pregnancy (e.g. carbohydrates) were associated with an increased chance of chronic mastitis by six-fold (Grant *et al.*, 2016). Adequate energy levels are not only important during pregnancy, particularly in the last 6 weeks when the foetus puts 70% of its birthweight on, but also during lactation so the ewe can meet the lambs demand for milk (Caroprese *et al.*, 2015). These demands can almost double the ewe's energy requirements. Inadequate milk supply may lead to hungry lambs biting or butting for more food causing teat lesions and predisposing the udder to infections and mastitis (Grant *et al.*, 2016; Huntley *et al.*, 2012). Milk quality and yield from ewes fed either a medium level (0.93 milk forage units) or a low level (0.64 milk forage units) of energy during pregnancy resulted in significant differences in somatic cell counts during lactation, with those fed low energy feed having higher SCC levels (Sevi *et al.*, 1998). This indicates that negative energy balance is not only an issue during pregnancy but persists into the early stages of lactation (Sevi *et al.*, 1998). Investigations have also found that under feeding energy, and energy depletion due to nematode infection, in lactation is associated with a two-fold risk of chronic mastitis in the subsequent pregnancy (Grant *et al.*, 2016) and pre-disposed ewes to mastitis (Mavrovgianni *et al.*, 2014).

2.5.1.3 Protein levels

Protein is also an important nutritional requirement during pregnancy and lactation that is associated with mastitis. Growth of the foetus and development of the mammary gland both demand increased levels of protein from the ewe (Grant *et al.*, 2016). Increased protein intake in sows has been demonstrated to have a positive effect on mammary growth of lactating sows (Kim *et al.*, 2009). Underfeeding protein in pregnancy was associated with a 4-fold increase in the rate of acute mastitis (Grant *et al.*, 2016). In addition, although not focussing only on protein, primiparous ewes that were exposed to undernourishment (60% of nutritional plane) in pregnancy had decreased alveoli cellular proliferation and overall mammary gland weight than those that were fed the required levels or higher (Neville *et al.*, 2013; Swanson *et al.*, 2008). In contrast, Sevi *et al.* (2006) found no significant effect of feeding low levels of dietary protein (13% versus 16% crude protein) during mid-lactation on milk yield in ewes. Evidence suggests that ewes only exhibit mammary growth and development through puberty, pregnancy and at the very beginning of lactation (Anderson, 1975; Neville *et al.*, 2013), compared to other species of animal such as pigs, that also display growth in lactation. This may explain why protein has less of a detrimental effect during lactation in ewes than if lacking during puberty and pregnancy. Therefore, if nutritional needs for protein are not met in pregnancy then it is possible the mammary gland does not develop properly, reduced alveoli will reduce the ability to produce milk. The ewe may be unable to provide enough milk for hungry lambs and be more susceptible to teat injuries from lambs butting and biting for more

food, providing an entry point for bacteria and predisposing the ewe to mastitis (Winter, 2001, Grant *et al.*, 2016). Protein and energy are linked, for example in the rumen the microbial community uses both to support maintenance of tissues, therefore both levels together should be taken into consideration.
There is sufficient evidence that energy and protein levels are very important and have a significant impact on mastitis levels. Further work is needed in this area especially as it is possible that the current guidelines for energy requirements in feed may be out of date and insufficient (Grant *et al.*, 2016).

2.5.1.4 Vitamin A and E, beta-carotene and selenium levels

Vitamin A is essential for a healthy immune system and the maintenance of skin integrity that provides a barrier against infections (Giadinis *et al.*, 2011). Beta-carotene is a pre-cursor for vitamin A and an anti-oxidant that protects the body from free radicals. Omission of vitamin A from the diet was associated with increased rates of clinical mastitis (Koutsoumpas *et al.*, 2013, Gelasakis *et al.*, 2015), and subclinical mastitis (Koutsoumpas *et al.*, 2013, Caroprese *et al.*, 2015, Gelasakis *et al.*, 2015). In a study by Giadinis *et al.* (2011) lower vitamin A blood levels were significantly associated with increased mastitis incidence rates in dairy ewes, caused by any pathogen, compared to concentrations found in healthy ewes. Vitamin A is involved with the integrity and function of the epithelium of the teat; therefore, any deficiency may increase the susceptibility of the teat skin to infections and the consequential development of mastitis (Giadinis *et al.*, 2011, Koutsoumpas *et al.*, 2013, Caroprese *et al.*, 2015). Vitamin A has also been reported to have a direct effect on the immune response, perhaps enhancing lymphocyte activity (Koutsoumpas *et al.*, 2013). This interaction has not been investigated in suckler ewes, although the integrity of the mammary gland skin is as important to suckler ewes as dairy ewes, because damage can occur from lambs or milking equipment.

Vitamin E is an antioxidant and has been reported to have positive effects on the activity of phagocytes (Caroprese *et al.*, 2013). Although no significant association was reported in the studies, retrieved from this systematic review, between vitamin E blood concentration and mastitis incidence, lower blood concentrations of vitamin E have been identified in dairy ewes that developed acute mastitis caused by *Staphylococcus aureus,* compared to concentrations found in healthy ewes (Giadinis *et al.*, 2011). In the same study by Giadinis *et al.* (2011) lower selenium blood levels were significantly associated with increased mastitis incidence rates in dairy ewes, caused by any pathogen. Other studies have investigated the effects of vitamin E and selenium together on mastitis. Administration of vitamin E and selenium during the dry period of dairy sheep resulted in lower SCC in the first stage of lactation although no differences in clinical cases was observed (Morgante *et al.*, 1999, Bergonier *et al.*, 2003).

Whether the effect was due to selenium or vitamin E or both is unknown because both were administered together. However, as demonstrated in the study by Giadinis *et al*. (2011) higher selenium blood concentrations were associated with reduced mastitis incidence and reduced blood concentrations were found in ewes with mastitis associated with *Staphylococcus aureus*, *Mycoplasma agalactiae* and coagulase-negative staphylococcus. This association was not found with vitamin E, which led the authors to conclude that selenium deficiency may predispose ewes to develop mastitis, through impaired cellular defences.

Reviews on the effects of nutrition on SCC in sheep milk and microbial diseases also highlight the importance of correct integration of vitamin A (or beta-carotene), vitamin E and selenium into diets, particularly if ewes are fed mainly conserved fodders where the levels of these micronutrients might decline with storage (Pulina *et al*., 2006, Caroprese *et al*., 2015).

Ewes fed on alfalfa hay and a concentrate with no added vitamin A were either given supplementary injections of vitamin A or left untreated. The ewes that received vitamin A supplementation had lower SCC during lactation than those left untreated (Koutsoumpas *et al*., 2013). However, the role of vitamin E and selenium on SCC is more ambiguous. Dairy ewes given parenteral injections of vitamin E and selenium around 1 month before lambing had reduced SCC compared to those that did not receive the supplementation injection (Morgante *et al*., 1998). However, dairy ewes that were fed supplements of vitamin E and selenium, from two weeks before lambing up to 60 days after parturition, had lower SCC than those supplemented with just vitamin E (Pulina *et al*., 2006). This suggests, similar to above, that selenium has a more protective role than vitamin E, although it may be that the nutrients work synergistically, but this is not discussed in the studies. However, in a nutrition review by Pulina *et al.* (2006) a study by Chiofaol *et al*. (1998) (original paper reported in Italian) is discussed, the results of which identified that ewes fed concentrates with supplemented vitamin E alone had decreased milk SCC than those that did not.

One paper did not report a change in SCC after administration of vitamin E and selenium; however, the sample size was very small (16 ewes) so whether this is a true representation could come into question (Pulido *et al*., 2012). Pulido *et al*. (2012) also looked at the effect of lengthening the milking period as well as vitamin E and selenium supplementation on SCC. There was a trend that supplementation when lengthening the milking interval between machine milking actually increased SCC. This was thought to be a consequence of vitamin E being an antioxidant, meaning it could have adverse effects under oxidative stress, such as during the milking period.

Although all the work on vitamins and minerals has been carried out on dairy ewes, suckler sheep are often deficient in micronutrients (Govasmark *et al.,* 2005, Gelasakis *et al.*, 2015) and these risks could be important to suckler ewe mammary gland health. Work in this area could warrant more attention.

2.5.1.5 Other supplements

Other supplements have been investigated for an association with mastitis. Bovine somatotropin (BST) is a peptide hormone used to increase milk yield in dairy cattle, as the genetic makeup of this peptide is extremely close to that of ovine somatotropin (which is not available) so studies have used BST in sheep. The systematic search only identified one study on BST and its impact on the risk of mastitis in sheep. This may be because the use of growth hormones is not registered or approved for livestock within EU countries. The small study on dairy ewes (22 Chios ewes) identified that BST administration was associated with increased milk yields throughout lactation but also demonstrated an association with increased SCC and subclinical mastitis levels compared to the non-treated ewes (Brozos *et al.*, 1998).

The hormone oxytocin is involved in lactation and is critical for the milk ejection reflex in suckling or artificial milking. It causes the contraction of the cells around the mammary alveoli, pushing milk into the ducts and cistern resulting in the expulsion of milk. Studies have been carried out to investigate the influence of oxytocin on milk yield because this can drop dramatically in dairy sheep after weaning, possibly because lambs stimulate release of oxytocin and this therefore stops after weaning (Zamiri *et al.*, 2001). The results of a small study of 25 ewes suggested that ewes injected with oxytocin had lower SCC and higher milk yields than ewes that were untreated (Zamiri *et al.*, 2001). The study did not suggest that those injected with oxytocin will have lower levels of mastitis, but taking into consideration the significant decrease in SCC it is possibly something to consider, although the effect of dilution with increased milk yields must also be recognised.

Unprotected conjugated linoleic acid was supplemented to dairy ewes but in this form increased SCC and significantly decreased milk yield and quality (Oliveira *et al.*, 2012). Ewes have also been supplemented with fatty acids using olive cake, which is characterised by high levels of monosaturated fats such as oleic acid. There were higher milk yields and reduced SCC, however, lower SCC only occurred when the diets were supplemented with vitamin E and showed a trend towards higher SCC in the supplement without vitamin E than in the controls. Perhaps the antioxidant effects of vitamin E were preventing high levels of SCC or promoting lower levels (Chiofalo *et al.*, 2004). More research focused on the effects this supplement has on the ewe, rather than the milk quality, is warranted.

Feed additives are used to try to manipulate the rumen to get as much nutritional utilisation and possibly increase ewe performance. One family of additives is essential oils. SCC were lower in ewes that experienced an addition of essential oils (150mg/kg of concentrate) than control ewes that were fed the same mixed ration but untreated with essential oils (Giannenas *et al.*, 2011). This led to a hypothesis that essential oils possess anti-bactericidal properties; however this needs further investigation as the study only involved 80 ewes (Giannenas *et al.*, 2011). In a review article on the interactions between nutrition and microbial diseases and the defence mechanism of small ruminants, the incorporation of glutamine in the diet of lactating animals reduced the inflammatory reaction (reduced secretion of IL-10) to a humoral response challenge when under environmental stress and enhanced immune responses when the animal was under homeostatic challenges (Caroprese *et al.*, 2015). During lactation ewes are subjected to a number of stressors, therefore the addition of glutamine to the diet may be of a benefit.

In summary, there is a large amount of evidence on aspects of nutrition having significant associations on the risk of mastitis occurrence and incidence. This includes the role of energy and protein levels in the diet, challenging the current recommended levels and ratios of these.

2.5.2 Hygiene related risk factors

The next sections discuss the identified risk factors related to hygiene in more detail. A breakdown on the subtopics and papers concerning these is detailed in Appendix 3.

2.5.2.1 Housing

Increased levels of clinical mastitis (Cooper *et al.*, 2016), subclinical mastitis (Tietze *et al.*, 2001), increased total bacterial counts (TBC) (Tietze *et al.*, 2001) and increases in SCC (Casamassima *et al.*, 2001, Tietze *et al.*, 2001, Caroprese, 2008, Sevi and Caroprese, 2012) have been associated with housed sheep.

Numbers of bacterial species and the loads of these bacteria in an area increase when ewes are housed indoors (Caroprese, 2008). The optimal conditions for bacteria to survive and grow varies, however in general, a warm, moist, humid condition protected from ultraviolet exposure (Marogna *et al.*, 2010) will aid in bacterial multiplication and survival. A build-up of faeces, wet litter and potential mould increases the chances for bacterial survival (Bergonier and Berthelot, 2003, Bergonier *et al.*, 2003). This situation allows more exposure of the udder to environmental mastitis-causing pathogens such as *Escherichia coli* and coagulase negative staphylococcus (Albenzio *et al.*, 2002). An 8-week study conducted on 40 housed dairy ewes identified that ewes housed on straw

bedding that was renewed (completed at 4 weeks in this study) or/and had absorbing products such as bentonite added (0.5kg/m^2 of litter) had increased milk yield and reduced milk SCC and bacterial counts compared to ewes on bedding that was not renewed or treated with an absorbing product (Sevi *et al.*, 2003a). Addition of bedding on alternate days was associated with halving the risk of clinical mastitis occurring while ewes were housed at lambing compared with bedding that was added at a frequency of less than 2 days (Cooper *et al.*, 2016).

What is used as flooring or bedding substrate may also influence mastitis. Authors have suggested that hardcore flooring is associated with lower levels of clinical mastitis (Cooper *et al.*, 2016). This may, however, be a reflection of the greater depth of bedding farmers give as it is a rougher flooring, or it could be due to its improved drainage qualities. Therefore insufficient or ineffective bedding, lack of regular and efficient manure removal, and lack of disinfection can predispose ewes to mastitis (Gelasakis *et al.*, 2015).

2.5.2.2 Air quality/hygiene

Increased stocking density per ewe increases bacterial counts, SCC in milk (Sevi *et al.*, 2001c), incidence of subclinical mastitis and reduces milk yield (Sevi *et al.*, 1999). This could also be a sign of stress as ewes that are offered access to an external paddock when housed had lower SCC levels than those without (Caroprese *et al.*, 2009). As the stocking density increases this can affect the ambient hygiene if no adjustments are made, i.e. area/volume per animal, ventilation and humidity and litter quality of the surrounding area and environment.

Ewes with higher volume airspace/animal have been associated with lower SCC, bacterial counts and incidence of subclinical mastitis (Sevi *et al.*, 2001a, Caroprese, 2008). A study of 36 dairy ewes identified that providing anything less than 7m^3 volume allocation per ewe reduced milk yield and quality, and increased airborne microorganisms and risk of subclinical mastitis (Sevi *et al.*, 2001a).

Current recommendations for space allowance of housed sheep after lambing are 2.0-2.2m^2 for lowland ewes and 1.8-2m^2 for hill ewes with lambs (Defra – recommendations for the welfare of livestock: Sheep). Space allocations below 2m^2 were associated with increased levels of mastitis and worsened udder health (Sevi *et al.*, 1999). However, this study did not leave the lambs with the ewes, as they were also looking at the effect on milk yield of these dairy ewes. Even so it is possible the current Defra recommendations do not allocate enough space per ewe.

Climatic conditions offer natural ventilation when outdoors (although this may not always be optimal), however, this is not the case indoors. With an increased number of bodies respiring and expelling products, providing ventilation for the housed animals, especially during hot spells, can play a role in sustaining health and performance (Bergonier and Berthelot, 2003, Bergonier *et al.*, 2003). Housing hygiene is associated with udder predisposition to mastitis, shown by low ventilation programmes being associated with increased levels of mastitis. Providing a ventilation rate of 66m^3/h per animal in the summer and 47m^3/h per animal in the winter has been reported to help in reducing any unnecessary addition to this risk (Caroprese, 2008). In contradiction a six-week trial where 36 ewes were divided into different ventilation systems resulted in no association between ventilation and SCC levels. This was conducted in the winter and on a small number of animals so these factors could have affected this. The study did show, however, that the low ventilation rate was unable to remove moisture and gases and if the ventilation is too high this can cause an increase in dust particles in the air (Sevi *et al.*, 2003b).

2.5.2.3 Season

The season may exacerbate the issue of hygiene and its effect on mastitis. A rise in SCC was reported in ewes that lambed in the winter (January - February) compared to ewes that lambed in the autumn (October – November) (Sevi *et al.*, 2004). Authors hypothesised that this association may have been a consequence from a rise in ambient temperature and worsening of air and litter hygiene during the summer months (Sevi *et al.*, 2004). Summer can provide warm, humid conditions suitable for the growth of some microorganisms, especially in warmer climates such as the Mediterranean and Middle East, where levels of clinical mastitis and SCC is higher in the summer months and the most common bacteria are *Staphylococcus aureus* or *E. coli* (Sulaiman and Al-Sadi, 1992, Lafi *et al.*, 1998, Matutinovic *et al.*, 2011, Sevi and Caroprese, 2012, Narenji Sani *et al.*, 2015). This has been attributed, not only because the conditions favour bacterial growth, but also due to the stress of the increased temperature on the ewe's physiological functions. Consequently, temperatures inside housing must be regulated and checked to ensure that it is not too hot and adequate ventilation and humidity levels are maintained. *Mannhaemia haemolytica* (a known pathogen of mastitis) favours cold, wet conditions and can survive up to 24 hours (longer if sheep are present), which could be of particular problem during wet periods, as bedding stays wet for long periods of time (Burriel, 1997, Sevi *et al.*, 2004, Omaleki *et al.*, 2011, Arias *et al.*, 2012).

2.5.2.4 Hygiene and techniques around milking

The vast majority of research and literature captured by this systematic review is in relation to the dairy sheep industry so this incorporates hygiene protocols or

techniques of milking equipment. Some of these techniques and theories can be easily transferable to other sheep industries, such as suckler and wool as the equipment can be thought of as sucking lamb/s.

Method of milking dairy sheep

The prevalence of subclinical mastitis has been reported to be lower in those flocks that are milked mechanically than those milked by hand (Las Heras *et al.*, 1999). Hand milking has also been seen to elicit TBC (Gonzalo *et al.*, 2006, de Garnica *et al.*, 2013), SCC (Sinapis, 2007), bulk tank somatic cell count (BTSCC) (Gonzalo et al., 2005, Olechnowicz and Jaoekowski, 2012), bulk milk somatic cell count (BMSCC) (Molina *et al.*, 2010) and intramammary infections (IMI) (Menzies and Ramanoon, 2001) than mechanical parlour systems. Hand milking systems have a reported 62% increased risk of bacterial positivity compared to mechanical systems (Marogna *et al.*, 2010, Olechnowicz and Jaoekowski, 2012). It has been hypothesised that this could be a consequence of the milker's hands acting as vectors passing the bacteria from ewe to ewe (Albenzio *et al.*, 2003, Bergonier and Berthelot, 2003, Gonzalo *et al.*, 2006, Sinapis, 2007, de Garnica *et al.*, 2013, Gelasakis *et al.*, 2015).

The use of gloves is likely to be higher in the dairy community than the meat industry, with reports of 80% of milkers wearing gloves during milking in north east Greece in 2010 (Alexopoulos *et al.*, 2011). Farmers of suckler sheep may check if ewes are lactating, when they give birth, offering an opportunity of spreading bacteria between ewes if hands are not washed or clean gloves are not used between animals, and if the milk is squirted onto the floor and/or bedding. The use of gloves for milking has been debated, with no association found if they are worn or not in a study by Carloni *et al.* (2015), however this may be attributed to a small sample size as only 5 of the 24 dairy farms involved actually wore gloves. It has also been commented that if gloves are worn there is a risk that it may be less obvious how soiled the gloves are in comparison to soiled hands, highlighting the importance of changing them regularly or between ewes (Albenzio *et al.*, 2003). The use of gloves has also been linked with increased mastitis levels, but this was considered a reflection on the fact that gloves are worn more when a mastitis outbreak occurs (Albenzio *et al.*, 2003), so this may represent an effect of mastitis rather than a cause.

Staphylococcus species and *Streptococcus* species have been identified at significantly higher levels in milk for hand milked flocks than machine milked (Menzies and Ramanoon, 2001, de Garnica *et al.*, 2013). Exceptions have been found where smaller farms with hand milking had better sanitation so therefore lower standard plate count (SPC) than those using mechanical systems (Zweifel *et al.*, 2005). Some have reported no significant difference between the two

practices (Carloni *et al.*, 2015), however the authors reported that this was due to good machine hygienic practices from the farms participating in the study. This study carried out by Carloni *et al.* (2015), involved questionnaires so it could also be that those who participated where already interested in hygiene and kept a high standard on their farm. In light, the hands of milkers could be considered a vector such as the mouths of lambs, which have also been identified as main carriers of staphylococci and streptococci compared to milking equipment (Albenzio *et al.*, 2003).

In addition, machine milking can offer a more reliable consistency of continuous pressure and technique while milkers can vary in experience and technique, possibly causing discomfort to the ewes and milking inefficiently (Sinapis, 2007).

Milking equipment

There are different levels of automatic or machine milking. For those that do not want to have a dedicated parlour or do not see the benefits of installing a fully automated system, bucket milking and portable milking devices can be used. This can eliminate some of the risk of the milkers hands acting as vectors, however bucket milking is associated with higher SPC (Zweifel *et al.*, 2005), TBC (Gonzalo *et al.*, 2006), BTSCC (Gonzalo *et al.*, 2005), worsened udder health (Gonzalo *et al.*, 2005) and a 40% higher risk of bacterial infection (Olechnowicz and Jaoekowski, 2012) than parlour milking with fully automated fixed plant systems. Small portable devices have also shown a strong correlation with the spread of *Streptococcus uberis*, a mastitis causing pathogen, in a flock (Marogna *et al.*, 2010). Portable devices have lower performance characteristics than fixed plants, which may affect the consistency of vacuum and pressure causing discomfort or entry for bacteria. They are also more prone to wear as they milk only two ewes at a time, tears and cracks may offer conditions for bacteria to grow and pass between the ewes (Marogna *et al.*, 2010).

Hygiene and cleanliness of all equipment is crucial. Improper cleaning of milking equipment has been associated with a 3-fold increase in levels of *Mycoplasma agalactiae*, which is the main pathogen of contagious agalactia, a cause of mastitis (Al-Momani *et al.*, 2008). Adequate quality control of the water that is used to clean the machines is also important; failure to do this has been linked to infection outbreaks in *Pseudomonas aeruginosa* (Contreras *et al.*, 2007). It is important to remove accumulation of dirt, debris and build-up of bacteria that can be a risk for infections. Using disinfectant as well as water to clean the machines is advisable with suggestions of this being carried out twice a day (Bergonier *et al.*, 2003).

Parlours can use dead-ended or looped milklines, there has been no significant difference found between the systems in relation to BTSCC (Gonzalo *et al.*, 2005) but higher TBC have been associated with dead-ended milklines (Gonzalo *et al.*, 2006). The height of the milking line, which can have an effect on the cost of the machine, has not been associated with any increase in SCC or affecting yield (Díaz *et al.*, 2004).

Liners in the teat cups can induce trauma to the teats predisposing the ewe to mastitis and act as a vector of bacteria between the ewes (Bergonier and Berthelot, 2003). Over use of liners can lead to an increase in bacterial and pathogen concentrations and load (Bergonier and Berthelot, 2003). Therefore, it is suggested to change rubber liners every year and for silicon liners every two years to try to prevent them getting worn out and increasing the risk of bacterial invasion and mastitis (Bergonier *et al.*, 2003, Gelasakis *et al.*, 2015). An analogy in suckler ewes would be the mouths of lambs, evidence suggests that lambs are a reservoir of bacteria and transmit bacteria to the teat of the ewe, which if the conditions are favourable may cause mastitis (Fragkou *et al.*, 2011).

Vacuum levels, pulsation rates and traction systems

Overmilking and milk retention should be avoided as they promote bacterial multiplication in the udder (Gelasakis *et al.*, 2015). These factors can be controlled via adequate vacuum levels, pulsation rates and traction control. Although studies have not always found an association between vacuum level and pulsation rate with SCC or IMI rates (Peris *et al.*, 2003a, Peris *et al.*, 2003b, Sinapis *et al.*, 2006), those that have or seen a trend, pose recommendations of more pulsations (180 cycles/min) and low vacuum levels (34 to 36 kPA) for optimum udder health and milk yields (Gonzalo *et al.*, 2005, Sinapis *et al.*, 2006). Traction systems have been seen to have an association with increased falling of teat cups, a doubled risk of acute mastitis and a trend of increased SCC levels (Peris *et al.*, 1995). The same concept concerns suckler ewes as milking systems mimic a sucking lamb, and suckling is thought to predispose the teat to infections (Gougoulis *et al.*, 2008). The manner in which lambs suck can cause damage to the teats and affect udder health and increase the risk of developing mastitis (Mavrogianni *et al.*, 2006).

Overmilking and cluster removal

Overmilking could be linked to timely cluster removal. An experienced operator will be able to time this, however, as the industry grows more and more farmers may start to use automatic cluster removal systems. It has been suggested that the inflexibility of a machine may lead to increased cases of mastitis or increase SCC levels, due to overmilking, although there is no significant evidence of this as of yet. Automatic cluster removal appears, however, effective at reducing oedema, which could lead to increased udder health (Peris *et al.*, 2003a, Bueso-

Ródenas *et al.*, 2015). Minimising mammary massage and machine stripping could also aid in reducing the possibility of mastitis and IMI (Bergonier *et al.*, 2003). There are suggestions that foremilking (stripping) may remove milk with high concentrations of bacteria so prevent the entry of this in the bulk milk. However, papers captured by this systematic review found no benefit either way in regards to SCC level, but overmilking was reduced by 33% when stripping was omitted (McKusick *et al.*, 2003). Although no associations of this have been found to be significant in regards to mastitis levels, SCC or SPC, there have been observations of lower *Staphylococcus aureus* counts in milk from those that carry out stripping (D'Amico and Donnelly, 2010). The benefit to the actual incidence of mastitis and its welfare impact on the sheep is questionable.

2.5.2.5 Milking frequency

When omitting or reducing the number of milkings, mixed results have been reported. Milk obtained from milking episodes separated by short intervals has been associated with higher SCC values than those milked with more than 16 hour intervals (Castillo *et al.*, 2008; McKusick *et al.*, 2002). In contrast, studies have reported a significant increase in SCC when milking frequencies were decreased from twice to once a day milking (Nudda *et al.*, 2002, Pulido *et al.*, 2012). Conversely, other studies have reported no significant association with SCC when omitting 1-2 milkings per week (Hervás *et al.*, 2006, Castillo *et al.*, 2009). Caution in the interpretation of these results is necessary as a reduction in frequency of milkings can also reduce milk yield, which may be another reason the SCC level appears affected. With decreased yields the concentration of SCC will seem higher. It has been suggested that a milking order, with those ewes with infections going last, may help in preventing transmission in the flock (Bergonier *et al.*, 2003, Gelasakis *et al.*, 2015).

2.5.2.6 Pre- and post-teat disinfection

Teat dipping or spraying before or after milking, which is commonplace in dairy cows but not so much in sheep, had been suggested to reduce IMI incidence and prevent new infections (Bergonier *et al.*, 2003, Contreras *et al.*, 2007, Gelasakis *et al.*, 2015). Post milking disinfection has been seen to reduce BMSCC (Molina *et al.*, 2010). However, it has been noted that the quality of teat dip is important as it could act as a source of infection causing sporadic outbreaks of mastitis (Contreras *et al.*, 2007, Gelasakis *et al.*, 2015). A targeted approach has been suggested for post milking teat disinfection, to be completed during times of increased prevalence of clinical mastitis and/or teat problems, which generally coincide with the start of the milking period for dairy ewes when the lambs are removed (Bergonier and Berthelot, 2003, Gelasakis *et al.*, 2015).

Whatever the equipment or technique used, if the practices are performed by untrained personnel this could predispose the ewe to mastitis (Gelasakis *et al.*, 2015).

In summary, although many of the papers have a dairy focus, hygiene is important across all sheep industries. In dairy cow literature there is a vast amount of evidence that hygiene has an impact on mastitis levels. Historically, in cow research, mastitis was thought of as contagious (animal to animal) or environmental. However, as many pathogens can behave as both (Bradley and Green, 2001; Sommerhauser *et al.*, 2003), it may be helpful when considering hygiene as a risk factor to think about it in this way. Key risk factors identified from the papers in this systematic review include: the exposure to housing environments that favour bacterial survival and growth such as dirty bedding that is not renewed with fresh bedding or does not contain any absorbing products, poor ambient hygiene (ventilation and air space) and below recommended space allocation at lambing. Hand milking ewes or using ill-maintained and unhygienic milking equipment also poses a risk to increased mastitis levels compared with machine milking and regularly checking and cleaning equipment.

2.6 Conclusion

The two key areas where risks for mastitis have been identified were hygiene and nutrition. A comprehensive review of the literature regarding these areas highlighted the importance of energy and protein levels and the possible role of supplements and additives in feed on the prevalence and incidence of mastitis in ewes. Multiple publications emphasise the impact housing and milking equipment hygiene may have on mastitis levels, mostly through controlling bacterial levels the ewes are exposed to. More work is needed in both areas, to better understand the nutritional requirements of the ewe to ensure that current feed requirements are suitable, and what management practices can help reduce mastitis risk during indoor housing.

Chapter 3. An intervention study to investigate the impact of "best practice" hygiene methods to minimise intramammary disease in indoor lambing suckler ewes

3.1 Introduction

Mastitis is an important endemic disease of sheep impacting heavily on health and welfare. It is usually caused by bacterial infection of the mammary gland and presents as sub-clinical infection or clinical disease. Clinical disease can be further characterised as acute or chronic mastitis. In acute mastitis the gland becomes hot, red and swollen and there might be an abnormal discharge. In chronic mastitis there are masses of abnormal consistency in the mammary gland.

The annual cost of mastitis in Texel sheep in the UK has been estimated to be £2.7 million (Conington *et al.*, 2008). The costs to the whole UK sheep industry is far above this figure. Losses from mastitis are both direct and indirect. Direct costs arise from increased deaths and culling of ewes affected with mastitis, and antibiotics for treatment. Indirect costs include decreased milk yield and lower lamb growth rates (Huntley *et al.*, 2012, Grant *et al.*, 2016).

A range of risk factors for intramammary infections have been identified. A systematic review (Chapter 2) looking at risk factors for mastitis identified the top five areas by frequency as; hygiene, nutrition, parity, stage of lactation and the drying off procedure respectively. Although this suggests that hygiene has been investigated widely, the systematic review (Chapter 2) also identified a dearth of knowledge regarding hygiene and indoor lambing suckler ewes.

In the dairy cattle industry the importance of hygiene on reducing mastitis levels was identified in the early 1960s which led to strategies being set in place. This includes disinfection of teats after milking, maintenance of udder health during the dry period to reduce exposure to environmental pathogens, and maintenance of udder and hind leg cleanliness by clean, dry bedding and disinfecting stalls (Neave *et al.*, 1969; Elbers *et al.*, 1998 and Green *et al.*, 2007).

Factors that have been associated with poor hygiene in the sheep industry include; poor housing ventilation affecting humidity and airspace, and dirty, wet bedding or equipment (Albenzio *et al.*, 2002; Bergonier *et al.*, 2003). An increased incidence rate of clinical mastitis has been associated with sheep being kept

indoors during lambing compared to those lambing outdoors or a combination of both (Cooper *et al.*, 2016). In the UK it is common practice for sheep to be housed during lambing, reflecting the importance of this time period in relation to the prevalence and incidence of clinical mastitis. Therefore, the importance of hygiene around this time should also be considered and investigated.

The frequency of indoor lambing in the UK, the reduced information on the importance of hygiene around this time and number of applied studies meant it was decided to focus entirely on hygiene around the lambing period rather than a more holistic approach.

3.2 Aims

The aim of this study was to investigate the impact of an improved hygiene protocol from the time a ewe started lambing until turnout (typically 1– 5 days) on the prevalence and incidence of acute and chronic mastitis.

3.3. Materials and methods

3.3.1 Study population

The study was approval by the University of Warwick's ethics committee (AWERB.29/16-17, revised, 19/01/17). The farmer was recruited in October 2016 and informed consent from the farmer was obtained before the study started. He was compensated for inconvenience of hosting the trial at the end of the study.

The study was carried out from January – September 2017 (Figure 3.1) in a commercial flock in Northamptonshire, England. The flock size was approximately 960 ewes, with a range of breeds, 85% of the ewes were Scottish and North country mule. Ewes lambed indoors between 20th February and 5th April 2017. Two researchers were present on farm from 19th February 2017 to 5th April 2017 (excluding 17th – 20th March 2017, rest bite period) and at least one researcher was present for 24 hours each day. There were 814 multiparous ewes due to lamb from 20th February and 150 first parity ewes due to lamb from the 20th March.

Data on the date, time, unique ewe identification number, body condition score (BCS 1 – 5 in 0.5 increments; Defra PB1975) and the presence/absence of IMM in each udder half and lambing data were collected over five periods during the study (Table 3.1, Figure 3.1). BCS was measured by placing a hand across the loin region, behind the last rib, and feeling the level of fat and muscling over and around the vertebrae. Depending on how prominent and sharp the horizontal and transverse processes were determines the score given (1-very prominent and sharp; 5-cannot feel either). The flock was inspected by the same two trained

researchers on each occasion. For each visit both researchers recorded data on the first 15 ewes to ensure consistency in the recording of IMM and BCS. The researchers swapped roles every 50 ewes. Data were recorded on a handheld data logger (Agrident APR 500) using project specific software (Border Software Ltd, UK).

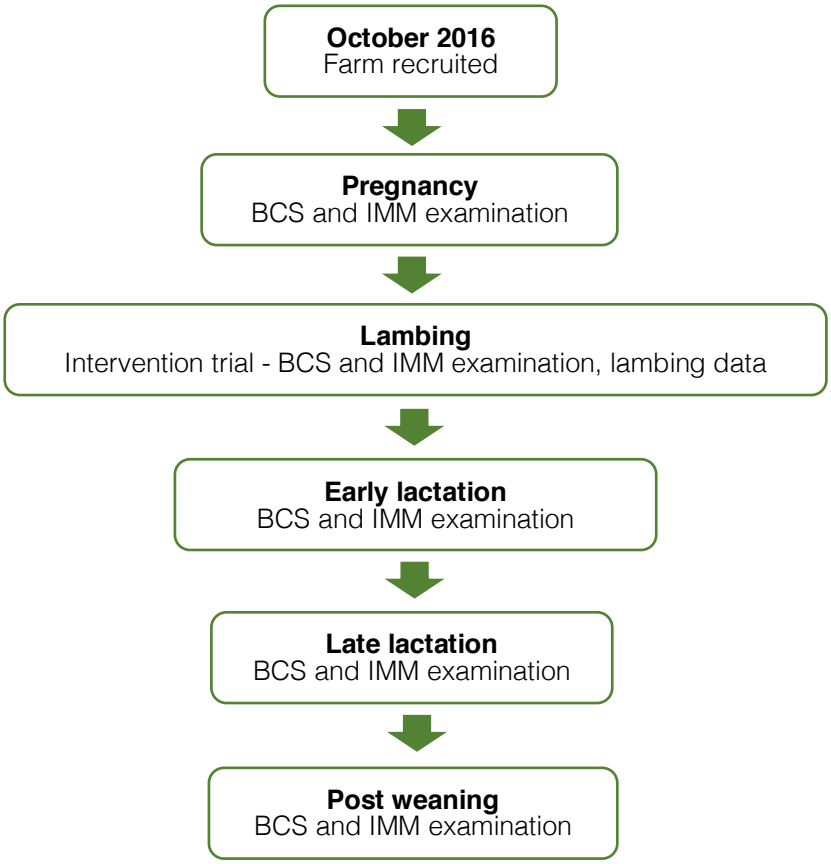

Figure 3.1 – Flow diagram of study and what measurements are taken when. BCS = body condition score, IMM = intramammary masses

Table 3.1 – Visit periods for the intervention study and data collected

Visit	Stage of production	Dates	Data collected
1	6 weeks before lambing	18th-19th January & 20th February	Body condition score (BCS) and presence of intramammary masses (IMM)
2	Lambing	19th February – 5th April	Biological samples*, lambing data, body condition score and presence of IMM
3	4 weeks after lambing	24th – 25th April	BCS and presence of IMM
4	End of lamb weaning period	3rd August & 9th August	BCS and presence of IMM
5	Before the ewes were mated	20th September	BCS and presence of IMM

* Swabs (vagina, teat, nose, lambs' mouth, handler's hands), milk and bedding samples

3.3.2 Detection of intramammary masses and body condition scoring

Ewes were examined for IMM on five occasions (Table 3.1). The ewes were examined upright in a race (single file walk way) whilst being restrained by a clamp (Ritchie equipment Ltd 'Combi-Clamp', Figure 3.2) except at lambing when examination was completed individually in a pen. Data collected were: date, time, unique ewe identification number, BCS and the presence/absence of IMM in each udder half. IMM were detected as a mass in the mammary tissue of either udder half that was a different consistency from the rest of the mammary tissue. The researchers estimate that the smallest masses detectable were approximately 1cm in diameter. If the entire gland was one hard mass it was categorised as severe chronic mastitis.

Figure 3.2 Set-up during detection of IMM and BCS scoring. Ewe is restrained upright by a clamp in a single file walkway.

3.3.3. General farm management at lambing

3.3.3.1 Housing and management during pregnancy and at lambing

All ewes were housed in one of two barns (lambing and second barn), in groups of 150 - 200 ewes, for six weeks before the flock was due to start lambing. The rams were left in with the ewes during mating for two oestrus cycles, so the ewes had two chances of getting pregnant. Due to this some ewes were due to lamb earlier than others. Ewes had been marked with coloured spray during scanning, to indicate the number of lambs expected and if due to lamb in the first or second flock. The second barn was split into 4 sections with approximately 150 ewes per section with the ewes closest to parturition housed in the sections nearest to the lambing barn. The lambing barn was divided into three sections (Figure 3.3); one third was a large yard containing approximately 200 ewes where the ewes lambed, another third was comprised of three small yards, two of these were used for approximately 20 ewes with newborn lambs and one was a “hospital” yard for sick ewes; this yard was closest to the preparation area and ewes could be observed for any behaviours that needed attention. Some lambs remained with their dam in the hospital yard, however if a ewe was very ill her lambs were removed and raised as orphans. The final third of the lambing barn contained 98 pens for individual ewes with lambs. Ewes lambed in the lambing yard of the lambing barn, under the supervision of the researchers and farm workers.

If a ewe had been straining to give birth for over half an hour she was checked and lambing assistance given if deemed necessary.

As ewes lambed and the number of ewes in the lambing yard reduced, the ewes that were expected to lamb next were moved from the second barn to the lambing yard of the lambing barn. Ewes that lambed unexpectedly in the second barn were walked or transported (when the mud between the barns was too much for the ewes to walk through) with her lambs to the after-lambing pens (described in section 3.3.3.2).

3.3.3.2 Housing and management after lambing

Once a ewe had lambed, she and her lamb(s) were left for approximately 15 minutes (to allow bonding to begin) and then moved to a ‘wet pen’ where the lambs were given 1ml of Spectam (Spectinomycin dihydrochloride pentahydrate, an oral aminocyclitol antibiotic against neonatal diseases, such as watery mouth) and their navels sprayed with iodine. A ‘wet pen’ (Figure 3.3 - orange shading) was 6m x 5m pens made from black, rigid, plastic sheeting. The concrete floor was sprayed with disinfectant before a layer of sawdust, and then a layer of straw was added to bed these pens. The purpose of the ‘wet pen’ was for the ewe to pass her placenta. It was also possible to observe whether the lambs were sucking from their mother. Most ewes cleansed after approximately 2

- 4 hours. The ewe and lamb(s) were then moved to a 5m x 5m individual pen (Figure 3.3 - blue shading). There were 15 wet pens and 83 individual pens. The concrete floors of the individual pens were also sprayed with disinfectant before a layer of straw bedding was added. The ewes and lamb(s) stayed in the individual pens for approximately 24 hours, until the lamb(s) were sucking successfully and had bonded with the ewe. Any lambs that had not been seen to have sucked or looked weak were bottle fed cows' colostrum and powdered milk (Lamlac ewe milk replacer). If they were very weak and cold then they were put into a heated box and medication was administered if necessary.

Lambs were castrated and tail-docked using rubber rings (Defra Code of recommendations for the welfare of livestock – sheep) before the ewe and lamb(s) were moved from the individual pens to a post-lambing group yard. 'Wet' and individual pens were completely cleaned between occupants, all straw bedding and waste was removed.

Post-lambing group yards housed approximately 20-50 ewes for an average of 4 days. Group yards were bedded with straw which was topped up on three occasions per the farmers discretion. Once the lambs were considered strong enough to survive outside, the ewes and lamb(s) were transported to pasture. The post-lambing group yards were located in the lambing barn initially but as the ewes lambed, the free sections in the lambing barn were used as post-lambing yards.

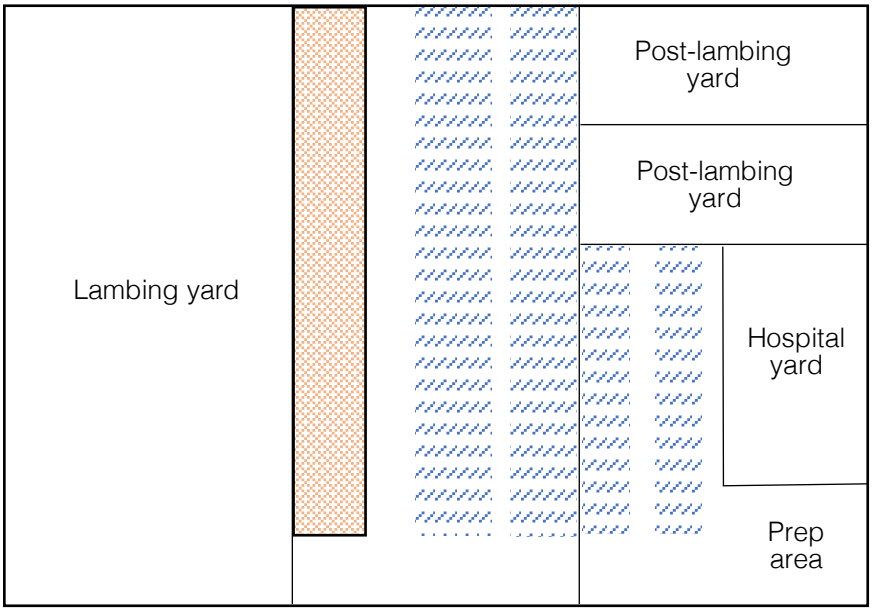

Figure 3.3 – Lambing barn layout including positioning of wet pens (orange shading, 6m x 5m pens) and individual pens (blue shading, 5m x 5m).

3.3.3.3 Housing and management for ewes that fostered orphan lambs

Orphan and triplet lambs were fostered onto healthy ewes which either had no live lambs or a single lamb. This was achieved by rubbing the ewe's placenta on the foster lamb when she had just given birth or by using an adopter pen, which restrains the ewe's head in a yoke to prevent her from attacking the foster lamb(s). Ewes remained in adopter pens for up to 4 days to increase the chance that she accepted the lamb(s). Adopter pens were the same size and bedded and cleaned out the same way as individual pens, and also had the adopter device (yoke).

3.3.3.4 Ewe nutrition

Pregnant ewes had ad libitum access to silage, and after lambing ewes had ad libitum access to hay. In addition, all ewes were fed twice a day, either with 0.5kg concentrate/ewe/day, or 0.5kg/ewe/day barley and rolled barley. Water was available to ewes ad libitum, through an automatic water system or buckets.

3.3.4. Intervention additional hygiene protocols

3.3.4.1 Selection of intervention ewes

The intervention consisted of additional hygiene procedures to the general ewe housing and managements listed in section 3.3.3 which is what the control ewes received. The additional procedures for the intervention ewes are listed in Table 3.2 and outlined in sections 3.3.4.2 to 3.3.4.5 below. At the first sign of lambing ewes were assigned alternately to either the control or intervention group. Ewes recruited into the intervention group were managed and handled by the researchers. Ewes in the control group were managed by the farm staff. The researchers wore clean clothes (overalls were disinfected with Fam 30 (Evans Vanodine International, iodophor disinfectant to kill bacteria, viruses and fungi) at the end of each shift and dried) and boots were disinfected each day at the end of the shift also using Fam 30.

3.3.4.2 Management differences for intervention and control ewes at lambing

If intervention ewes required assistance during lambing, the researcher sanitised their hands and arms using antibacterial gel before touching the ewe and washed their hands and arms thoroughly with warm water and soap afterwards. If the lamb needed assistance after birth (e.g. opening or removing the water bag) then the researcher would sanitise their hands first; on a small number of occasions this procedure was not followed because the lamb needed urgent attention (this was recorded). No sanitisation procedures were carried out by the farm workers for the control ewes and no time limit was instilled before intervention was deemed necessary. For the intervention ewes, the researchers waited at least 30 minutes before deciding it was necessary to assess the ewe, farm workers would intervene before this time period. On some occasions, only the researchers were present in

the lambing barn when control ewes required full or partial lambing assistance. Partial assistance were occasions when the lambing sac needed to be removed from the mouth and/or nose of the lambs to allow them to be able to breathe. These events were noted.

When the ewe and lamb(s) were being moved from the lambing yard to a 'wet pen', shoulder length gloves were worn to carry lambs in the intervention group

Table 3.2 – Comparison of hygiene procedure between intervention and control groups of sheep

Procedure	Control group	Intervention group
Moving the lambs and ewes after lambing to wet pens	Carried out without gloves or cleaning hands	Disposable shoulder length gloves were worn and changed between ewes
Treating the navels of lambs with iodine	Iodine sprayed on navels of lambs at birth	Navel dipped in iodine using a teat cup, which covered the cord in iodine up to the body wall at birth. A second dip was administered within 4 hours of the first. Researchers wore gloves for the first iodine dip and either wore gloves or sanitised their hands for the second time.
Handling ewes or lambs (including lambing assistance)	No hygiene procedure in place	Hands were sanitised using antibacterial gel before handling sheep and at least 30 mins waiting time before assistance unless and emergency
'Wet' pen bedding	Sawdust and straw only	Addition of 50g of bedding powder (Quill Ultra Dri Powder Disinfectant) spread on dry disinfected flooring below the sawdust and straw bedding
Individual pen bedding	Straw only and no cleaning was carried out until the ewe and lambs left the pen	Addition of 50g of bedding powder below the straw on dry disinfected floor. Wet bedding and waste removed daily and bedding topped up with fresh straw
Post-lambing group yards	Bedding was added on 3 occasions.	Bedding powder used. Bedding was checked daily and very dirty/mouldy bedding was removed. Clean straw was added on 3 occasions over the course of the experiment

3.3.4.3 Housing and management for intervention ewes and lambs after lambing

Control ewes were managed as described in section 3.3.3.2. For intervention ewes hands were sanitised with antibacterial gel (or gloves were worn) before any handling of the ewes or lambs. In the 'wet pens', the naval of the lambs was dipped rather than sprayed with iodine and a second dip was administered within 4 hours of the first.

Intervention ewes had the addition of 50g of a sanitising powder disinfectant (Quill productions, Ultra Dri Powder - BioVX disinfectant) on top of the dried disinfected floor (under the bedding) for both the 'wet' and individual pens. Bedding was checked daily in the individual pens and heavily soiled bedding was removed and topped up with fresh bedding.

During the first week of lambing control and intervention ewes were grouped together in a post-lambing yard. From the second week of lambing intervention and control ewes were grouped separately. Approximately 1kg of bedding powder was dispersed over the dirty straw and then the pen was topped-up with clean bedding. The intervention and control ewes were housed in groups of approximately 20 ewes with lambs.

3.3.4.4 Housing and management differences for intervention ewes that fostered orphan lambs

Wet bedding and manure were removed daily from the pens of intervention ewes with fostered lambs, and the bedding was topped up with fresh straw, this was not carried out for the control ewes.

3.3.5 Data collected during the study period in addition to the intramammary mass detection visits

Ewe deaths and cases of acute mastitis for the duration of the experiment were recorded. The ewe BCS, lamb health problems and the presence of IMM were recorded for all ewes as they lambed. Data collected during lambing also included; parity (multiparous, primiparous (first time lamber), multiparous but lambed later than expected), non-functioning udder (cannot express milk from one or both halves), assistance required at lambing, ewe fostered orphan lamb(s), number and health status of lamb(s), 'wet' and individual pen identification, lambing location (lambing yard, second barn or in field) and days housed after lambing before turn out to pasture. On occasions control ewes were left in the location they lambed and then taken straight to individual pens rather than to a 'wet pen' first, these incidences were recorded.

3.3.6 Data management

All data were downloaded from the datalogger as CSV files and converted to Microsoft Excel (Microsoft Excel for Mac Version 16.10) files. All data were combined into a single database for analysis. Additional relevant information (e.g. handwritten notes in a lambing notebook) was added to this database. The data were cleaned, duplicate values, data where ewe identification number was missing, and ewes that experienced both treatments (n= 10) were excluded. Ewes that experienced both treatments were; intervention ewes that were selected as suitable to foster a lamb(s) onto and moved to a control adoption pen,

intervention ewes that were still in pens during the respite period so were then managed by the farm staff, and ewes that came back in from the field as their lambs had died. On five occasions a control ewe was brought back through the indoor housing system after being turned out into the field, or from group housing, due to lamb loss. These ewes were excluded from analyses as they had experienced housing conditions twice. This resulted in 737 ewes (315 intervention and 422 control) remaining in the analysis.

3.3.7 Statistical analysis

Microsoft Excel and SPSS (IBM SPSS Statistics Version 24) were used for preliminary data analysis. Only ewes that lambed during the intervention trial period and were allocated into the control or intervention groups were included in the analysis. After examining frequencies by category, body condition score categories were combined into four groups; <2, 2.5, 3.0 and >3.5. The change in BCS was calculated by subtracting the BCS at the sampling point by the BCS reported at the previous sampling point. Change in BCS was categorised into 5 groups; +2-2.5, +0.5-1.5, 0, -0.5-1.5, -2-2.5. Days housed was also combined into three categories; 1-3 days, 4-8 days and 9+ days. 'Wet pen' use was coded as yes or no. Ewes were coded into flock 1 or 2 depending on their lambing due date, ewes that were identified during scanning to be due to lamb in the first flock but ended up lambing with the second flock was coded as 3. On occasions intervention ewes did not receive the correct intervention protocol, for example not sanitising hands before breaking the birthing bag off a lambs nose or no bedding powder being used. These were identified and coded for in the data. Week was categorised into 4 week groups; 1, 6-12, 15 and 30-31 and included in all 2-level analyses.

Chi - squared tests were used to investigate associations between the treatment group, parity and observational data collected across the study and at each sampling point. Where sampling point was investigated, only those ewes present at that time point were included in the analysis.

3.3.8 Binomial random effects multi-level models

To account for the clustering in the data, multi-level models were used with sampling point clustered within ewe. Unique ID code for each ewe (ewe unique identification number) and the sampling point were used to group the data to account for the repeated measures. The following factors were investigated for significant associations: identification of a non-functioning udder, presence of intramammary masses, development of acute mastitis and ewe death.

One single-level binomial random effects model was used to investigate associations with a non-functioning udder at lambing. The model took the form:

$$\text{Logit}(\pi_i) = \beta_0 + \beta x_i + u_i$$

where $\text{Logit}(\pi_i)$ is the log odds of the probability of a ewe having a non-functioning udder, β_0 is the constant, βx are the fixed effects that vary at *i* (ewe unique identification number) and μ_i the residual variance estimates at ewe unique identification number.
Three two-level binomial random effects models were built to explore factors associated with the presence of intramammary masses, acute mastitis and ewe death. The model took the form:

$$\text{Logit}(\pi_{ij}) = \beta_0 + \beta x_j + \beta x_{ij} + \mu_j$$

where $\text{Logit}(\pi_{ij})$ is the log odds of the probability of IMM or AM being present or ewe death, β_0 is the constant, βx are the fixed effects that vary at *j* (ewe unique identification number) and *i* (sampling point), and μ_j the residual variance estimates at ewe unique identification number. The outcome variable (acute mastitis or death, depending on the model) was right censored which was achieved by coding as 0 at each sampling point up to the point the event occurred, where it was then coded as 1. Where a ewe developed acute mastitis between sampling points she was coded as 1 on the sampling visit after the onset, any further sampling points after the event were left blank and data for this ewe were therefore excluded from this point. Where a ewe died between sampling points this was coded as 1 on the previous sampling point.

Concerning the outcome variable, presence of IMM, for the explanatory variable, acute mastitis, the observations were censored after the time of the occurrence, acute mastitis censored (AM censored). IMM data were lagged to investigate the impact of the previous observation (IMM at a sampling t-1).

All models were run in MLwiN version 3.01 (Charlton et al., 2019) with Restrictive Iterative Generalised Least Squares estimation (RIGLS). Any variables where the number affected was zero were removed from the analysis. After identifying variables that had a significant effect on the presence of the outcome variable at the univariable stage, forward stepwise model building was used to develop the multivariable model. Variables were considered significant if the 95% confidence intervals did not contain 1 (Wald's test). Where any variables were correlated with each other the most biologically plausible variable was retained in the model.

3.4 Results

3.4.1. Flock and treatment group sizes, disease rates and frequency of recorded events

Data collection occurred from January to October and a total of 946 ewes were observed at least once. Not every ewe was present at every sampling point, a summary of the number of ewes observed at each point is listed in Table 3.3. there were 742 ewes (315 intervention and 427 control) included in the intervention trial. The percentage of ewes in each treatment group observed by study week is displayed in Figure 3.4.

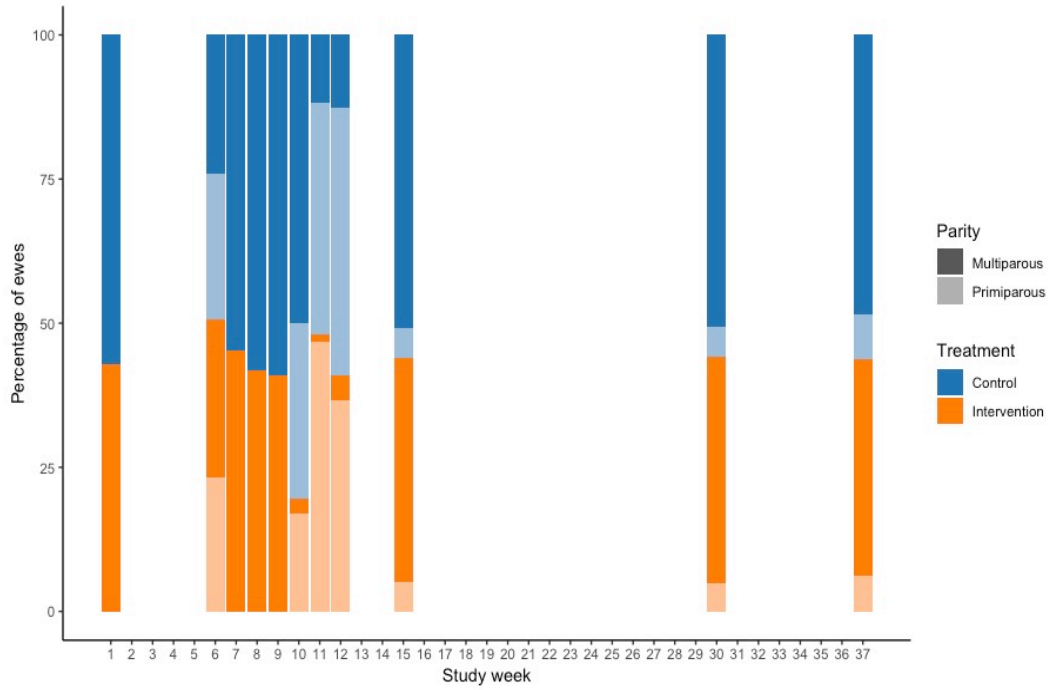

Figure 3.4 Percentage of control and intervention ewes at each week in the study – during weeks 6-12 the ewes are lambing, percentages relate to those still in housing

The flock lambed as two groups; the first flock consisted of 631 multiparous ewes (359 control and 272 intervention), and the second group had 98 primiparous ewes (57 control and 41 intervention) and 13 multiparous ewes (11 control and 2 intervention). There were 1208 lambs at turn out from 682 ewes, a lambing percentage of 1.8% (control 1.7% and intervention 1.8%). The percentage of ewes by treatment group that lambed each week is presented in Figure 3.5.

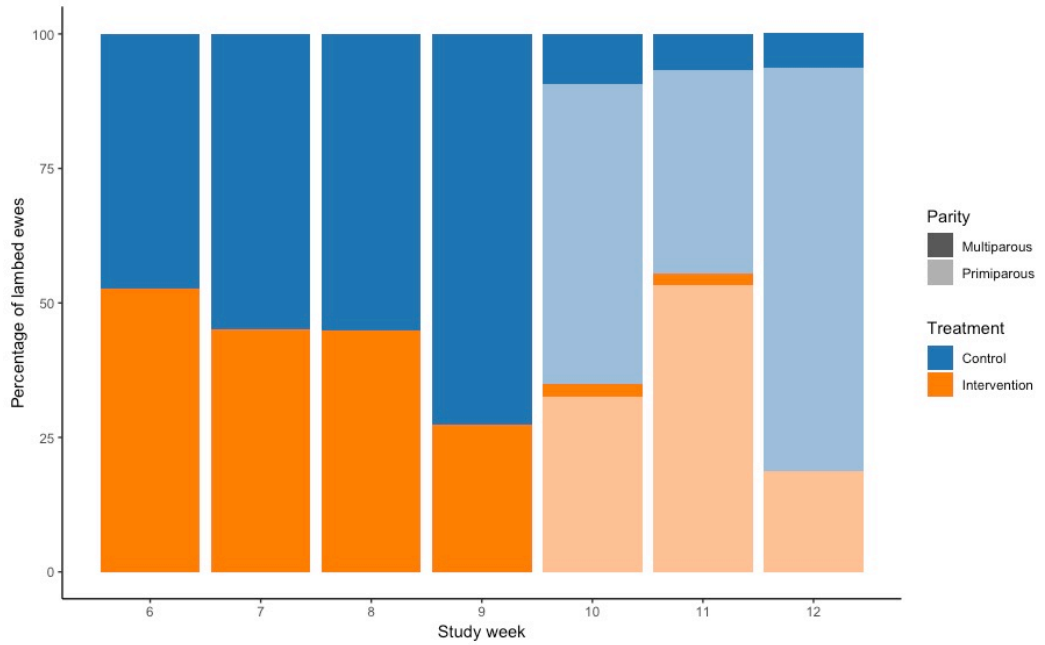

Figure 3.5 Percentage of control and intervention ewes at week lambed

Normally the ewes lambed in the lambing yard, however, 48 ewes (22 control and 26 intervention) lambed in a different location. From these 48 ewes, 24 (50%) lambed in the first barn, 20 (41.7%) lambed in a wet pen and 4 (8.3%) lambed in the field. From the 718 ewes that lambed either in the designated lambing yard or in the first barn, on 29 occasions (28 control and 1 intervention) the ewe was left in the lambing yard to cleanse and then moved straight to an individual pen, rather than cleansing in a wet pen with her lambs and then moving to an individual pen.

Acute mastitis was recorded in 5.3% of experimental ewes. Intramammary masses (IMM) were detected at least once in 37.5% of ewes. Non-functioning udders at lambing were detected in 2.7% of ewes. Approximately 30% of ewes were assisted at lambing; control ewes were assisted on 30.9% and intervention ewes on 27.6% of occasions. Significantly more ewes in the control group died over the study compared with ewes in the intervention group, 12 (2.8%) and 2 (0.6%) respectively (Table 3.4). During the lambing period 13 ewes (7 control and 6 intervention) were moved to the hospital yard.

Table 3.3 Number and percentage of ewes observed at each sampling point by intervention and control group

Sampling time point		Date of sampling	Control group			Intervention group		
			Total	n	%	Total	n	%
1	Udder examination 6 weeks prior to lambing	18-19/01/17 & 20/02/17	422	395	93.6	315	304	96.5
2	Lambing	19/02/17 - 04/04/17	422	392	92.9	315	311	98.7
3	~ 24 hours after lambing	19/02/17 - 04/04/17	420	358	85.2	315	291	92.4
4	Leaving indoor housing (av. 4 days after lambing)	19/02/17 - 04/04/17	419	388	92.6	315	288	91.4
5	Udder examination 4 - 8 weeks into lactation	24-25/04/17	415	376	90.6	314	294	93.6
6	Udder examination 18-22 weeks into lactation	03/08/17 & 09/08/17	404	385	95.3	310	301	97.1
7	Udder examination 6 weeks after weaning	20/09/2017	342	313	91.5	262	243	92.7

Table 3.4 Number and percentage of ewes with negative events by treatment group

Recorded event	Control			Intervention			Overall		
	Total	n	%	Total	n	%	Total	n	%
Acute mastitis	422	21	5.0	315	18	5.7	737	39	5.3
IMM	414	150	36.2	312	126	40.4	726	276	38.0
Non-functioning udder at lambing	422	12	2.8	315	7	2.2	737	19	2.6
Ewes with fostered lambs	422	39	9.2	315	16	5.1	737	55	7.5
With fostering device	39	30	76.9	16	9	56.3	55	39	70.9
Without fostering device	39	9	23.1	16	7	43.7	55	16	29.1
Lambing assistance	345	131	38.0	301	87	28.9	646	218	33.7
Died	422	12	2.8	315	2	0.6	737	14	1.9
Culled	422	69	16.4	315	51	16.2	737	120	16.3

IMM = Intramammary mass

3.4.1.1 Number of days in housing

The number of days ewes were housed after lambing varied by treatment group (Table 3.5); control ewes stayed in housing statistically longer than intervention ewes (p=0.00).

Table 3.5. Number and percentage of control and intervention ewes by length of housing

Days housed	Total ewes	Control		Intervention	
		n	%	n	%
1-3	359	169	40.0	190	60.3
4-8	206	128	30.3	78	24.8
9+	60	45	10.7	15	4.8
Unknown	112	80	19.0	32	10.2
Total	737	422		315	

n and % refer to the number and percentage of ewes.

3.4.1.2. Body condition score

Ewes had a higher BCS 6 weeks prior to lambing and 6 weeks after weaning than at lambing and during lactation (Figure 3.6). Overall there were no differences in BCS between the treatment groups (Table 3.6).

Table 3.6 Number and percentage of ewes by body condition score (BCS), treatment group and sampling point

Sampling point	Treatment	Body condition score								Total
		<2		2.5		3		>3.5		
		n	%	n	%	n	%	n	%	
6 weeks prior to lambing	Control	31	7.8	79	20.0	106	26.8	179	45.3	395
	Intervention	31	10.2	65	21.5	71	23.4	136	44.9	303
At lambing	Control	102	27.3	128	34.3	85	22.8	58	15.5	373
	Intervention	92	30.1	93	30.4	77	25.2	44	14.4	306
4–8 weeks into lactation	Control	127	33.9	119	31.7	82	21.9	47	12.5	375
	Intervention	89	30.3	73	24.8	72	24.5	60	20.4	294
18-22 weeks into lactation	Control	82	21.3	90	23.4	79	20.5	134	34.8	385
	Intervention	72	23.9	65	21.6	54	17.9	110	36.5	301
6 weeks after weaning	Control	21	6.7	57	18.2	79	25.2	156	49.8	313
	Intervention	11	4.5	40	16.5	60	24.8	131	54.1	242

n and % refer to the number and percentage of ewes.

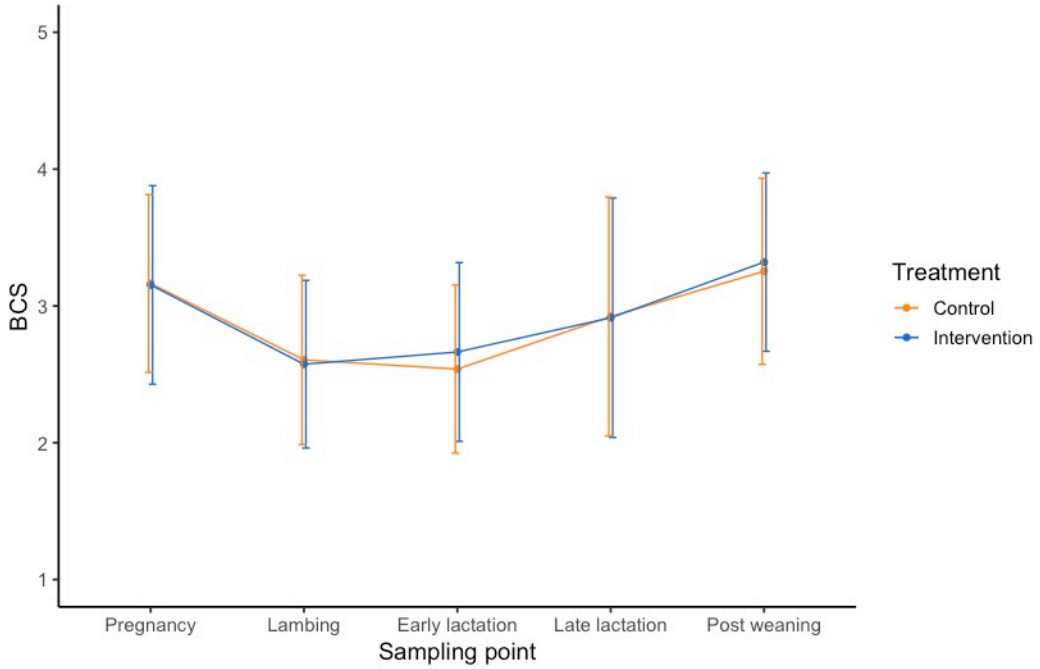

Figure 3.6 – Mean body condition score (BCS) by treatment group over time (sampling point) with 95% confidence intervals

3.4.2. Presence of non-functioning udders at lambing in the flock

There were 19 ewes with one or two non-functioning udder halves, 11/19 (57.9%) had an IMM present in pregnancy and 9/11 (81.8%) were in the non-functioning half. Only variables measured or known at pregnancy where used for this analysis (treatment, BCS, the presence of IMM at lambing and parity). There was no significant difference between treatment groups. The results from the binomial mixed effects regression model of the presence of a non-functioning udder at lactation were that the presence of a IMM in pregnancy and a multiparous ewe who lambed after their due date, >1 parity (late), increased the odds of ewe having a non-functioning udder at lactation (Table 3.7).

Table 3.7 – Multivariable binomial random effects regression model of variables associated with the presence of a non-functioning teat in 737 ewes

		Frequency of detection				
Explanatory variable	Category	No.	%	OR	Lower 95% CI	Upper 95%CI
IMM during pregnancy	Absent	8	1.3	Ref		
	Present	11	11.5	10.18	3.91	26.48
Ewe parity	Multiparous	15	2.4	Ref		
	Primiparous	2	2.0	1.49	0.32	6.96
	Multiparous (late)	2	15.4	8.76	1.53	50.22
Random effects						
Variance (Ear tag)				2.72		

Significance is based on the 95% confidence intervals not containing 1 – Wald test
IMM = Intramammary mass. No. and % refer to the number and percentage of ewes. OR = odds ratio. CI = confidence interval. Ref = reference value for comparison

3.4.3. Presence of intramammary masses in the flock

Overall 38% of the ewes had an IMM present at some point during the study. IMM were not consistently present in a ewe over time (Table 3.8). However, the presence of IMM at any point was associated with an increased percentage of ewes having an IMM at a future sampling point (Table 3.8 and Figure 3.7).

Table 3.8 - Number (%) of control and intervention ewes (n = 405) with or without an IMM present at every sampling point

IMM present 6 weeks prior to lambing	IMM present at lambing	IMM present 4-8 weeks into lactation	IMM present 18-22 weeks into lactation	IMM present 6 weeks after weaning
No = 358 (88.4)	No = 335 (93.6)	No = 305 (91.0)	No = 289 (94.8)	No = 272 (94.1)
				Yes = 17 (5.9)
			Yes = 16 (5.2)	No = 8 (50.0)
				Yes = 8 (50.0)
		Yes = 30 (9.0)	No = 23 (76.7)	No = 23 (100)
				Yes = 0 (0)
			Yes = 7 (23.3)	No = 4 (57.1)
				Yes = 3 (42.9)
	Yes = 23 (6.4)	No = 12 (52.2)	No = 10 (83.3)	No = 9 (90.0)
				Yes = 1 (1.0)
			Yes = 2 (16.7)	No = 2 (66.7)
				Yes = 1 (33.3)
		Yes = 11 (47.8)	No = 6 (54.6)	No = 6 (100)
				Yes = 0 (0)
			Yes = 5 (45.4)	No = 2 (40.0)
				Yes = 3 (60.0)
Yes = 47 (11.6)	No = 42 (89.4)	No = 31 (73.8)	No = 30 (96.7)	No = 27 (90.0)
				Yes = 3 (10.0)
			Yes = 1 (3.3)	No = 1 (100)
				Yes = 0 (0)
		Yes = 11 (26.2)	No = 6 (54.5)	No = 6 (100.0)
				Yes = 0 (0)
			Yes = 5 (45.5)	No = 1 (20.0)
				Yes = 4 (80.0)
	Yes = 5 (10.6)	No = 4 (80.0)	No = 4 (100)	No = 4 (100)
				Yes = 0 (0)
			Yes = 0 (0)	No = 0 (0)
				Yes = 0 (0)
		Yes = 1 (20.0)	No = 0 (0.0)	No = 0 (0)
				Yes = 0 (0)
			Yes = 1 (50.0)	No = 0 (0)
				Yes = 1 (100)

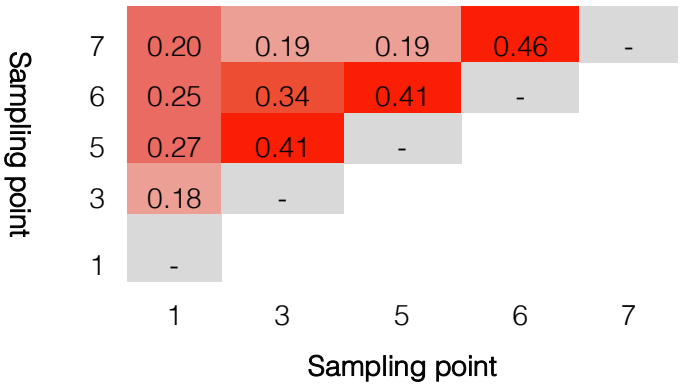

Figure 3.7 Correlation matrix of the presence of an IMM at a sampling point on IMM presence a previous sampling point

The percentage of IMM varied over time and this was true for both treatment groups (Figure 3.8) and parity groups (Table 3.9). Although the intervention ewes had a higher percentage of IMM compared with the control ewes (40.0% and 35.6% respectively), this was not a significant difference (Table 3.10). The percentage of primiparous ewes with IMM did seem to be increasing at a larger rate than the multiparous from lactation onwards however, the percentage of ewes with IMM detected at each sampling point was not significantly different between the primiparous and multiparous ewes (Table 3.10).

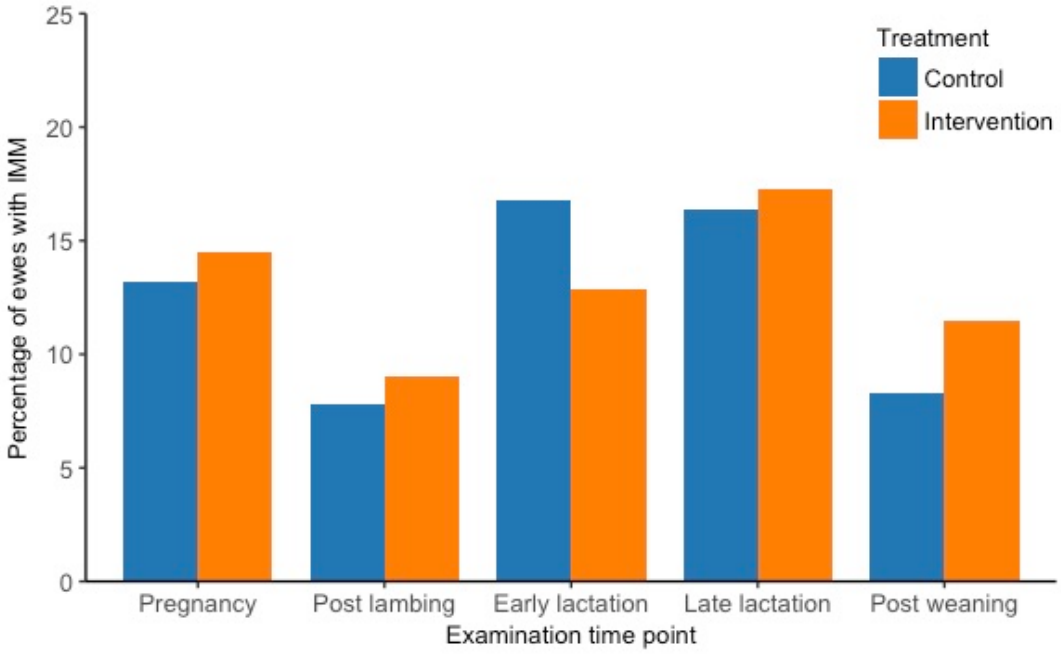

Figure 3.8 – Percentage of control and intervention ewes with intramammary masses at each sampling point

Table 3.9 Number and percentage of ewes with IMM by parity

Parity	IMM pregnancy		IMM ~24hrs after lambing		IMM 4-8 wks into lactation		IMM 18-22 wks into lactation		IMM 6wks after weaning	
	n	%	n	%	n	%	n	%	n	%
Multiparous	90	14.4	50	9.4	92	15.3	99	16.6	45	9.4
Primiparous	6	8.2	2	2.2	9	12.9	16	18.2	9	11.4

Wks = weeks. Multiparous includes Multiparous and Multiparous late variable

Table 3.10 Univariable binomial random effects survival data regression model of variables associated with the presence of IMM in 4085 observations of 737 ewes

Explanatory variable	Category	No. affected	% affected	OR	Lower 95% CI	Upper 95% CI
Treatment	Control	160	13.5	Ref		
	Intervention	204	13.7	0.99	0.75	1.29
Flock parity	Multiparous	327	14.1	Ref		
	Primiparous	33	10.3	0.71	0.46	1.09
	Multiparous (late)	4	9.1	0.63	0.20	2.02
Acute mastitis	No	333	12.7	Ref		
	Yes	21	67.7	13.28	5.87	30.00
IMM at the previous sampling point	Absent	182	11.1	Ref		
	Present	76	32.2	2.97	2.12	4.15
Non-functioning udder	No	338	13.0	Ref		
	Yes	26	38.8	4.54	2.31	8.90
Adopter	No	207	12.8	Ref		
	Yes	113	14.8	1.17	0.71	1.92
Lambing assistance	No	244	12.6	Ref		
	Yes	127	13.8	1.17	0.87	1.58
Days housed after lambing	0 days	7	9.7	0.70	0.29	1.70
	1-3 days	187	13.6	Ref		
	3-8 days	103	13.3	0.98	0.72	1.34
	9+ days	38	18.8	1.53	0.96	2.44
Body condition score at pregnancy	<2	24	10.7	0.71	0.40	1.27
	2.5	76	14.2	Ref		
	3	93	13.8	0.96	0.65	1.43
	>3.5	161	13.5	0.94	0.66	1.34
Body condition score at lambing	<2	105	14.7	1.18	0.83	1.68
	2.5	103	12.7	Ref		
	3	77	12.8	1.01	0.69	1.47
	>3.5	54	14.4	1.16	0.76	1.77
Body condition score 4-8 weeks into lactation	<2	110	13.2	1.16	0.80	1.66
	2.5	85	11.7	Ref		
	3	76	12.8	1.12	0.75	1.66
	>3.5	70	17.0	1.56	1.03	2.38
Body condition score 18-22 weeks into lactation	<2	68	12.0	0.76	0.51	1.13
	2.5	90	15.4	Ref		
	3	80	15.8	1.03	0.69	1.53
	>3.5	111	11.9	0.74	0.52	1.06

Change in body condition score from last sampling point	> +1.5	6	18.8	1.17	0.43	3.17
	+0 up to 1.5	84	14.7	0.87	0.59	1.29
	Stayed the same	54	16.4	Ref		
	- 0 down to -1.5	66	16.4	1.00	0.66	1.51
	- 2 down to -2.5	**6**	**40.0**	**3.44**	**1.05**	**11.31**
Wet Pen	Control	150	13.4	Ref		
	Intervention	156	13.5	1.01	0.75	1.34
	Other	21	20.2	1.61	0.85	3.06
Individual Pen	Control	176	14.0	Ref		
	Intervention	157	13.3	0.95	0.72	1.25
Group Pen	Control	170	13.7	Ref		
	Intervention	147	13.0	0.94	0.71	1.26
	Hospital pen	11	25.0	2.26	0.92	5.56
Lambing location	Lambing yard	334	13.9	Ref		
	Other	17	11.0	0.76	0.42	1.39
Intervention ewe hygiene issue*	No	281	13.3	Ref		
	Yes	70	15.5	1.20	0.85	1.69
Live lambs	None	2	25.0	1.99	0.26	15.03
	Single	79	3.0	Ref		
	Twins	240	13.7	1.18	0.86	1.62
	Triplets	17	15.6	1.35	0.68	2.68
Lamb health problem	No	315	13.5	Ref		
	Yes	21	16.7	1.26	0.70	2.29

Significance is based on the 95% confidence intervals not containing 1 and indicated by bold text – Wald's test. IMM = Intramammary mass. AM (censored) = Acute mastitis. No. and % refer to number and percentage of cases. OR = odds ratio. CI = confidence interval. Ref = reference value for comparison * Occasions where the intervention hygiene protocols could not be fully implemented, e.g. putting bedding on top of a still damp freshly disinfected concrete floor, or no sanitising powder was added before the bedding

3.4.3.1 Binomial random effects model of variables associated with the presence of IMM at a time point

The univariable analysis for the presence of IMM at any time point are shown in Table 3.10. There was no effect of treatment on the presence or absence of IMM. Ewes that had an IMM at a previous sampling point, a non-functioning udder at lambing, developed acute mastitis, lost 2 or more body condition scores from the last sampling point or BCS greater than 3.5 4-8 weeks into lactation were associated with the presence of IMM. These variables were analysed further using multivariable analysis.

Study week was also included in the final model as a polynomial term. A drop in BCS and study week appeared to confound (Appendix 4) each other so BCS was removed. Two variables remained significant in the multivariable model (Table 3.11). Ewes that had an IMM at a sampling point had increased odds of having an IMM at the previous sampling point. Ewes were also more likely to have an IMM if they were to go on to develop acute mastitis.

Table 3.11 Multivariable binomial random effects survival model of variables associated with the presence/absence of IMM from 4085 observations of 737 ewes

Explanatory variable	Category	OR	Lower 95% CI	Upper 95% CI
Fixed effects				
Acute mastitis	No	Ref		
	Yes	15.1	6.52	35.05
IMM at the previous sampling point	Absent	Ref		
	Present	2.94	2.04	4.23
Week	Week	6.29	2.57	15.41
	Week^2	0.41	0.23	0.75
Random effects				
Variance (Unique ewe ID)		1.20		
Variance (Sampling point)		2.72		

Significance is based on the 95% confidence intervals not containing 1– Wald's test IMM = Intramammary mass. AM (censored) = Acute mastitis. OR = odds ratio. CI = confidence interval. Ref = reference value for comparison

3.4.4. Presence of acute mastitis in the flock

Acute mastitis was observed in 5.3% of the experimental flock. There was no difference between treatment groups; 5.0% control and 5.7% intervention ewes developed acute mastitis over the experimental period. Acute mastitis cases occurred throughout lactation (Figure 3.9). One ewe died from acute mastitis and 15 ewes were culled due to acute mastitis, the remaining 23 ewes are considered to have survived but not all ewes were seen after the case of mastitis, although these ewes may have just been missed it may also be that their death was not recorded.

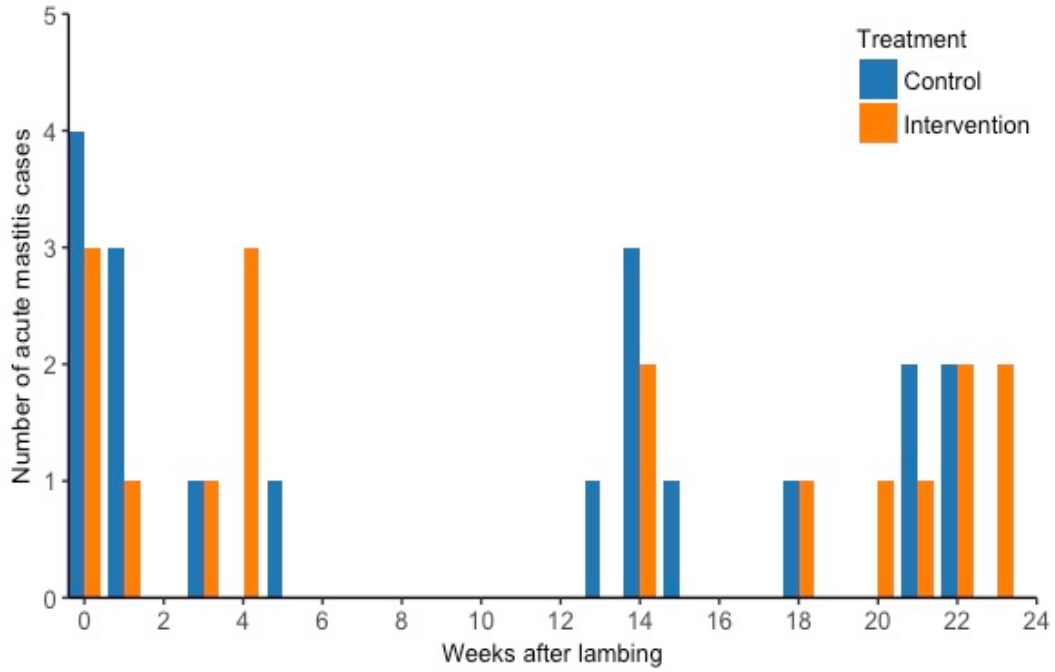

Figure 3.9 – Number of control and intervention ewes that developed acute mastitis by week of lactation for 36 ewes (17 control and 17 intervention)

Among the 39 ewes that had acute mastitis, 32 (82.1%) had an IMM at any sampling point, 23 (59%) had IMM present before the case of acute mastitis and 7 (17.9%) had an IMM before and after the acute case. From the 30 ewes that had an IMM before the case of acute mastitis, 19 (63.3%) had an IMM at the sampling point immediately before the acute case was diagnosed. IMM was present after the case of acute mastitis in 2 ewes (5.1%) (Table 3.12).

Table 3.12 Presence of IMM in the 39 ewes with acute mastitis

Time IMM was detected in the ewe in relation to AM onset	Number of ewes (%)
IMM detected before AM case	19 (48.7)
IMM detected before but not after AM case	4 (10.3)
IMM detected before and after AM case	7 (17.9)
IMM detected only after AM case	2 (5.2)
No IMM detected at any sampling points	7 (17.9)

IMM = Intramammary mass. AM = Acute mastitis

Factors associated with occurrence of acute mastitis in the univariable random effects survival model were the presence of IMM, a non-functioning udder at lambing, housed > 9 days and in the hospital pen (Table 3.13). Many of the variables were correlated, with correlation ranging from -0.20 to 1.00 (Appendix 4). In the multivariable model, week was included as a term and two variables

remained significant, these were that the odds of a ewe developing acute mastitis was higher when a ewe had IMM at any sampling point before the case of acute mastitis, and if the udder was not functioning at lambing (Table 3.14).

Table 3.13 Univariable random effects survival model of variables associated with the occurrence of acute mastitis from 4085 observations of 737 ewes

Explanatory variable	Category	No. affected	%	OR	Lower 95% CI	Upper 95% CI
Treatment	Control	21	0.9	Ref		
	Intervention	18	1.0	1.03	0.54	1.97
Flock parity	Multiparous	33	0.9	Ref		
	Primiparous	5	1.0	1.12	0.43	2.87
	Multiparous (late)	1	1.6	1.72	0.23	12.78
IMM at any previous sampling point	Absent	10	0.4	Ref		
	Present	**21**	**5.9**	**14.44**	**6.76**	**30.83**
IMM at the previous sampling point	Absent	22	1.3	Ref		
	Present	**8**	**3.5**	**2.73**	**1.21**	**6.20**
IMM pregnancy	Absent	26	0.8	Ref		
	Present	**11**	**2.1**	**2.53**	**1.21**	**5.27**
IMM ~24 hours after lambing	Absent	25	0.8	Ref		
	Present	5	1.7	2.32	0.88	6.13
IMM 4-8 weeks into lactation	Absent	18	0.5	Ref		
	Present	**12**	**2.1**	**3.86**	**1.85**	**8.05**
IMM 18-22 weeks into lactation	Absent	12	0.4	Ref		
	Present	**21**	**3.3**	**9.13**	**4.48**	**18.64**
Non-functioning udder at lambing	No	28	0.7	Ref		
	Yes	**11**	**12.8**	**21.43**	**10.26**	**44.79**
Adopter	No	37	1.0	Ref		
	Yes	2	0.7	0.71	0.17	2.97
Lambing assistance	No	20	0.8	Ref		
	Yes	13	1.1	1.34	0.66	2.70
Intervention ewe hygiene issue*	No	29	0.9	Ref		
	Yes	7	1.0	1.15	0.50	2.64
No. of live lambs per ewe	1	11	1.1	Ref		
	2	24	0.9	0.82	0.40	1.69
	3	1	5.9	0.53	0.07	4.13
Lamb health problem	No	33	0.9	Ref		
	Yes	2	1.0	1.11	0.27	4.68
Wet Pen	Control	15	0.9	Ref		
	Intervention	17	1.0	1.13	0.56	2.26
	Other	1	0.6	0.71	0.09	5.42
Individual Pen	Control	18	0.9	Ref		
	Intervention	17	1.0	1.04	0.53	2.02
Group Pen	Control	15	0.8	Ref		
	Intervention	13	0.8	0.73	0.34	1.53
	Hospital pen	**3**	**4.6**	**6.00**	**1.69**	**21.23**
Days housed after lambing	1-3 days	14	0.7	Ref		
	3-8 days	9	0.8	1.13	0.49	2.61
	9+ days	**8**	**2.5**	**3.86**	**1.61**	**9.26**
Body condition score at pregnancy	<2	2	0.6	0.52	0.11	2.42
	2.5	9	1.1	Ref		
	3	11	1.1	0.99	0.41	2.39
	>3.5	15	0.8	0.70	0.30	1.63

Body condition score at lambing	<2	8	0.7	0.75	0.31	1.84
	2.5	12	1.0	Ref		
	3	9	1.0	1.02	0.43	2.42
	>3.5	7	1.2	1.26	0.50	3.23
Body condition score 4-8 weeks into lactation	<2	8	0.6	0.77	0.30	2.01
	2.5	9	0.8	Ref		
	3	2	0.2	0.27	0.06	1.26
	>3.5	11	1.8	2.21	0.91	5.37
Body condition score 18-22 weeks into lactation	<2	11	1.3	1.62	0.62	4.20
	2.5	7	0.8	Ref		
	3	5	0.7	0.83	0.26	2.62
	>3.5	10	0.7	0.37	0.14	0.98

Significance is based on the 95% confidence intervals not containing 1 and indicated by bold text – Wald's test. IMM = Intramammary mass. No. and % refer to number and percentage of affected cases. OR = odds ratio. CI = confidence interval. Ref = reference value for comparison. * Occasions where the intervention hygiene protocols could not be fully implemented, e.g. putting bedding on top of a still damp freshly disinfected concrete floor, or no sanitising powder was added before the bedding

Table 3.14. Multivariable binomial random effects survival model of variables associated with the acute mastitis from 4085 observations in 737 ewes

Explanatory variable	Category	OR	Lower 95% CI	Upper 95% CI
Fixed effects				
Non- functioning udder at lambing	No	Ref		
	Yes	22.6	6.67	76.33
IMM present at any sampling point before acute mastitis	Absent	Ref		
	Present	13.1	5.81	29.33
Week	6-12			
	15	1.18	0.37	3.83
	30-31	3.1	1.09	8.79
Random effects				
Variance (Unique ewe ID)		3.3		
Variance (Sampling point)		2.7		

Significance is based on the 95% confidence intervals not containing 1- Wald's test
IMM = Intramammary mass (lump in the udder of unusual consistency indicative of chronic mastitis). OR = odds ratio. CI = confidence interval. Ref = reference value for comparison

3.4.5 Ewe deaths

There were 14 ewe on farm deaths over the course of the experiment. A significantly higher number of control ewes 12/422 (2.8%) died than did intervention ewes 2/315 (0.6%) (Figure 3.10).

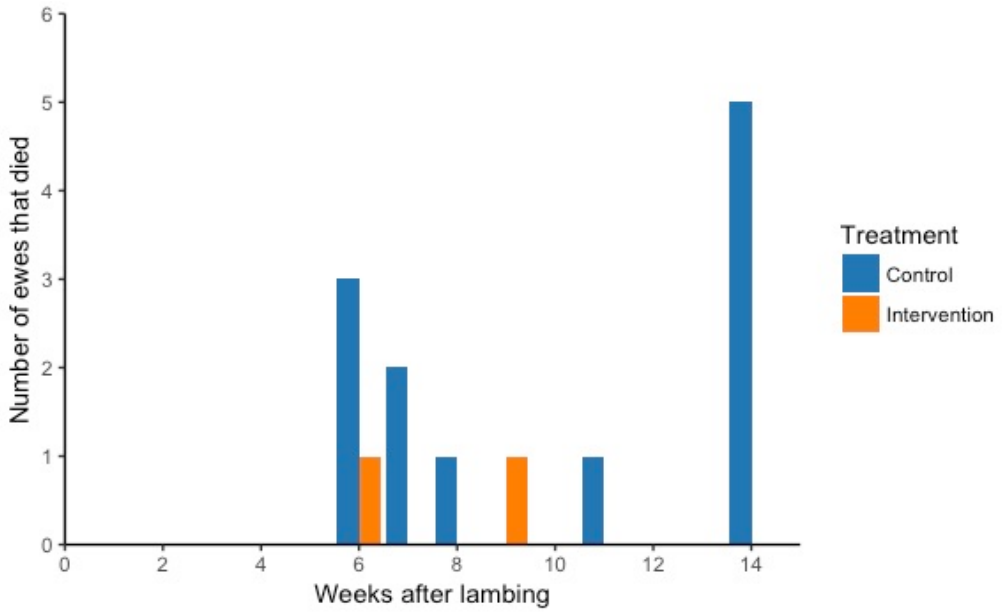

Figure 3.10 – Number of control and intervention ewes that died over the weeks after lambing

Assistance during lambing was given to 218 (29.1%) ewes; 132/422 (31.3%) control and 87/315 (27.6%) intervention ewes. It was the most common cause of death in both the control and treatment groups, 5/12 (41.7%) control ewes and 2/2 (100%) intervention ewes.

More orphan lambs were adopted on control (39, 70.9%) than intervention ewes (16, 29.1%). Of the 39 control ewes, 30 (76.9%) fostered lambs using a fostering device. A lower percentage of intervention ewes (9, 56.3%) used the fostering device.

Ewes that fostered lambs were more likely to die (Table 3.15). The three ewes that fostered lambs and died were all control ewes that used a fostering device.

Table 3.15 - Death of ewe by adopter and lambing assistance status

			Died	
		N	n	%
Adopter	Yes	55	3	5.5
	No	682	11	1.6
Assistance given	Yes	218	7	3.2
	No	428	2	0.5

3.4.5.1 Binomial random effects survival model of factors associated with ewe death

The univariable random effects survival model results (Table 3.16) for ewe death were that control ewes, multiparous ewes, lambing late, acute mastitis, assistance during lambing, death or removal of lambs, and retained in the lambing yard to pass the placenta after lambing were all associated with an increased odds of ewe death.

These explanatory variables were correlated (Appendix 4) which resulted in two possible multivariable models (Table 3.17 and Table 3.18). It was decided to use model 1 for the final model because the variables remaining in model 1 were related to aspects adapted by the experiment. The variables in model 2 included multiparous ewes lambing late and ewes dying from acute mastitis. In addition to the smaller numbers of affected ewes this model is based on, ewes lambing late are more likely to already have a health condition that may lead to death and acute mastitis is known to occasionally cause death.

Using model 1 as the final multivariable model, a ewe was more likely to die if they had assistance during lambing and if the wet pen type was “other”. Week was also included in the final model.

Table 3.16 - Univariable binomial random effects survival data regression model of variables associated with ewe death from 4085 observations of 737 ewes

Explanatory variable	Category	No. affected	%	OR	Lower 95% CI	Upper 95% CI
Treatment	Control	12	0.5			
	Intervention	**2**	**0.1**	**0.21**	**0.05**	**0.95**
Flock parity	Multiparous	10	0.3	Ref		
	Primiparous	2	0.4	1.43	0.31	6.54
	Multiparous (late)	**2**	**3.1**	**11.16**	**2.40**	**51.97**
IMM at a previous sampling point	No	5	0.2	Ref		
	Yes	1	0.3	1.27	0.15	10.91
Acute mastitis	No	13	0.3	Ref		
	Yes	**1**	**2.6**	**8.33**	**1.06**	**65.36**
IMM at the previous sampling point	No	5	0.3	Ref		
	Yes	1	0.4	1.41	0.16	12.15
Adopter	No	11	0.3	Ref		
	Yes	3	1.0	3.53	0.98	12.72
Lambing assistance	No	2	0.2	Ref		
	Yes	**7**	**0.6**	**7.21**	**1.50**	**34.64**
Days housed after lambing	1-3 days	4	0.2	Ref		
	3-8 days	3	0.3	1.31	0.29	5.88
IMM pregnancy	Absent	13	0.4	Ref		
	Present	1	0.2	0.49	0.06	3.75
IMM ~24 hours after lambing	Absent	9	0.3	Ref		
	Present	1	0.3	1.21	0.15	9.61
IMM 4-8 weeks into lactation	Absent	5	0.2	Ref		
	Present	1	0.2	1.12	0.13	9.60
Body condition score at pregnancy	<2	2	0.6	1.19	0.22	6.50
	2.5	4	0.5	Ref		
	3	2	0.2	0.40	0.07	2.18
	>3.5	6	0.3	0.68	0.19	2.40
Body condition score at lambing	<2	2	0.2	0.56	0.10	3.09
	2.5	4	0.3	Ref		
	>3.5	3	0.5	1.63	0.36	7.28
Body condition score 4-8 weeks into lactation	<2	5	0.4	4.39	0.51	37.61
	2.5	1	0.1	Ref		
Wet Pen	Control	5	0.3	Ref		
	Intervention	2	0.1	0.40	0.08	2.05
	Other	**2**	**1.2**	**4.37**	**0.84**	**22.70**
Individual Pen	Control	7	0.4	Ref		
	Intervention	2	0.1	0.31	0.07	1.51
Group Pen	Control	10	0.5	Ref		
	Intervention	2	0.1	0.22	0.05	1.02
Intervention ewe hygiene issue*	No	9	0.3	Ref		
	Yes	1	0.1	0.53	0.07	4.18
Live lambs	**0**	**1**	**7.1**	**26.34**	**2.51**	**276.72**
	1	3	0.3	Ref		
	2	6	0.2	0.76	0.19	3.04
Lamb health problem	No	8	0.2	Ref		
	Yes	1	0.5	2.30	0.29	18.51

Significance is based on the 95% confidence intervals not containing 1 and indicated by bold text -Wald's test. IMM = Intramammary mass (lump in the udder of unusual consistency indicative of chronic mastitis). No. and % refer to number and percentage of affected cases. OR = odds ratio. CI = confidence interval. Ref = reference value for comparison. * Occasions where the intervention hygiene protocols could not be fully implemented, e.g. putting bedding on top of a still damp freshly disinfected concrete floor, or no sanitising powder was added before the bedding

Table 3.17 - Multivariable binomial random effects survival data regression model of variables associated with ewe death from 4085 observations of 737 ewes (model 1)

Explanatory variable	Category	OR	Lower 95% CI	Upper 95% CI
Model 1				
Fixed effects				
Lambing assistance	No	Ref		
	Yes	7.85	1.58	39.07
Wet Pen	Control	Ref		
	Intervention	0.47	0.45	0.09
	Other	6.26	6.95	1.26
Week	6-12	Ref		
	15	3.33	0.88	12.61
	30-31	-		
Random effects				
Variance (Unique ewe ID)		1.00		
Variance (Sampling point)		2.72		

Significance is based on the 95% confidence intervals not containing 1 – Wald's test. OR = odds ratio. CI = confidence interval. Ref = reference value for comparison.

Table 3.18 - Multivariable binomial random effects survival data regression model of variables associated with ewe death from 4085 observations of 737 ewes (model 2)

Explanatory variable	Category	OR	Lower 95% CI	Upper 95% CI
Model 2				
Fixed effects				
Flock parity	Multiparous	Ref		
	Primiparous	1.25	0.27	5.75
	Multiparous (late)	11.6	2.45	55.43
Acute mastitis	No	Ref		
	Yes	1.59	0.20	12.40
Week	6-12	Ref		
	15	2.36	0.81	6.89
	30-31	-		
Random effects				
Variance (Unique ewe ID)		1.00		
Variance (Sampling point)		2.72		

Significance is based on the 95% confidence intervals not containing 1 – Wald's test. OR = odds ratio. CI = confidence interval. Ref = reference value for comparison.

3.5 Discussion

3.5.1 The impact of hygiene and intramammary masses on the occurrence of acute and chronic mastitis

The increase in hygiene procedures around lambing were not significantly associated with reduced occurrence of acute mastitis or intramammary masses. This flock had a history of acute mastitis and 38% of the flock had IMM present at some point during the study. It is possible that pre-existing intramammary masses prevented any beneficial effects from the increased hygiene regimes, dominating the cause of acute mastitis in this flock.

A key result from this study is the strong association between intramammary masses and acute mastitis; with the presence of one heavily influencing the presence of the other. This association has been previously reported in a longitudinal study over two years (Grant *et al.*, 2016). It has been established that IMM in suckler ewes are abscesses in the udder, which have been identified to include the presence of mastitis causing pathogens, such as *Staphylococcus aureus* (Smith *et al.*, 2015). Abscesses typically mature and rupture (Cheng *et al.*, 2011). When these IMM rupture, the bacteria that was resident in the IMM have the chance to dominate the udder environment, either due to already high numbers achieved in the abscess or by multiplying in the udder, and then have the opportunity to cause an infection. Therefore, even if the increased hygiene protocols experienced by the intervention ewes in this study decreased their exposure to external bacterial pressures, from human hands or bedding, the additional hygiene protocols did not provide enough protection against these bursting internal abscesses. As the percentage of IMM was not significantly different between the groups this would be an explanation why no difference was seen between the acute mastitis cases in the treatment groups. The bacteria in the udder not only has an opportunity to spread and cause acute infection but to reform another abscess, which could be elsewhere in the udder environment (Cheng *et al.*, 2011; Grant *et al.*, 2016). This would explain the strong association between the risk of an IMM being present increasing if an IMM had been detected previously (Grant *et al.*, 2016).

It has been suggested that IMM may be a consequence of previous acute infection (Grant *et al.*, 2016), therefore it would be expected that primiparous animals would not have IMM. However, a novel result from this study is that there was no significant difference between the multiparous and primiparous ewes regarding the presence of IMM, suggesting IMM may also result from other means, such as a challenge from environmental bacteria. Grant *et al.* (2016) also found an association between underfeeding energy and IMM. This study found an association between loss of body condition and IMM, which may indicate that

they have not had enough nutrition to maintain condition leaving them susceptible to infections and IMM presence as suggested by Grant *et al*. (2016).

3.5.2 The impact of acute and chronic mastitis on mammary gland function

IMM in pregnancy were associated with ewes that had non-functioning udders. Fibrotic scars have been reported after abscess drainage in organs (Cheng *et al*., 2011). The act of growing, bursting and fibrotic scar formation may cause irreparable damage to the udder, leaving the udder unable to function normally and express milk. As this damage does not always appear on the outside of the udder this damage could go unseen. Therefore, it may be that these ewes that could not express at lambing in this study had previously had IMM, that led to the loss of function or currently had an abscess that prevented milk let down.

Ewes identified with a non-functioning udder at lambing were also associated with increased odds of a case of acute mastitis. Perhaps this increased risk is linked to the fact the ewes with non-functioning udders were more likely to have IMM which are heavily associated with AM cases. Another possibility is that any suckling lambs may have been hungrier and more likely to perform excessive butting and biting. This may have caused trauma to the teats, allowing entry of bacteria into the udder environment to go on to cause an infection, as the presence of teat lesions have been reported to be high in ewes with clinical mastitis (Mørk *et al*., 2007).

3.5.3 The effect of lambing assistance and hygiene on ewe death

The association between lambing assistance and death is an important finding. The provision of assistance at lambing varies between farming systems and flocks. In this study over 30% of the flock received lambing assistance. There has been little published work on the effects of human intervention during lambing, on ewe welfare, and the cost implications of such actions and none that report on deaths associated with this. However, there are studies that provide both support for lambing assistance, and also raise concerns in its use regarding ewe and lamb health and welfare (Waterhouse, 1996; Fisher and Mellor, 2002). The results from this study imply that human intervention during lambing should be approached with caution, as this was the most common known reason for death in the ewes, and was highly associated with death in the resulting multi-level model. This is an important potential finding for farmers, as most will assist ewes during lambing with the belief that this reduces the possibility of issues and deaths arising during lambing. However, provision of assistance was only known for 9 out of the 14 ewes that died, due to this small number further investigation into this hypothesis is required.

The number of control ewes that died was significantly higher than the number of intervention ewes. As the percentage of ewes receiving lambing assistance in each group was not significantly different (38% control and 28.9% intervention ewes) this difference must be attributed to other factors. The actual cause of the difference in death rates between the groups in this study cannot be identified from the data collected. However, it can be hypothesised that the additional hygiene protocols could be related to this reduced mortality rate in the intervention group. The use of antibacterial hand gel and daily cleaning of pens may have reduced the bacterial load, by creating a sub-optimal environment for bacterial growth, or preventing transmission events and therefore reducing the risk of bacteria multiplying and challenging the ewe, causing disease. The benefits of hand hygiene and reduced bacterial transmission has been well demonstrated in human health care (Traub-Dargatz *et al*., 2006). In addition, the intervention ewes overall spent significantly less time in housing, which may have reduced exposure to bacterial challenges associated with housing and disease.

However, it should be noted that due to the different people conducting the interventions between the control and intervention ewes there was a difference between technical approach. This variation may well have affected mortality and therefore should not be ignored.

3.6 Study limitations and future studies

Due to the nature of conducting an experiment on a working sheep farm, some ewes were missed at different sampling points and data recording points were overlooked resulting in unknowns in the data set. These unknowns may have skewed the data or prevented a complete picture of associations to be made.

Although the ewes in the intervention group did receive the additional hygiene protocols while housed after lambing, the pens and yards they resided in, were located in the same barn as the control ewe pens, so some cross-contamination may have occurred. Also, mixing the ewes post-housing perhaps removed any beneficial effects of the additional hygiene over lambing, as mastitis is a contagious disease so could have moved throughout the flock regardless of treatment group.

Further investigation is needed regarding these issues. More information is needed on intramammary masses so we can get a fuller picture of their role with acute mastitis and reoccurring episodes of disease. And further work is need to understand what aspects of lambing assistance and hygiene over lambing are associated with ewe death.

3.7 Conclusion

There were no significant associations between increased hygiene procedures around the peri-parturient period and the occurrence of acute mastitis and intramammary masses. However, the flock had a history of acute mastitis and 38% of the flock had IMM present at some point during the study. It is possible that pre-existing intramammary masses may have prevented any beneficial effects from the increased hygiene regimes, dominating the cause of acute mastitis in this flock. Therefore, even if the additional intervention hygiene procedures did reduce the exposure of the intervention ewes to external bacteria for example, from bedding or human hands, there was no additional protection against the bursting abscesses. As the percentage of IMM was not significantly different between the groups this would be one explanation why no difference in the incidence of acute mastitis was identified. The results of the study highlight the importance of intramammary masses and the strong influence they appear to have on acute mastitis and the productivity of the flock.

Another finding from the study is the link between lambing assistance and death, suggesting the decision to intervene at lambing should not be taken lightly. However, further investigation into this is warranted. And that significantly fewer intervention ewes died than control ewes. However, as the number of ewes assisted during lambing in each group was not significantly different, the differences in numbers of deaths between the groups must be due to another factor. The actual cause of the difference in death rates between the groups in this study cannot be identified from the data collected suggesting further investigation into this is needed.

Chapter 4. Investigation of transmission and persistence of bacteria associated with mastitis in ewes housed over lambing using MALDI-ToF-MS

4.1 Introduction

Mastitis is a contagious disease, with evidence of both acute and chronic disease spreading through flocks (Mørk *et al.*, 2007; Grant *et al.*, 2016). Although our knowledge of the pathogens associated with mastitis has increased over the years, there is still a dearth of information regarding the transmission routes and persistence of bacteria in suckler ewes. Potential transmission routes and reservoirs for bacteria have been identified and considered risk factors for mastitis, of which are discussed in Chapters 1 and 2.

As described in Chapter 1, MALDI-ToF is recognised as an accurate and rapid method of identifying bacterial strains (Singhal *et al.*, 2015). It is being used increasing in clinical and veterinary medicine (Barreiro *et al.*, 2010*;* Neville *et al.*, 2011) and has been used successfully in numerous studies to identify mastitis causing pathogens in both cows and sheep (Braga *et al.*, 2018; Archer *et al.*, 2017; Cooper 2015).

A greater understanding of how bacteria move between ewes and the involvement of lambs, humans and the environment in this transmission would be of value. This knowledge could aid the development of effective control strategies for mastitis.

4.2 Aim

The aim of this study was to investigate the transmission and persistence of bacterial strains within and between ewes and lambs and the influence of additional hygiene protocols during indoor lambing on transmission. This was achieved by culturing samples taken from ewe nose, teat, milk, vagina, lamb mouths and the hands of handlers during the intervention study carried out in Chapter 3, and using MALDI-ToF-MS to identify the bacteria isolated from these samples. Analytical techniques, such as cluster analysis, were used to identify strains and investigate possible transmission pathways, bacterial reservoirs and persistence.

4.3 Materials and methods

4.3.1 Study ewe selection

Seventy pregnant ewes from Chapter 3 were randomly selected as study ewes. The only criterion for selection was that ewes had been scanned as having twins, to remove any confounding impact that the number of lambs would have on the occurrence of mastitis. Selected sheep were identified and spray marked with a unique number over three randomly allocated mornings during the lambing period, and an initial set of samples were collected.

Ewes were allocated into treatment groups as described in section 3.3.4. Only 63/70 ewes lambed during the intervention trial, resulting in 32 control and 31 intervention ewes. In addition, samples were taken from 30 ewes that developed acute mastitis during the trial at the point of diagnosis.

4.3.2 Sample collection

The 63 study ewes were sampled repeatedly (Table 4.1) resulting in 1812 swabs associated with these animals (64 vaginal, 129 nasal, 506 teat, 259 lambs' mouth and 124 hand samples), 252 milk and 478 bedding samples collected for microbiological analyses. Of the 1812 samples, 56 were duplicates or extras. The collection of 71 samples were missed, reasons for this included bedding being cleaned out before collection, ewes leaving indoor housing earlier than normal protocol and non-functioning udders. A further 109 samples (60 teat swabs and 49 milk) were taken from 30 ewes that developed acute mastitis. Nasal swabs were taken from the farmer and the two researchers at the start and end of the trial. All samples were taken with gloved hands and gloves were changed between each sample. Once the samples were taken, they were stored at -20°C. All samples were transported on ice according to UN3373 regulations to the University of Warwick and stored at -20°C until further analysis.

All samples were taken by trained researchers, except for 42 teat and milk samples from 11 ewes that developed acute mastitis during the experiment when the researchers were not present on farm. The farmer was trained to take these samples, which were also stored at -20°C until collection by the researchers.

Table 4.1 Summary of samples collected during lambing (duplicates and extras)

Sampling time point	Vagina	Teats	Ewe nose	Lambs' mouth	Handler hands	Milk	Bedding
Pre-lambing	63	124	63				
Immediately post-lambing		124		123 (4)			124
Moving pen							230
~24 hours post-lambing		122 (8)			122 (2)	120 (7)	
Leaving housing	(1)	120 (8)	63 (3)	121 (11)		119 (6)	118 (6)

Moving pen = The timepoint when the ewes and lambs were moving between pens in housing (detailed in chapter 2)

4.3.2.1 Procedure for collecting samples

Vaginal swabs

The ewe was restrained by one researcher while the second researcher collected the sample. The outer lips of the vulva were first wiped with an ethanol wipe to remove any soiling. One hand was used to part the lips of the vulva while a charcoal swab was inserted 2cm into the vagina and rotated up and down once on the mucosal walls.

Teat swabs

One side of a charcoal swab was used to sample one side of the teat, from the base to the tip. The swab was then rotated and the other side used to sample the other side of the teat (Figure 4.1). The orifice of the teat was not sampled over but the tip of the teat was used to hold the teat steady. This was then repeated on the other teat, teats were swabbed as separate samples.

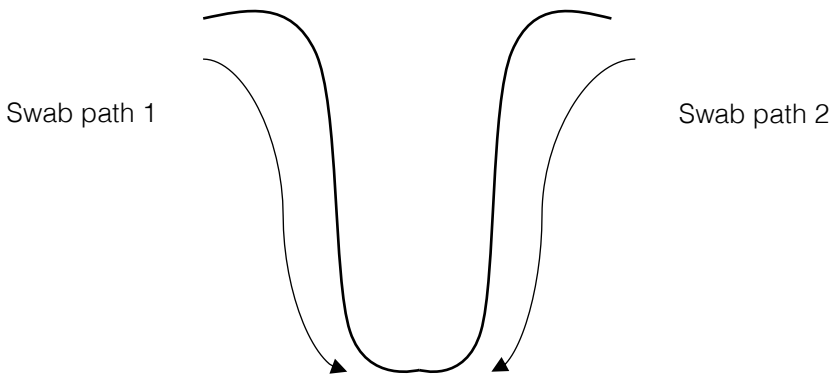

Figure 4.1 Teat swab path

Ewe nasal cavity swabs

The ewe's head was held still by one researcher so a charcoal swab could be inserted approximately 1cm into the ewe's nasal cavity and rubbed gently up and down twice on the outside mucosal walls.

Lamb mouth swabs

Lambs were marked at birth to link them to their mother and identify them as first or second-born. The mouth of the lamb was held open by inserting the index and middle fingers of one hand into the lamb's mouth and a charcoal swab was inserted approximately 2cm and rubbed gently up and down twice on the mucosal walls.

Handler hand swabs

Hand swabs were taken from a farm worker for control ewes and a researcher for intervention ewes. Approximately 24 hours after lambing the ewe's udder would be touched by a farm worker or researcher, this was performed before any other samples that were to be collected at the same time point. After touching, a charcoal swab was swept across the palm as shown in Figure 4.2. This was repeated on the other hand with a new swab.

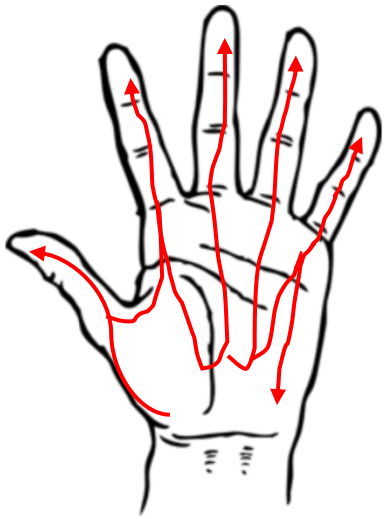

Figure 4.2 Handler hand swab path

Aseptic milk samples

An ethanol wipe was used to wipe the teat and the teat was left to dry before sampling. The foremilk was discarded and approximately 5ml of milk was collected into the universal tube without the tube touching the teat. This was then repeated with the other udder half.

Bedding samples

A handful of bedding that appeared dry was picked up using a small clean plastic bag. This method was repeated with a fresh bag for bedding that appeared wet from animal fluid (urine, milk, vaginal discharges). When bedding samples were being taken immediately post-lambing in the group lambing yard, bedding from and around where the ewe gave birth was taken.

Acute mastitic ewes

Any cases of acute mastitis were recorded and the ewe sampled (teat swabs and aseptic milk samples if possible). It was recorded if it wasn't possible to collect any milk from either or both udder halves. The farmer was trained to collect teat swabs and aseptic milk samples and continued to take samples from any ewes that developed acute mastitis after lambing.

4.3.3 Laboratory analysis of samples

Category II microorganisms were involved in this study therefore relevant safety procedures were followed.

4.3.3.1 Sample selection

Culturing samples and preparing them for MALDI-ToF-MS analysis is a time-consuming procedure, therefore, only swab and milk samples from 10 randomly selected study ewes (5 control and 5 intervention ewes) were processed. This gave a total of 216 samples (175 swabs and 41 milk) that were analysed further for species and strain typing of bacteria present. From the 30 acute mastitic ewes that were sampled only those that lambed during the clinical trial (between 20th February and 5th April 2017), were allocated to a treatment group, and had a full set of four samples (2 teat and 2 milk samples) were processed. This resulted in 12 ewes going through to sample processing.

4.3.3.2 Culturing

Swab samples

Each charcoal swab was thawed at room temperature. Once thawed the swab tip was added to a pre-labelled 1.5ml microcentrifuge tube containing 300µl of buffer (PBS + 10% (70%) glycerol) and vortexed for 5 seconds.

A second pre-labelled 1.5ml microcentrifuge tube was placed in a tube holder and positioned in the bottom of a 50ml falcon tube. A 2ml empty syringe was inserted through the falcon tube lid so that the end of the syringe sat inside the 1.5ml microcentrifuge tube to retain the sample supernatant. The swab was added to the assembled column (Figure 4.3). using sterile tweezers and centrifuged at 1600g for 8 minutes. The supernatant was pipetted back into the

original tube, vortexed for 5 seconds and then centrifuged at 14,000rpm for 5 seconds. A 100µl aliquot of the centrifuged supernatant was pipetted onto a labelled sheep's blood agar (SBA) plate containing 7% (v/v) sterile sheep's blood. The sample was spread over the plate and left to dry for approximately 30 seconds before being inverted and incubated at 37°C for 48 hours. The remainder of the sample supernatant was stored at -80°C.

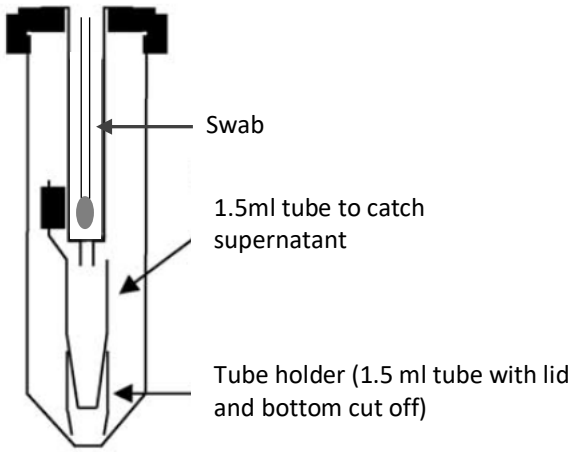

Figure 4.3 Column assembly

Aseptic milk samples

Milk samples were thawed at room temperature and 10µl streaked on a pre-labelled SBA plate using inoculating loops. A new loop was used for each new set of lines (Figure 4.4) to reduce the possibility of overgrowth and increase the ease of isolate selection. Plates were inverted and incubated at 37°C for 48 hours.

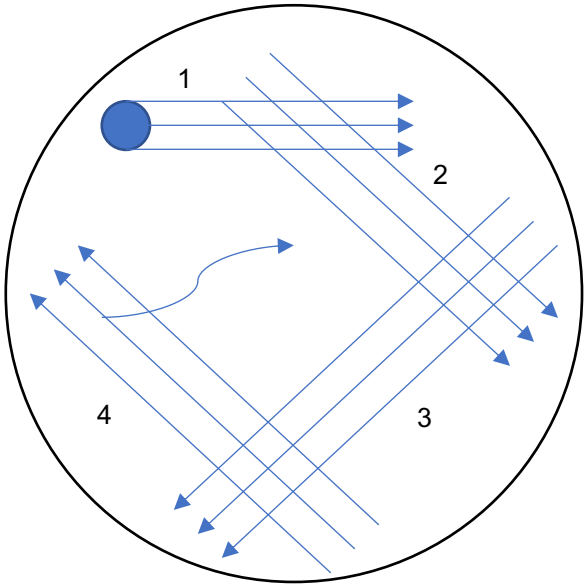

Figure 4.4 Sterile sample streaking method

Isolate selection

After incubation the SBA plates were removed from the incubator and using a 1µl/5µl inoculating loop (depending on how close the colonies had grown together) a single colony from each morphologically unique isolate was picked and placed into a pre-labelled 1.5ml microcentrifuge tube containing 300µl of ultrapure water (Invitrogen). The resulting mixture was vortexed to ensure thorough mixing to achieve a good suspension to which 900µl of 100% ethanol (Fisher Scientific) was then added. The isolate was stored at -20°C until further analysis by MALDI-ToF-MS.

Any plates that had excessive growth, or had colonies that were too close together to pick separately, were quarter plated. To quarter plate, a single colony was picked and sub-cultured onto one quadrant of a pre-labelled SBA plate (Figure 4.5). After a 24-hour incubation period at 37°C, a single colony from any isolates that had grown were picked for further analysis.

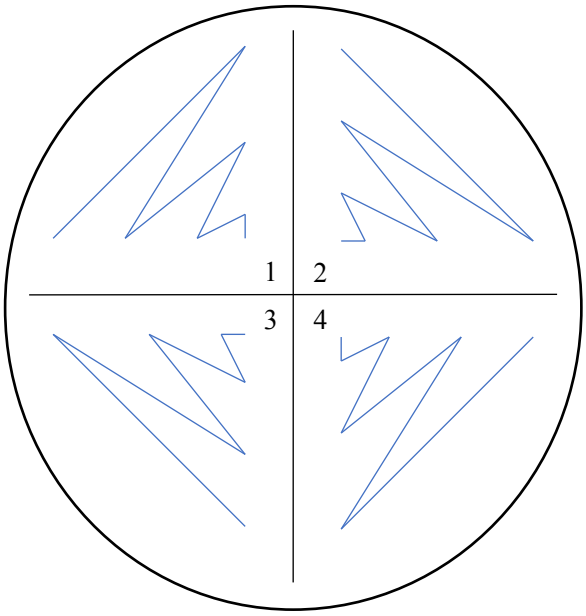

Figure 4.5 Quarter plate example – each numbered quarter would have a morphologically unique isolate streaked onto it

4.3.3.3 Analysis of isolates using MALDI-ToF-MS

Sample preparation

A formic acid protein extraction method was used to prepare isolates for MALDI-ToF-MS analysis. This method has been identified to achieve a better spectral result than the direct transfer method, reducing background noise and increasing peak intensity (Alatoom *et al.*, 2011; Cooper, 2015), which is important when looking at strain types rather than at species alone. The specialised target plates (MTP 384 polished steel BC targets, Bruker Daltronik GMBH, Germany) used for this analysis were thoroughly cleaned between screenings using a multi-step cleaning process with Propan-2-ol and Trifluoroacetic acid (Fisher Scientific) to prevent cross-contamination of isolates.

The isolates selected for analysis were brought up to room temperature, centrifuged at 14,000xg for 2 minutes and the supernatant discarded. This step was repeated and the residual ethanol removed from the resulting pellet between centrifugation steps. The pellet was left to air dry for 30 minutes. The isolates were separated by pellet size into small (equivalent of 10µl), medium (equivalent of 20µl), large (equivalent of 30µl) and extra-large (equivalent of 40µl).

Between 10µl-40µl (depending on pellet size) of 70% formic acid and the equivalent volume of pure acetonitrile were added and mixed thoroughly by pipetting up and down. Each isolate was then centrifuged again at 14,000xg for 2 minutes and1µl of the supernatant was spotted onto a MALDI target plate and

allowed to air dry. To prepare the matrix solution 1ml organic solvent was prepared from 500µl pure acetonitrile, 475µl ultrapure water (Invitrogen) and 25µl pure Trifluoroacetic acid. 250µl of organic solvent was pipetted into a HCCA matrix portioned tube (Bruker HCCA portioned) and vortexed until all the matrix crystals had completely dissolved. After air drying, 1µl of matrix solution was overlaid within 30 minutes and air dried.

Samples were transported in a dark container at 2 - 4°C for MALDI-ToF-MS analysis at Quality Milk Management Services Ltd (QMMS), Somerset.

Production of sample spectra

The target plates were inserted into the MALDI-ToF-MS biotyper and set to analyse each target spot three times according to the manufacturer's instructions. Once all the spectra had been produced they were compared to an updated mastitis pathogen database resulting in species identification and confidence scores being assigned to isolates. The biotyper uses a matching algorithm to compare the spectra to the known spectra in the database and computes a score value against the most relevant match. A higher score represents a more closely matched spectrum. As the species identification relies on the spectra having already been found and imported to the database not every spectrum will generate a valid species identification.

4.3.3.4 MALDI-ToF-MS species identification

The Bruker biotyper uses a matching algorithm to identify the species of an isolate by comparing the isolate mass spectra fingerprint to a database of known species fingerprints. A list of potential candidates with confidence scores is then produced with the most likely matching species at the top. This top scoring species name was taken forward. Duplicates were removed for the species counts. A duplicate was defined as any species identified more than once in an individual sample. These duplicates were not removed from the overall database because of the possibility that the two isolates identified in the same sample may actually be different strains. Retaining this level of detail was important for further analysis.

4.3.3.5 Mass spectra processing

All spectral data were saved for analysis to assess how similar the isolates were to each another. Occasionally the spectral data will be referred to as a fingerprint, meaning the peaks present and intensity of these peaks detected in each isolate's mass spectral data. Each bacterial species has its own set of peaks, intensities vary by strain (Figure 4.6).

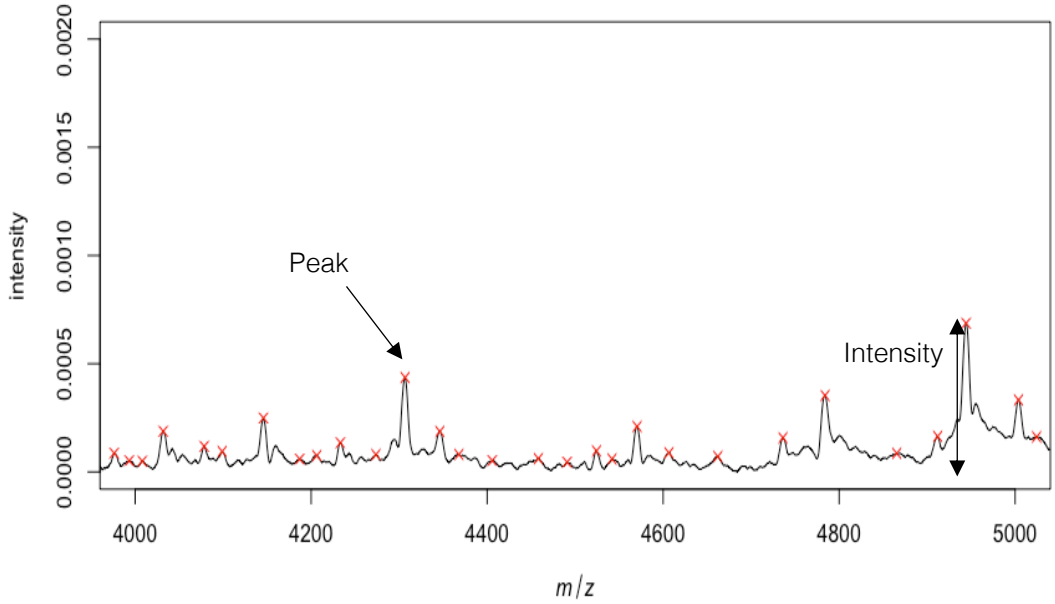

Figure 4.6 Mass spectra fingerprint – example of peak and intensity labelled

Identification of faulty spectra

Before the isolates could be compared to each other the spectral data had to be processed. The spectral data were first loaded into R (version 1.1.447) for pre-processing (Palarea-Albaladejo *et al.*, 2018). The data was screened for potentially faulty, low quality raw mass spectra, using a method to compute upper and lower thresholds for the identification of atypical mass spectra. Each spectrum was assigned an atypicality score based on a weighted function of two aspects; a robust location - free scale estimate (more efficient than the median absolute deviation) of the scaled spectra and the median intensity of the spectral signals. This score was then used to label potentially faulty spectra. Any spectra that were labelled as potentially faulty were removed from the data. Inclusion of this additional quality control step ensured later analyses were robust and accurate.

Processing the spectral data

The data was square root transformed (Gibb and Strimmer, 2012) and smoothed using the 21-point Savitzky-Golay-Filter (Savitzky and Golay, 1964, Figure 4.7a). Alignment of the data is necessary to match peaks and identify similarities accurately. This was achieved by: correction of the baseline using the SNIP algorithm (Ryan *et al.*, 1988, Figure 4.7b&c), the spectral intensity values were then normalised and the mass values recalibrated using a warping algorithm (He *et al.*, 2011 and Wang *et al.*, 2010).

Peak detection

The final processing step was peak detection: the signal-to-noise ratio was estimated and this estimate was used as a threshold to remove background noise (Figure 4.7d). The data were calibrated using an algorithm adapted from next generation sequencing data calibration (Anders and Huber, 2010; Gibb and Strimmer, 2012). The resulting peak intensity matrix was then ready for comparison using unsupervised hierarchical clustering.

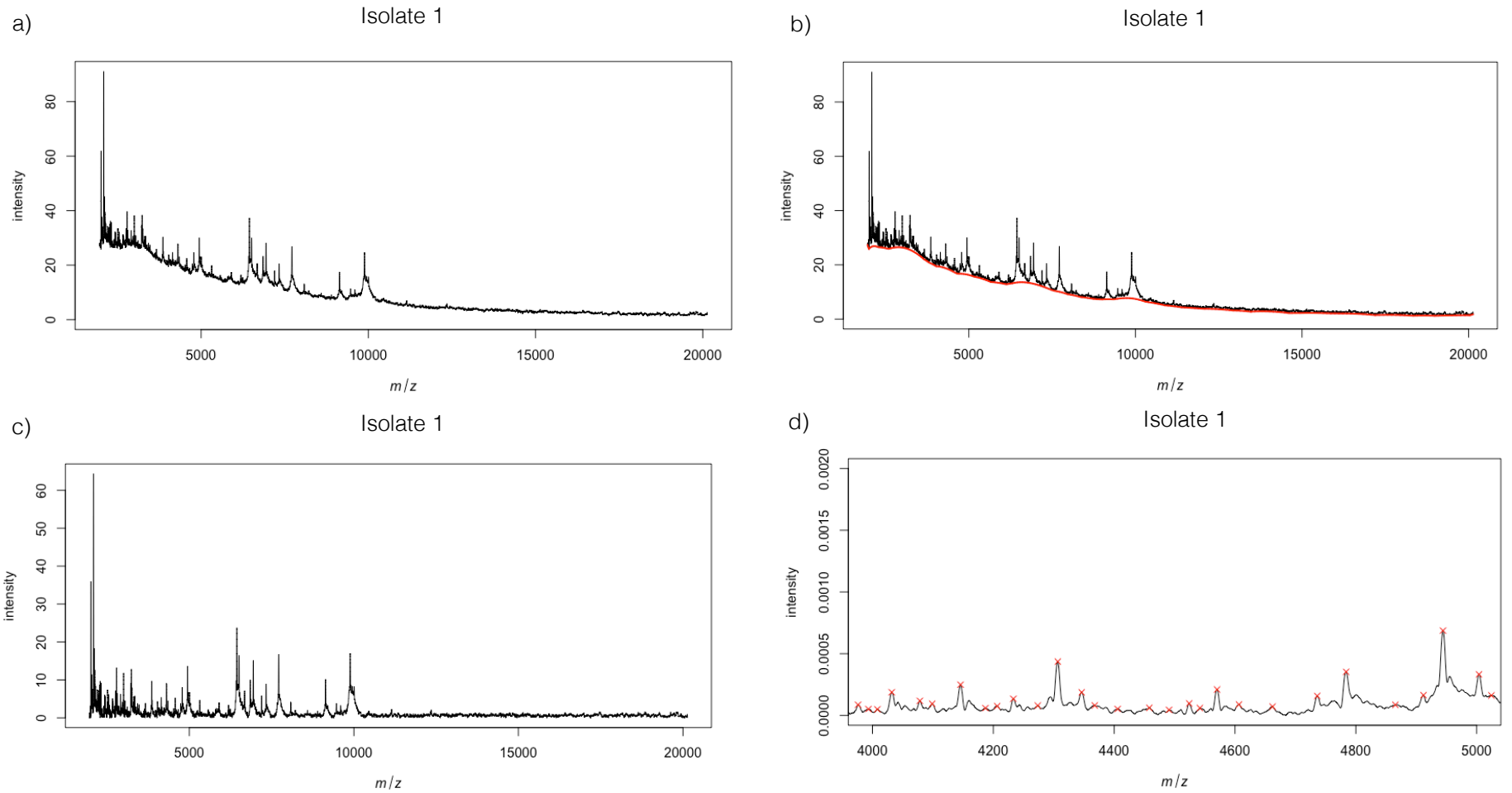

Figure 4.7 Spectra processing steps. a) Smoothed spectra, b) Baseline inserted, c) Baseline corrected and d) Peaks detected after normalisation and recalibration

4.3.3.6 Unsupervised hierarchical cluster analysis

To measure the similarities between the isolates unsupervised agglomerative hierarchical clustering was used, which is a method of cluster analysis. A distance matrix was computed using the Euclidian distances between isolates from the peak intensity matrix. The distance matrix was then analysed for similarities and dissimilarities using the ward.D2 method (Murtagh and Legendre, 2014). This method implements a clustering criterion where the dissimilarities are squared before clustering. The results were visualised as a dendrogram, with isolates clustered by similarity. Shorter branches of the dendrogram tree represented isolates that were more similar to one another.

Data for the study ewes and mastitic ewes were analysed separately and together using the above method. The combined data for the study and mastitic ewes were only used to investigate shared strains.

4.3.3.7 Strain differentiation

To be able to investigate the movement of bacteria, it is necessary to be able to identify isolates to strain level. Identifying the presence of a species at different locations, or at the same location on multiple occasions, is not enough evidence to confidently suggest that a transmission or persistence event has occurred.

The method for strain differentiation was based on Rupf *et al.*, 2005 and Sauer *et al.*, 2008. Replicates (biological and technical) were used to determine a cut-off Euclidean distance value on a dendrogram of isolates. Under the determined cut-off any isolates clustering were deemed to be of the same bacterial strain. Biological replicates were isolates taken from colonies grown from different SBA plates, i.e. different samples, that looked morphologically identical. This was to check similarities across different plates and samples. Technical replicates were isolates taken from the same SBA plate and same bacterial colony. This was to establish the similarity between isolates taken from an exact replicate. It has been reported that the Biotyper struggles to clearly identify spore forming bacteria (Wenning *et al.*, 2014), so several technical replicates were used from different species, including spore formers. The length of the dendrogram branches for each replicate was measured and the mean for each calculated. To improve the clustering of replicates, so therefore sensitivity of strain typing, it was decided that replicates that fell in ±0.5 standard deviations from the replicate group means (groups being technical or biological) would be used, this resulted in 10 technical and 5 biological replicates (ranging from 3-6 isolates per replicate). Taking these replicates forward a cut-off value of 0.0023 for biological and 0.0018 for technical replicates was identified (Figure 4.8). It was decided to use the technical value only as this was a more discriminatory. Finally, all the isolates were identified by

a cluster number (which from here will be referred to as a strain) for further statistical analysis.

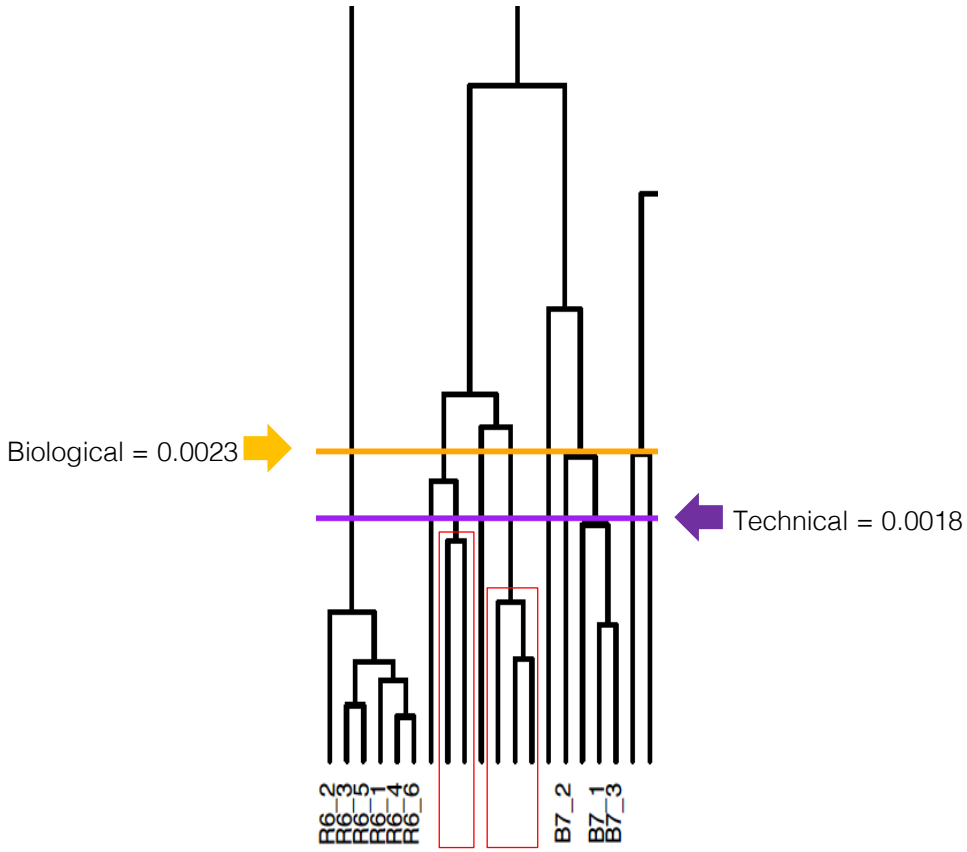

Figure 4.8 Section of the resulting dendrogram with only the replicates and thresholds labelled. R6 = technical replicate, B7 = biological replicate. The two red boxes are examples of isolates considered to be of the same strain.

4.3.3.8 Statistical analysis and modelling

The resulting database from the strain differentiation in R was exported to Microsoft Excel. The isolate ID's were matched back up to their more in-depth information, for example the ewe ID, sample type and sampling time point.

Multilevel regression models were used to determine associations between persistence and possible transmission of bacteria and different ewe factors. As the focus of this study was the transmission and persistence of bacteria, only those strains that occurred in more than one sample were taken forward to modelling.

All models were run in MLwiN version 3.01 (Charlton *et al.*, 2019). To account for clustering in the data, as samples were taken over time within sheep, two-level models were used. Two separate models were built, the first looking at the presence of multiple occurring or shared strains and, the other, the proportion of multiple occurring or shared strains per sample. Variables such as treatment

group, sampling site and sampling time point were included as fixed effects. Variables were considered significant if the Z ratio was greater than 2 and the 95% confidence intervals did not contain 1. Where variables were correlated with each other the most biologically plausible variable was retained in the model.
The models took the form:

$$\mathrm{Logit}(\pi_{ij}) = \beta_0 + \beta x_j + \beta x_{ij} + \mu_j$$

where $\mathrm{Logit}(\pi_{ij})$ is either the log odds of the probability of a multiple occurring strain being present or the proportion of the multiple occurring strains present, β_0 is the constant, βx are the fixed effects that vary at j (ewe unique identification number) and i (sample type) and μ_j the residual variance estimates at ewe unique identification number. Level 1 variance followed a binomial error distribution.

4.3.3.9 Dice-Sørensen coefficient of similarity

The Dice-Sørensen coefficient of similarity (Dice, 1945 and Sørensen, 1948) was used to compare bacterial communities at different sampling sites. Unlike other similarity coefficients this calculation considers both mutual presences and absences, and the size of the number of bacterial strains detected. Vectors of the strains isolated for each sample were produced. Using R, the vectors were compared for similarities using the formula: (2c)/(a+b), where a and b are the number of strains in each vector and c is the number of shared strains (Determan Jr., 2017). The function returns a value between 0 and 1, where 0 indicates no overlap between the vectors and 1 that the two vectors are identical. A 'for loop' was created in R, to run the calculation between all the samples and create a final matrix with all the similarity values for interpretation.

A PCA plot was created with each isolate plotted to ensure that no one sample or ewe would skew a combined sample analysis. After this was determined PCA plots were created to visualise the community similarities using samples grouped together. The use of ANOSIM and SIMPER was attempted to determine significant relationships between sites, however the data was not suitable to be used in these methods. Therefore, the similarities between the communities will be described using the Dice-Sørensen coefficient only.

4.4 Results

4.4.1 Descriptive summary of the collected samples and the isolates identified

A total of 1020 isolates were analysed using MALDI-ToF-MS, 835 isolates originated from 10 study ewes (5 control and 5 intervention ewes) and 160 from 12 ewes (7 control and 5 intervention) that developed acute mastitis. The number of isolates that grew on the SBA (sheep blood agar) plate varied between sampling sites. A higher number of vaginal and teat swabs from both study and mastitic ewes had no growth, and lower overall average count of isolates (Table 4.2) compared to the other sample sites. The highest number of morphologically unique isolates was identified from ewe noses and lamb mouths (Figure 4.9). The mastitic teat and milk samples had lower numbers of unique isolates than the clinically healthy teat and milk samples (Figure 4.9). A summary of the number of ewes, sampling site and whether spectra was successfully identified for the isolates is shown in Table 4.2 (sample study ewes) and Table 4.3 (mastitic ewes).

Table 4.2 Summary of samples taken and number of isolates and spectra produced by sampling site for the study ewes (n=10) and handler hands (n=8)

		Sampling site						
		Vagina	Ewe nose	Teat	Milk	Lamb mouths	Handler hands	Total
Samples	Growth on SBA plate	5	20	51	35	37	16	163
	No growth on SBA plate	5	0	29	5	3	4	47
	Total	10	20	80	40	40	20	210
Isolates	Spectra produced	10	138	190	122	270	63	793
	No spectra produced	0	7	8	8	11	8	42
	Total	10	145	198	130	281	71	835

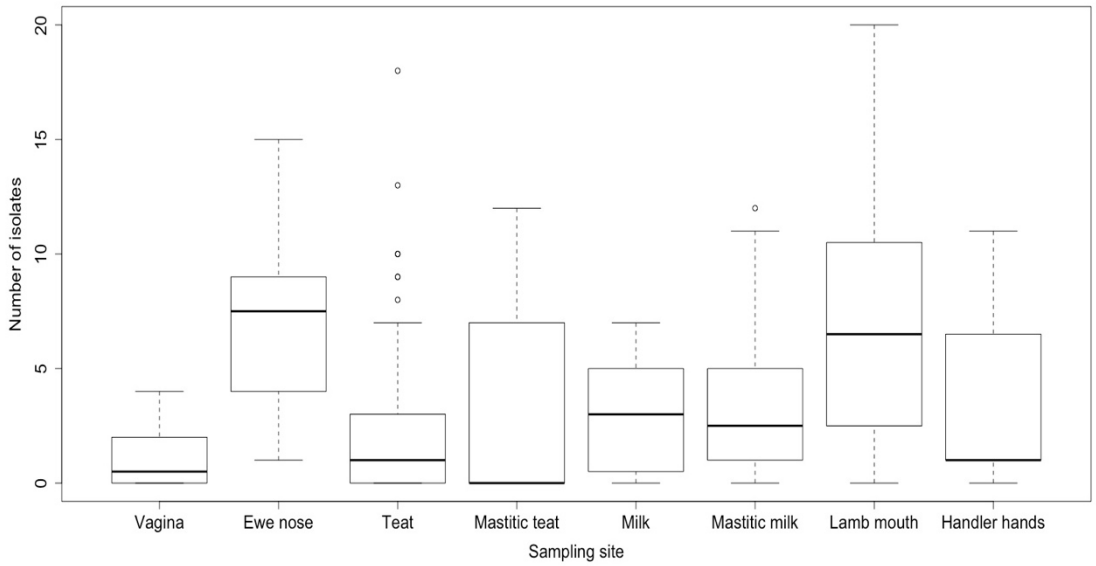

Figure 4.9 Number of isolates by sampling site per ewe for both study ewes (n=10) and mastitic ewes (n=12)

Table 4.3 Summary of samples taken, and number of isolates and spectra produced by sampling site for ewes that developed acute mastitis (n=12)

		Sampling site		
		Teat	Milk	Total
Samples	Growth on SBA plate	11	17	28
	No growth on SBA plate	13	7	20
	Total	24	24	48
Isolates	Spectra produced	72	82	154
	No spectra produced	4	2	6
	Total	76	84	160

4.4.2 MALDI-ToF-MS identified bacterial species

Removal of species duplicates, as detailed in section 4.3.3.4, reduced the number of isolates for the sample study from 793 to 552 and the mastitic isolates from 154 to 85.

4.4.2.1 MALDI-ToF-MS identified species from the study and mastitic ewe samples

As the species identification relies on that species' fingerprint having already been categorised and saved into the database (QMMS March 2017 update, 5,989 isolates), not all the isolates were identified. Approximately a fifth of the both the study ewe (n=110, 19.9%) and mastitic ewe (n= 18, 21%) isolates could not be reliably identified using the Biotyper.

From the 552 study ewe isolates, 58 species were identified. The most frequently identified species of bacteria from all sites were *Aerococcus viridans*, *Staphylococcus lentus*, *Bacillus licheniformis*, *Staphylococcus equorum and Staphylococcus sciuri*. Thirty-four (59.6%) of the bacterial species identified in the study ewes were detected at more than one sampling site. *Aerococcus viridans*, *Bacillus licheniformis*, *Corynebacterium stationis*, *Staphylococcus equorum*, *Staphylococcus lentus*, *Staphylococcus scuiri* and *Staphylococcus succinus* were identified at all sampling sites except for the vagina. *Staphylococcus auricularis* was the most frequently isolated species from teat and milk samples. *Aerococcus viridans*, *Bacillus licheniformis*, *Staphylococcus lentus* and *Staphylococcus sciuri* were isolated in high numbers in the ewe nose, handler's hand and lamb mouth swabs. *Escherichia coli*, which is associated with toxic mastitis in ewes, was one of the most commonly isolated species in the mouths of the lambs.

From the 85 mastitic isolates 28 species were identified. The most frequently isolated bacterial species from ewes that developed acute mastitis were *Staphylococcus aureus* (12.9%) followed by *Staphylococcus auricularis* (10.6%). Unlike the study ewes, only teat and milk samples were taken from ewes that developed acute mastitis. *Staphylococcus aureus*, was found in greater numbers in the milk samples. *Staphylococcus auricularis* was isolated at higher frequencies in both teat and milk samples. Of the 28 species identified, 9 (32.1%) were isolated from both teat and milk samples, including *Bacillus* sp., *Corynebacterium* sp. and coagulase negative staphylococci. Although not statistically significant, the number of species isolated from mastitic teats (20) and milk (17) was lower than the number isolated from clinically healthy teats (31) and milk (25).

Twenty species were isolated from both the study and mastitic ewes. *Staphylococcus auricularis* and *Staphylococcus lentus* were frequently isolated from both groups of ewes. In contrast, the most frequently isolated species from the mastitic samples, *Staphylococcus aureus*, was not isolated or identified in the study ewes at all, the only other location *Staphylococcus aureus* was isolated from was the farmers nose. A full list of bacterial species identified can be found in the appendix (Appendix 5).

Of the 20 species that were identified in both the study and mastitic ewes, a number of species were isolated at larger percentages from mastitic teats than from teats of clinically healthy ewes, particularly *Arthrobacter gandavensis* (12.2% mastitic and 0.8% healthy) and *Solibacillus silvestris* (9.8% mastitic and 1.6% healthy). The percentage of isolates identified as *Staphylococcus auricularis* was higher in mastitic milk (13.6%) than in milk from the clinically healthy study ewes (7.3%). In contrast, other species were identified at lower percentages in the mastitic samples compared to the clinically healthy samples including *Bacillus licheniformis* (2.4% mastitic and 6.9% healthy), *Corynebacterium stationis* (2.4% mastitic and 4.9% healthy), *Staphylococcus equorum* (2.4% mastitic and 6.3% healthy) and *Staphylococcus sciuri* (2.4% mastitic and 5.4% healthy). A full list of the bacterial species identified by sampling sites can be found in Appendix 6.

4.4.3 Strain identification using computational methods

Using the method described in section 3.3.3.6, strains were identified from the 793 study isolates and 154 mastitic isolates. As this method does not depend on the Biotyper species naming of isolates, all the isolates were included.

There were 947 isolates that produced spectra (Table 3.2), and of these 769 from study ewes and 149 from mastitic ewes were of sufficient quality for unsupervised cluster analysis (section 3.3.3.5). Using the technical threshold level as described in the methods section 3.3.3.6, isolates clustering together were identified. Duplicates, identified as the same sample (e.g. left teat, from Ewe 06, taken post-lambing) in the same cluster were removed which reduced the number of study ewe isolates to 741 and mastitic isolates to 125. The final cleaned dataset resulted in 541 strains identified from the 748 study ewe isolates (Appendix 7), and 100 strains from the 125 mastitic ewe isolates.

4.4.4 Defining strain persistence and transmission

To investigate strain persistence and transmission, only strains that occurred in more than one sample were analysed. These will be referred to as multiple occurring or reoccurring strains for the study ewes and shared strains for the mastitic ewes. The sample study ewe and mastitic ewe datasets were analysed separately with duplicates being defined slightly different for each, as described below.

4.4.4.1 Study ewes definition of multiple occurring strains

For the study ewes multiple occurring strains were defined as any strain that was detected more than once across the samples within and between ewes, this reduced the number of strains analysed from 541 to 128 (Table 4.4).

4.4.4.2 Mastitic ewes definition of shared strains

Regarding the mastitic samples, strains that were identified in both the study ewes and mastitic ewes were analysed (Table 4.4). When the databases were combined 35 strains were seen in both mastitic and study ewes. These 35 shared strains were analysed further.

Table 4.4 Number of isolates, strains and reoccurring for all samples

	No. of isolates with spectra	No. of isolates passing QC step	No. of isolates after duplicates removed	No. of strains identified	No. of multiple occurring or shared strains
Study ewes (n=10)	793	769	741	541	128
Mastitic and study ewes (n=22)	947	913	872	622	35

4.4.5 Study ewe strain persistence and possible transmission events

4.4.5.1 Frequency and location of multiple occurring strains

The number of occurrences ranged from 2 – 8, with 57% of the strains occurring twice and very few occurring more than four times (Table 4.5).

Table 4.5 Number of strains by frequency of occurrences

Frequency of occurrences	Number of strains
2	73
3	38
4	12
5	3
6	1
8	1
Total	128

The presence and proportion of these 128 multiple occurring strains in the study ewes varied between sample sites and sampling time points. The samples from lambs' mouths and ewe noses had higher proportions of multiple occurring strains (Figure 4.10a) compared to other sample sites. Samples taken immediately post-lambing were also associated with higher proportions of multiple occurring strains (Figure 4.10b) compared to the other sampling time points.

To look at potential persistence and transmission events the 128 multiple occurring strains were analysed within ewes, within ewes and their lambs, between ewes and between ewe-lamb-handler units (a ewe, her lambs and the handler).

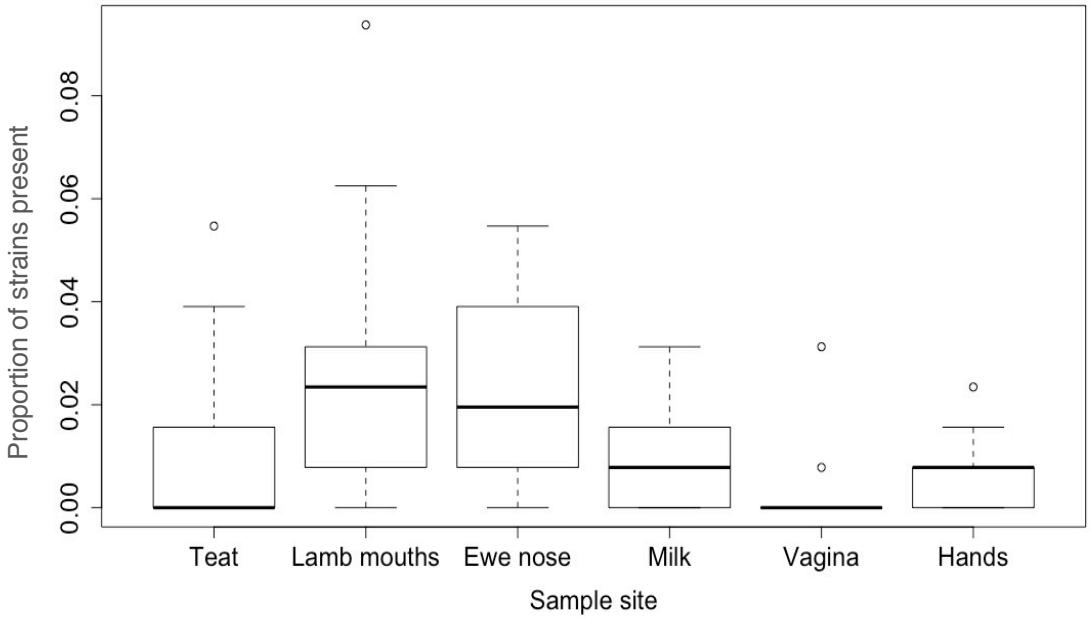

Figure 4.10a. Proportion of multiple occurring strains per sample by site in the 10 sample study ewes

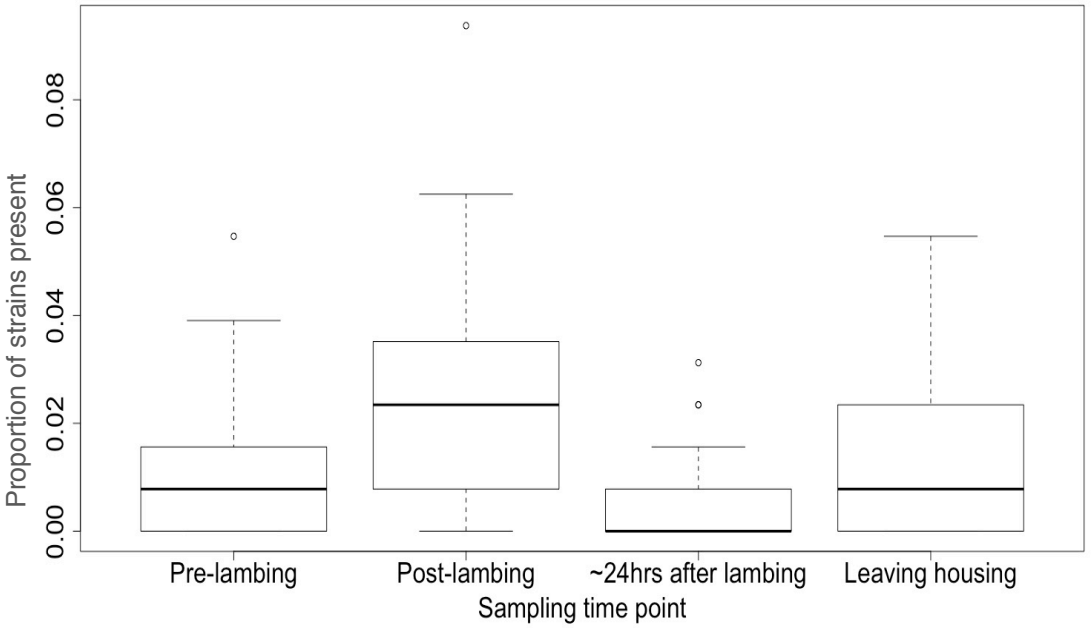

Figure 4.10b. Proportion of multiple occurring strains per sample by sampling time point in the 10 sample study ewes

4.4.5.2 Within-ewe strain persistence

To investigate within-ewe strain persistence only strains that occurred more than once in at least one ewe, were retained for analysis and considered to be persisting strains. These 16 persisting strains were plotted by time and site (Figure 4.11 and Appendix 8). There were no multiple occurring strains in 30% (3/10) of ewes (two intervention and one control ewe). The remaining 70% (7/10) had between one and five strains that occurred more than once, over time or in different sites. From the 16 strains, 14 (87.5%) occurred twice and two (12.5%) occurred three times. No ewes shared the same multiple occurring strain. Strains occurring at the same time point were more often present in/on both udder halves of the same site, e.g. present in the left and right milk sample. Strains that reoccurred at two time points were more likely to be in the same exact site e.g. isolated from the right teat at both time points (Table 4.6).

Table 4.6 Frequency of the 16 strains identified to reoccur in individual ewes by occurrences at time points and sites

	Occurred at same site	Occurred in the other udder half	Occurred at different sites
Present at same time point	-	6	1
Present at two consecutive time points	4	1	0
Present at two non-consecutive time points	1	1	2
Present at three consecutive time points	1	0	0

From the 16 persisting strains, six reoccured on the teats of ewes, four persisted on the teats over time and two occurred on both teats at the same time point. From the four strains that persisted on teats over time, three were over two consecutive time points (pre- and post-lambing), the ewe was in the same lambing yard for both of these sampling time points. There was only one strain that persisted on the same teat of one ewe at three consecutive time points (*Staphylococcus sciuri* - strain 36). MALDI-ToF-MS identified species that persisted on teats as: *Corynebacterium stationis*, *Bacillus lichenformis*, *Staphylococcus auricularis* and *Staphylococcus sciuri*.

Five strains were seen in more than one milk sample per ewe, this was both over different time points (2 strains) and the same time point (3 strains). These reoccuring strains were only seen in milk samples. All the named species from the identified persisting milk strains were coagulase negative staphylococci (CNS); *Staphylococcus auricularis*, *Staphylococcus vitulinus*, *Staphylococcus lentus* and *Staphylococcus equorum*.

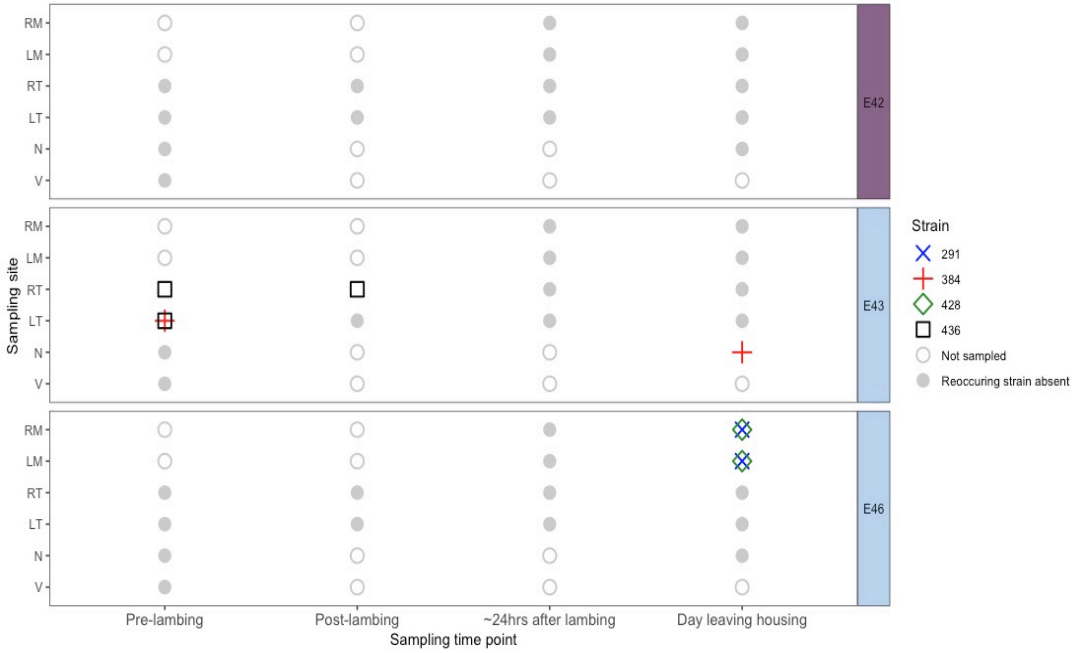

Figure 4.11 Three ewes (2 control and 1 intervention) as an example of strain persistence within ewes by sampling site and time. V=vagina, N=Ewe nasal cavity, LT=left teat, RT=Right teat, LM=Left milk, RM=Right milk. Purple=Intervention ewes, Blue=Control ewes

Teats appear to be more affected by the housing environment than milk. More milk samples had a strain persist over different housing environments than teat and ewe nose samples, perhaps suggesting stability in the milk samples compared to the nose and teats.

4.4.5.3 Between ewe strain persistence and possible transmission events

From the 128 multiple occurring strains the number identified between ewes was higher than the number of multiple occurring strains within the same ewe, with 54 (42.2%) compared to 16 (12.5%) respectively. Of the resulting 54 strains, 39 were identified across two ewes, 13 strains across three ewes and 2 strains across four ewes. For this analysis, strains were considered to reoccur between ewes if they occurred between at least three ewes instead of two, to increase the robustness of the definition of persistence and decrease any effects from local contamination. This reduced the number of strains to 15; 2 (13%) occurred over the same time point, 9 (60%) were identified between ewes over two time points, and 4 (27%) over three time points (Table 4.7).

Table 4.7 Frequency of strains identified to reoccur between three to four ewes by occurrences at time points and sites

Occurrences		No. of strains	Occurrences at same time point			Occurrences over 2-3 time points		
			Different sites	Same site	Same half	Different sites	Same site	Same half
Same time point		2	1	1	1	-	-	-
Two time points	Consecutive	6	2	3	3	4	1	4
	Non-consecutive	3	1	0	2	3	0	0
Three time points	Consecutive	1	0	0	0	1	1	0
	Non-consecutive	3	2	0	0	3	2	0

The frequency of multiple occurring strains associated with each sampling time point was similar, ranging from 6-9 strains. However, samples taken while the ewe and lambs were in individual pens had the fewest strains. The ewe nose and teat samples had an increased number of strains associated with them (Figure 4.12), potentially indicating that these sites are more unstable, with faster changing or larger communities than other sites.

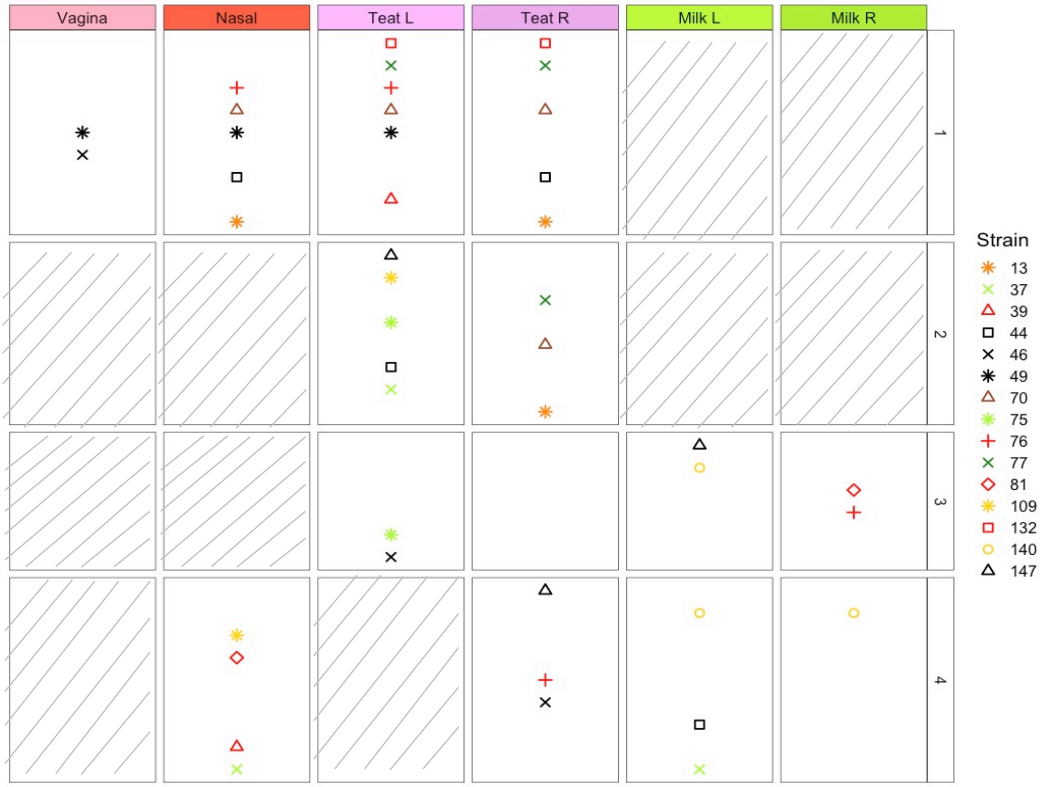

Figure 4.12. Between ewes multiple occurring strains by sampling site and sampling time point 1=Pre-lambing, 2=Immediately post-lambing, 3=~24 hrs after lambing, 4=Day leaving housing. Shading = No sample taken

4.4.5.4 Within ewe-lamb-handler unit strain persistence and possible transmission

To investigate within ewe strain persistence and the role of lambs and humans, ewes, their twin lambs, and their handler's hands were considered as one unit. Strains that occurred between a ewe, her lambs or handler were selected for analysis which resulted in 34 strains. There were no instances that included the handler, so the units are known as ewe-lamb units. The number of multiple occurring strains per ewe increased, compared to within ewes only, with every ewe having at least one strain that reoccurred within the ewe-lamb unit. Of the 34 strains, 28 (82.4%) occurred twice, 5 (14.7%) three times and 1 (2.9%) four times. One strain occurred in two ewes, which was the same strain that occurred 4 times (strain 35). The inclusion of lambs increased the number of multiple occurring strains at every sampling point and every site apart from the vagina (Figure 4.13a and 4.13b). The number of multiple occurring strains associated with the ewe nasal cavity increased in particular, perhaps illustrating the transfer of bacteria during maternal cleaning and bonding with the lambs. Several strains were isolated from ewes in a following sampling time point after being isolated from that ewe's lamb samples, perhaps again suggesting a transmission event for this strain. (Table 4.8)

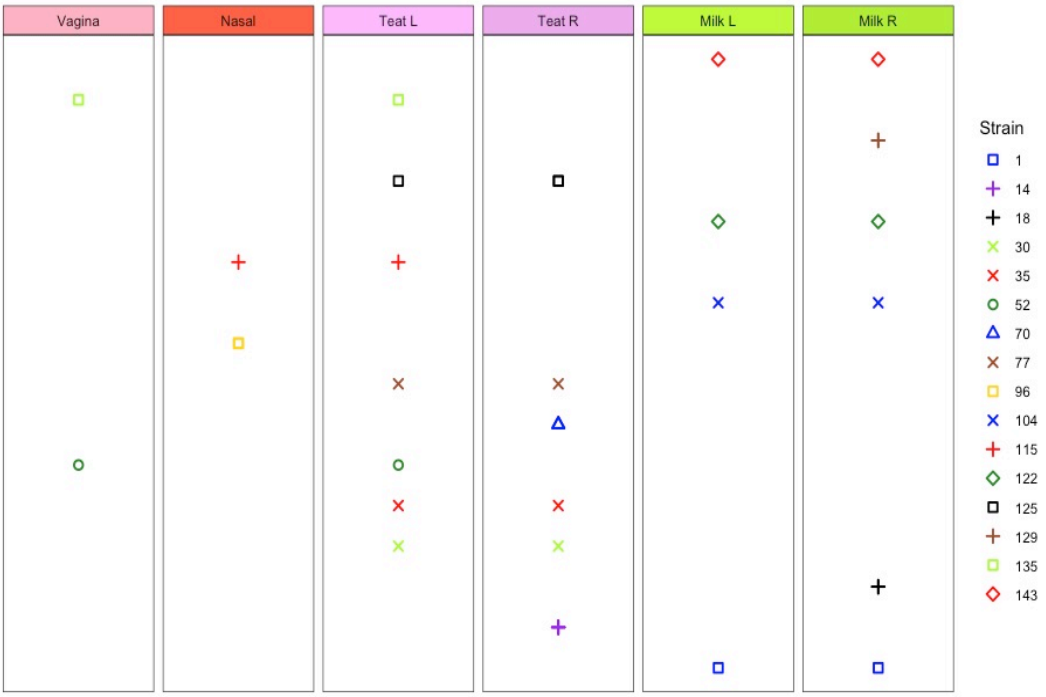

Figure 4.13a. Within ewe multiple occurring strains by sampling site

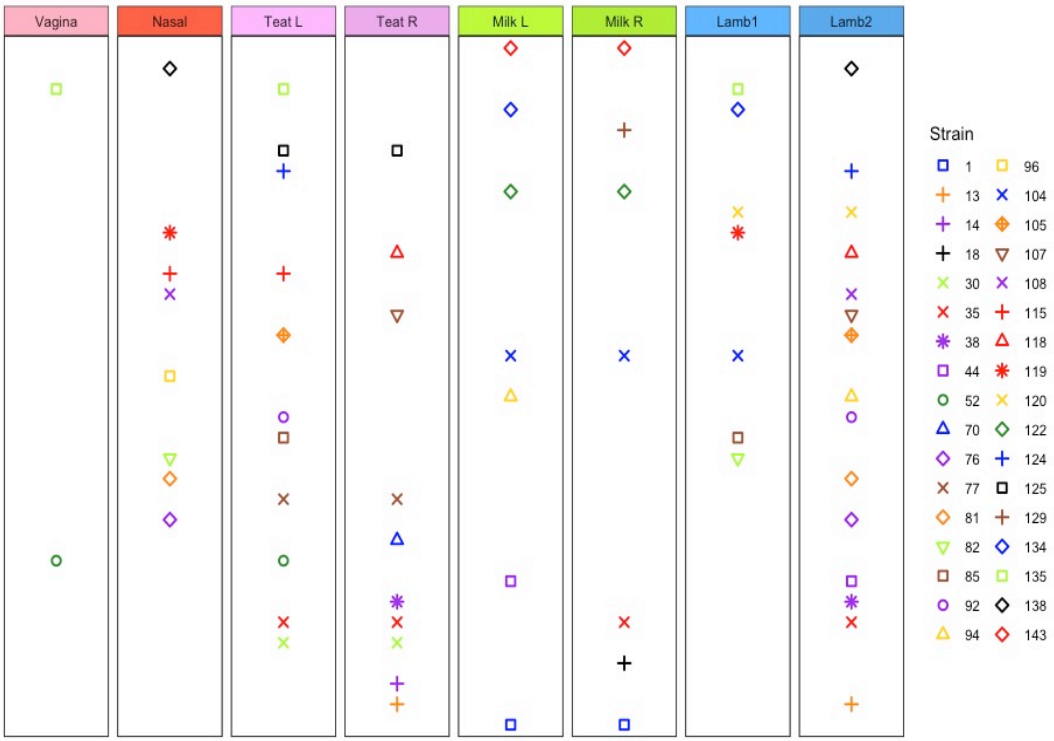

Figure 4.13b. Within ewe-lamb units multiple occurring strains by sampling site

Table 4.8 Frequency of 34 multiple occurring strains in ewe-lamb units by occurrences in ewe and lamb/s

	Frequency of strains	Number of ewe-lamb units positive
Strain isolated in ewe then in lamb/s	7	7
Strain isolated in lamb/s then in ewe	6	5
Strain isolated in ewe and lamb/s at same time point	8	6

These results suggest that lambs play an important role in bacterial persistence and transmission, however, the role of common elements such as the environment cannot be dismissed.

4.4.5.5 Between ewe-lamb-handler units strain persistence and possible transmission

From the 128 multiple occurring strains identified in the study flock, 116 strains occurred between the ewe-lamb-handler units. This was the most strains seen to occur across the four analysis groups (sections 4.4.5.2, 4.2.5.3 and 4.4.5.4).

Over half the strains (76, 65.5%) occurred in two units, however 30 strains occurred in 3 units and 10 between 4 ewe-lamb-handler units. Those that occurred in at least three units (40 strains) were analysed further. This was more than twice the number of strains that occurred between 3 or more ewes only. When looking at multiple occurring strains between ewe-lamb-handler units, handlers had multiple occurring strains associated with them, which was not the case for strains reoccurring within ewe-lamb-handler units (section 4.4.5.4). This could indicate the involvement of human interaction with the movement of bacteria between animals rather than within the animal. Again, the teats, ewe nose and lambs' mouths appear to be associated with the most multiple occurring strains (Figure 4.14a and 4.14b). These results further support the theory that lambs play an important role in bacterial persistence and transmission, increasing the chances of a strain of bacteria detected on multiple occasions.

When comparing time points, samples taken in the individual pens had the least strains associated with them. Half of the strains (20) reoccurred over two time points, whether this be at consecutive or non-consecutive time points, 11 occurred over three time points, eight at the same time point and one over all four time points.

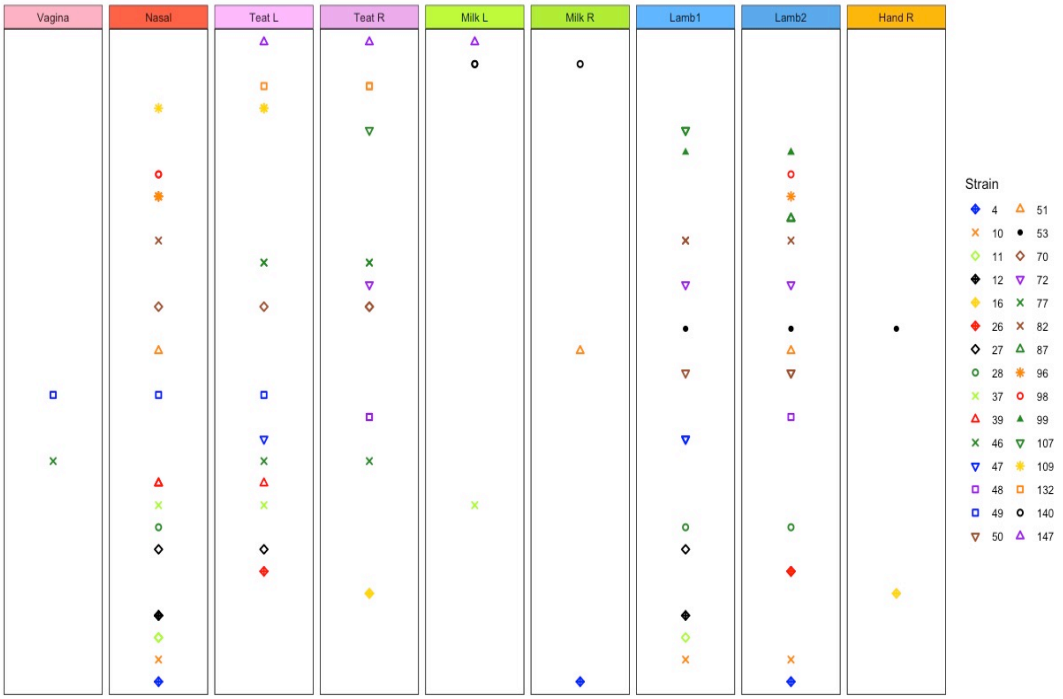

Figure 4.14a: Strains identified between three ewe-lamb-handler units by site

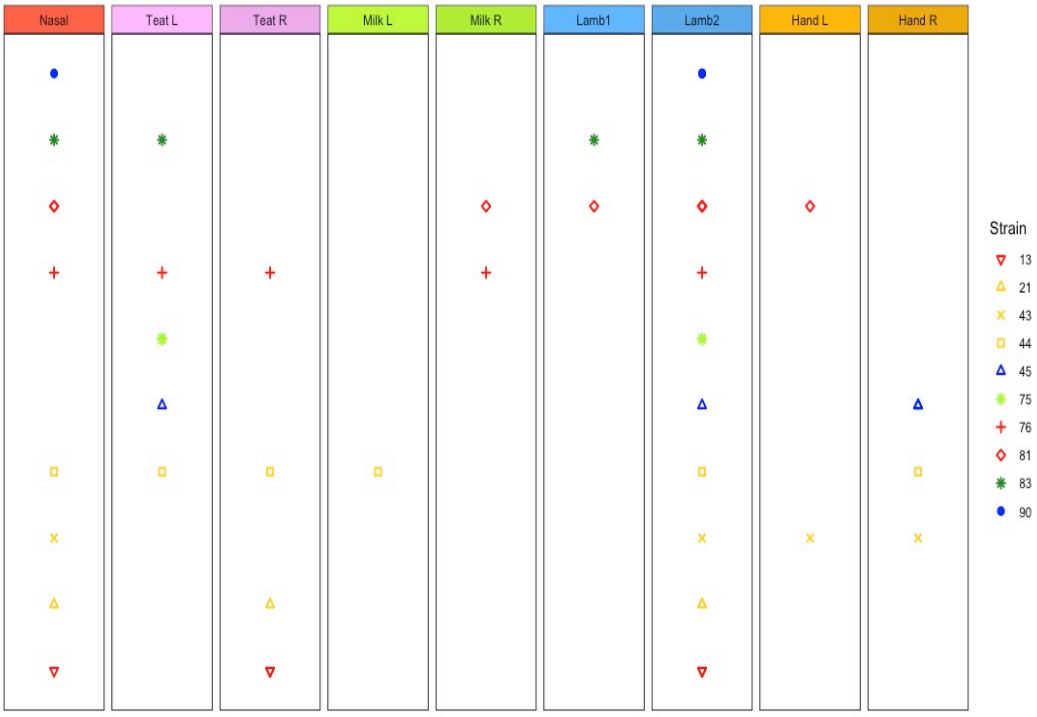

Figure 4.14b: Strains identified between 4+ ewe-lamb-handler units by site

4.4.5.6 Effect of sampling site and time point on strain persistence and transmission

The number of isolates harvested, and strains identified, differed between the sampling sites. The mouths of the lambs, the ewe nose and teats had more strains than the other sites (Figure 4.15). The same pattern was seen for the number of multiple occurring strains per site indicating that strains that reoccur within and between the ewes, lambs and handlers were not associated with one particular site (Figure 4.15).

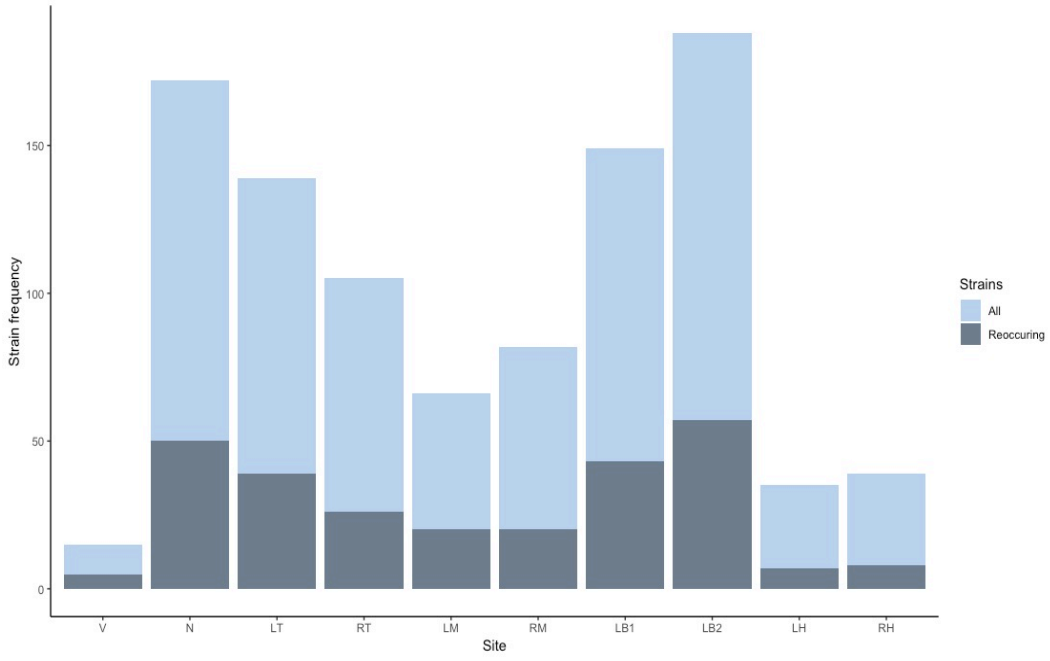

Figure 4.15. Frequency of all strains and reoccurring strains by site. V=vagina, N= ewe nose, LT=left teat, RT=right teat, LM=left milk, RM=right milk, LB1=lamb 1, LB2=lamb2, LH=left handler's hand, RH=right handler's hand

Of the 128 strains that occurred more than once in the study ewes, 38 (29.7%) strains appeared at 1 time point only, 75 (58.6) appeared at two time points, 14 (10.9%) over three time points and 1 (0.8%) over all four time points. There were 55 strains that reoccurred more than 3 times in the study ewes, these strains can be considered to be persisting, and were investigated further. The multiple occurring strains were isolated at larger frequency from samples taken immediately post-lambing and the day the ewe was leaving housing, but a larger proportion of the total isolated strains were identified to occur again in samples taken pre-lambing (Figure 4.16). Fewer strains were isolated when the ewes were in the individual pens. In addition, a smaller proportion of reoccurring strains (including those identified to have reoccurred at the same time point) were identified from samples taken while ewes were in individual pens (Figure 4.16). A larger frequency and proportion of strains are identified while the ewes are in

group yards; time points 1 and 2 (lambing yard), and 4 (treatment group post-lambing yard) (Figure 4.16).

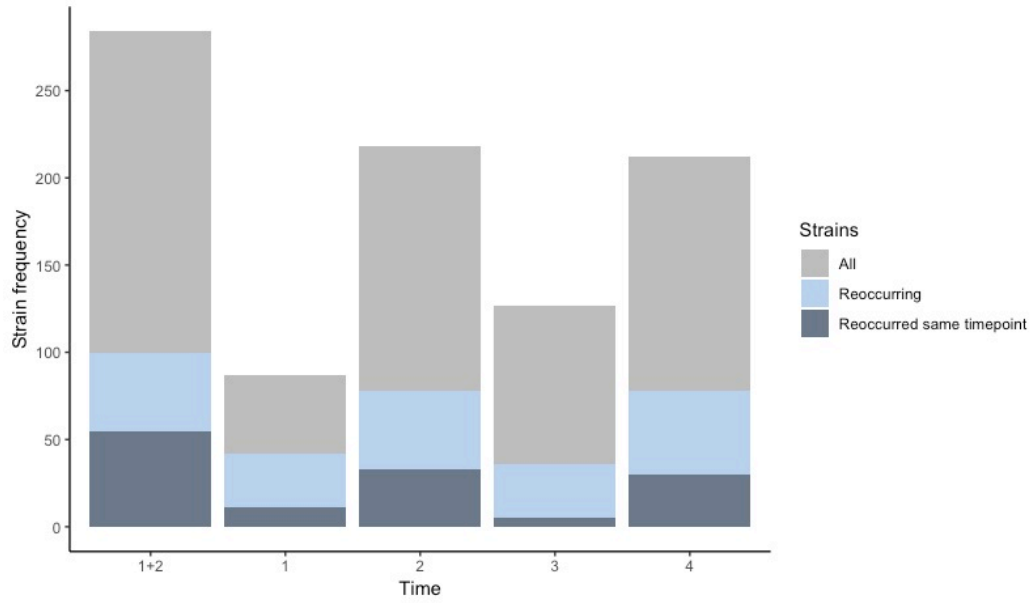

Figure 4.16. Frequency of all strains and reoccurring strains by time point 1=Pre-lambing, 2=Immediately post-lambing, 3=~24 hrs after lambing, 4=Day leaving housing

4.4.5.7 Effect of treatment group on reoccurring strains

Strains persisting within ewes varied between ewes and ewe-lamb units in both treatment groups. In the control group the number of strains reoccurring in ewes ranged from 0-3 with a mean of 1.6. For the intervention ewes, number of strains reoccurring ranged from 0-5 with a mean of 1.6. For ewe-lamb units the range increased for both groups, with strain frequencies ranging from 1-4 with a mean of 2.8 for the control ewes and ranging between 2-8 with a mean of 4.2 for intervention ewes.

Multiple occurring strains in control ewes ranged from 11-19 per ewe with a mean of 13.2, compared to intervention ewes that ranged from 6-22 with a mean of 13.8. There was an increased frequency of strains detected between ewe-lamb-handler units, with control ewes ranging from 26-34 strains per ewe with a mean of 30.8 compared to intervention ewes which ranged from 23-47 strain per ewe with a mean of 32.2. One study ewe, E46, increased the range and mean for the intervention ewes in both cases.

The average number of strains identified at each site per sample differed between the treatment groups, however the only marked differences were a reduced

number of strains on the hands of the handlers of the intervention ewes, and on the teats of the intervention ewes compared to the control ewes (Figure 4.17).

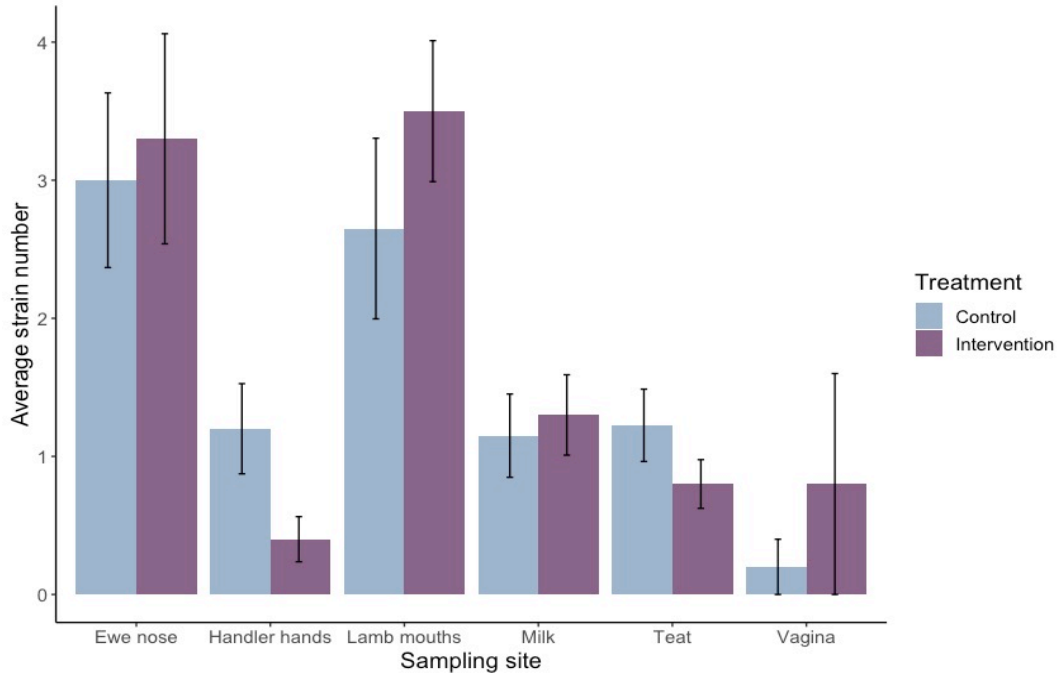

Figure 4.17. Average number of multiple occurring strains (n=128) with standard error at each sampling site by treatment group for the 10 study ewes

In both treatment groups fewer strains were seen in samples taken while the ewes were in individual pens. This difference was more marked for the intervention ewes (Figure 4.18).

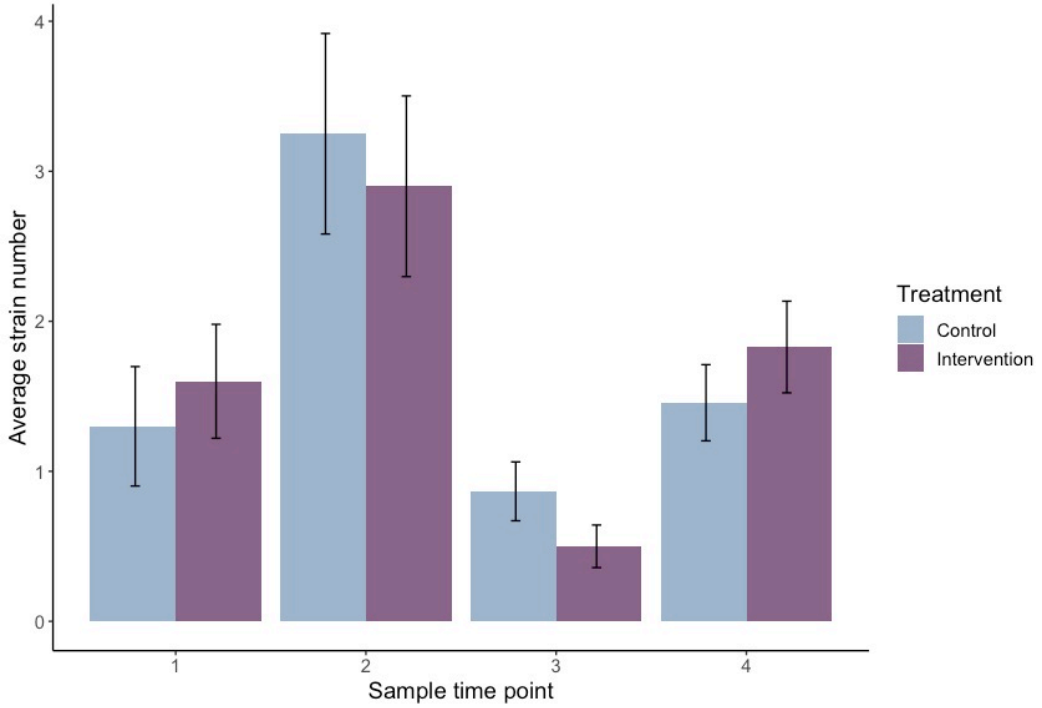

Figure 4.18 Average number of multiple occurring strains (n=128) with standard error at each sample time point by treatment group for the 10 study ewes. 1= Pre-lambing, 2=Immediately post-lambing, 3=~24hrs after lambing and 4=Day leaving housing.

4.4.6 Strains isolated from both mastitic ewes and study ewes

More species and strains of bacteria were isolated from the mastitic samples from control ewes than from the mastitic samples taken from intervention ewes (Table 4.9).

Table 4.9 Number of bacterial species and strains isolated from mastitic samples

	No. of species	No. of strains
Control	15	30
Intervention	10	22

From the 947 isolates, 126 had strains isolated that were in both the mastitic ewes and the study ewes. From the mastitic samples, 57 strains were identified to occur more than once and 35 of these strains occurred in both the study ewes and the mastitic ewes. 13 were strains that had not been seen to reoccur in the study ewes alone, the remaining 22 had been seen to reoccur at least twice in the study ewes. All ten study ewes shared strains with 9 (7 control and 2 intervention) out of the 12 mastitic ewes.

The presence of shared strains varied between the sample sites (Figure 4.19). No shared strains were associated with the samples taken from the vagina. Higher percentages of shared strains were seen in the samples taken from the ewe nose and lamb mouths.

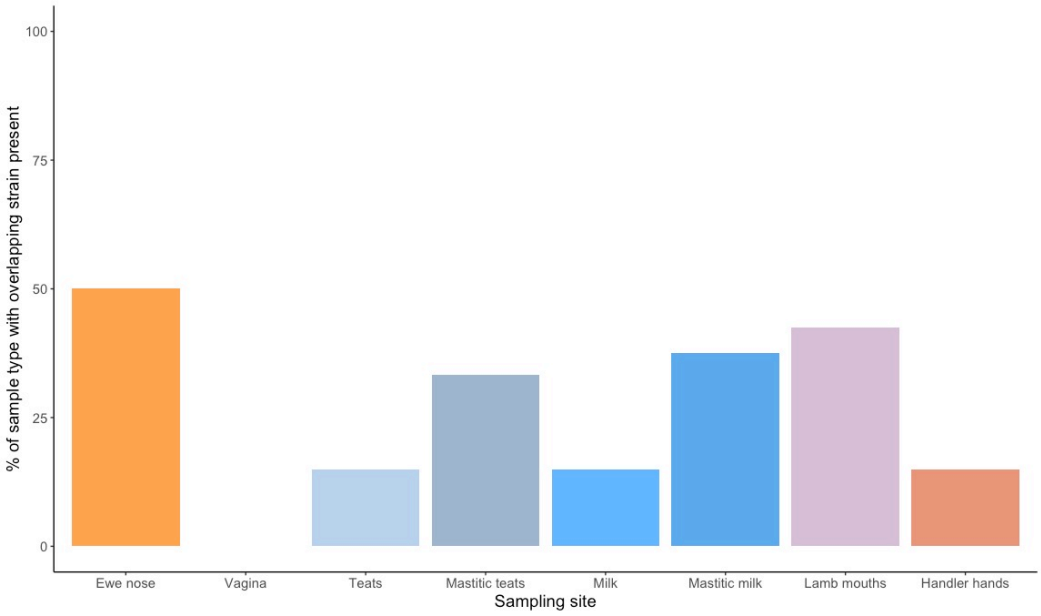

Figure 4.19 Percentage of each sampling site with and shared strain present

4.4.7 Multi-level modelling of flock data

It was not possible to statistically model the transmission pathways of the bacterial strains but it was possible to statistically investigate the variables that affected the presence and proportions of bacterial strains that occurred more than once in the samples.

4.4.7.1 Two-level binomial random effects models for the presence and proportion of the 128 multiple occurring strains in the study ewe isolates

All the sampling sites except the vagina were more likely to have a multiple occurring strain present compared to teats (Table 4.10). Samples taken while the ewes were in the individual pens were less likely to have a multiple occurring strain present compared to samples taken pre-lambing (Table 4.10).

Table 4.10 Two-level multivariable binomial random effects model of the presence of the 128 multiple occurring strains in the study ewe isolates

Explanatory variable	Category	No. samples with strain present	%	OR	Lower 95% CI	Upper 95% CI
Fixed effects						
Sampling site	Teat	37	46.3	Ref		
	Lamb mouth	**34**	**85.0**	**5.6**	**1.94**	**16.25**
	Handler hand	**11**	**55.0**	**3.9**	**1.14**	**13.20**
	Milk	**24**	**60.0**	**3.3**	**1.32**	**8.25**
	Ewe nose	**19**	**95.0**	**21.3**	**2.62**	**172.82**
	Vagina	2	20.0	0.2	0.03	1.13
Sample time point	Pre-lambing	23	57.5	Ref		
	Post-lambing	31	77.5	1.4	0.41	4.51
	Individual pen	**26**	**43.3**	**0.2**	**0.07**	**0.80**
	Leaving housing	47	67.1	0.5	0.17	1.56
Random effects						
Variance	(Ewe tag)			1.4		
Variance	(Sample type)			2.7		

Significance is based on the 95% confidence intervals not containing 1 and indicated by bold text

Five explanatory variables stayed in the multivariable model that investigated variables associated with the proportion of multiple occurring strains present. Higher proportions of multiple occurring strains were associated with lamb mouth swabs, ewe nose swabs and milk samples compared to teat swabs. Samples taken immediately post-lambing were associated with higher proportions of multiple occurring strains compared to samples taken pre-lambing, whereas samples taken while the ewe was housed in an individual pen were associated

with lower proportions of reoccurring strains compared to samples taken pre-lambing. As the study week increased so too did the proportion of present reoccurring strains (Table 4.11).

Table 4.11. Two-level multivariable binomial random effects model of the proportion of the 128 multiple occurring strains in the study ewe isolates

Explanatory variable	Category	OR	Lower 95% CI	Upper 95% CI
Fixed effects				
Study week		**1.1**	**1.01**	**1.16**
Sampling site	Teat	Ref		
	Lambs	**2.2**	**1.65**	**2.99**
	Hand	1.9	0.99	3.49
	Milk	**1.9**	**1.25**	**2.73**
	Nasal	**3.4**	**2.36**	**4.84**
	Vagina	0.5	0.19	1.22
Sample time point	Pre-lambing			
	Post-lambing	**1.7**	**1.12**	**2.56**
	Individual pen	**0.4**	**0.26**	**0.76**
	Leaving housing	0.8	0.52	1.11
Random effects				
Variance (Ewe tag)		1		
Variance (Sample type)		2.7		

Significance is based on the 95% confidence intervals not containing 1 and indicated by bold text

4.4.7.2 Two-level binomial random effects models for the presence and proportion of the 35 strains isolated from both mastitic ewes and clinically healthy study ewes

Swabs from the mouths of the lambs and ewe nose were more likely to have a shared strain with mastitic samples present (Table 4.12) and were more likely to have higher proportions of shared strains with mastitic samples (Table 4.13). Samples taken immediately post-lambing were also more likely to have higher proportions of shared strains with mastitic samples (Table 4.13).

Table 4.12 Two-level multivariable binomial random effects model of the presence of shared strains

Explanatory variable	Category	No. samples with strain present	%	OR	Lower 95% CI	Upper 95% CI
Fixed effects						
Sampling site	Teat	12	15.0	Ref		
	Mastitic milk	9	56.3	7.3	2.2	23.7
	Mastitic teat	9	56.3	7.3	2.2	23.8
	Lamb mouth	21	52.5	6.3	2.6	15.0
	Handler hand	3	15.0	1.0	0.3	3.9
	Milk	7	17.5	1.2	0.4	3.3
	Ewe nose	10	50.0	5.7	1.9	16.5
Random effects						
Variance	(Ewe tag)			1.1		
Variance	(Sample type)			2.7		

Table 4.13 Two-level multivariable binomial random effects model of the proportion of shared strains

Explanatory variable	Category	OR	Lower 95% CI	Upper 95% CI
Fixed effects				
Sampling site	Teat	Ref		
	Mastitic milk	15.1	5.2	44.0
	Mastitic teat	12.3	4.2	36.5
	Lamb mouth	3.0	1.6	5.8
	Handler hand	1.5	0.3	6.5
	Milk	1.5	0.6	3.6
	Ewe nose	6.2	2.9	13.5
Sample time point	Pre-lambing	Ref		
	Post-lambing	3.0	1.2	7.6
	Individual pen	1.0	0.3	3.3
	Leaving housing	1.6	0.7	3.6
	AM case	1.0	1.0	1.0
Random effects				
Variance (Ewe tag)		1.3		
Variance (Sample type)		2.7		

4.4.8 Strain community similarities

Results from the strain community analysis for the 10 study ewes (Table 4.14 and Figure 4.20), identified that sampling sites showed similarity to each other over time. Ewe nose and milk samples clustered close together, perhaps suggesting these sites had a more stable community. Only the Dice-Sørensen matrix for the similarities between the sample site communities over time is shown to illustrate how the PCA is combing and visualising these results considering all similarity indexes for each site and time. For example, the Dice-Sørensen index for the ewe nose and milk samples over time were 0.18 and 0.4 respectively. With values closer to 1 indicating pure overlap, it appears that the high similarities between the ewe nose and lambs' mouths samples pulled the ewe nose samples closer together on the PCA. Whereas the teat samples were more dissimilar to each other and more associated with other sampling sites (e.g. lamb mouths). The pre-suckling lamb mouth communities showed high similarity to the ewe nose samples, whereas the samples taken post-suckling showed similarity with both the ewe teats and milk samples.

Considering the site strain community similarities within the treatment groups and halves, in both treatment groups. Again, the teat communities appeared more unstable and fluctuated, compared to the other sampling sites, showing similarity to the mouths of the lambs, handler's hands and milk samples (Figures 4.21a and 4.21b).

The control ewes display a similar pattern to the flock community shown in Figure 4.20. In addition, the hands of the handlers of the control ewes also showed similarity to the ewe nose and mouths of the lambs as well as the teats (Figure 4.21a and Appendix 9).

The teat sample strain communities from the intervention ewes also show dissimilarity to each other, on two occasions there was high similarity with the mouths of the lambs and the right hand of the handler. Whereas, the milk samples show similarity to each other across halves and over time. The pre-suckling mouths of the lambs show similarity to the ewe nose but also to the milk and teat samples. Like the control ewes, the hands of the handlers also show similarity to the nose of the ewe as well as a teat sample (Figure 4.21b and Appendix 10).

With the strains shared in both the mastitic and study ewes it is interesting how highly similar the strain community is in the mouths of the lambs from the sample study ewes and the milk from the mastitic ewes (Figure 4.22 and Appendix 11).

	N_1	N_4	V_1	T_1	T_2	T_3	T_4	M_3	M_4	L1_2	L1_4	L2_2	L2_4	H_3
N_1	1.00													
N_4	0.18	1.00												
V_1	0.06	0.00	1.00											
T_1	0.23	0.13	0.18	1.00										
T_2	0.20	0.13	0.00	0.24	1.00									
T_3	0.00	0.00	0.36	0.17	0.11	1.00								
T_4	0.05	0.00	0.20	0.06	0.21	0.10	1.00							
M_3	0.04	0.04	0.00	0.11	0.08	0.00	0.24	1.00						
M_4	0.08	0.12	0.07	0.05	0.15	0.00	0.05	0.40	1.00					
L1_2	0.18	0.21	0.00	0.04	0.23	0.00	0.00	0.09	0.04	1.00				
L1_4	0.14	0.09	0.10	0.00	0.04	0.00	0.13	0.06	0.11	0.05	1.00			
L2_2	0.23	0.31	0.00	0.10	0.27	0.04	0.10	0.07	0.09	0.31	0.14	1.00		
L2_4	0.18	0.13	0.00	0.12	0.12	0.09	0.25	0.11	0.05	0.04	0.24	0.10	1.00	
H_3	0.20	0.05	0.11	0.13	0.17	0.10	0.07	0.06	0.11	0.14	0.00	0.14	0.06	1.00

Table 4.14. Dice-Sørensen similarity matrix between the sample site communities over time for the strains isolated from the study ewes. N=Ewe nose, V=vagina, T=Teat, M=Milk, L1=Lamb1 mouth, L2=Lamb 2 mouth, H=Handler hand, 1=Time point 1, 2=Time point 2, 3=Time point 3, 4=Time point 4.

Figure 4.20 Principal component analysis of the Dice-Sørensen similarity matrix between the sample site communities over time for the strains isolated from the study ewes T1=Pre-lambing, T2=Immediately post lambing, T3-~24hrs after lambing, T4=Leaving housing. Similarities highlighted by coloured shapes.

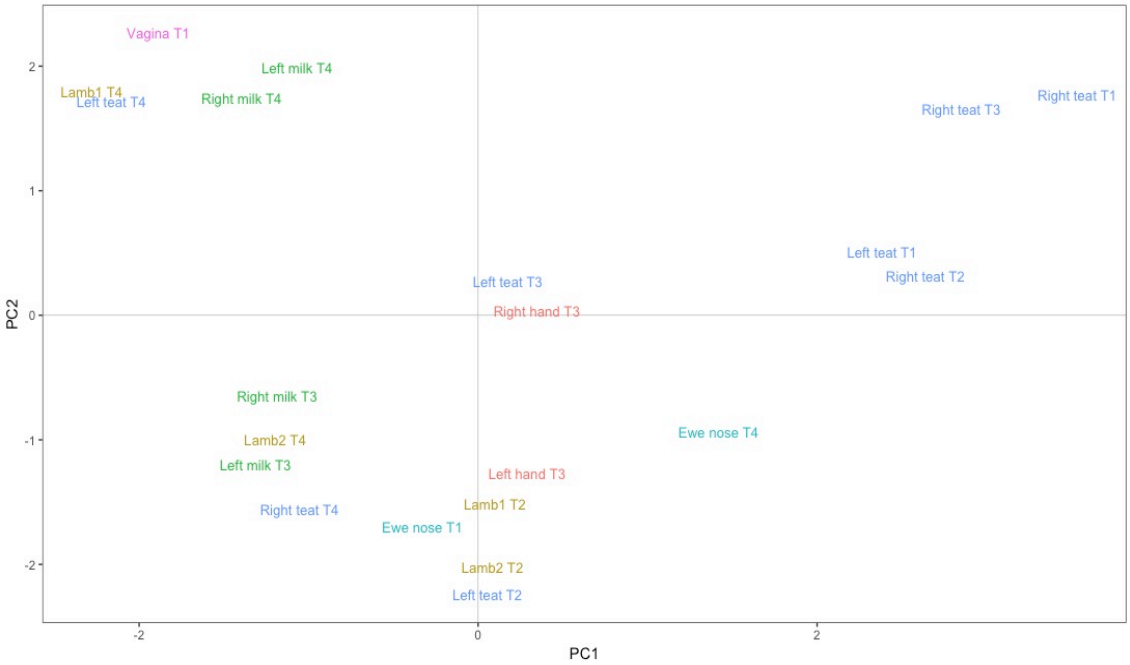

Figure 4.21a. Principal component analysis of the Dice-Sørensen similarity matrix between the sample site communities over time for the strains isolated from the control group study ewes. T1=Pre-lambing, T2=Immediately post lambing, T3=~24hrs after lambing, T4=Leaving housing.

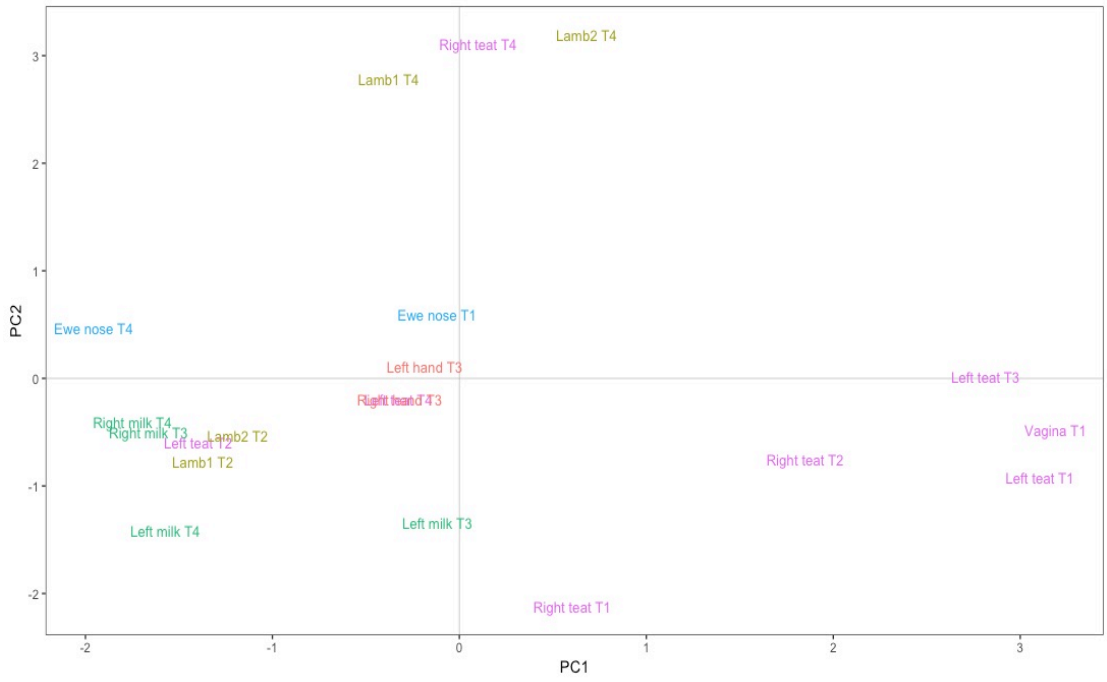

Figure 4.21b. Principal component analysis of the Dice-Sørensen similarity matrix between the sample site communities over time for the strains isolated from the intervention group study ewes T1=Pre-lambing, T2=Immediately post lambing, T3=~24hrs after lambing, T4=Leaving housing.

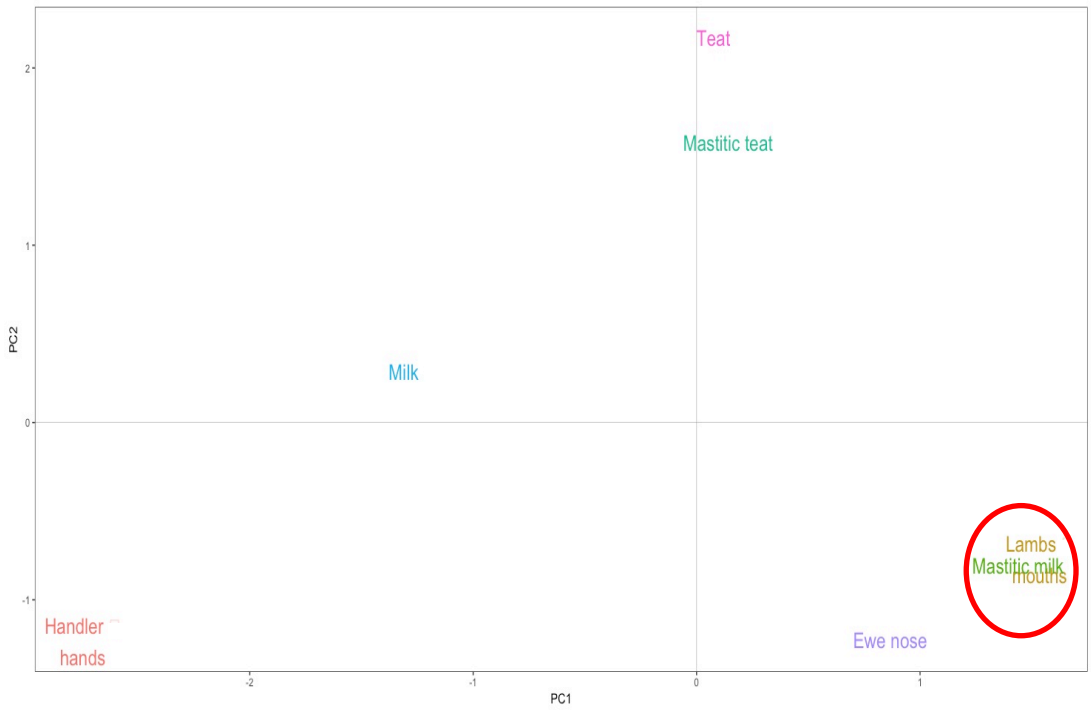

Figure 4.22 Principal component analysis of the Dice-Sørensen similarity matrix between the sample site communities over time for strains isolated in both mastitic ewes and study ewes. Similarities highlighted by coloured circle.

4.5 Discussion

The aim of this study was to investigate the effect of additional hygiene protocols during indoor lambing on the transmission and persistence of bacteria within ewes, and between ewes and lambs, at strain level using MALDI-ToF-MS.

Over recent years the theory that the mammary gland is a sterile environment has been challenged. Instead the concept of it being a diverse, complex community of bacteria is favoured and supported by data (Monaghan, 2015; Oikonomou *et al.*, 2012). A large number of bacterial species and strains were isolated from the milk samples of the clinically healthy ewes in the current study, supporting this newer theory. Over 50 species of bacteria were identified from the study ewe isolates in this study, with the most frequent species, all being associated with mastitis; *Aerococcus viridans*, *Bacillus licheniformis*, *Escherichia coli* and various coagulase negative staphylococci. This supports previous work, that not only does a healthy udder contain a diverse community, but that a normal community (microbiota) includes pathogenic microorganisms (Braem *et al.*, 2012; Oikonomou *et al.*, 2014).

This PhD study found no significant difference between the treatment groups regarding the presence or proportion of multiple occurring strains. A key finding from this study was that strains of bacteria were identified to persist in ewes, and possible transmission pathways were recognised. The same strains of bacteria were isolated from the same site over time on the same ewe indicating persistence, whereas other strains of bacteria were isolated from different ewes or lambs over the same and different time periods, indicating possible transmission events. Patterns and relationships were identified between ewes, lambs and handlers regarding persistence, possible transmission events and reservoirs of bacteria.

4.5.1 The role of lambs and humans as vectors of infection in the persistence, and possible transmission routes of bacteria

The inclusion of the lambs in the analysis dramatically increased the number of strains seen to occur in more than one sample. This suggests that lambs are heavily involved in the transmission and persistence of bacteria within the ewe and flock. The results present evidence of strains of bacteria appearing in a ewe that were not detected in that ewe at the previous sampling point, but was for the lambs and vice versa (Table 4.8), which suggests that transmission of bacteria occurred between ewes and lambs. Previous studies have reported lambs acting as a source of infection and associated with changing bacterial populations on the teats of ewes (Fragkou *et al.*, 2011; Gougoulis *et al.*, 2008; Cooper, 2015). One study also suggests that suckling predisposes the teat to infections (Gougoulis *et al.*, 2008). The Dice-Sørensen coefficient results from this study

indicate that the strain community in the mouths of the lambs is more similar to the strain community in the milk samples, than of any of the other samples taken. This higher similarity between the lambs and the milk samples implies that transmission is likely to have occurred, as the lambs have shared bacteria with ewes. The lambs may also provide a route for bacteria to move from the environment to milk. Perhaps, as suggested, sucking makes the udder more susceptible, opening the otherwise closed teat sphincter, and allowing bacteria to enter and provide an opportunity for bacteria to flourish and dominate. Bacteria identified as the same strain were isolated from lambs and ewes other than their dam. It could be hypothesised that cross-suckling may have occurred or that some other common element, for example shared housing or lambs licking milk off each other's faces, may have provided an opportunity for transmission.

The strain community of the mouths of the lambs was not only highly similar to that of the milk samples but also to the ewe nose strain communities. Pre-sucking lamb mouth communities were highly similar to the ewe nose community, whereas the lamb mouth samples taken after sucking showed greater similarity with both the teat and milk samples. This perhaps highlights the transmission of bacteria during mothering behaviour, such as licking the lambs, as well as during sucking.

The hands of the handlers were not associated with any strains that persisted in a ewe-lamb unit (a ewe and her twin lambs), but were involved with strains that were discovered to transmit between ewes. This suggests the involvement of human interaction with the movement of bacteria between animals, rather than within the animal. It has been suggested in other studies that the hands of farm workers milking ewes, act as vectors passing bacteria between ewes (Albenzio *et al.*, 2003). Fewer strains were isolated from intervention handler hands, in the current study, although there was no statistical difference between the treatment groups regarding the presence or proportion of multiple occurring strains in the final model. This non-significant result may be due to a lack of data concerning the handler hands, as they were only sampled once. Strains that occurred in three or more sites in the intervention ewes did not involve the hand swabs, whereas these strains for the control ewes were isolated from the control handler hands. Possibly the use of the anti-bacterial hand gel before handling events, in the case of the intervention ewes, caused this reduced number of isolated strains and hand involvement. However, further study would be needed to confirm this hypothesis.

3.5.2 The role of lambs as vectors of infection of/from mastitic ewes

In human health local and systemic inflammatory responses have been identified to be affected by dysbiosis (disruption of the normal commensal bacteria by increased numbers of potential pathogens) of the gut microbiota (Grigg and Sonnenberg, 2017). This may also be true for the mammary gland microbiota,

there is a theory that fluctuations in the mammary gland bacterial community aids the opportunity for a species of bacteria to dominate and cause infection (Fragkou *et al*., 2007; Monaghan, 2015; Kuehn *et al*., 2013). This appears to be reflected in the results of this study as the dominance of bacteria differed between healthy and diseased states. In this study, the mastitic samples had a reduced bacterial diversity compared to the clinically healthy ewes, although this was not significant. Over a fifth of the mastitic isolates were either identified by MALDI-ToF-MS as *Staphylococcus aureus* or *Staphylococcus auricularis*. This agrees with previous studies which have shown that the microbial diversity of mastitic milk in both humans and animals was lower than that in healthy individuals (Patel *et al*., 2017; Monaghan, 2015). This change in community profile in the diseased states may imply that the clinical mastitic state is characterised by a dominant bacterial species that out grows other bacteria.

An example from this study of a species that was isolated from both mastitic and clinically healthy ewes and appears to have dominated in diseased states, is *Staphylococcus auricularis*. *Staphylococcus auricularis* has been reported to be a major part of the human external ear microbiota (Kloos and Schleifer, 1983; Becker *et al*., 2014), however, it has also been reported to be a cause of mastitis in sheep (Rupp *et al*., 2009). The percentage of this bacteria on the clinically healthy study ewe teats was higher than that of their milk samples. In the mastitic samples the role is reversed, with higher percentages in the milk than on the teats. The other site that *Staphylococcus auricularis* was isolated from was the mouths of the lambs. Possibly the lambs aided the transfer of bacteria into the milk. *Staphylococcus auricularis* is also able to adhere to epithelial cells and form a biofilm (Szczuka *et al*., 2016), this may be another reason it is able to increase in numbers, cause infection and protect itself from host immune cells or possibly even antibiotics. Due to the consistent higher percentages of *Staphylococcus auricularis*, compared to the other cultured bacteria species, in all samples in this study, it may be acting as an opportunistic pathogen causing mammary dysbiosis resulting in infection in this flock.

The above example and the high similarity between the strain communities identified in milk from the mastitic ewes and mouths from the lambs of the study ewes suggest that lambs act as vectors as well as sources of infection, moving bacteria around the flock. This may be possible from lambs feeding from dams other than their mothers, resulting in lambs being a risk of infection and a source of transmission to all ewes not just their mothers. Lambs may facilitate mammary microbiota dysbiosis, by moving bacteria onto the teats and into the udder, increasing the risk of infection and disease. In addition, lambs can cause damage to teats by excessive butting or feeding, resulting in cuts and lesions, which offers bacteria another entry point to the udder environment.

4.5.3 Role of the different sites within a ewe and the persistence and possible transmission of bacteria

Studies in the dairy industry have identified that the same strain of bacteria was responsible for recurrent cases of mastitis in the same cow (e.g. Abureema *et al.*, 2014; Milne *et al.*, 2005; Davies *et al.*, 2016), providing evidence that bacteria do persist in the mammary gland. A finding from this study was that the bacteria isolated from milk samples appeared more associated with "within ewe" persistence than transmission events (section 4.4.5.2). The same bacterial strain was isolated from more than one milk sample in 5/10 ewes suggesting that bacteria, such as coagulase negative staphylococci (CNS), survive and persist in the udder of the ewe. Strains that persisted within ewes were more likely to originate from a milk sample than a teat or nose sample, and more milk samples from the same ewe had strains persist across different pens and yards than the other sample sites. This may further support the theory that the udder has its own microbiota, which is relatively stable.

However, the mammary gland is not an isolated environment, there is evidence from this study of transmission events, bacteria moving into and out of the gland. This invasion of bacteria could then alter the mammary gland environment causing disease. In the current study transmission events could be differentiated from new environmental infection by the involvement of a vector. For example, if a strain was not identified from a teat sample, but was detected in the mouths of lambs and then from a teat sample of the same ewe at a later sampling point this would suggest a transmission event. Whereas, the same strain identified on separate swabs from a ewe teat taken in the same environment, may indicate a repeated colonisation from the environment than persistence.

Although the nose and teat communities behave slightly differently, the study suggests that the ewe nose and teats may be acting as reservoirs or a source of overlap for bacteria, allowing transmission from other sites, such as the environment, lambs or humans, to the ewe. The ewe nose and teat strain communities appeared less stable, more variable and diverse than that of the milk. Both sites are also associated with higher frequencies of reoccurring strains and their bacterial communities overlap with those of other sampling sites. The overlapping of bacterial communities may suggest their possible involvement in transmission events.

Teats appear to be more affected by the housing environment than milk. More milk samples had a strain persist over different housing environments than teat and ewe nose samples, perhaps suggesting stability in the milk samples compared to the nose and teats or that true persistence only occurs inside the mammary gland. Strains that were identified at two time points, from the teat

samples, always came from samples that were taken in the lambing yard. The teats are more exposed to the immediate environment so colonisation on the udder skin may occur from bedding, explaining the similarity over these time points, this could indicate a repeat challenge from local contamination rather than persistence. This may also explain the difference in strain communities seen over time with the teat samples, as they are exposed to different bacterial challenges, in the different housing environments. Therefore, the definition of persistence in this study; that the strain only had to be seen on two occasions within a ewe for it to be considered persisting, may be too low to robustly define persistence because the possibility of repeated colonisation of bacteria from the same environment rather than truly persisting. The results of the study also indicate that fewer strains were identified on the teats of the intervention ewes compared to the control ewes, this may be a consequence of the additional hygiene steps followed by the intervention handlers. The additional hygiene protocols may have reduced the risk of teat contamination from bedding or handlers by reducing bacterial load or presence. However, the presence of the bacteria on teats does not always mean that it will enter the udder, as the udder has various defences to protect itself from invading bacteria (Winter and Colditz, 2002, Fragkou *et al.*, 2007), which may explain the lower similarity of strain communities between the milk and teat samples.

4.5.4 The environment as a reservoir for bacteria

The descriptive data and results from the multi-level modelling from the current study demonstrate that strains, which occurred in more than one sample, are associated with the environment the ewe/lamb is exposed to. Multiple occurring strains were less likely to be present in samples taken while the ewes were in the individual pens. Samples taken at this time point (time point 3) were also associated with reduced proportions of reoccurring strains, particularly those occurring again at the same time point (Figure 4.16). In addition, strains that occurred at the most sites in the intervention ewes were not identified in samples taken in the individual pens. Whereas, the largest proportion of strains that occurred repeatedly in the flock, were in samples taken immediately post-lambing and were associated with an increase in experimental weeks, so therefore older bedding. Furthermore, there appeared to be similarity between time points and samples across ewes (Figure 4.20). These results suggest that the housing environment acts as a source of bacteria and that management of this environment can impact the transmission or persistence of bacteria. During modelling, persistence was defined in the current study as a strain that was detected more than once in the flock. However, if a strain was detected at time point 1 on, for example, both teat samples or a strain was detected at time point 1 and then again at time point 2, this may actually reflect repeat colonisation from the environment. Perhaps demonstrating environmental persistence rather than

ewe transmission and persistence. Previous studies have suggested that poor hygiene, including litter quality, ventilation and stocking density can contribute to increased risks of mastitis occurring (Mavrogianni *et al.*, 2007; Caroprese, 2008) because levels of pathogens rise in indoor conditions allowing greater exposure of the udder to these pathogens (Alexopoulos *et al.*, 2011; Albenzio *et al.*, 2002). There was a greater proportion of reoccurring strains at the same time point for time point 1 and 2, while the ewes were in the same overall location, the lambing yard and at time point 4, the group post-lambing yards. Stocking density, duration of stay and exposure to older bedding would have been increased, in the lambing yard in particular, therefore the results agree with previous research that the ewe is at greater risk of exposure to pathogens under these conditions. Transmission and local contamination would be reduced whilst the ewes were in individual pens as there was no access to other ewes and the pens were cleaned out after every ewe. Although there was no significant difference overall between the intervention and control ewes regarding the presence or proportion of multiple occurring strains, the provision of bedding powder and the removal of wet litter may have provided an environment that bacteria did not find as optimal as in areas where the bedding was not changed, had less bedding powder and increased stocking densities. The addition of absorbing products, like the disinfectant sanitising powder used in this study, has been reported to lower milk bacterial counts and inflammatory responses, suggesting reduced infection, in housed ewes (Sevi *et al.*, 2001c). However, the role of bedding as a reservoir of bacteria can only be hypothesised in the current study as the bedding samples were not processed. Therefore, the association of the presence and greater proportion of multiple occurring strains in the group housing may be an effect of direct ewe-ewe transmission or the role or lambs in transmission rather than the environment.

4.5.5 The use and limitations of Matrix assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-ToF-MS) and cluster analysis for species and strain identification

A limitation of MALDI-ToF-MS is its dependence on the library database used to compare the spectra from the sample isolate to known identified reference spectra. If the organism is not in the library database then the unknown isolate cannot be reliably identified. However, for this project this limitation was overcome. Instead of relying on the species identification by the Bruker Biotyper, the spectral fingerprint was used for strain typing using cluster analysis and strains determined using threshold cut-offs from technical replicates. Cluster analysis and the use of threshold cut-offs have been used successfully in other studies (Fernández-Álvarez *et al.,* 2017; Dubois *et al.*, 2010; Decristophoris *et al.*, 2011). However, these studies used another method to confirm the strains or used known strains to start with, whereas in the current study all the isolates were unknown and no other method of strain typing was used. As no other method was

used to strain type the isolates, such as sequencing, currently, there is no way to positively identify any of the strains in this study.

4.5.6 Limitations of culture-dependent work and study design

Limitations of culture-dependant work has been previously documented, suggesting that this method does not reflect the actual bacterial community (Theron and Cloete, 2000) because it favours faster growing bacteria, and is affected by factors such as incubation conditions and media. However, culture is considered the primary diagnostic for identifying bacteria present in milk (Rovai *et al.*, 2014, Smith *et al.*, 2011) as it is less time consuming and cheaper than other methods such as pulse-field gel electrophoresis. Culturing techniques were used to grow and isolate bacteria from the samples in this study, therefore, it may be that just because a bacterium has not grown from one sample, does not mean that the bacterium was not present. It may just have failed to grow at that time, leading to false negatives and lower specificity. The samples from the vagina of the ewe had little growth and consequently had reduced association with multiple occurring strains compared to the other sites. This difference may be reflected by true community diversity differences or by the limitations of aerobically culturing bacteria, as the vagina is known to have more anaerobic bacteria present (Ravel *et al.*, 2011) than other sites.

There was no statistical difference between the treatment groups, this may be due to many reasons, such as the length of the intervention period not being long enough to detect an effect, and cross contamination between ewes. Some ewes only experienced the intervention protocols for as little as two days, there was limited housing space during peak lambing periods, therefore ewes and lambs looking strong enough to survive outside went out very soon after lambing. The group yard that the intervention ewes were housed in, after moving from the individual pens, was originally a yard where the ewes scanned as having triplets were housed before lambing. The triplet-bearing ewes would have been on the same bedding for up to 6 weeks, although the bedding would have been topped up on occasions. When this yard was empty, the bedding had antibacterial sanitising powder added to it and then another layer of fresh bedding added before the intervention ewes entered the yard. Ideally this would have been completely cleaned out at the beginning and during the experiment. This was unavoidable, but may have affected the starting bacterial loads and potentially removed any benefit of additional hygiene protocols implemented and increasing the potential for bacterial movement and invasion from the bedding. However, not cleaning out between groups of ewes is standard practice on many farms. If farmers are going to improve hygiene at lambing they may need to address the whole process from group housing onwards.

4.5.7 Future research

Due to time and labour constraints only a proportion of the total collected samples were processed. Processing further samples and increasing the data set would give a more complete picture of persistence and transmission. An increased number of isolates may also allow the threshold for persistence to be drawn higher than occurring at least twice.

Future research could also include processing the environmental samples to provide a fuller picture of the impact and involvement of the environment. To investigate the role of human hands, more samples should be taken from the ewe udder and teat and the handler's hands, over time so any effect between the handler and ewe can be seen. In addition to further understand the impact and role of the lamb in the possible transmission of bacteria between ewes, samples could be taken from the mouths of lambs, and milk and teat samples from a whole flock of ewes and their lambs at regular intervals over the course of a lactation period.

Validation of the strain differentiation method used in this study perhaps through repeating the method and sequencing the same samples would also be a useful addition. Using a threshold value for each species rather than one across all samples may aid to decrease any false strain identifications, although this would be time-consuming as every morphologically unique species would need to be replicated in order to do this.

4.6 Conclusion

A key finding from the study is the evidence that lambs may act as vectors moving bacteria, including pathogenic bacteria, around the flock. The number of reoccurring strains dramatically increased when the lambs were included in the analysis. Furthermore, the community of bacteria isolated from the mouths of the lambs was highly similar to the community of bacteria isolated from both the study and mastitic ewe milk samples. Persisting bacterial strains were more likely to be isolated from milk samples than the other sample types. This may suggest persistence only occurs in the udder and not at other sites, such as the nose or mouth. However, this may also just highlight the transient quality of sites such as the nose and mouth compared to the inner udder.

There is also evidence that the environment may act as a reservoir of bacteria. Reduced presence and proportions of bacteria were isolated from ewes when housed individually with their lambs and on bedding that was more frequently changed.

Chapter 5. The relationship between intramammary masses and bacterial communities

5.1 Introduction

IMM are pus-filled abscesses (Smith *et al.*, 2015) protecting themselves from the ewe's immune defences and normal udder microbiota via a host/pathogen protective wall (Cheng *et al.*, 2011). As these abscesses burst, they may cause changes to the ewe mammary gland microbiota through the introduction of different or increased numbers of strains of bacteria into the community, and cause an infection challenge to the ewe. As identified in Chapter 3 (section 3.4.3 and 3.4.4) and previously reported by Grant *et al.*, (2016), there is a strong association between intramammary masses (IMM) and acute mastitis. There is also evidence of IMM persistence; with the risk of having an IMM increasing if one has been previously detected. However, the role of abscesses in the development of acute mastitis, or the effect abscesses have on the community of bacteria in the gland is unknown. Increasing our understanding of the role of intramammary masses in the udder is therefore important.

5.2 Aim

The aim of this study was to investigate associations between the presence versus absence of intramammary masses over time with changes in the bacterial communities of collected samples from both clinically healthy ewes and ewes diagnosed with acute mastitis.

5.3 Methods

Data on the 10 study (5 intervention and 5 control) and 12 mastitic ewes from Chapters 3 and 4 were combined into site specific Excel databases. Data from Chapter 3 included the presence or absence of intramammary masses at the five different udder examinations (pregnancy, ~24 hours after lambing, 8 - 12 weeks after lambing, 22 - 24 weeks after lambing and 4 weeks before mating). Data concerning only the study ewe milk, teat and lamb mouth samples were extracted from Chapter 4. In Chapter 4 the data is reported at udder half and individual lamb level (when appropriate for the sample site) for each sampling time point (pre-lambing, post-lambing, ~24 hours after lambing and leaving housing). For this

analysis, species and strains counts were reported at ewe or lamb pair level, for example, species or strains that were isolated from milk samples collected from both udder halves at the same time point would only be counted once, not twice. Initially, only the counts relating to the milk samples for both the mastitic and study ewes were analysed. For any significant results the teat and lamb mouth samples from the same sampling time point were also investigated.

Central tendency of the number of species and strains per sample were calculated for all samples split by sampling site and the presence or absence of IMM overall and for each individual sampling site. The variables were non-parametric, therefore the results reported are expressed as medians, minimum, maximum and interquartile ranges. Differences between the median number of species or strains identified per sample in relation to the presence versus absence of IMM, and differences between the study and mastitic ewes, were assessed using the Mann-Whitney U test. Time point 4 was chosen to compare the milk and teat samples from the study ewes against the mastitic samples, as this was the closest in time to when the mastitic samples were taken. Results were considered significant when $p<0.05$. All data were analysed using R (version 1.1.447).

5.4 Results

5.4.1 Presence of intramammary masses and the diversity of bacteria isolated from 10 study and 12 mastitic ewes

Data for 160 samples from 10 study ewes and 48 samples from 12 mastitic ewes were combined for analysis (Table 5.1).

Table 5.1 Number (n) of samples collected from the 10 study (n = 160) and 12 mastitic ewes (n = 48)

	Study ewes		Mastitic ewes	
	Intervention	Control	Intervention	Control
Teat	40	40	10	14
Milk	20	20	10	14
Lamb mouth	20	20	-	-

There were 7 study and 8 mastitic ewes that had an IMM detected at least once. The sampling point with the highest number of ewes with an IMM was early lactation for study ewes and late lactation for mastitic ewes. The sampling point with the smallest number of ewes with an IMM was at lambing for both study and mastitic ewes (Table 5.2).

Table 5.2 Presence and absence of IMM in the 10 study and 12 mastitic ewes by udder examination time point

		Study ewes	Mastitic ewes
		n	n
IMM anytime	Present	7	8
	Absent	3	4
IMM pregnancy	Present	3	3
	Absent	7	9
IMM lambing	Present	1	2
	Absent	9	10
IMM early lactation	Present	5	4
	Absent	5	8
IMM late lactation*	Present	3	6
	Absent	6	6
IMM pre-tupping#	Present	1	0
	Absent	5	12

IMM = intramammary mass, n and % = number and % of ewes affected, *1 ewe died, #3 ewes culled

5.4.1.1 Species and strains identified per sample from 10 study ewes (n = 160) and 12 mastitic ewes (n = 48)

Although there was a lower median reported for the number of species isolated from the milk of mastitic ewes compared to the study ewes this was not significant. There was also no significant difference between the median number of strains isolated from the milk of mastitic ewes compared to the study ewes (Figure 5.1).

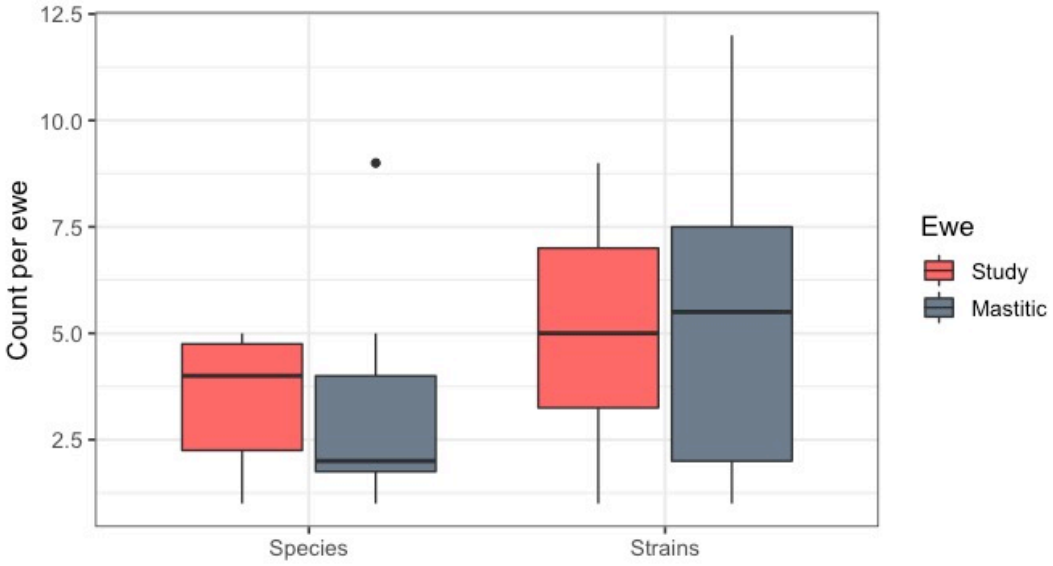

Figure 5.1 Counts of species and strains isolated from milk samples collected from 10 study and 12 mastitic ewes.

5.4.1.2 Species and strains identified per sample from 10 study ewes (n = 160) and the presence versus absence of intramammary masses

Study ewe milk samples taken at time point 3 (~24 hours after lambing in individual pens) from ewes that had IMM detected at least once throughout the study, had a significantly lower median number of species (median = 4, p = 0.021) and strains (median = 4, p = 0.030) identified per udder than ewes that never had IMM detected (species median = 7, strain median = 10). There were no significant differences reported between the presence versus absence of IMM at the individual udder examination points and the number of species or strains identified (Figure 5.2 and Figure 5.3).

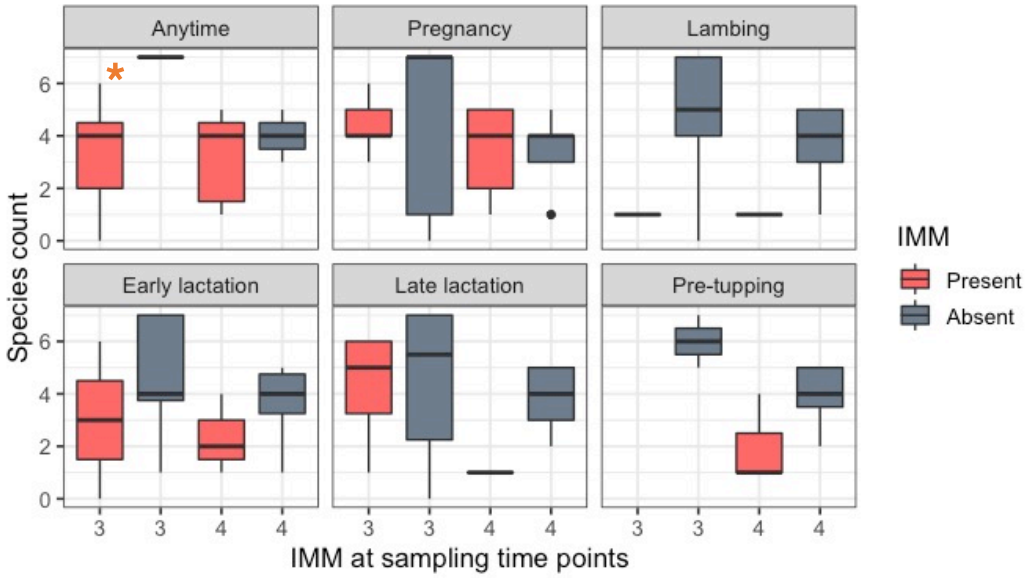

Figure 5.2 Counts of species isolated from milk samples collected from 10 study ewes by sampling time point and grouped by presence versus absence of intramammary masses 5% significance indicated by * .

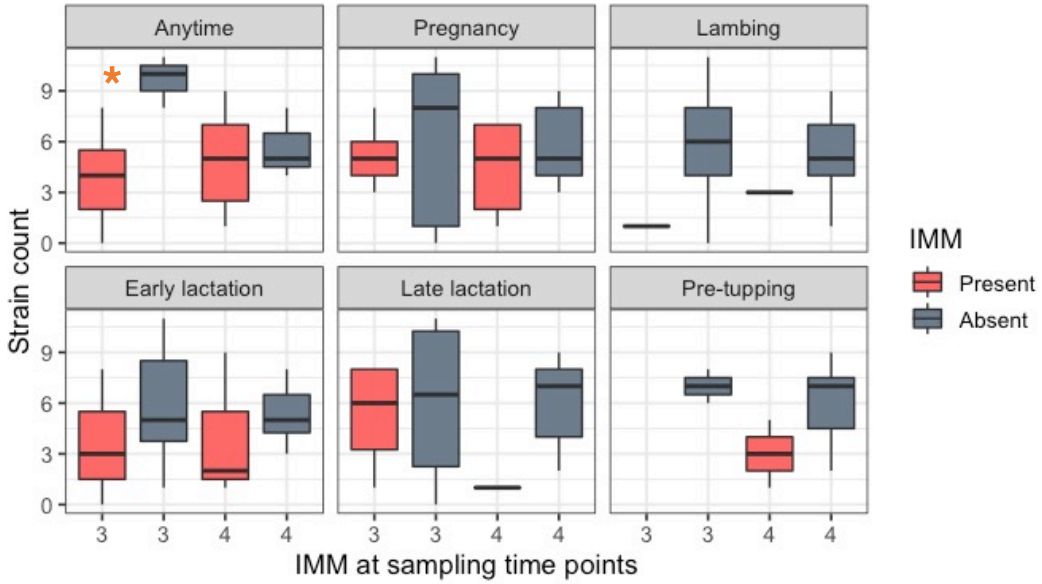

Figure 5.3 Counts of strains isolated from milk samples collected from 10 study ewes by sampling time point and grouped by presence versus absence of intramammary masses 5% significance indicated by * .

5.4.1.2 Species and strains identified per sample from 12 mastitic ewes (n = 48) and the presence versus absence of intramammary masses

Although the median value for species and strains isolated from the milk of mastitic ewes who had IMM detected at the individual udder examination points, were lower than the median value of species and strains isolated from ewes with no IMM detected, the differences were not significant (Figure 5.4 and Figure 5.5). However, mastitic ewes that had an IMM detected in pregnancy, late lactation or anytime had significantly lower median numbers of species and strains isolated than ewes that did not have an IMM detected at these examinations at the 10% significance level for the pregnancy (Figure 5.4 and Figure 5.5).

The median number of strains isolated per mastitic ewe was significantly lower for ewes that had IMM detected at any time over the study (median = 3, p = 0.026) compared to ewes that did not have IMM detected at any time point (median = 8.5). There were no significant differences between strain medians and the presence versus absence of IMM for the lamb mouth or teat samples.

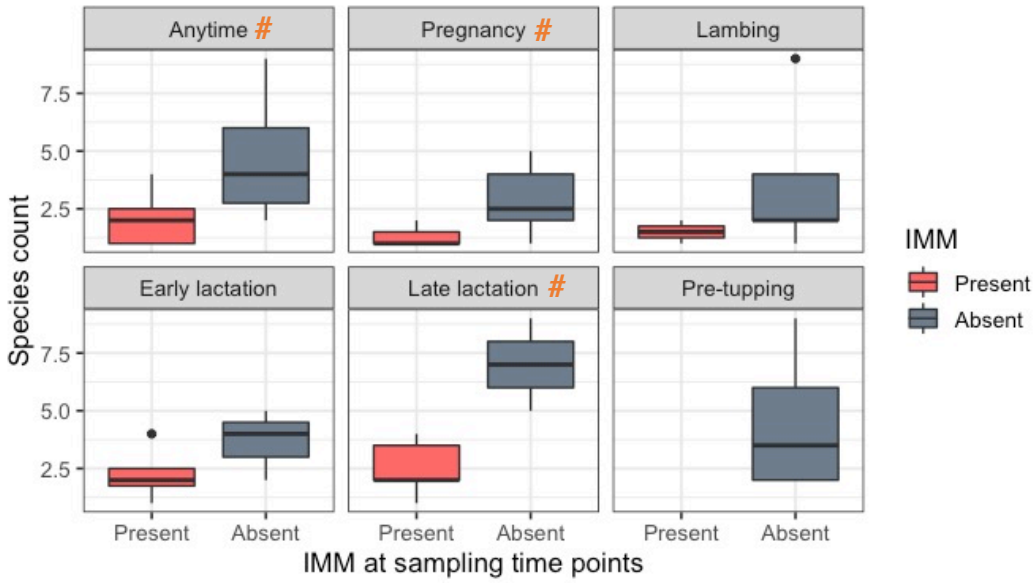

Figure 5.4 Counts of species isolated from milk samples collected from 12 mastitic ewes grouped by presence versus absence of intramammary masses. 10% significance indicated by # .

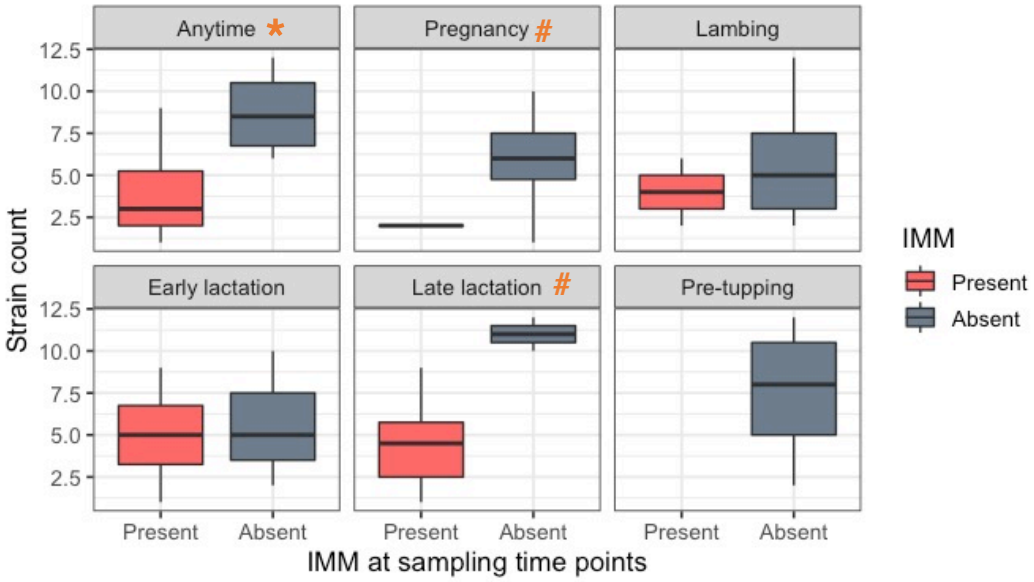

Figure 5.5 Counts of strains isolated from milk samples collected from 12 mastitic ewes grouped by presence versus absence of intramammary masses. 5% significance indicated by * , 10% significance indicated by #

5.5 Discussion and conclusion

The aim of this study was to investigate any associations between the presence versus absence of intramammary masses in clinically healthy and diseased ewes, and in the number of bacterial species and strains isolated from milk samples.

As mentioned in Chapter 4, the difference in microbiota between diseased and healthy samples has been reported (Oikonomou *et al.*, 2014) and in particular a reduction in the number of species in samples relating to disease has been identified by various studies (Hunt *et al.*, 2011; Kuehn *et al.*, 2013; Smith *et al.*, 2015). Surprisingly, the results of this study do not support this. Although the median number of species isolated from mastitic milk samples was lower compared to the clinically healthy ewes, this difference was not significant. However, this may be attributed to low statistical power due to a small sample size and perhaps because the study samples and mastitic samples were taken at different time points, with as much as two months between sampling dates.

Recent work has challenged the suggestion that abscesses are generally polymicrobial (Brook, 2002) with results indicating there was no evidence of mixed species in 60% of IMM cases examined (Smith *et al.*, 2015). The life cycle of an abscess is to grow, burst and reform. When these abscesses burst this would allow the spread of bacteria into the udder environment. This rapid addition of bacteria into the mammary gland microbiota could cause dysbiosis (disruption of the bacterial community) and allow any dominating species that was

encapsulated in the abscess an opportunity to dominate the udder environment, therefore lowering the diversity of resident bacteria in milk. The results from the current analysis somewhat supports the theory of an association between the presence of intramammary masses (IMM) and reduced diversity in ewes' milk bacterial community. In general, a lower median number of identified species and strains per study ewe appeared to be more common in ewes that had an IMM detected. However, this was only a significant difference for milk samples collected while the study ewe was housed individually, approximately 24 hours after lambing, and not for milk samples taken from ewes in post-lambing group yards (Figure 5.2 and 5.3). The ewes, when housed individually, were on fresh bedding and had no opportunity to interact with other ewes or lambs. Whereas, in group yards the ewes were housed with approximately 15 other ewes and their lambs, on bedding that was topped up on three occasions over the lambing period but never completely cleaned out. This may suggest that the presence of IMM reduces the diversity of bacteria in the udder but that the environment may also play a role, which supports Chapter 4 (section 4.5.4) which suggests that bacterial contamination enters the udder.

Mastitis is caused by bacterial infection and the presence of IMM appears to have driven acute mastitis development in the flock of ewes that participated in the intervention study (Chapter 3). The results from this current chapter's analysis, identified that mastitic ewes that had an IMM detected at any time were associated with a lower median number of strains isolated. This may imply that acute mastitis was caused by a dominating strain of bacteria that was encapsulated in an IMM, that when ruptured, had the opportunity to dominate the udder, cause disease and consequently lower the strain diversity in the milk (Figure 5.5). *Staphylococcus aureus* was the most isolated bacterial species from the mastitic ewes in this study (Chapter 4, section 4.4.2.1) and is known to form abscesses (Cheng *et al*., 2011), therefore IMM may be acting as reservoirs of *Staphylococcus aureus*.

To conclude, the relationship between IMM, acute mastitis and the udder microbiota is complex. Although no firm conclusions can be drawn from this study, the pattern of results support a theory that IMM effect the udder microbiota and play a role in the development of acute mastitis. However, further work with a larger sample and flock size would have to be carried out to investigate these hypotheses further.

Chapter 6. General discussion and future research

6.1 Overall study aim and key research findings

The overall aim of this project was to improve our understanding of mastitis in suckler ewes by exploring persistence and transmission routes of mastitis-causing bacteria. In order to address this aim a systematic review on the risk factors for mastitis was conducted. The systematic review formulated a hypothesis that an improved hygiene regime over lambing would reduce the prevalence and occurrence of acute and chronic mastitis in an indoor lambing suckler ewe flock. An intervention study was designed and conducted to test the impact of hygiene regimes on the occurrence of chronic and acute mastitis and to investigate the persistence and transmission of bacteria.

Key findings from the intervention study were as follows:

- Pre-existing intramammary masses (IMM) were associated with acute mastitis in the experimental flock.
- Lambs mouths and ewe milk shared bacterial species indicating transmission of bacteria.
- Reduced proportions of species, strains and reoccurring strains were associated with individual housing and more frequent bedding renewal. This may indicate the association of the environment with persistence and transmission of bacteria.
- Reduced isolated numbers of species and strains were associated with ewes that had an IMM detected compared to those that did not have an IMM detected. This may suggest an association between intramammary masses and a decrease in milk bacterial community diversity.

6.2 Discussion of key research findings

A key finding from this study is the importance of IMM and their possible role in causing incidences of acute mastitis (Chapter 3). Increased hygiene around the lambing period for indoor lambing ewes did not have a significant impact on the occurrence and prevalence of acute and chronic mastitis in this study flock. However, a highly significant association between chronic and acute mastitis, and future IMM, were recognised in the current study, which also

supports previous work that suggested IMM may be a result of a previous clinical or sub-clinical infection (Grant *et al.*, 2016). Almost 38% of the flock had an IMM detected at some point over the course of the experiment. The percentage of IMM present at each examination ranged from 8.4% to 16.8%, because abscesses develop, burst and reform the true percentage of ewes that had an IMM is unknown. In addition, as there was no significant difference between the prevalence of IMM in the treatment groups, this further suggests that the pre-existing infections (IMM) was the main cause of acute mastitis in this flock.

In humans and sheep, intramammary abscesses have been demonstrated to contain polymicrobial (Hai-jin Yu *et al.*, 2016) or monomicrobial species cultures (Smith *et al.*, 2015). In the current study there was a reduced diversity of bacteria in milk samples in ewes that had an IMM at any point compared with ewes that did not have an IMM at any point (chapter 5), although, this was not significantly different. However, the difference between the median number of strains in milk samples of mastitic ewes with and without an IMM at any point was significantly lower. These results support the idea that IMM encapsulate species of bacteria that, when released into the udder, change the normal community of bacteria. This may allow a species to dominate and cause disease.

There is no evidence of a particular composition of mammary gland microbiota being protective against disease (Monaghan, 2015). It may be that the mammary gland bacterial community, perhaps due to an idiosyncratic balance between host and bacterial needs, may constantly fluctuate without disease occurrence (Cabrera-Rubio *et al.*, 2012). However, a reduced diversity of bacteria has been detected in milk samples from diseased individuals compared to milk samples taken from clinically healthy individuals (Hunt *et al.*, 2011; Kuehn *et al.*, 2013; Smith *et al.*, 2015). Although the results of chapter 4 and 5 agree with previous studies of mastitic ewes displaying a lower diversity of species of bacteria, this was not a significant result. However, the milk samples from the clinically healthy study ewes were collected during housing over lambing, whereas, the milk samples from the mastitic ewes were collected on detection of acute mastitis, during lactation out on pasture. The location/environment of sampling (see below) can affect numbers and presence of bacteria in samples, so this may explain why no statistical difference was identified.

There is evidence from Chapter 3 that IMM dominated the cause of acute mastitis (Table 3.12). However, this does not eliminate the possible impact of the environment on the development of mastitis. Key findings from the results

of both Chapters 4 and 5 identified that the location of where the sample was taken (i.e. group pre-lambing yard or individual post-lambing pen) impacted the presence and prevalence of bacteria isolated. Leading to hypothesise that location may have a role in the bacterial community composition. In addition, the management of the bedding may impact the presence and prevalence of bacteria. Studies have commented on the influence the immediate environment has on gut colonisation in calves and piglets either reared in groups or individually (Fonty *et al.*, 1987; Thompson *et al.*, 2008). Whether the results were concerning overall numbers of species or strains, prevalence or mean numbers of reoccurring or shared strains, the outcome was always lowest for sampling time point 3 in the current study. It was at this time point that all ewes, irrespective of treatment group, were housed individually with their lambs. These results may be explained by the reduction in transmission possible between ewes, and other dam's lambs because the ewes are housed individually, but it may also suggest that the bedding management reduced bacterial presence, prevalence and persistence. The bedding was removed, flooring disinfected and new bedding added between every ewe, and for the intervention ewes this included the addition of an antibacterial absorbing powder. These actions would reduce local contamination and, presumably, recolonisation of bacteria from the bedding environment.

Similarities in strain communities for sites at the same time point in the study support local contamination from the environment. Other studies support this theory suggesting that poor hygiene, such as litter/bedding quality can contribute to increased levels of pathogens in indoor conditions and allow greater exposure of the udder to these (Alexopoulos *et al.*, 2011; Albenzio *et al.*, 2002). Similarities in strain communities between time points from the same location (time point 1 and 2 were both collected while the ewe was in the pre-lambing group yard) support bacterial persistence in the environment that colonise the ewe or lambs and possibly transmit between time points or recolonise at the second time point. The bedding in both group yards (pre-lambing and post-lambing) would have been older perhaps providing time and a more optimum environment for bacteria to multiple and flourish. Evidence for persistence came from study week which was significantly associated with the proportion of reoccurring strains, as study week increased the proportion of isolated reoccurring strains also increased, possibly demonstrating bacterial persistence in milk.

The intervention ewes, had significantly fewer reoccurring strains at time point 3 than control ewes (Figure 4.18). Further supporting a hypothesis that the environment, in this studies case, the bedding was acting as a reservoir of bacteria that colonised the ewes. A study investigating the effect of adding an

absorbing powder to bedding identified that bacterial counts and somatic cell counts were reduced in ewes that had the addition of the absorbing powder compared to those that did not (Sevi *et al.*, 2001c). Both that study and the current study highlight the importance of the immediate environment and the risk this may pose to the ewe. However, as suggested in Chapter 4, the role of the environment as a reservoir of bacteria, facilitating persistence and recolonisation of bacteria can only be hypothesised from the current study because, due to labour and time constraints, the bedding samples were not analysed. The difference may also be due to handling at this time point and the hygiene measures followed by the researchers before any handling events compared to the farm workers. However if this was the case, significant results would also be expected at time point 4, this was not identified,

Bacteria may enter the ewe's udder via the teat end through contact with contaminated bedding or by sucking lambs (Sevi *et al.*, 2003a, Fragkou *et al.,* 2011). During suckling of lambs the teat end dilates making it easier for bacteria to enter. The results from Chapter 4 identified that the mouths of lambs had a wide range of bacterial species and strains present, and was one of the most prolific sites that had larger proportions of reoccurring strains associated with it (Figure 4.15). This suggests that lambs could be a source of transmission and persistence of bacteria within the ewe and flock. In addition, strains, not previously identified in the ewe, appeared in a ewe's lamb and in that ewe at a later sampling point suggesting transmission of bacteria between ewe and lamb. Transmission of bacteria between ewe and lamb has been documented before (Gougoulis *et al.*, 2008; Fragkou *et al.*, 2011), including mastitis causing pathogens such as *Mannheimia haemolytica* (Fragkou *et al.*, 2011). The strain community similarity of the mouths of lambs and the ewe milk also provides evidence that transmission may be occurring due to the high similarity identified between the sites. However, the lamb mouth strain communities were also highly similar to the ewe nose strain community, this suggests that transmission of bacteria also occurs from mother behaviours, such as licking the lamb. Therefore, if bacteria enters the nasal cavity of the ewe via close contact to contaminated bedding or through respiration, this may then be passed to the lambs, and then via the lambs into the udder of the ewe.

Lambs may also be involved with moving bacteria between infected and healthy ewes. Chapter 4 provided evidence of overlapping similarity between the strain communities of milk from mastitic ewes and the mouths of lambs from the study ewes (Figure 4.22). Suggesting cross-sucking between dams had occurred and that the lambs had acted as vectors moving pathogenic bacteria around the flock. It may be that the mastitic ewes in the current study were

producing less milk, or preventing the lamb/s from suckling due to pain, which led to her lambs going to other dams to “steal” milk.

An interesting finding from Chapter 4 investigating persistence of bacteria within ewes, identified that persisting strains were more likely to come from a milk sample, than teat or nose samples (section 4.4.5.2). Furthermore, a greater number of strains that persisted over time and location were isolated from milk samples rather than teat or nose samples. The teat and nose communities appear more diverse and variable compared to the milk strain communities. Perhaps suggesting a hypothesis that persistence only occurs in the udder and not outside the gland perhaps in part due to the more transient nature of the communities in other sites, such as the nose and mouth. A study that investigated the bacterial flora of the ovine teat duct and mammary gland supports this claim, persistently isolated mastitis causing pathogens from a ewe, indicating that bacteria do persist in the mammary gland (Mavrogianni *et al.*, 2007). Studies in cows have identified recurrent cases of mastitis in the same cow, suggesting that persisting strains may cause recrudescence of disease (Abureema *et al.*, 2014; Milne *et al.*, 2005; Davies *et al.*, 2016).

6.3 Conclusions

In conclusion, the results from the systematic review and subsequent intervention study have contributed to our understanding of acute and chronic mastitis in ewes, and of bacterial persistence and transmission in an indoor lambing flock of suckler ewes.

The results of this thesis indicate that the presence of pre-existing intramammary masses drive acute mastitis. Evidence for this is the highly significant association between the two types of disease presented in Chapter 3. There was also evidence that lambs are an important source of bacterial transmission and possibly act as vectors of disease, moving pathogenic bacteria around the flock. The presence and proportion of reoccurring strains increases when lambs are included and, the bacterial communities between the mouths of lambs and ewe milk and nose samples was the most similar, reflecting the intimate and frequent contact between these sites and an excellent route for transmission of bacterial strains. The environment also appears to impact on the presence and proportion of bacterial strains detected. For example, when ewes were individually housed with their lambs the presence and proportion of species, strains and reoccurring strains of bacteria isolated were reduced compared to samples taken when the ewes and lambs were housed in groups. The origin of IMM development may have

been due to invasion of bacteria from the environment, which then allowed one or more species of bacteria to form an abscess. We hypothesize that this invasion could be directly from contaminated bedding or facilitated through lambs feeding. Therefore, the environment will affect the development of chronic and acute mastitis through not only the development of IMM but also direct infection. Therefore, the environment, pre-existing infections (IMM) and ewe/lamb contact appear to be important in the persistence and transmission of bacteria that could cause mastitis.

6.4 Possible future research

Future research could focus on determining the impact of IMM on acute mastitis and the role of lambs in transmission. This could be achieved through a longitudinal study of a flock. For a better understanding of the intramammary masses and the bacterial community of the ewe, analysis of samples from more ewes and over longer time periods but with shorter duration between sampling may be able to aid to this understanding. For example, more milk samples from the whole flock taken over the course of lactation, and examining ewes weekly for IMM during lactation to try and capture periods of IMM presence and absence and any effect this has on the udder bacterial community profile. In addition, teat swabs and samples taken from the mouths of all the lambs could be taken to investigate the role of lambs in the transmission of bacteria between lactating ewes and any effect IMM has on the transmission or changes in the community of bacteria in the mouths of lambs. The inclusion of culture results from dissected udders of ewes from the flock with and without IMM would also add to the understanding of the role of IMM and their impact on intramammary infections. Intramammary abscesses of, either/both elected culls or ewes that had died naturally. Culturing isolates from all the samples and using whole genome sequencing to differentiate between strains, bacterial presence and communities could be identified, strain locations could then be mapped and transmission events identified. Alternatively, metagenomics could be used to describe both the diversity and abundance of the microorganisms present in the samples. This method does not need pure cultures and identifies to a strain resolution unlike other methods such as 16s rRNA sequencing. However, it is time-consuming and expensive method that can require greater read depth as mammalian cells are not removed during sample preparation. Either method may provide evidence of IMM communities and whether that community affects the mammary gland and possibly even reaches the mouths of lambs to be moved around the flock.

Further work on this study could include processing the remaining samples for the study ewes, including the bedding samples. This would add power to the

results already identified and could help with the understanding of the role of the environment. Whether bacteria persist outside the ewe, for example in contaminated bedding, and if this is the case whether that does actually pose a risk to the udder microbiome of the ewe.

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Appendix 1 – Sentinel papers

Barbagianni, M. S., E. Giannenas, E. Papadopoulos, I. G. Petridis, S. A. Spanos, P. G. Gouletsou, I. Valasi, and G. C. Fthenakis. 2015. Pregnancy toxaemia in ewes: Development of an experimental model and potential interactions with gastrointestinal nematode infections. Small Ruminant Research 133:102-107.

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Caroprese, M. 2008. Sheep housing and welfare. Small Ruminant Research 76(1-2):21-25.

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Appendix 2 – Nutrition papers by sub-category

Subcategory	Papers
Body condition score	Huntley *et al.*, 2012; Arsenault *et al.*, 2008; Karagiannis *et al.*, 2014; Marogna *et al.*, 2010; Grant *et al.*, 2016
Energy levels	Bouvier-Muller *et al.*, 2016; Karagiannis *et al.*, 2014; Grant *et al.*, 2016; Barbagianni *et al.*, 2015; Mavrogianni *et al.*, 2014; Fthenakis *et al.*, 2015; Gelasakis *et al.*, 2015; Caroprese *et al.*, 2015; Sevi *et al.*,1998
Protein levels	Grant *et al.*, 2016; Winter, 2001
Vitamin A and E, Beta-carotene and Selenium levels	Bergonier and Berthelot, 2003; Pulina *et al.*, 2006; Giadnis *et al.*, 2011; Koutsoumpas *et al.*, 2013; Caroprese *et al.*, 2015; Morgante *et al.*, 1999; Bergonier *et al.*, 2003; Pulido *et al.*, 2012; Gelasakis *et al.*, 2015
Supplements	Brozos *et al.*, 1998; Zamiri *et al.*, 2001; Oliveira *et al.*, 2012; Chiofalo *et a*l., 2004; Giannenas *et al.*, 2011; Caroprese *et al.*, 2015

Appendix 3 – Hygiene papers by sub-category

Subcategory	Papers
Housing	Bergonier *et al.*, 2003; Tietze *et al.*, 2001; Cooper *et al.*, 2016; Casamassima *et al.*, 2001; Caroprese, 2008; Sevi and Caroprese, 2012; Sevi *et al.*, 1999; Sevi *et al.*, 2001c; Caroprese *et al.*, 2009
Stocking density and ambient hygiene	Mavrogianni *et al.*, 2007; Sevi *et al.*, 2001a; Caroprese 2008; Gelasakis *et al.*, 2015; Bergonier *et al.*, 2003; Bergonier and Berthelot, 2003; Sevi *et al.*, 2003b; Sevi *et al.*, 1999; Alexopoulos *et al.*, 2011; Albenzio *et al.*, 2002; Marogna *et al.*, 2010; Sevi *et al.*, 2001c; Sevi *et al.*, 2003a; Cooper *et al.*, 2016; Gelasakis *et al.*, 2015
Season	Sevi *et al.*, 2004; Sulaiman and Al-Sadi, 1992; Lafi *et al.*, 1998; Matutinovic *et al.*, 2011; Sevi and Caroprese, 2012; Narenji Sani *et al.*, 2015; Burriel, 1997, Omaleki *et al.*, 2011; Arias *et al.*, 2012
Physiological stress through hygiene	Sevi *et al.*, 1999; Caroprese *et al.*, 2009; Cooper *et al.*, 2016; Alexpoulos *et al.*, 2011
Hygiene and techniques around milking	Las Heras *et al.*, 1999; Gonzalo *et al.*, 2006; de Garnica *et al.*, 2013; Sinapis, 2007; Gonzalo *et al.*, 2005; Olechnowicz and Jaoekowski, 2012; Molina *et al.*, 2010; Menzies and Ramanoon, 2001; Marogna *et al.*, 2010; Albenzio *et al.*, 2003; Bergonier and Berthelot, 2003; Gelasakis *et al.*, 2015; Alexopoulos et al., 2011; Carloni *et al.*, 2015; Zweifel *et al.*, 2005; Al-Momani *et al.*, 2008; Contreras *et al.*, 2007; Bergonier *et al.*, 2003; Díaz *et al.*, 2004; Peris *et al.*, 2003a; Peris *et al.*, 2003b; Sinapis *et al.*, 2006, Peris *et al.*, 1995; Buesdo-Ródenas *et al.*, 201; McKusick *et al.*, 2003; D'Amico and Donnelly, 2010; Castillo *et al.*, 2008; Hervás *et al.*, 2006; Castillo *et al.*, 2009; Nudda *et al.*, 2002; Pulido *et al.*, 2012

Appendix 4 – Associations between variables tested in binomial random effects models

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1																								
2																								
3		0.24																						
4	-0.07																							
5	-0.08			0.11																				
6	-0.12			0.25																				
7		-0.04	-0.04		0.14	0.07																		
8		0.32	0.38	0.05	0.05	0.07	-0.04																	
9		0.30	0.36				-0.09	0.11																
10	-0.05	0.39	0.44		0.04			0.13	0.22															
11		0.42	0.32			0.05		0.09	0.14	0.30														
12	0.06	0.36	0.19			-0.06		0.13	0.09	0.13	0.41													
13	-0.03						-0.12																	
14				0.09	-0.05			0.05	-0.06	-0.05			0.32											
15	0.10			0.05	-0.06	-0.06	-0.07	0.09				-0.06	0.21	0.30										
16				0.05		-0.16	-0.09	0.05		0.05	-0.12		0.27	0.21	0.43									
17	0.05					-0.12				0.07	-0.06	-0.09	0.23	0.19	0.34	0.58								
18	0.73			-0.05	-0.12	-0.10	-0.05	0.07	0.04	-0.04		0.08		0.07	0.11		0.07							
19	1.00			-0.09	-0.10	-0.12	0.10			-0.06		0.05	-0.06		0.09	-0.05	0.05	0.73						
20	0.87			-0.03	-0.05	-0.14	-0.04		0.04				-0.06	-0.10	0.05		0.05	0.59	0.87					
21	0.46						-0.16		0.06		0.03		0.10	0.06	0.08			0.35	0.45	0.39				
22				-0.12		0.06	-0.20				0.04	0.04		-0.18	-0.21	-0.17	-0.18	-0.07		0.05	0.06			
23	-0.08			0.25	0.08	0.10		0.04			0.05	0.05		-0.05	0.04	0.08	0.05	-0.05	-0.09	-0.06	-0.05	-0.14		
24		0.16	0.05			0.04		0.04		0.06	0.11													

1= Treatment, 2 = IMM at any point, 3 = IMM at previous point, 4 = Adopter, 5 = Assisted, 6 = Days housed, 7 = Flock parity, 8 = IMM in pregnancy, 9 = IMM ~24hrs after lambing, 10 = IMM in 4-8 wks into lactation, 11 = IMM in 18-22 wks into lactation, 12 = IMM in 6wks after weaning, 13 = BCS in pregnancy, 14 = BCS ~24hrs after lambing, 15 = BCS 4-8 wks after lambing, 16 = BCS 18-22 wks after lambing, 17 = BCS 6wks after weaning, 18 = Wet pen, 19 = Individual pen, 20 = Group pen, 21 = Intervention ewe hygiene issue, 22 = No. of live lambs per ewe, 23 = Lamb health problem, 24 = acute mastitis

Appendix 5 – Number and percentage of species identified from the study ewe (n=552) and mastitic ewe isolates (n=85)

Species identified	Study ewes		Mastitic ewes	
	Number	%	Number	%
Acinetobacter johnsonii	1	0.2	0	0
Acinetobacter lwoffii	3	0.5	0	0
Acinetobacter tandoii	0	0	1	1.2
Advenella incenata	1	0.2	0	0
Aerococcus viridans	**41**	**7.4**	**1**	**1.2**
Alcaligenes faecalis	3	0.5	0	0
Arthrobacter gandavensis	**4**	**0.7**	**5**	**5.9**
Bacillus clausii	3	0.5	0	0
Bacillus licheniformis	**38**	**6.9**	**2**	**2.4**
Bacillus niacini	1	0.2	0	0
Bacillus oleronius	1	0.2	0	0
Bacillus pumilus	2	0.4	0	0
Bacillus safensis	1	0.2	0	0
Bacillus simplex	**1**	**0.2**	**1**	**1.2**
Bacillus siralis	**2**	**0.4**	**1**	**1.2**
Bacillus subtilis	4	0.7	0	0
Bacillus vietnamensis	0	0	1	1.2
Bacillus weihenstephanensis	0	0	2	2.4
Cellulosimicrobium cellulans	0	0	1	1.2
Corynebacterium afermentans	1	0.2	0	0
Corynebacterium ammoniagenes	1	0.2	0	0
Corynebacterium casei	4	0.7	0	0
Corynebacterium freneyi	5	0.9	0	0
Corynebacterium glutamicum	1	0.2	0	0
Corynebacterium pilosum	1	0.2	0	0
Corynebacterium stationis	**27**	**4.9**	**2**	**2.4**
Corynebacterium xerosis	**12**	**2.2**	**2**	**2.4**
Curtobacterium flaccumfaciens	2	0.4	0	0
Empedobacter brevis	9	1.6	0	0
Enterococcus faecalis	**4**	**0.7**	**1**	**1.2**
Enterococcus faecium	8	1.4	0	0
Enterococcus gallinarum	1	0.2	0	0
Enterococcus hirae	5	0.9	0	0
Escherichia coli	**16**	**2.9**	**1**	**1.2**

Kocuria carniphila	7	1.3	0	0
Lysinibacillus sphaericus	1	0.2	0	0
Mannheimia haemolytica	0	0	2	2.4
Micrococcus luteus	**1**	**0.2**	**1**	**1.2**
Micrococcus terreus	1	0.2	0	0
No reliable identification	110	19.9	18	21.2
Nocardiopsis alba	3	0.5	0	0
Paenibacillus amylolyticus	**4**	**0.7**	**1**	**1.2**
Paenibacillus barengoltzii	1	0.2	0	0
Pseudomonas graminis	1	0.2	0	0
Rhodococcus coprophilus	**1**	**0.2**	**4**	**4.7**
Rothia amarae	0	0	1	1.2
Rothia nasimurium	**1**	**0.2**	**0**	**0**
Solibacillus silvestris	**4**	**0.7**	**4**	**4.7**
Staphylococcus aureus	0	0	11	12.9
Staphylococcus auricularis	**25**	**4.5**	**9**	**10.6**
Staphylococcus capitis	2	0.4	0	0
Staphylococcus chromogenes	5	0.9	0	0
Staphylococcus cohnii	**7**	**1.3**	**2**	**2.4**
Staphylococcus epidermidis	1	0.2	0	0
Staphylococcus equorum	**35**	**6.3**	**2**	**2.4**
Staphylococcus fleurettii	1	0.2	0	0
Staphylococcus gallinarum	3	0.5	0	0
Staphylococcus lentus	**39**	**7.1**	**4**	**4.7**
Staphylococcus schleiferi	1	0.2	0	0
Staphylococcus sciuri	**30**	**5.4**	**2**	**2.4**
Staphylococcus simulans	3	0.5	0	0
Staphylococcus succinus	19	3.4	0	0
Staphylococcus vitulinus	**29**	**5.3**	**1**	**1.2**
Staphylococcus xylosus	**8**	**1.4**	**1**	**1.2**
Streptococcus entericus	6	1.1	0	0
Streptococcus uberis	0	0	1	1.2

Species in **bold** identified in both study ewes and mastitic ewes. Number= Number of isolates and % = percentage from total isolates

Appendix 6 – Percentage of identified species by sampling site for study ewe (n=552) and mastitic ewe isolates (n=85)

Identified bacteria species	Vagina	Ewe nose	Teats	Mastitic teat	Milk	Mastitic milk	Lamb mouths	Handler hands
(Number of isolates)	(98)	(49)	(164)	(24)	(96)	(24)	(127)	(7)
Acinetobacter johnsonii	0	0	0.8	0	0	0	0	0
Acinetobacter lwoffii	0	1	0	0	0	0	0.6	2
Acinetobacter tandoii	0	0	0	2.4	0	0	0	0
Advenella incenata	0	0	0	0	0	0	0.6	0
Aerococcus viridans	**14.3**	**4.1**	**0.8**	**2.4**	**5.2**	**0**	**7.3**	**14.3**
Alcaligenes faecalis	0	0	0	0	0	0	1.2	2
Arthrobacter gandavensis	**0**	**2**	**0.8**	**12.2**	**0**	**0**	**0.6**	**0**
Bacillus clausii	0	0	0.8	0	0	0	0.6	2
Bacillus licheniformis	**0**	**8.2**	**10.2**	**2.4**	**4.2**	**2.3**	**6.1**	**6.1**
Bacillus niacini	0	1	0	0	0	0	0	0
Bacillus oleronius	0	0	0.8	0	0	0	0	0
Bacillus pumilus	0	0	0.8	0	0	0	0	2
Bacillus safensis	0	0	0	0	0	0	0	2
Bacillus simplex	**0**	**0**	**0**	**2.4**	**0**	**0**	**0**	**2**
Bacillus siralis	**0**	**1**	**0.8**	**2.4**	**0**	**0**	**0**	**0**
Bacillus subtilis	14.3	2	0	0	0	0	0.6	0
Bacillus vietnamensis	0	0	0	2.4	0	0	0	0
Bacillus weihenstephanensis	0	0	0	2.4	0	2.3	0	0
Cellulosimicrobium cellulans	0	0	0	0	0	2.3	0	0

Corynebacterium afermentans	0	0	0.8	0	0	0	0	0
Corynebacterium ammoniagenes	0	1	0	0	0	0	0	0
Corynebacterium casei	0	0	0	0	3.1	0	0	2
Corynebacterium freneyi	14.3	0	0	0	1	0	1.2	2
Corynebacterium glutamicum	0	0	0.8	0	0	0	0	0
Corynebacterium pilosum	0	0	0	0	1	0	0	0
Corynebacterium stationis	**0**	**6.1**	**8.7**	**2.4**	**2.1**	**2.3**	**3.7**	**4.1**
Corynebacterium xerosis	**0**	**2**	**0.8**	**2.4**	**1**	**2.3**	**4.9**	**0**
Curtobacterium flaccumfaciens	0	1	0.8	0	0	0	0	0
Empedobacter brevis	0	1	0.8	0	0	0	4.3	0
Enterococcus faecalis	**0**	**0**	**0.8**	**0**	**0**	**2.3**	**1.8**	**0**
Enterococcus faecium	0	4.1	1.6	0	0	0	1.2	0
Enterococcus gallinarum	0	0	0	0	1	0	0	0
Enterococcus hirae	0	1	0	0	0	0	0.6	6.1
Escherichia coli	**0**	**4.1**	**0.8**	**0**	**2.1**	**2.3**	**5.5**	**0**
Kocuria carniphila	0	0	3.1	0	2.1	0	0.6	0
Lysinibacillus sphaericus	0	0	0.8	0	0	0	0	0
Mannheimia haemolytica	0	0	0	0	0	4.5	0	0
Micrococcus luteus	**0**	**0**	**0**	**2.4**	**1**	**0**	**0**	**0**
Micrococcus terreus	0	0	0.8	0	0	0	0	0
Nocardiopsis alba	0	1	0	0	0	0	0.6	2
Paenibacillus amylolyticus	**0**	**3.1**	**0**	**2.4**	**1**	**0**	**0**	**0**
Paenibacillus barengoltzii	0	0	0.8	0	0	0	0	0
Pseudomonas graminis	14.3	0	0	0	0	0	0	0

Rhodococcus coprophilus	**0**	**0**	**0**	**9.8**	**1**	**0**	**0**	**0**
Rothia amarae	0	0	0	2.4	0	0	0	0
Rothia nasimurium	0	0	0	0	1	0	0	0
Solibacillus silvestris	**0**	**2**	**1.6**	**9.8**	**0**	**0**	**0**	**0**
Staphylococcus aureus	0	0	0	2.4	0	22.7	0	0
Staphylococcus auricularis	**0**	**0**	**11**	**7.3**	**7.3**	**13.6**	**2.4**	**0**
Staphylococcus capitis	0	0	0	0	0	0	0.6	2
Staphylococcus chromogenes	14.3	0	0.8	0	2.1	0	0.6	0
Staphylococcus cohnii	**0**	**0**	**1.6**	**2.4**	**3.1**	**2.3**	**0.6**	**2**
Staphylococcus epidermidis	0	0	0.8	0	0	0	0	0
Staphylococcus equorum	**0**	**4.1**	**4.7**	**0**	**18.8**	**4.5**	**3**	**4.1**
Staphylococcus fleurettii	0	0	0	0	0	0	0.6	0
Staphylococcus gallinarum	0	1	0	0	0	0	1.2	0
Staphylococcus lentus	**0**	**7.1**	**4.7**	**2.4**	**5.2**	**6.8**	**9.8**	**10.2**
Staphylococcus schleiferi	0	0	0	0	1	0	0	0
Staphylococcus sciuri	**0**	**7.1**	**8.7**	**2.4**	**2.1**	**2.3**	**4.3**	**6.1**
Staphylococcus simulans	0	0	0	0	3.1	0	0	0
Staphylococcus succinus	0	4.1	2.4	0	2.1	0	4.3	6.1
Staphylococcus vitulinus	**0**	**5.1**	**3.1**	**0**	**6.3**	**2.3**	**8.5**	**0**
Staphylococcus xylosus	**0**	**4.1**	**0**	**0**	**1**	**2.3**	**1.8**	**0**
Streptococcus entericus	0	3.1	0	0	0	0	1.8	0
Streptococcus uberis	0	0	0	0	0	2.3	0	0
Total	5	26	31	20	25	17	31	19
No reliable identification	**28.6**	**18.4**	**23.6**	**22.0**	**20.8**	**20.5**	**18.3**	**20.4**

Species in **bold** identified in both study ewes and mastitic ewes.

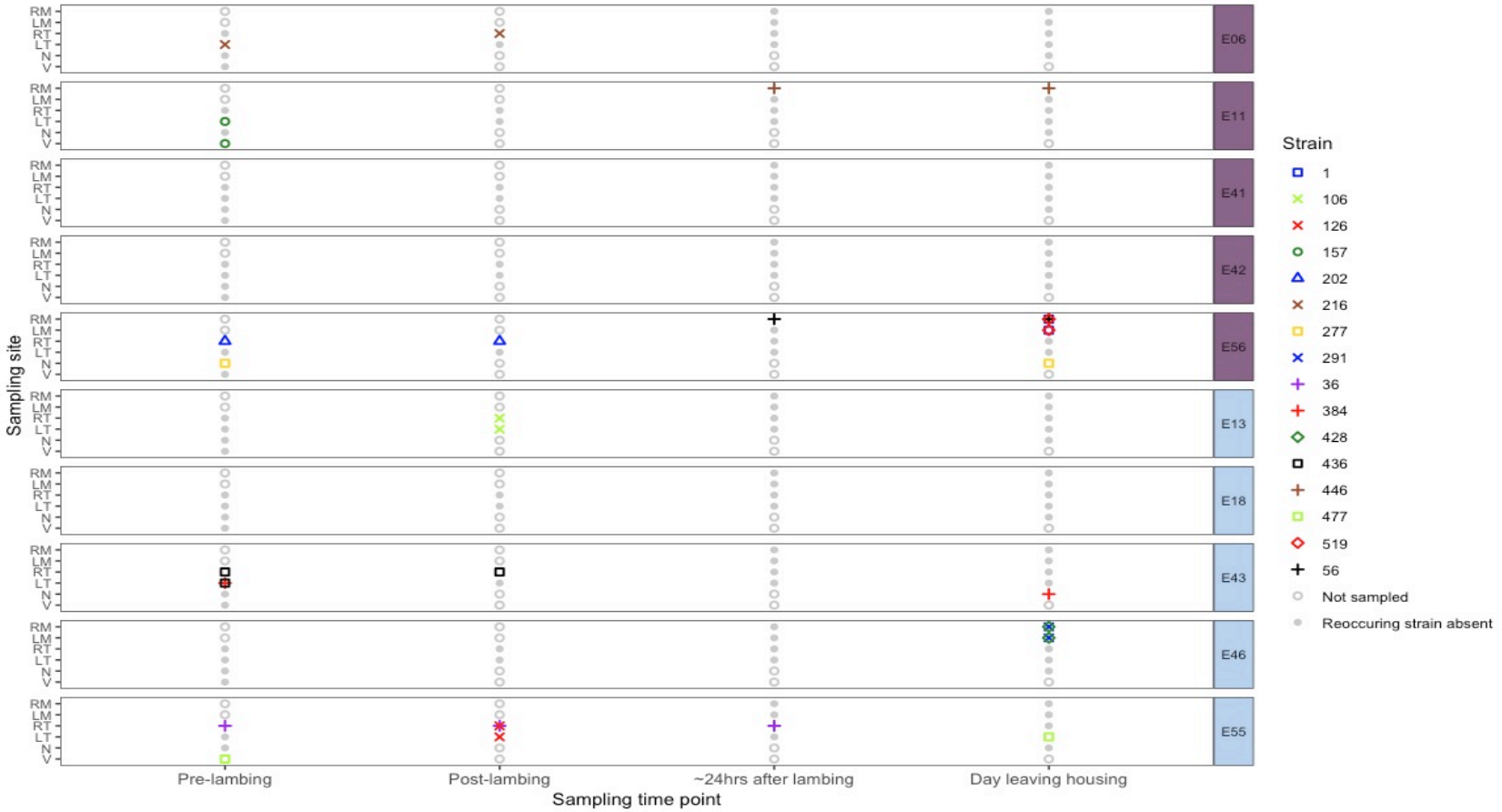
Appendix 7 – 540 strains identified in the 750 study ewe isolates, by the 58 bacterial species identified by MALDI-ToF-MS

Identified species	Frequency	Strain count
Acinetobacter johnsonii	1	1
Acinetobacter lwoffii	3	3
Advenella incenata	1	1
Aerococcus viridans	48	20
Alcaligenes faecalis	3	2
Arthrobacter gandavensis	4	3
Bacillus clausii	3	3
Bacillus licheniformis	50	36
Bacillus niacini	1	1
Bacillus oleronius	1	1
Bacillus pumilus	2	2
Bacillus safensis	1	1
Bacillus simplex	1	1
Bacillus siralis	2	2
Bacillus subtilis	4	4
Corynebacterium afermentans	1	1
Corynebacterium ammoniagenes	1	1
Corynebacterium casei	4	4
Corynebacterium freneyi	6	4
Corynebacterium glutamicum	1	1
Corynebacterium pilosum	1	1
Corynebacterium stationis	29	15
Corynebacterium xerosis	19	15
Curtobacterium flaccumfaciens	1	1
Empedobacter brevis	13	9
Enterococcus faecalis	4	1
Enterococcus faecium	8	5
Enterococcus gallinarum	1	1
Enterococcus hirae	5	4
Escherichia coli	19	10
Kocuria carniphila	7	6
Lysinibacillus sphaericus	1	1
Micrococcus luteus	1	1
Micrococcus terreus	1	1
No reliable identification	220	164
Nocardiopsis alba	3	3

Paenibacillus amylolyticus	4	4
Paenibacillus barengoltzii	1	1
Pseudomonas graminis	1	1
Rhodococcus coprophilus	1	1
Rothia nasimurium	1	1
Solibacillus silvestris	3	1
Staphylococcus auricularis	25	17
Staphylococcus capitis	2	2
Staphylococcus chromogenes	6	3
Staphylococcus cohnii	7	7
Staphylococcus epidermidis	1	1
Staphylococcus equorum	51	38
Staphylococcus fleurettii	1	1
Staphylococcus gallinarum	4	4
Staphylococcus lentus	48	36
Staphylococcus schleiferi	1	1
Staphylococcus sciuri	37	25
Staphylococcus simulans	3	2
Staphylococcus succinus	26	26
Staphylococcus vitulinus	39	27
Staphylococcus xylosus	9	8
Streptococcus entericus	8	5

One duplicate isolate as the strain was identified as two species

Appendix 8 – Strain persistence within ewes by sampling site and time



V=vagina, N=Ewe nasal cavity, LT=left teat, RT=Right teat, LM=Left milk, RM=Right milk. 1=Pre-lambing, 2=Immediately post-lambing, 3=Individual pens and 4=leaving housing. Yellow=Intervention ewes, Blue=Control ewes

Appendix 9 – Dice-Sørensen similarity matrix between the sample site communities over time for the strains isolated from the control study ewes

	N1	N4	V1	LT1	LT2	LT3	LT4	RT1	RT2	RT3	RT4	LM3	LM4	RM3	RM4	L12	L14	L22	L24	LH3	RH3
N1	1.00																				
N4	0.00	1.00																			
V1	0.00	0.00	1.00																		
LT1	0.00	0.32	0.00	1.00																	
LT2	0.13	0.00	0.00	0.00	1.00																
LT3	0.00	0.00	0.00	0.00	0.00	1.00															
LT4	0.00	0.00	0.50	0.00	0.00	0.00	1.00														
RT1	0.00	0.00	0.00	0.25	0.00	0.00	0.00	1.00													
RT2	0.08	0.08	0.00	0.13	0.08	0.00	0.00	0.31	1.00												
RT3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	0.18	1.00											
RT4	0.08	0.00	0.00	0.00	0.17	0.00	0.18	0.00	0.11	0.00	1.00										
LM3	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.31	1.00									
LM4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00								
RM3	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.17	0.00	1.00							
RM4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.44	0.00	1.00						
L12	0.08	0.08	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00					
L14	0.09	0.00	0.25	0.00	0.00	0.00	0.20	0.00	0.00	0.00	0.00	0.00	0.17	0.14	0.18	0.00	1.00				
L22	0.05	0.21	0.00	0.00	0.20	0.00	0.00	0.00	0.06	0.00	0.06	0.07	0.00	0.00	0.00	0.18	0.13	1.00			
L24	0.17	0.10	0.00	0.00	0.00	0.00	0.20	0.00	0.12	0.00	0.13	0.17	0.00	0.14	0.00	0.12	0.14	0.06	1.00		
LH3	0.18	0.10	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00	1.00	
RH3	0.10	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.13	0.00	0.00	0.00	0.20	0.00	0.00	0.00	0.00	0.07	0.00	0.00	1.00

N=Ewe nose, V=vagina, LT=Left Teat, RT=right Teat, LM=Left Milk, RM=Right Milk, L1=Lamb1 mouth, L2=Lamb 2 mouth, LH=Left Handler hand, RH=Right Handler Hand, 1=Time point 1, 2=Time point 2, 3=Time point 3, 4=Time point 4

Appendix 10 – Dice-Sørensen similarity matrix between the sample site communities over time for the strains isolated from the intervention study ewes

	N1	N4	V1	LT1	LT2	LT3	LT4	RT1	RT2	RT4	LM3	LM4	RM3	RM4	L12	L14	L22	L24	LH3	RH3
N1	1.00																			
N4	0.13	1.00																		
V1	0.12	0.00	1.00																	
LT1	0.10	0.00	0.33	1.00																
LT2	0.00	0.18	0.00	0.00	1.00															
LT3	0.00	0.00	0.50	0.17	0.00	1.00														
LT4	0.00	0.00	0.00	0.00	0.00	0.00	1.00													
RT1	0.00	0.00	0.00	0.15	0.00	0.00	0.00	1.00												
RT2	0.00	0.00	0.00	0.33	0.00	0.00	0.00	0.22	1.00											
RT4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00										
LM3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.00	0.00	1.00									
LM4	0.00	0.08	0.00	0.00	0.17	0.00	0.00	0.15	0.00	0.00	0.00	1.00								
RM3	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00							
RM4	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.31	1.00						
L12	0.18	0.16	0.00	0.07	0.08	0.00	0.00	0.00	0.00	0.00	0.08	0.07	0.09	0.00	1.00					
L14	0.09	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.00	1.00				
L22	0.11	0.19	0.00	0.06	0.00	0.07	0.08	0.14	0.00	0.00	0.00	0.06	0.07	0.06	0.18	0.12	1.00			
L24	0.09	0.14	0.00	0.11	0.00	0.14	0.00	0.00	0.14	0.31	0.00	0.00	0.00	0.10	0.00	0.21	0.00	1.00		
LH3	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	
RH3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.00	1.00

N=Ewe nose, V=vagina, LT=Left Teat, RT=right Teat, LM=Left Milk, RM=Right Milk, L1=Lamb1 mouth, L2=Lamb 2 mouth, LH=Left Handler hand, RH=Right Handler Hand, 1=Time point 1, 2=Time point 2, 3=Time point 3, 4=Time point 4

Appendix 11 – Dice-Sørensen similarity matrix between the sample site communities over time for the strains isolated from the mastitic and study ewes

	Hands	Lambs	Mmilk	Mteat	Milk	Nose	Teat
Hands	1.00						
Lambs	0.17	1.00					
Mmilk	0.08	0.65	1.00				
Mteat	0.11	0.43	0.16	1.00			
Milk	0.22	0.29	0.34	0.26	1.00		
Nose	0.24	0.50	0.49	0.39	0.18	1.00	
Teat	0.00	0.26	0.38	0.46	0.24	0.16	1.00

Mmilk=Mastitic milk, Mteat=Mastitic teat