The role of intramammary masses on the transmission and persistence of mastitis-associated pathogens, flock prevalence and the live weight of lambs

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Table of Contents

List of Figures ..... **v**

List of Tables ..... **vii**

Acknowledgements ..... **ix**

Declaration ..... **x**

Summary ..... **xi**

List of abbreviations ..... **xii**

Chapter 1 Introduction **1**

1.1. Definitions and prevalence of intramammary infections *1*
1.1.1. Acute clinical and subclinical mastitis 1
1.1.2. Chronic mastitis 2

1.2. The mammary microbiome and mastitis-associated pathogens *4*
1.2.1. The mammary microbiome 4
1.2.2. Mastitis associated pathogens 6

1.3. Transmission and persistence of mastitis-associated pathogens *8*
1.3.1. Sources and transmission routes of mastitis-associated pathogens 8
1.3.2. Persistence of bacterial strains in the mammary gland 10

1.4. Factors affecting disease occurrence and pathogenesis *11*
1.4.1. Host risk factors 11
1.4.2. Environment and management factors 12

1.5. Economic and welfare concerns *13*

1.6. Methods used to investigate mammary gland health *14*
1.6.1. Bacteriological analysis of milk samples 14
1.6.2. Identification of cultured isolates at species and strain level 15
1.6.3. Different study designs 16

1.7. Summary and conclusions from current knowledge *16*

1.8. Aims and hypotheses *17*

1.9. Thesis structure *17*

Chapter 2 A longitudinal study investigating the role of chronic intramammary masses in mastitis prevalence and somatic cell count **19**

2.1. Introduction *19*

2.2. Methods *20*
2.2.1. Ethical approval 20
2.2.2. Study farm 20
2.2.3. Data collection and management 20
2.2.4. Intervention design 23
2.2.5. Data analysis 24

2.3. Results *29*
2.3.1. Descriptive analysis 29
2.3.2. Chronic intramammary mass results 32
2.3.3. Acute mastitis results 44
2.3.4. Somatic cell counts 49

2.4. Discussion *53*
2.4.1. Strengths and limitations of the study 55
2.4.2. Conclusions from Chapter 2 56

Chapter 3 A longitudinal study of the effect of mastitis on the live weight of lambs 57

3.1. Introduction *57*

3.2. Methods *58*
3.2.1. Study farm and data collection 58
3.2.2. Data analysis 58

3.3. Results *62*
3.3.1. Descriptive analysis 62
3.3.2. Linear mixed effect model of lamb weight up to weaning 65
3.3.3. Generalised additive mixed effect model (GAMM) of lamb weight over the whole study 67
3.3.4. Linear regression model of age at slaughter 71

3.4. Discussion *73*
3.4.1. Strengths and limitations of the study 75
3.4.2. Conclusions from Chapter 3 75

Chapter 4 Epidemiological analysis of mastitis-associated pathogens in sheep flocks using MALDI-ToF MS and Whole Genome Sequencing 76

4.1. Introduction *76*

4.2. Methods *78*
4.2.1. Study farm and milk collection for bacteriological analysis 78
4.2.2. Growth and selection of bacterial isolates 79
4.2.3. Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-ToF) mass spectrometry 81
4.2.4. Data storage and management 82
4.2.5. Data analysis of bacterial species isolated from milk 83
4.2.6. Strain typing using MALDI-ToF for investigation of transmission and persistence 84
4.2.7. Whole Genome Sequencing of *Staphylococcus aureus* isolates 86
4.2.8. Defining persistence and transmission 87
4.2.9. Validating MALDI -ToF MS strain typing 87

4.3. Results *88*
4.3.1. Identification of bacterial species in milk samples from healthy ewes, ewes with chronic mastitis, and ewes with acute mastitis 88
4.3.2. Investigation into transmission and persistence of *Staphylococcus aureus* using Whole Genome Sequences 95
4.3.3. Validation of MALDI-ToF MS strain typing 99

4.4. Discussion *105*
4.4.1. Bacterial species presence 105
4.4.2. Persistence of *Staphylococcus aureus* within ewes 106
4.4.3. Transmission of *Staphylococcus aureus* within flocks 107
4.4.4. Validation of MALDI-ToF MS as a strain-typing method for *Staphylococcus aureus* isolates 108
4.4.5. Strength and limitations of the study 109
4.4.6. Conclusions from Chapter 4 110

Chapter 5 General discussion 111

5.1. Key findings *111*

5.2. Discussion of key findings *111*

5.3. Limitations and future work *113*

5.4. Conclusions of thesis *114*

5.5. Conclusions for industry *115*

References **116**

Appendix **127**

Figure A- 1: IMM occurrence by ewe part 1. ..... *127*

Table A- 1: Univariable linear mixed effect model of lamb weight up to weaning *132*

Table A- 2: Univariable generalised additive mixed model *134*

Table A- 3: Univariable linear mixed effect model of lamb age at slaughter (days) *136*

Table A- 4: Univariable binomial mixed effects regression model of presence of an IMM in the same year as the sample *138*

Table A- 5: Univariable binomial mixed effects regression model of acute mastitis in the same year as the sample *140*

List of Figures

Figure 1-1: Intramammary mass visible and palpable in the ewe's right gland 2

Figure 1-2: Mammary glands sliced in parasagittal sections showing a range of abscess morphologies. 3

Figure 1-3: Schematic diagram of the teat, showing the entry point for bacteria. 8

Figure 1-4: Host, environmental, and pathogen factors that can affect disease 11

Figure 2-1: Scores used to measure a) teat angle, b) teat position c) degree of separation between the two halves of the udder d) udder drop 24

Figure 2-2: Body condition score over the study by group at examination centred by lambing date. 31

Figure 2-3: IMM incidence centred by lambing date. 33

Figure 2-4: Proportion of ewes with IMM by group at detection. All ewes entered the study with no group (grey), and subsequently were moved into the IMM group if an IMM was detected. 34

Figure 2-5: Proportion of ewes with an IMM at the monthly examination centred by lambing date. 35

Figure 2-6: Relative proportion of IMM by size, ... 36

Figure 2-7: Percentage of ewes with one or both udder halves classified as firm or unsure 37

Figure 2-8: Number of ewes with acute mastitis by week following lambing. 45

Figure 3-1: Lamb weight up to 220 days, with a line of best fit showing linear growth for the first 90 days. 59

Figure 3-2: Lamb weight from birth to weaning 66

Figure 3-3: Residual plots for linear mixed regression model of lamb weight 66

Figure 3-4: Fitting different regression lines to the lamb growth curve 68

Figure 3-5: Fitted GAMM final model residual statistics 69

Figure 3-6: Lamb weight over the whole study. 71

Figure 4-1: Preparation of cultures by streaking (A) schematic of method, (B) example. 80
Figure 4-2: Pipeline for processing mass spectra to generate peak lists. 85
Figure 4-3: Species detected in milk samples ordered by percentage. 92
Figure 4-4: Staphylococcus aureus isolates are concentrated into flock lineages. 98
Figure 4-5: Percentage reproducibility of A: Technical samples, B: Biological samples. 100
Figure 4-6: Comparison of isolate clusters calculated by MALDI-ToF MS spectra and by cgMLST profiles using WGS. 102
Figure 4-7: MALDI-ToF MS calculated clusters mapped onto unrooted trees with fixed branch length. 103

List of Tables

Table 2-1: Data sets generated on FarmIT (Border Software Ltd.) 21
Table 2-2: Combined categories in categorical explanatory variables 25
Table 2-3 Explanatory variables used in regression models in Chapter 2 26
Table 2-4: Categorical variables for ewes examined in March 2019 separated by group during lambing 29
Table 2-5: Number of ewes in the control group and in the IMM group 32
Table 2-6: Number of observations recorded IMM positive over the study period ... 35
Table 2-7: Associations between explanatory variables in the IMM data set 38
Table 2-8: Univariable binomial logistic mixed effect model of factors associated with intramammary masses over the study period in 570 ewes 39
Table 2-9: Multivariable binomial logistic mixed effect model of factors associated with intramammary masses over the study period in 570 ewes 41
Table 2-10: Univariable binomial logistic mixed effect model of factors associated with intramammary masses detected after lambing in 570 ewes 42
Table 2-11: Multivariable binomial logistic mixed effect model of factors associated with intramammary masses detected after lambing in 570 ewes 44
Table 2-12: Variables associated with acute mastitis 46
Table 2-13: Univariable binomial logistic model of factors associated with acute mastitis in 570 ewes 47
Table 2-14: Multivariable binomial logistic model of factors associated with acute mastitis in 570 ewes 49
Table 2-15: Summary of log10 SCC measurements 49
Table 2-16: Univariable linear mixed effect model of log10 somatic cell count in 354 udder halves 50
Table 2-17: Multivariable linear mixed effect model of log10 somatic cell count in 354 udder halves 51

Table 2-18: Estimated marginal means of log10 somatic cell count and estimated marginal geometric means....52
Table 3-1: Summary statistics for categorical ewe variables....62
Table 3-2: Associations between categorical variables....63
Table 3-3: Associations between categorical variables and lamb birth weight and lamb age at slaughter using Chi-squared tests....64
Table 3-4: Multivariable linear mixed effect model of lamb weight up to weaning ...67
Table 3-5: AIC comparison of three model types....68
Table 3-6: Multivariable generalised additive mixed model....70
Table 3-7: Multivariable linear mixed effect model of lamb age at slaughter (days) 72
Table 4-1: Milk samples used in bacteriological study....79
Table 4-2: Sampling time points in the study....79
Table 4-3: Bruker Daltonik MALDI Biotyper given meanings for score values, taken from Bruker classification results....82
Table 4-4: Summary of milk samples and isolates detected by flocks and sampling times....89
Table 4-5: Summary of ewe disease data over the study period....91
Table 4-6: Genus of bacteria isolated from udder halves and ewes over the study 93
Table 4-7: Multivariable binomial mixed effects regression model of presence of an IMM in the same year as the sample....94
Table 4-8: Multivariable binomial mixed effects regression model of acute mastitis in the same year as the sample....94
Table 4-9: Distribution of *S. aureus* isolates by flock, ewe and udder....95
Table 4-10 Disease status of ewes used in this study....95
Table 4-11: MLST profiles for each sequence type as given on pathogen.watch....96
Table 4-12: MALDI-ToF MS clustering compared to cgMLST as a gold standard.104

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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 Professor Laura Green and Dr Kevin Purdy and has not been submitted in any previous application for any degree.

Summary

Mastitis is endemic in suckler sheep flocks in the UK and has major implications for farm sustainability and sheep health and welfare. This study aimed to investigate intramammary masses (IMM), their association with acute mastitis and their role in transmission pathways of mastitis-associated pathogens. Ewes with IMM were separated to investigate the success of separation in reducing IMM prevalence and the effect of IMM on lamb growth rates was investigated.

Previous studies have identified a strong association between IMM and acute mastitis and hypothesised that ewes with IMM provide a reservoir of mastitis-associated pathogens.

A 12-month longitudinal study of 570 ewes was carried out. New and reoccurring IMM were recorded each month and ewes with IMM were separated from ewes with no IMM. Somatic cell counts (SCC) and udder conformation investigated at shortly after lambing. Ewes remained separate throughout the study and their lambs were weighed at birth and at regular periods until slaughter. Milk samples were collected during a second two-year longitudinal case-control study and cultured aerobically. Morphologically unique isolates were selected and identified using Matrix-assisted laser desorption ionization time-of-flight (MALDI-ToF MS). Whole genome sequencing (WGS) was carried out on a subset of *Staphylococcus aureus* samples for molecular epidemiological analysis.

Separating ewes with IMM did not reduce the number of new IMM identified in the flock, indicating that culling will not successfully reduce IMM risk in a flock. SCC were significantly higher in ewes with acute mastitis or intramammary masses than ewes with no mastitis. In the second study, *S. aureus* was significantly associated with IMM and acute mastitis compared to healthy ewes. WGS analysis evidenced transmission of strains within a flock and persistence within the mammary gland. Lambs of ewes with IMM or acute mastitis had significantly lower growth rates compared to healthy ewes all the way until slaughter, significantly increasing the age at slaughter.

List of abbreviations

AHDB	Agriculture and Horticulture Development Board
AIC	Akaike information criterion
AM	Acute mastitis
AWERB	Animal Welfare Ethical Review Body
BCS	Body condition score
cgMLST	Core genome MLST
CI	Confidence interval
CMT	California Mastitis Test
CNS	Coagulase negative staphylococci
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
E. faecium	*Enterococcus faecium*
EID	Electronic identification number
EL	Early lactation
EMM	Estimated marginal means
GAMM	Generalised additive mixed model
HCCA	α-Cyano-4-hydroxycinnamic acid
IMI	Intramammary infection
IMM	Intramammary mass
IQR	Interquartile range
K. pneumoniae	*Klebsiella pneumoniae*
LL	Late lactation
M. haemolytica	*Mannheimia haemolytica*
MADLI-ToF	Matrix-assisted laser desorption ionization time-of-flight
MLST	Multilocus sequence typing
MLVA	multiple locus variable number tandem repeat analysis
MS	Mass spectrometry
OR	Odds ratio
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PPE	Potential persistence event
PTE	Potential transmission event
QMMS	Quality Milk Management Services Ltd.
RAPD	random amplification of polymorphic DNA
RFID	Radio-frequency identification logger
S. aureus	*Staphylococcus aureus*
S. chromogenes	*Staphylococcus chromogenes*
S. cohnii	*Staphylococcus cohnii*
S. epidermidis	*Staphylococcus epidermidis*
S. lentus	*Staphylococcus lentus*
S. marcesens	*Serratia marcescens*
S. sciuri	*Staphylococcus sciuri*
S. simulans	*Staphylococcus simulans*
S. succinus	*Staphylococcus succinus*
S. xylosus	*Staphylococcus xylosus*
SBA	Sheep blood agar
SCC	Somatic cell count
SD	Standard deviation
SE	Standard error
ST	Sequence type
WGS	Whole genome sequencing

Chapter 1 Introduction

Mastitis is an inflammation of the mammary gland, usually caused by a bacterial infection. The intramammary infection (IMI) has a wide clinical spectrum, including no clinical signs, acute disease, and the presence of intramammary masses (IMM).

Below the current knowledge on bacterial mastitis in meat sheep is contextualised, with a focus on chronic mastitis where appropriate. Three main themes are presented: an overview of mastitis, the potential causative agents and their transmission pathways, and the economic importance, with a focus on the effect on lamb production.

1.1. Definitions and prevalence of intramammary infections

1.1.1. Acute clinical and subclinical mastitis

Acute mastitis is defined by the rapid onset of clinical signs. These include a hot or cold, swollen, and painful udder. It is accompanied with reduced and abnormal milk, which can be watery, blood filled, or contain clotted secretions (Khan et al. 2006).

Cooper et al. (2016) estimated that the incidence rate of acute mastitis in suckler ewes in the UK was 1.2 % p.a. (range: 0-19%). Other studies have estimated incidence of acute mastitis using researcher-led trials and report incidence from 0 – 5.0 %, with flock incidence ranging between 0 – 37 % (Onnasch et al., 2002; Arsenault et al., 2008; Grant et al., 2016). Although the reported incidence rates are low, there is high variability in flocks, and the economic impact on the sheep farming industry is significant (Conington et al., 2008).

Subclinical mastitis, where infection is present without obvious clinical signs, is usually diagnosed by bacteriological analysis of milk or individual somatic cell counts (SCC) (Bergonier and Berthelot, 2003). SCC provides a measurement of the number of inflammatory and epithelial cells in a milk sample, indicating if an immune response has begun (Conington et al., 2008). There is no common agreement on the level of SCC that indicates infection in ewes, but recent studies have identified samples over 400,000 cells/ml as potential subclinical mastitis (Huntley et al., 2012; Esteban-Blanco et al., 2019). Regardless of a given threshold, higher SCC have been associated with reduced milk production, lower lamb weights and decreased

microbial diversity (Fthenaskis and Jones, 1990b; Huntley et al., 2012; Esteban-Blanco et al., 2019). In this thesis, a significantly higher SCC compared to a control group is considered important.

1.1.2. Chronic mastitis

Lumps, nodules, masses, abscesses and fibrous lesions within the mammary tissue are all signs of chronic mastitis in ewes (Menzies and Ramanoon, 2001; Bergonier and Berthelot, 2003; Marogna et al., 2010; Smith et al., 2015; Grant et al., 2016). Farmers usually detect these udder abnormalities by palpating the udder tissue during routine inspections, for example before weaning or mating, (Smith et al., 2015; Cooper et al., 2016). Occasionally intramammary masses (IMM) are visible to the eye without palpation, with sizes ranging from small (1-2cm) to very large (>15cm) (Figure 1-1).

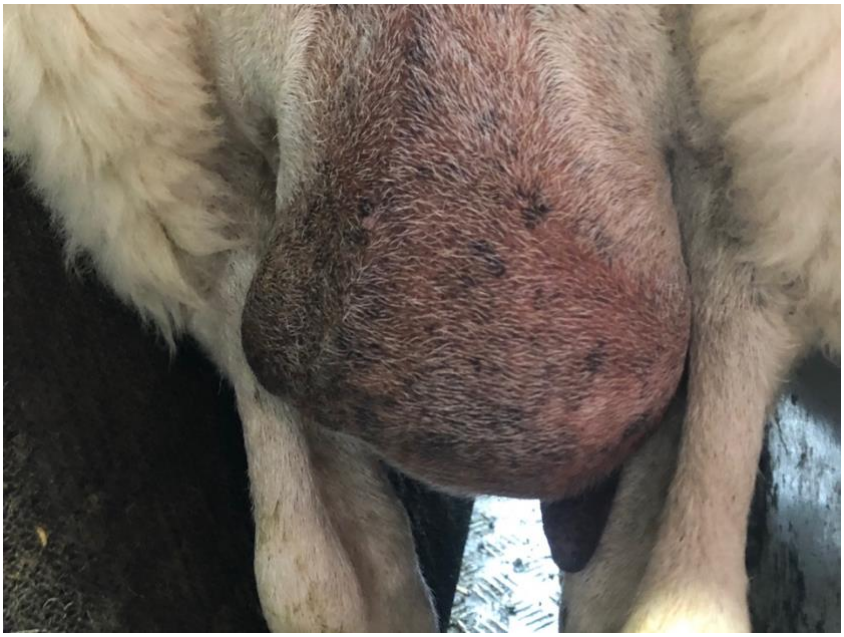


Figure 1-1: Intramammary mass visible and palpable in the ewe's right gland

In a study of 10 suckler sheep flocks in Great Britain, Grant et al. (2016) detected IMM in 4.7% of ewes in pregnancy and 10.9% of ewes in lactation. In New Zealand, Griffiths et al. (2019) reported the prevalence of ‘abnormal’ udders at palpation before mating and at weaning at 6% and 7.4% respectively. Both studies focused on specific times in the farming year; raising the possibility that the prevalence of IMM could change significantly in other months. This may be important for framing

new farmer management practices, as the time of year a farmer checks the ewes could affect the outcome of any mitigation practices.

IMM are detected intermittently in ewes. For example, after being present in one examination they may not be present in the subsequent examination, but reappear in future examinations (Grant et al., 2016). It is thought that IMM undergo a rupture-reform process that can result in fibrotic scars and different abscess morphologies (Figure 1-2) (Smith et al., 2015). Early inoculation studies found that *Staphylococcus* infection could cause abscesses and the formation of fibrous tissue within the udder, whilst the ewe appeared otherwise healthy (Fthenakis and Jones, 1990a; de la Fuente et al., 1993), similar to symptoms of naturally acquired chronic mastitis.

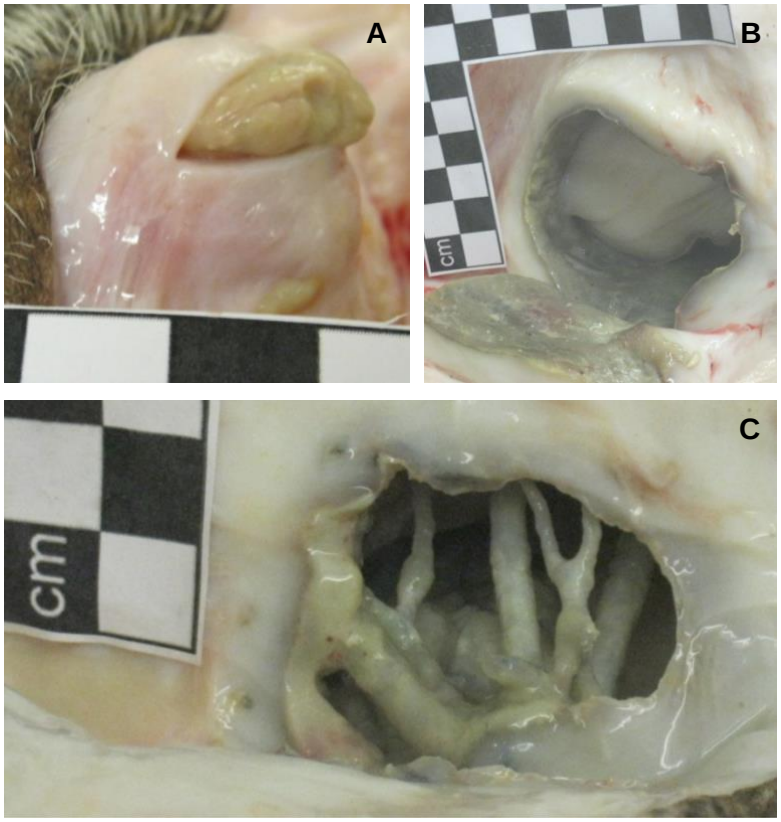


Figure 1-2: Mammary glands sliced in parasagittal sections showing a range of abscess morphologies. A) Pus-filled abscess, B) Intramammary void, C) Intramammary void intersected by fibrous strands. Images from Smith et al., 2015

IMM are also strongly associated with acute mastitis, with 12-fold odds of presence in lactation when a ewe had acute mastitis, suggestive of IMM formation following an episode of acute mastitis (Grant et al., 2016). IMM risk has also been associated

with previous IMM and flock percentage of IMM. Risk associated with flock percentage implies that ewes with IMM may a source of infection and bacterial dissemination into the environment, as a higher percentage of ewes with IMM within a flock has the potential to increase pathogen presence in the surroundings (Gelasakis et al., 2015; Grant et al., 2016).

1.2. The mammary microbiome and mastitis-associated pathogens

1.2.1. The mammary microbiome

Traditionally, a healthy mammary gland was considered a sterile environment. Until recently, studies investigating pathogens associated with mastitis in sheep used culture dependent methods and some took samples only from diseased sheep (Onnasch et al., 2002; Mørk et al., 2007). Culture dependent methods are limited as only cultivable bacteria can be detected and identification methods can be laborious and costly, often resulting in small sample sizes. Samples from apparently infected glands are often reported with no bacteriological growth, providing evidence that culture alone cannot detect all bacteria capable of causing disease in the mammary gland (Kuehn et al., 2013).

Samples with more than two colony types are generally considered contamination, and removed from analysis (Albenzio et al., 2002; Arsenault et al., 2008; Rovai et al., 2014), and samples from healthy mammary glands with bacterial growth are considered evidence for subclinical mastitis (Arsenault et al., 2008; Marogna et al., 2010). The limitations of this methodology mean that authors can only associate a single bacteria to each case of mastitis.

Recent culture independent studies in dairy cattle and humans have detected diverse microbial communities in healthy and diseased mammary glands using molecular methods (Martín et al., 2007; Hunt et al., 2011; Oikonomou et al., 2014; Patel et al., 2015). These form the hypothesis that rather than mastitis occurring following the introduction of a single pathogen into a sterile environment, a dysbiosis of the mammary microbiota causes the disease (Oikonomou et al., 2014). Diseased mammary glands of dairy cattle and humans show distinct communities and dominating pathogens in comparison to healthy glands, which forms the evidence for dysbiosis of the microbiota (Oikonomou et al., 2014; Patel et al., 2015;

Derakhshani et al., 2018). Furthermore, a common finding across studies is that mastitis results in a reduction in microbial diversity within the mammary gland (Ganda et al., 2016; Lima et al., 2018; Esteban-Blanco et al., 2019). This may also explain why many culture dependent studies cultivate a large number of single microbe samples, as the microbiome diversity has reduced sufficiently to allow a single pathogen to dominate culture plates.

Few studies have investigated the mammary microbiota of sheep, and those that do focus on dairy sheep (Castro et al., 2019; Esteban-Blanco et al., 2019). Esteban-Blanco et al. (2019) associated an increased SCC with a decreased diversity and a significant increase in the relative abundance of *Staphylococcus*. However, Castro et al. (2019) did not report a consistently distinct microbiota between ewes with a history of mastitis compared to those without. Conclusions from these recent studies cannot be drawn on whether dysbiosis occurs in the sheep mammary gland as has been suggested in cattle and humans. Furthermore, the husbandry of meat and dairy ewes are different enough that it is likely to influence their mammary microbiota (section 1.4.2).

The results of molecular studies challenge the assumption of a sterile mammary gland. However, the existence of a mammary microbiota is controversial and there are still many questions about its existence and formation (Oikonomou et al., 2020). Rainard (2017) thoroughly reviewed the evidence for a mammary microbiome in dairy cattle and concluded that although a teat apex microbiota was likely to exist, a mammary microbiome was ‘fiction’. Regardless of whether a study is culture dependent or independent, sample collection methods are the same and are prone to contamination. Many culture dependent studies discount samples with three or more species as a way of managing potential contamination, while culture independent studies assume the existence of multiple genera of bacteria to be part of the microbiome (Rovai et al., 2014; Esteban-Blanco et al., 2019). Rainard (2017) suggests that this potential for contamination is evidence against a microbiome. Contamination may arise from the udder skin, teat apex, or from the sampler themselves. Hunt et al. (2011) found microbial communities in milk samples from women contrasted with the known skin microbiota, suggesting these results were not the result of skin contamination. Furthermore, Metzger et al. (2018) added to conventional sampling techniques by collecting milk using cisternal puncture to bypass the teat canal and teat apex, with the unexpected result of higher PCR amplification of bacterial DNA compared to the conventional samples. Smith et al. (2015) also successfully cultured bacteria from milk collected from mammary glands

post-mortem directly by using aspiration. This is a strong indication that bacteria are within the mammary gland, and results of the mammary microbial communities are not a result of external contamination.

1.2.2. Mastitis associated pathogens

Using culture dependent methods, many pathogens have been associated with mastitis in sheep. The most prevalent agents differ between production systems, region and disease presentation (acute, subclinical or chronic) (Gelasakis et al., 2015). Some authors classify bacteria into 'major' or 'minor' pathogens, where major pathogens are commonly detected in cases of acute mastitis, and minor pathogens are less commonly detected, or detected in cases of subclinical mastitis (Conington et al., 2008; Hawari et al., 2014). Other authors categorise pathogens by their transmission model: contagious, opportunistic, or environmental (Zadoks et al., 2011; Bergonier et al., 2014; Achek et al., 2020). With the improved understanding of mastitis, it is clear most bacterial species are not confined to these classifications. For example, many species of coagulase negative staphylococci (CNS) have been detected in both acute mastitis and subclinical mastitis (Gelasakis et al., 2015; Derakhshani et al., 2018; Vasileiou et al., 2019). Pathogens considered traditionally contagious may also have environmental reservoirs, for example, Zadoks et al. (2011) reviewed the evidence for *Staphylococcus aureus* transmission in cattle and found that in some herds there are multiple strains which suggests its profile is that of an environmental pathogen. It is likely that multiple transmission patterns exist for other pathogens in both sheep and cattle.

In suckler ewes, commonly detected pathogens are *S. aureus* and CNS (Onnasch et al., 2002; Mørk et al., 2007; Arsenault et al., 2008). Other bacteria that have been associated with mastitis in sheep are: *Mannheimia haemolytica*, *Streptococcus* species, *Enterobacteriaceae*, *Pseudomonas* species, *Corynebacterium* and *Bacillus* species (Arsenault et al., 2008; reviewed by Bergonier and Berthelot, 2003; Omaleki et al., 2011; Smith et al., 2015). These studies focus on culture dependent methods and either only investigate samples from diseased glands or assume samples positive for potentially pathogenic bacteria are from ewes with subclinical mastitis (Mørk et al., 2007; Arsenault et al., 2008; Marogna et al., 2010). Without also examining the milk of healthy ewes, it is not known if these bacteria always cause disease, or if they can exist commensally in the mammary microbiota. *S. aureus* and CNS are regularly identified in milk samples from all types of mastitis and are

therefore likely to be important in causing disease, so are discussed in more detail below.

1.2.2.1. Evidence for *Staphylococcus aureus* as a causative agent

All types of mastitis have been frequently associated with *S. aureus* and a number of studies have reported it as the most frequently isolated species from infected ewes (Onnasch et al., 2002; Bergonier and Berthelot, 2003; Mørk et al., 2007; Koop et al., 2010). It has also been detected in glands with no signs of clinical disease (Smith et al., 2015; Vasileiou et al., 2018). Vasileiou et al. (2018) suggested *S. aureus* isolation from clinically healthy ewes might indicate subclinical disease, early stages of acute mastitis, or effective host defences resulting in limited invasion. Mørk et al. (2012) investigated 520 milk samples taken only from ewes without acute mastitis and detected *S. aureus* in 1.5% of samples, despite isolating *S. aureus* in 60% of nasal swabs from the same ewes. A bacteriological study of dairy ewes in Portugal only found 17.9% *S. aureus* positive samples from diseased mammary glands (Queiroga, 2017). The authors suggested the disparity between their results and common findings was due to the majority of studies investigating high incidence severe outbreaks. Although these results suggest *S. aureus* is associated with outbreaks of mastitis and is not associated with sporadic disease or healthy mammary glands, more research into the bacterial species present in milk of healthy and infected ewes is necessary to confirm *S. aureus* is a contagious causative agent.

1.2.2.2. Evidence for Coagulase Negative Staphylococcus (CNS) as causative agents

CNS are difficult to distinguish with traditional bacteriological methods (Onni et al., 2010), which results in some studies specifying individual CNS and some collecting CNS under a single term when investigating possible mastitis causative agents

CNS are commonly isolated from sheep with subclinical mastitis (Fthenakis et al., 1994; Onni et al., 2010; Alekish et al., 2018; Vasileiou et al., 2018; Achek et al., 2020). Each species of CNS is thought to have a different effect on the health of the udder, for example a longitudinal study on dairy cattle found different CNS had varying effects on SCC (Supré et al., 2011). In ewes, the most prevalent CNS isolated is *S. epidermidis* (Marogna et al., 2010; Onni et al., 2010; Castro et al.,

2019), with *S. chromogenes*, *S. simulans*, and *S. xylosus* also commonly isolated (Bergonier and Berthelot, 2003; Gelasakis et al., 2015).

CNS have been isolated from cases of acute mastitis in both dairy and suckler flocks, although less commonly than *S. aureus* (Vasileiou et al., 2019). In dairy ewes, studies have found CNS in 10 – 50% of acute mastitis cases (Bergonier and Berthelot, 2003). In meat sheep, Arsenault et al. (2008) isolated CNS from 17% of clinically affected glands while Mørk et al. (2007a) found CNS in 2.9% of effected glands. Further to this, in an experimental inoculation study, *S. chromogenes* was reported to result in acute mastitis (Fthenakis and Jones, 1990a). Collectively, these studies show varying presence of CNS associated with both subclinical and acute mastitis. As many studies have not identified individual species of CNS, it is difficult to know which species are actually associated with disease.

1.3. Transmission and persistence of mastitis-associated pathogens

1.3.1. Sources and transmission routes of mastitis-associated pathogens

It is widely thought that pathogenic bacteria gain entry to the mammary gland via the teat orifice (Gougoulis et al., 2007). The teat canal acts as a defensive barrier to microbe entry, but because it dilates during suckling or artificial milking and takes

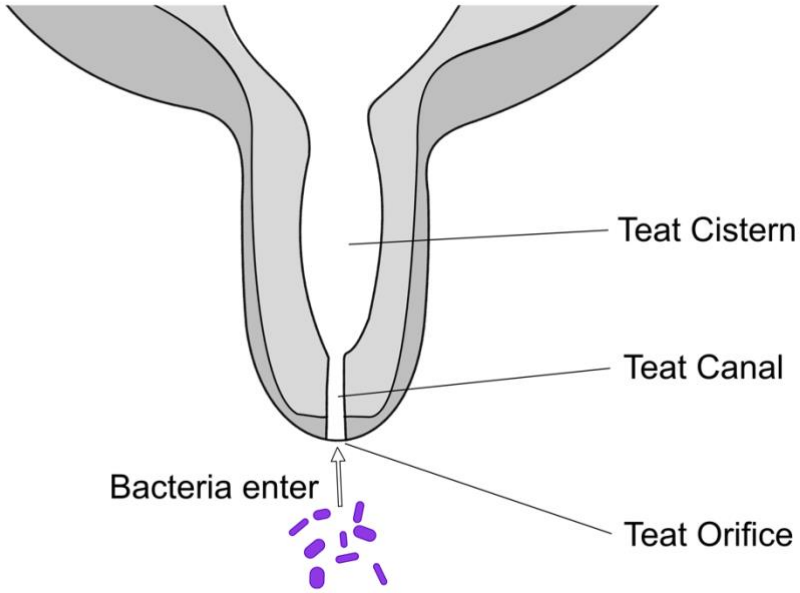


Figure 1-3: Schematic diagram of the teat, showing the entry point for bacteria.

some time to close after, this facilitates movement, or even pushes, bacteria into the teat (Gougoulis et al., 2008; Derakhshani et al., 2018) (Figure 1-3).

Staphylococcus are able to colonise the teat canal as normal flora, and a change in circumstance may allow some of these bacteria to act as opportunistic pathogens and cause mastitis (Fthenakis et al., 2004; Fragkou et al., 2007a; Mavrogianni et al., 2007). Events that could cause this change are mostly host-mediated; the onset of lactation, damage to the udder or teat physical defences, or reduction of immunological defences due to nutritional stress or other disease factors (Gelasakis et al., 2015).

As discussed above, bacteria are generally considered to come from an environmental or contagious source and understanding the transmission route is key to reducing infection rates. Outbreaks of acute mastitis in sheep flocks (Bergonier and Berthelot, 2003) suggest a single contagious pathogen has been transmitted between sheep. In dairy flocks, the contagious transmission point is considered to be milking, whereas in suckler ewes, 'milk robber' lambs are thought to carry pathogens between ewes as they search for milk around the flock (Bergonier and Berthelot, 2003). *M. haemolytica* has been isolated from both the mouths of lambs and ewes and the udder skin shortly after lambing (Scott and Jones, 1998). Gougoulis et al. (2008) isolated *M. haemolytica* from the teat duct following suckling, indicating suckling lambs as a source of infection. Similarly, *S. aureus* has been isolated from udder skin, nasal cavities, and lambs' tonsils, although it is also considered a common udder skin commensal (Bergonier and Berthelot, 2003; Koop et al., 2010; Mørk et al., 2012). IMM have been suggested as a source of infection to other ewes (Smith et al., 2015; Grant et al., 2016). Smith et al. (2015) isolated closely related strains from the milk and abscesses of the same gland and hypothesised affected ewes could be a reservoir of *S. aureus*.

The detection of bacteria in sheep milk that are commonly detected in the environment suggests the environment is a source of bacteria (Burriel, 1998; Albenzio et al., 2002). CNS, enterobacteria, and pseudomonads are all present in the environment (e.g. bedding) and have been associated with IMI (Bergonier and Berthelot, 2003). Omaleki et al. (2016) used molecular techniques to analyse isolates from an outbreak of *M. haemolytica* in South East Australia and reported distinct clusters of isolates. This finding suggests the particular outbreak was caused by environmental or host factors, similar to Fthenakis et al. (2004), where a high prevalence of mastitis was associated with nutritional factors.

A good example of the impact of different transmission routes of mastitis-associated pathogens is the varying success of control measures in dairy cattle. Control measures such as teat disinfectants before and after milking, antimicrobial treatment of diseased cows, and culling chronically infected cows rely on reducing contagious transmission from cow to cow (Barkema et al., 2009). These measures have been successful in reducing incidence of mastitis in some UK herds by about 20% (Green et al., 2007). However, there are cases where these control measures do not reduce incidence in a herd, which indicates an environmental reservoir of pathogens not affected by the control measures implemented (Sommerhäuser et al., 2003; Anderson and Lyman, 2006).

1.3.2. Persistence of bacterial strains in the mammary gland

Understanding persistence of strains causing mastitis is important for disease management and treatment, because ewes carrying pathogenic bacteria have the potential to transmit contagious bacteria to other ewes or shed pathogens to the environment. In ewes, chronic infections are usually associated with abscess formation and IMM, although this may not be necessary for a mammary gland to carry a bacterial strain across lactations. IMM are detrimental to ewe health and increase risk of acute mastitis (Grant et al., 2016), highlighting the importance of understanding persistent bacteria. Wente et al. (2020) used RAPD PCR to strain-type recurrent infections in a 4 year longitudinal study in dairy cattle, finding 11% of recurrent infections were caused by an identical strain. It is important to note that 32% of recurrent infections were caused by the same pathogenic species, but not strain, and therefore suggests reinfection of a different strain in two-thirds of cases rather than persistence. This finding highlights that strain-typing is necessary to differentiate persistence versus reinfection of a pathogen.

To the author's knowledge there are no longitudinal studies investigating bacterial strains in ewes. Smith et al. (2015) used MALDI-ToF analysis to investigate strains in a post-mortem study of milk and abscesses, and reported a high species overlap between milk and abscesses in the same hierarchical clusters. These findings show that the same bacterial strain within intramammary abscesses can also be detected in the milk, suggesting a potential mechanism for persistence. In dairy cattle *Escherichia coli*, *S. aureus*, CNS, and *Streptococcus uberis* can persist in the mammary gland (Döpfer et al., 1999; Bradley and Green, 2001; Anderson and Lyman, 2006; Taponen et al., 2007; Leelahapongsathon et al., 2020), so there is

good evidence to suggest these bacteria may also have mechanisms for persisting in ewe mammary glands.

1.4. Factors affecting disease occurrence and pathogenesis

The development of mastitis is dependent on the interaction between the host, the pathogen and the environment. Together, these can affect the likelihood of disease and the level of severity. Figure 1-4 outlines the main factors that determine mastitis risk. Below, host risk factors and management factors are discussed in more detail. Pathogen factors are briefly discussed above, but examination of virulence factors and antibiotic resistance are beyond the scope of this thesis.

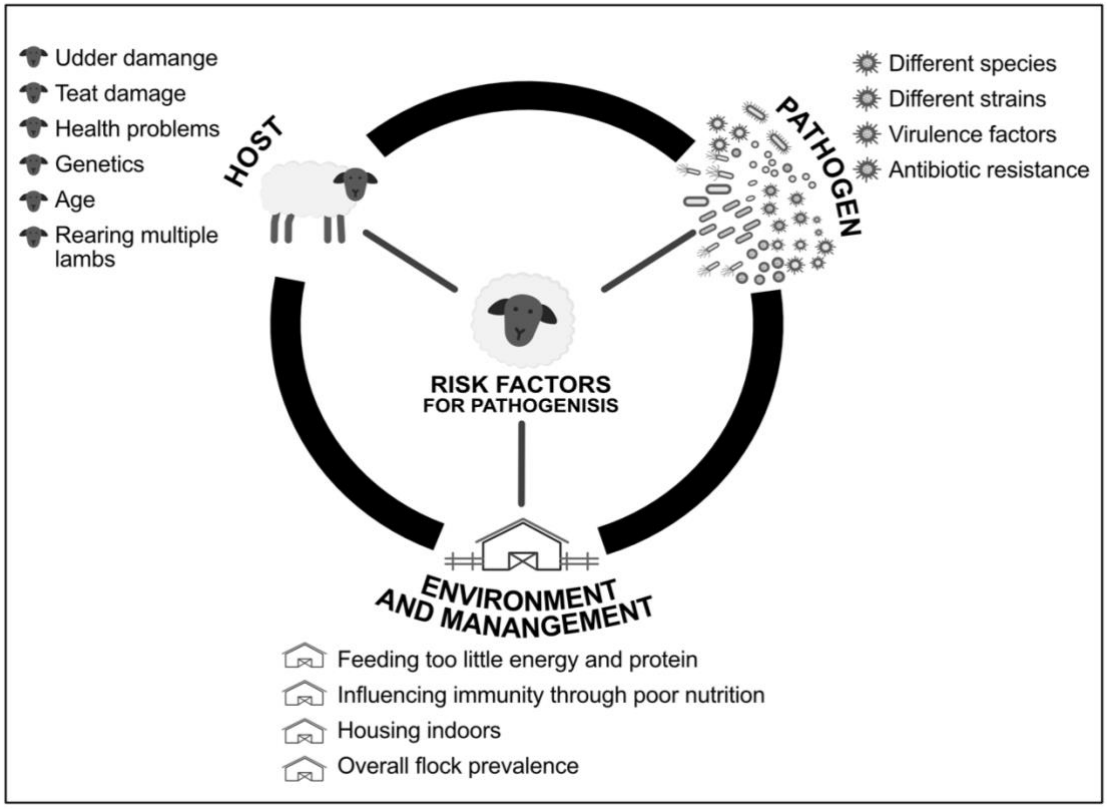


Figure 1-4: Host, environmental, and pathogen factors that can affect disease

1.4.1. Host risk factors

Host risk factors have been well examined in suckler ewes. Several studies have reported an association with udder or teat damage and acute mastitis. Fragkou et al. (2007b) found teat lesions increased colonisation of the teat duct, and Grant et al. (2016) reported a higher risk of mastitis associated with non-traumatic teat lesions. Further, poor flock udder conformation has been associated with higher

prevalence of acute mastitis (Cooper et al., 2016), and individual poor udder and teat conformation had been linked to an increase in SCC (Huntley et al., 2012).

Individual host immunity can be influenced by health problems or high production and is likely to have a significant effect on the pathogenesis of mastitis (Gelasakis et al., 2015). Waage and Vatn (2008) reported that dystocia was associated with increased risk of acute mastitis in meat sheep, and an experimental study of dairy ewes in Greece found that pre-existing pregnancy toxaemia predisposed ewes to mastitis when they were inoculated with *M. haemolytica* (Barbagianni et al., 2015).

Genetic factors and breed play a role in the level of susceptibility to mastitis. Fragkou et al. (2007c) compared an indigenous Greek sheep breed and a high-production breed, reporting the former maintained higher resistance to mastitis. Similarly, Waage and Vatn (2008) found that old Norwegian breeds were less likely to suffer acute mastitis compared to other breeds. A study on udder traits of Texel ewes in the UK reported that all examined traits were to some extent heritable, indicating that selective breeding could be used as a method to produce ewes with more resilience to mastitis though udder traits (Crump et al., 2018).

Sucking lambs can introduce bacteria into the teat duct (Gougoulis et al., 2008), and can cause teat and udder damage (Bergonier and Berthelot, 2003). There is an increased risk of mastitis associated with multiple lambs (Arsenault et al., 2008; Waage and Vatn, 2008; Koop et al., 2010; Grant et al., 2016), which could be explained by the additional pressure multiple lambs put on the udder. Other risk factors include increased ewe age and a lower body condition score (BCS), which is an indication of poor nutrition (Onnasch et al., 2002; Arsenault et al., 2008; Waage and Vatn, 2008; Grant et al., 2016).

1.4.2. Environment and management factors

Management factors such as housing and nutrition can also play a role. Flocks housed indoors for any period have a higher prevalence of mastitis than flocks kept outdoors, with risk increasing the longer ewes are kept indoors (Cooper et al., 2016). Indoor housing usually has an increased stocking density compared to outdoor housing, which is likely to lead to higher concentrations of bacteria within the environment (Sevi et al., 1999). Nutritionally, underfeeding protein and energy in pregnancy and lactation have been highly associated with both acute mastitis and IMM (Grant et al., 2016). A similar effect was reported by Barbagianni et al. (2015), where ewes given a low-energy diet had higher levels of mastitis.

Differences in mastitis disease dynamics between dairy cattle or ewes and suckler ewes may be caused by the milking process. Dairy animals are usually machine milked, with the entire mammary gland emptied each occasion. Suckler ewes are naturally sucked by their lambs throughout the lactation period until weaning.

1.5. Economic and welfare concerns

Costs associated with mastitis in meat ewes include ewe and lamb mortality, premature culling of ewes, medicine to treat mastitis, and reduced lamb growth rates, from a reduction in milk quality and quantity (Fthenakis and Jones, 1990b; Grant et al., 2016).

Although exact figures are not available, Menzies and Ramanoon (2001) estimated that between 13-50% of British ewes had mastitis or udder abnormalities at slaughter, and an Irish study found approximately 30% of ewes were culled because of mastitis (Onnasch et al., 2002). A questionnaire study found that up to 93% of ewes with a case of acute mastitis would be subsequently culled, strongly indicating that up to 8% of the UK flock may be culled each year due to mastitis (Cooper et al., 2016). Conington et al. (2008) estimated the annual cost of mastitis to the pedigree Texel sheep industry alone at £2.7 million, but there is no recent or accurate estimate of the full cost of mastitis to the UK sheep flock.

A recent study from New Zealand found ewes suffering from a case of acute mastitis had lambs 3 times more likely to not survive to weaning than ewes with no mastitis (Griffiths et al., 2019). This is consistent with previous findings, where a smaller study in Canada estimated lamb mortality was 5 times more likely when ewes had acute mastitis (Arsenault et al., 2008).

Lambs of ewes with acute mastitis are lighter in weight than those without acute mastitis. The 8-week weight was used to compare daily lamb growth in a study of UK flocks, reporting a difference of 40g/day when ewes had acute mastitis (Grant et al., 2016). Griffiths et al. (2019) reported a similar difference (38g/day) when using weaning weights to investigate daily lamb growth rates in Australia. Arsenault et al. (2008) similarly investigated lamb weaning weights, but only found a significant reduction when ewes with acute mastitis were also older than 4 or rearing multiple lambs. Reduction in lamb growth has been attributed to decreased milk production and ewes preventing suckling due to pain. More lambs or older age will likely

influence milk availability and production further due to the additional stress on the mammary gland, which could explain the result from Arsenault et al. (2008).

The effect of subclinical mastitis on lamb weight was first examined by Fthenakis and Jones (1990b), where experimentally induced subclinical mastitis resulted in lighter lambs, despite consuming more food when provided with a creep feed supplement than lambs of healthy dams. A high SCC, indicative of subclinical mastitis, has also been associated with lower lamb weight (Huntley et al., 2012). Similarly, a study using the California Mastitis Test (CMT) as proxy for SCC reported a 2.9kg difference in 8 week weight between the lowest scoring (no infection) and highest scoring (high level of somatic cells) ewes (McLaren et al., 2018).

The effect of IMM on lamb growth has been studied less. The presence of an IMM in lactation resulted in a 10g/day decrease in lamb daily weight gain (Grant et al., 2016). Griffiths et al. (2019) found ‘hard’ or ‘lump’ udder scores before lambing, at docking and at weaning were associated with reduced lamb growth, compared to ewes with a ‘normal’ score. However, they found pre-mating udder scores did not influence lamb weight, suggesting the timing of observations of abnormal udder scores was associated with their impact on growth rate (Griffiths et al., 2019).

1.6. Methods used to investigate mammary gland health

1.6.1. Bacteriological analysis of milk samples

In order to isolate individual strains for strain-typing from a milk sample, culture-based methods must be used. Culture dependent methods have a number of limitations, which include time, expense and being restricted to cultivable bacteria. Many studies report culture-negative milk samples, from both healthy and clinically diseased samples (Mørk et al., 2007; Arsenault et al., 2008; Smith et al., 2015). Recent culture-independent studies showing the presence of a mammary microbiota indicate that these culture-negative samples are not due to sterile milk, but rather a low level of culturable bacteria (Kuehn et al., 2013). Culture-based methods also rely on the correct media and conditions and these will affect the pathogens isolated. Despite these limitations, strain level analysis is required when investigating transmission and persistence, as detection of the same species does not always mean the same pathogen has been found (Zadoks et al., 2011; Wente et al., 2020).

1.6.2. Identification of cultured isolates at species and strain level

Traditional phenotypic identification techniques, including biochemical tests, morphology, and gram stains can be used to identify bacteria at the genus or species level. For a more discriminatory analysis at species level, polymerase chain reaction (PCR) can be used to amplify species-specific regions of DNA, often within the 16S rRNA gene. There are genomic targets for many mastitis-associated pathogens, including *S. aureus* (Attili et al., 2016) and many CNS, which were traditionally very difficult to differentiate even at species level (Onni et al., 2010).

To a strain level, a range of DNA-based methods can be applied to distinguish different isolates. This bacterial 'typing' is necessary when understanding pathogen epidemiology and ecology. Ribotyping, multilocus sequence typing (MLST), multiple locus variable number tandem repeat analysis (MLVA) and random amplification of polymorphic DNA (RAPD) PCR have all been previously used in mastitis studies (Aarestrup et al., 1999; Fitzgerald et al., 2000; Bergonier et al., 2014; Keane, 2016).

Whole genome sequencing (WGS) provides the highest resolution for typing by providing a full genome for comparison, which allows strain identification. WGS allows discriminative analysis of the emergence, evolution, and spread of a bacterial species, and has been used in the investigation of human and animal epidemics (Holden et al., 2013). Whole genome sequencing is still not in widespread use due to the expense, although this has been decreasing in recent years.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a relatively novel protein-based identification method, utilising species diversity between ribosomal proteins. MALDI-TOF MS is a fast, reliable and cost-effective identification technique that has begun to replace conventional phenotypic identification methods (Bizzini and Greub, 2010; Dierig et al., 2015). Bacterial colonies can undergo a protein extraction step to enhance identification or be placed directly on a target plate. This produces a unique 'fingerprint' spectrum of the mass peak profile, which is individual to each bacterial species. This spectrum can be compared to a database of reference bacterial and fungal peak profiles and an identification assigned (Dierig et al., 2015).

Several studies have investigated the use of MALDI-ToF MS as a typing tool, where spectra of the same species are compared and segregated into subtypes based on similarity. Despite concerns over a lack of common methodology, and variable results over different species, most consider MALDI-ToF MS-based typing as a

promising tool for epidemiological analysis (Spinali et al., 2014; Oberle et al., 2016; Sauget et al., 2017). Indeed, MALDI-ToF MS has been used to type *Streptococcus uberis* in bovine mastitis (Archer et al., 2017; Esener et al., 2018) and *S. aureus* in ovine mastitis (Smith et al., 2015).

1.6.3. Different study designs

Longitudinal studies follow a cohort of individuals over a period of time, taking repeated measurements and observations. They enable changes and patterns over time be examined. To investigate persistence of pathogens, samples must be taken from the same site at multiple occasions and then strain-typed in order to confirm the same pathogen was present at each time point. This has been successfully carried out in studies of persistence in dairy cattle mammary glands (Leelahapongsathon et al., 2020, Wente et al., 2020). An intervention study carried out over time is more informative, as improvements over time can be monitored. Longitudinal studies require multiple data collection time points, which usually restricts the number of farms the study can be carried out on, reducing sample sizes compared to cross sectional studies.

1.7. Summary and conclusions from current knowledge

Mastitis is a complex disease, with several presentations and various causative agents. Recent culture-independent studies have opened up the possibility of an udder microbiome and have changed the traditional view of a host-pathogen interaction. Nevertheless, some pathogens are commonly isolated from cases of mastitis and, in cattle, several studies have used typing methods to prove both transmission amongst a herd and persistence within an udder are possible. Persistence and transmission studies in ewes are rare and are often carried out in dairy flocks. In cattle, various transmission pathways and infection sources exist, both environmental and contagious, and it is likely the same applies in sheep.

Studies into the role of chronic mastitis in sheep are limited, but have shown a strong link to acute mastitis, and have suggested IMM as a potential reservoir of infection. It is unknown how IMM change throughout the year and there is not sufficient evidence to show if they follow a case of acute mastitis or exist beforehand. The effect of acute and subclinical mastitis on lambs has been well studied, but few studies investigate the effect of IMM on lambs. It is also uncommon for studies to continue to examine the effect on lambs beyond weaning, and

although lambs are no longer reliant on their mothers after this point, the effects of acute mastitis and IMM may continue to influence lamb weight.

1.8. Aims and hypotheses

The overall aim of the current study was to investigate the persistence of bacteria in flocks and investigate intramammary masses and their association with acute mastitis and their effect of lamb growth rate. This study also aimed to gain an understanding of within-flock transmission routes of pathogens associated with acute mastitis and IMM by developing a suitable strain-typing methodology using MALDI-ToF MS and comparing the results from MALDI-ToF-based typing with the results of whole genome sequencing of *Staphylococcus aureus* isolates.

Hence, this thesis aims to test the following hypotheses:

1. IMM are a reservoir for infection to other ewes in the flock, and isolation of ewes with IMM will reduce flock prevalence of acute mastitis and IMM
2. The presence of IMM before lambing is predictive of acute mastitis during lactation
3. Acute mastitis and IMM have a negative impact on lamb growth, before and after weaning
4. Mastitis associated pathogens are capable of persisting in the mammary gland including across lactations; in particular when associated with cases of acute and chronic mastitis
5. Bacterial strains are shared across ewes within flocks, indicating common reservoirs of bacteria or contagious transmission

1.9. Thesis structure

This thesis comprises of two cohort studies. A novel intervention study is described in chapters 2 and 3 and then microbiological research based on a previously investigated cohort study is described in chapter 4.

Chapters 2 and 3 describe the results of a 12-month cohort study on IMM and address the first two hypotheses. Chapter 2 describes the results of isolating ewes with IMM from the rest of the flock and the effect of the reproductive cycle on the presence, absence and size of IMM, as well as the associations between intramammary mass presence, SCC and acute mastitis. Chapter 3 presents the effect of IMM and acute mastitis on lamb growth rates up to slaughter.

Chapter 4 will address hypotheses 3 and 4. A well characterised set of milk samples is used to investigate persistence and transmission of bacterial strains over a two year longitudinal study, utilising MALDI-ToF MS to identify and strain-type bacterial species isolated from these milk samples. The results of whole genome sequencing a subset of *Staphylococcus aureus* isolates are also presented, describing genotypic differences between strains and their epidemiological associations to disease, flock and time.

Chapter 5 discusses the overall findings from this study.

Chapter 2 A longitudinal study investigating the role of chronic intramammary masses in mastitis prevalence and somatic cell count

2.1. Introduction

Intramammary masses (IMM) are defined as a palpable mass in udder tissue and are one presentation of chronic mastitis (Menzies and Ramanoon, 2001; Bergonier and Berthelot, 2003; Marogna et al., 2010; Smith et al., 2015; Grant et al., 2016). IMM are highly correlated with cases of acute mastitis in the same udder (Grant et al., 2016), but our knowledge of transmission of pathogens between glands and indeed which bacteria are causal pathogens is poor for both IMM and acute mastitis.

Presence of IMM has been associated with previous occurrence of IMM in a gland and positively associated with the flock percentage of IMM, possibly suggesting that ewes are a source of infection and bacterial dissemination to themselves and other ewes (Gelasakis et al., 2015; Grant et al., 2016). One route that this might occur is via lambs sucking several ewes other than their dam, and so transmitting pathogens between udders (Bergonier and Berthelot, 2003; Smith et al., 2015). However, evidence for this is limited to the isolation of mastitis-associated pathogens in lamb mouths, without evidence of the same strain within linked ewe udders (Scott and Jones, 1998; Mørk et al., 2012).

A study was designed where in one flock ewes were separated into two groups by presence / absence of IMM to test the hypothesis that ewes with IMM were a source of infection to those without. In addition, the relationships between acute mastitis, SCC and udder conformation were investigated in order to understand the associations between acute and chronic mastitis.

2.2. Methods

2.2.1. Ethical approval

This study was approved by the University of Warwick's Animal Welfare & Ethical Review Body (AWERB) prior to commencement.

2.2.2. Study farm

A 600 ewe indoor lambing flock in North Yorkshire, England was convenience selected for the study. The ewes were a mix of mule, Texel cross and Abermax and sire breeds were Texel, Aberfield, Abermax and Rouge de l'Ouest. They lambed over a four week period from mid March 2019. All ewes were in at least their second lactation because ewes in their first lactation were kept as a separate flock and were excluded from this study.

2.2.3. Data collection and management

Data collection occurred from September 2018 to September 2019. The whole flock was examined in the first week of each month except in November when ewes were running with rams and in April when lambing.

Data from each monthly flock inspection were collected electronically using a handheld data-logger (Argident APR500) using software written for the project (Border Software Ltd) and downloaded after each visit. Data collected at other times were collected using a handheld computer integrated with a radio-frequency identification logger (RFID) (PSION and MOTOROLA WORKABOUT PRO Handheld Computer with Agrident AIR300). Data from the handheld data-logger and handheld computer were uploaded onto a computer using the software package FarmIT (Border Software Ltd.).

At weekly intervals during lambing and the weeks proceeding, lambing data were uploaded and saved on the software FarmIT on farm servers. At the end of the study, data on ewes and lambs were exported from FarmIT (Border Software Ltd.) as comma separated value (csv) files. Datasets generated over this period are summarised in Table 2-1.

Table 2-1: Data sets generated on FarmIT (Border Software Ltd.)

Data set generated	Animals in dataset	Data
Lambing	Ewe & Lamb	Ewe EID, lamb(s) EID, birth date lamb(s) birth weight, litter size, lambing assistance, lamb sex
Ewes	Ewe	Ewe EID, age
Lambs	Lamb	Lamb EID, breed
Treatments	Ewe & Lamb	EID, treatment, reason for treatment
Deaths	Ewe & Lamb	EID, death, date of death
Lamb weights	Lamb	Lamb EID, weight, date of measurement
Market	Lamb	Lamb EID, date sent to market, market information (e.g. carcass score)

EID: Electronic identification number

2.2.3.1. Monthly examinations for intramammary masses and body condition score

Prior to the study onset researchers were trained on detecting intramammary masses (IMM) and on measuring body condition score (BCS). Every adult ewe was inspected at each visit, except when occasionally ewes escaped the holding pen and they were not examined. Ewes were held upright in a clamp and the udder palpated by one of two additional trained researchers (Kate Bamford with Kate Lewis or Naomi Prosser). Researchers examined ewes in batches of 50, with one carrying out measurements whilst the other recorded data, swapping roles at the end of each batch. At each visit the BSC (1-5 in 0.5 increments; Defra PB1875) and the presence of IMM were recorded. IMM were defined as a physically detectable mass of a different consistency to the rest of the mammary tissue, as described in Grant et al. (2016). Approximate size was recorded using fruit as a comparable scale; grape (<1 cm), plum (1-3 cm), kiwi (>3-5 cm), apple (>5-7 cm), bramley (>7-10 cm), melon (>10 cm). Additionally, the gland was scored as ‘firm’ if the entire gland was firm to touch; and ‘unsure’ if the researcher was unsure whether abnormal masses were large supramammary lymph nodes.

2.2.3.2. Lamb birth, rearing and weight data collection

Within 48 hours of birth lambs were identified with a numbered ear-tag, linked to their dam, and their weight and sex were recorded. The usual management of the study farm was to keep all ewes with no more than two lambs. When lambs were born as triplets, one lamb was removed and brought up as an orphan lamb with an automatic milk feeder. Occasionally a triplet was fostered to a ewe with a single

lamb to create a twin. In these cases the foster lamb was linked to the ewe soon after birth using the handheld data-logger to capture the ewe's EID. Lambs within the 'orphan lamb' group were not included in the analysis.

Weight was measured using a 10kg electronic scale using the animal weight setting where 16 measurements were taken over a 3 second period and averaged (KERN, HDB 10K10N). Lambs that died before being weighed were recorded as stillborn or live born but died. At the beginning of April, when lambs were approximately 2 weeks of age, 206 lambs from the control group and 150 lambs from the IMM group were weighed using a 50kg hanging scale (KERN CH 50K50) using the animal weight setting as above. Lambs were then weighed using a weigh crate at approximately 6, 8, 12 weeks of age, at weaning and then at least once a month until slaughter.

Lambs were weaned in mid-June (18/06/2019), when they were approximately 90 days old (mean: 89.89 days, range: 73 - 101 days). They were sent to market in batches when finished, selected by the farmer and the first batch was sent on the day of weaning. Data from lambs sent to market was collected until 30/12/2019, when 802 (87%) of lambs had been sent. The mean age at slaughter was 174 days (age range: 74 - 290 days).

2.2.3.3. Acute mastitis data

Cases of acute mastitis were recorded when the ewe had any of the clinical signs of disease, including abnormal milk, hot or cold mammary tissue and visible swelling of the tissue. Cases were recorded by researchers when they were on farm and by the farmer at other times when a case of acute mastitis was treated.

2.2.3.4. Milk for somatic cell count data

Milk samples were collected from 185 ewes from both groups on one occasion in mid-April 2019 when ewes were in their 3rd - 6th week of lactation. The number of ewes required for the somatic cell count (SCC) study was calculated using mean and standard deviation SCC from a previous sheep milk study (Mavrogenis et al. 1995). The authors reported that a change of 0.5 x 10^6 cells/ml resulted in a mean daily reduction of milk by 18g, and so this change was assumed to be biologically relevant for the purposes of sample size calculation. Using the figures from Mavrogenis et al. (1995), a sample size calculation for comparing two independent means was carried out using the online calculator Statulator (Dhand and Khatkar).

In order to detect at least a difference of 0.5 x 10^6 cells/ml at 5% significant with a power of 80% taking into account the distribution of ewes across the two groups, 74 ewes from the control group and 51 ewes from the IMM were required. Milk samples were collected from each udder half from the first 108 control ewes and first 77 IMM ewes entering the race.

SCC samples were couriered directly to an external laboratory (QMMS Ltd., Somerset, UK) for analysis using an automated flow cytometer and spectrometer.

2.2.3.5. Udder conformation scoring

In May 2019, when ewes were approximately 6 weeks postpartum, udder conformation data was scored on 282 ewes to investigate udder health in the context of IMM and to account for potential confounders. This included teat position, teat angle, udder drop, degree of separation of udder halves and teat size. The linear scoring system from Cooper et al. (2013) was used, which was originally developed from Casu et al. (2006). Sheep were examined whilst upright in a clamp, and the diagrams in Figure 2-1 were used to provide a discrete value between 1-9 for teat angle, degree of separation and udder drop, and between 1-7 for teat position. Additionally, the length and width of the left teat was measured in 0.5cm increments using a ruler.

At the same examination, all ewes (570 ewes) were scored on the additional traits udder symmetry, presence of udder lesions and presence of teat lesions. Lesions were recorded as traumatic when there was evidence of broken skin, for example where a lamb had bitten a teat, or non-traumatic in the case of warts, spots or orf-like lesions (Grant et al., 2016).

2.2.4. Intervention design

Ewes were examined monthly (section 2.2.3.1) for IMM presence. Ewes with manually detectable IMM were separated from the rest of the flock and managed separately. Ewes with manually detectable IMM entered the IMM group at the end of the monthly visit where IMM was first detected. Once placed in the IMM group, ewes remained in the IMM group until the end of the study, regardless of the result of future examinations. The two groups were kept physically separate in different areas of the farm including during the lambing period. Other farm husbandry management, such as feeding supplementary feed, was kept as similar as possible between the two groups and was carried out as usual by the farmer.

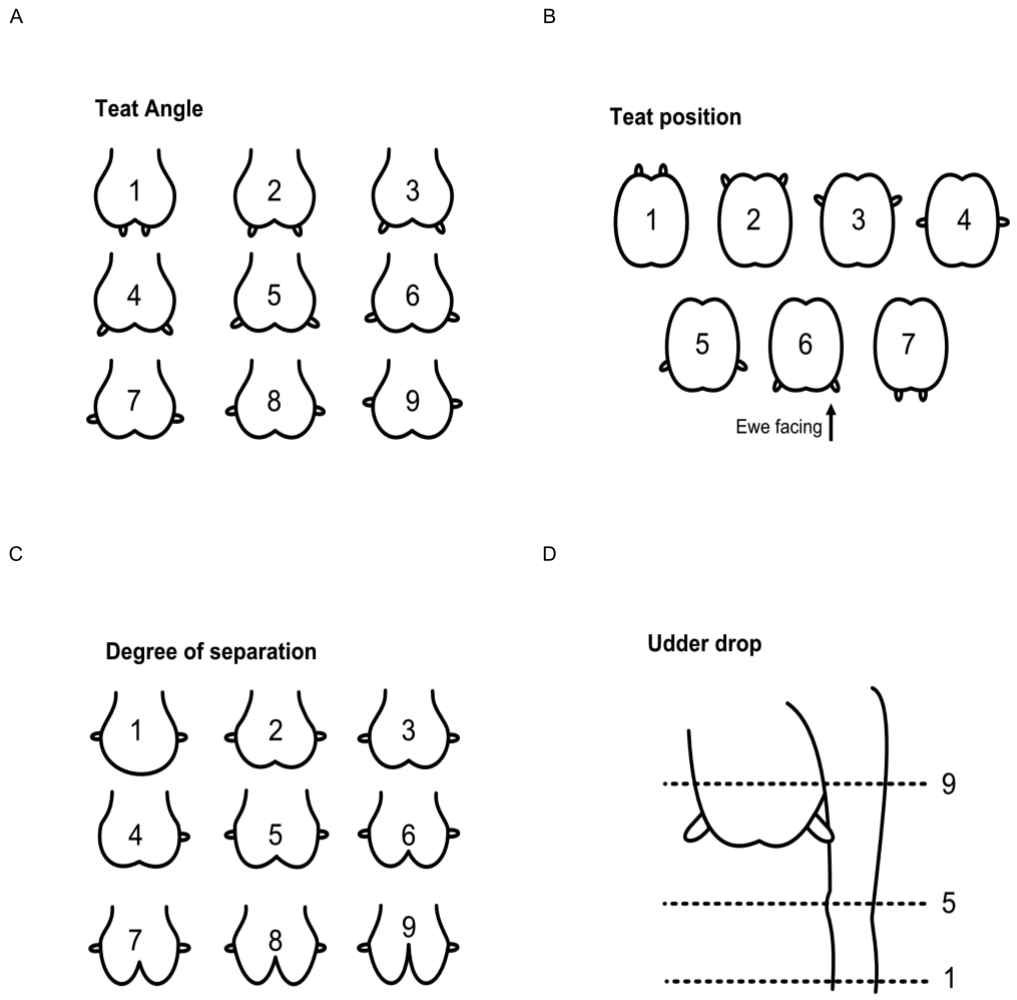


Figure 2-1: Scores used to measure a) teat angle, b) teat position c) degree of separation between the two halves of the udder d) udder drop (Casu et al., 2006)

2.2.5. Data analysis

All statistics were carried out in R studio version 1.2.5 using R version 4.0.2 (2020-06-22).

2.2.5.1. Statistical analysis of intramammary masses and acute mastitis data

Data management was done using the R package ‘dplyr’ (Wickham et al., 2020). Date of examination was subtracted from lamb birth dates in order to create time in days since birth. Months since birth were created by dividing number of days by 28. Date manipulation used the R package ‘lubridate’ (Grolemund and Wickham, 2011).

Associations between categorical variables were tested using Fisher's Exact Test for count data. For variables with more than two categories, a significant association between any category was considered a significant association between variables.

Where the number of observations in variable categories was less than 5, categories were combined (Table 2-2).

Table 2-2: Combined categories in categorical explanatory variables

Variables	Categories	Categories used in analysis
IMM Size	Grape, Plum	Small
	Kiwi, Apple	Medium
	Bramley, Melon	Large
Body condition score	< 2.5	Thin
	2.5 - 3.5	Healthy
	> 3.5	Fat
Teat Position	1, 2	1 - 2
	3	3
	4, 5, 6, 7	4 - 7
Teat Angle	1, 2, 3	1 - 3
	4	4
	5	5
	6	6
	7, 8, 9	7 - 9
Udder Drop	2, 3, 4	2 - 4
	5, 6	5 - 6
	7, 8	7 - 8
Degree of Separation	1, 2, 3	1 - 3
	4, 5, 6	4 - 6
	7,8,9	7 - 9
Teat Length	1 - 4 in 0.5cm increments	1, 2, 3, 4 (rounded to the nearest cm)
Teat Width	1 - 3 in 0.5cm increments	1, 2, 3 (rounded to the nearest cm)

IMM: intramammary mass

Table 2-3 Explanatory variables used in regression models in Chapter 2

Variables	Regression models
Month centred on month of lambing (-8 to 6)	a
Month centred on month of lambing (1 – 6)	b
Body condition score (BCS)	a, b
Previous month BCS	a, b
Average BSC before lambing	c
Average BSC after lambing	c
AM incidence at any point during the study (ewe level: either udder half)	a, b, d
AM incidence at any point during the study (at udder half level)	d
Ewe age	a, b, c, d
Litter size	d
IMM incidence at any point during the study	c
Previous month IMM	a, b
Previous month IMM and size	a, b
IMM ever before lambing	c
IMM ever after lambing	c
Biggest size of IMM ever before lambing	b, c
Biggest size of IMM ever after lambing	c
Group at lambing (i.e. IMM group had at least one IMM before lambing)	d
Group in September (i.e. IMM group had at least one IMM before September)	d
Future IMM (at udder half level)	d
Previous IMM (at udder half level)	d
Lactation week	d
Teat position	a, b, c
Teat angle	a, b, c
Udder drop	a, b, c
Degree of separation of udder halves	a, b, c
Teat Length	a, b, c
Teat Width	a, b, c
Symmetry	a, b, c
Udder lesion	a, b, c
Traumatic teat lesion	a, b, c
Non-traumatic teat lesion	a, b, c

IMM: intramammary mass

a: Binomial logistic mixed effect model of factors associated with intramammary masses over the study period; b: binomial logistic mixed effect model of factors associated with intramammary masses detected after lambing; c: binomial logistic mixed effect model of factors associated with acute mastitis; d: linear mixed model of $\log_{10}$ somatic cell counts in 354 udder halves.

All variables are at the ewe level unless otherwise stated. At the ewe level the maximum size of IMM in either udder half was used as the variable and an incidence of AM in either udder half was considered positive for AM.

2.2.5.2. Binomial mixed effect model of factors associated with intramammary masses

Data on IMM were analysed using binomial logistic mixed effect models. Two models were constructed, one with presence of at least one IMM at any time in the 12-month study as the response variable and a second with presence of at least one IMM in the 6 months following lambing as the response variable. Ewe and observation (visit) were set as random effects to account for repeated measures in the data. Udder half data were combined into a single observation of a ewe at visit. Where a ewe had an IMM on each udder half the larger IMM was used to classify the ewe (Table 2-2). Where only one udder half had an IMM that udder was used to classify the ewe.

All explanatory variables were tested in univariable models (Table 2-3) and variables significant at p<0.1 were taken forward for model selection. The final multivariable model was selected using a manual forward selection process (Dohoo et al., 2003). Variables were retained in the model when they were significant to p<0.05 and resulted in a lower akaike information criterion (AIC). Where there were highly correlated variables, the variable with the most biologically plausibility was retained. Models were constructed using the 'glmer' function from the package lme4 (Bates et al., 2015). Models took the form:

$$Logit(\Pi_{ij}) = \beta_0 + \beta x_j + \beta x_{ij} + u_j$$

where $Logit(\Pi_{ij})$ is the the log odds of the probability that IMM is present, β_0 is the constant, and βx_{ij} is a series of fixed effects varying at i (month) and j (ewe). Residual variance estimates were included at ewe (u_j). All non-significant variables were retested in the final model to investigate residual confounding (Cox and Wermuth, 1996).

2.2.5.3. Binomial model of factors associated with acute mastitis

Data on acute mastitis presence were analysed using binomial logistic models. Ewes were acute mastitis positive if any case of acute mastitis was recorded in the study year even if before the explanatory event. Again, udder half data were combined into one observation per ewe. The explanatory variables IMM before lambing, IMM after lambing, biggest size before lambing and biggest size after lambing were created to describe IMM presence. Models were constructed as described in section 2.2.5.2.

2.2.5.4. Statistical analysis of somatic cell count (SCC) data

SCCs were merged with sheep data using the R statistical package 'dplyr' (Wickham et al., 2020). SCCs were log transformed using the common logarithm in order to normalise the data. A linear mixed effect model was constructed for the outcome of $\log_{10}$ SCC with ewe as a random effect to account for lack of independence of milk samples being from each udder half of each ewe. All explanatory variables (Table 2-3) were tested in univariable models as fixed effects and variables significant at $p<0.1$ were taken forward for model selection. The final multivariable model was selected using a manual forward selection process (Dohoo et al., 2003). Variables were retained in the model when they were significant to $p<0.05$ and resulted in a lower akaike information criterion (AIC). Where correlated variables caused poor model fit, the variable with the most biologically plausibility was retained. Models were constructed using the 'lmer' function from the package lme4 (Bates et al., 2015). Model fit was tested by examination of residual plots. The model took the form:

$$y_{ij} = \beta_0 + \beta_1 x_{ij} + u_{kj} + e_i$$

where y_i was the continuous outcome variable of $\log_{10}$ common logarithm of SCC (cells/ml) from an udder half j, in ewe i. β_0 was the intercept, and βx_{ij} was a series of fixed effects varying at ij (ewe) and j (udder half). u_j and e_{ij} are the residual variance estimates. All non-significant variables were retested in the final model to investigate residual confounding (Cox and Wermuth, 1996).

Estimated marginal means (EMMs) (also known as least-squares means) were calculated using the final model to investigate the change in SCC, as any significant increase in SCC was considered important to ewe health. The R package 'emmeans' was used to provide predictions of the EMMs and their back-transformed geometric means (Lenth, 2020).

2.3. Results

2.3.1. Descriptive analysis

There were 776 ewes examined throughout the study period. 206 ewes did not have a lamb, due to culling, death, or not being in lamb following tupping. All ewes that had a lamb were retained in the data set, regardless of the number of observations. There were 5759 observations from 570 ewes during the study, with observations per ewe ranging from 1 - 11.

Before lambing began in March 2019 there were 183 (32.1%) ewes in the intramammary mass (IMM) group. These ewes were significantly older than the ewes in the control group: 31.1% were over 7 years old, compared to 16.3% in the control group ($p < 0.001$). The number of lambs born and the BCS were not significantly different between the two groups. (Table 2-4)

Throughout the study, the majority of ewes had a 'healthy' body condition score between 2.5 and 3.5, regardless of group or month (Figure 2-2).

Table 2-4: Categorical variables for ewes examined in March 2019 separated by group during lambing

Variable		Control N (%)	IMM N (%)	Total N (%)	p
Age	1-3	71 (18.3)	25 (13.7)	96 (16.8)	0.0003
	4-6	253 (65.4)	101 (55.2)	354 (62.1)	
	7+	63 (16.3)	57 (31.1)	120 (21.1)	
Number of lambs	1	92 (23.8)	44 (24.0)	136 (23.9)	0.778
	2	235 (60.7)	115 (62.8)	350 (61.4)	
	3	60 (15.5)	24 (13.1)	84 (14.7)	
BCS	Fat	8 (2.1)	7 (3.8)	15 (2.6)	0.072
	Healthy	305 (78.8)	129 (70.5)	434 (76.1)	
	Thin	74 (19.1)	47 (25.7)	121 (21.2)	
Teat Position	1-2	40 (36.0)	51 (29.8)	91 (32.3)	0.333
	3	44 (39.6)	65 (38.0)	109 (38.7)	
	4-7	27 (24.3)	55 (32.2)	82 (29.1)	
Teat Angle	1-3	23 (20.7)	29 (17.0)	52 (18.4)	0.006
	4	27 (24.3)	21 (12.3)	48 (17.0)	
	5	15 (13.5)	27 (15.8)	42 (14.9)	
	6	22 (19.8)	26 (15.2)	48 (17.0)	
	7-9	24 (21.6)	68 (39.8)	92 (32.6)	
Udder Drop	2-4	9 (8.1)	10 (5.8)	19 (6.7)	0.751
	5-6	69 (62.2)	109 (63.7)	178 (63.1)	

Variable		Control N (%)	IMM N (%)	Total N (%)	p
	7-8	33 (29.7)	52 (30.4)	85 (30.1)	
Degree of Separation	1-3	72 (64.9)	115 (68.0)	187 (66.8)	0.599
	4-6	36 (32.4)	52 (30.8)	88 (31.4)	
	7-9	3 (2.7)	2 (1.2)	5 (1.8)	
Teat Length	1	33 (29.7)	32 (18.8)	65 (23.1)	0.045
	2	55 (49.5)	95 (55.9)	150 (53.4)	
	3	20 (18.0)	28 (16.5)	48 (17.1)	
	4	3 (2.7)	15 (8.8)	18 (6.4)	
Teat Width	1	39 (35.1)	65 (38.7)	104 (37.3)	0.316
	2	66 (59.5)	87 (51.8)	153 (54.8)	
	3	6 (5.4)	16 (9.5)	22 (7.9)	
Symmetry	Symmetrical	291 (81.5)	102 (59.6)	393 (74.4)	<0.001
	Asymmetrical	66 (18.5)	69 (40.4)	135 (25.6)	
Udder Lesion	No	109 (98.2)	166 (97.1)	275 (97.5)	0.708
	Yes	2 (1.8)	5 (2.9)	7 (2.5)	
Traumatic Teat Lesion	No	338 (94.7)	156 (91.2)	494 (93.6)	0.134
	Yes	19 (5.3)	15 (8.8)	34 (6.4)	
Non-traumatic Teat Lesion	No	283 (79.3)	127 (74.3)	410 (77.7)	0.220
	Yes	74 (20.7)	44 (25.7)	118 (22.3)	

p: Fisher's exact test; IMM: intramammary mass; BCS: body condition score

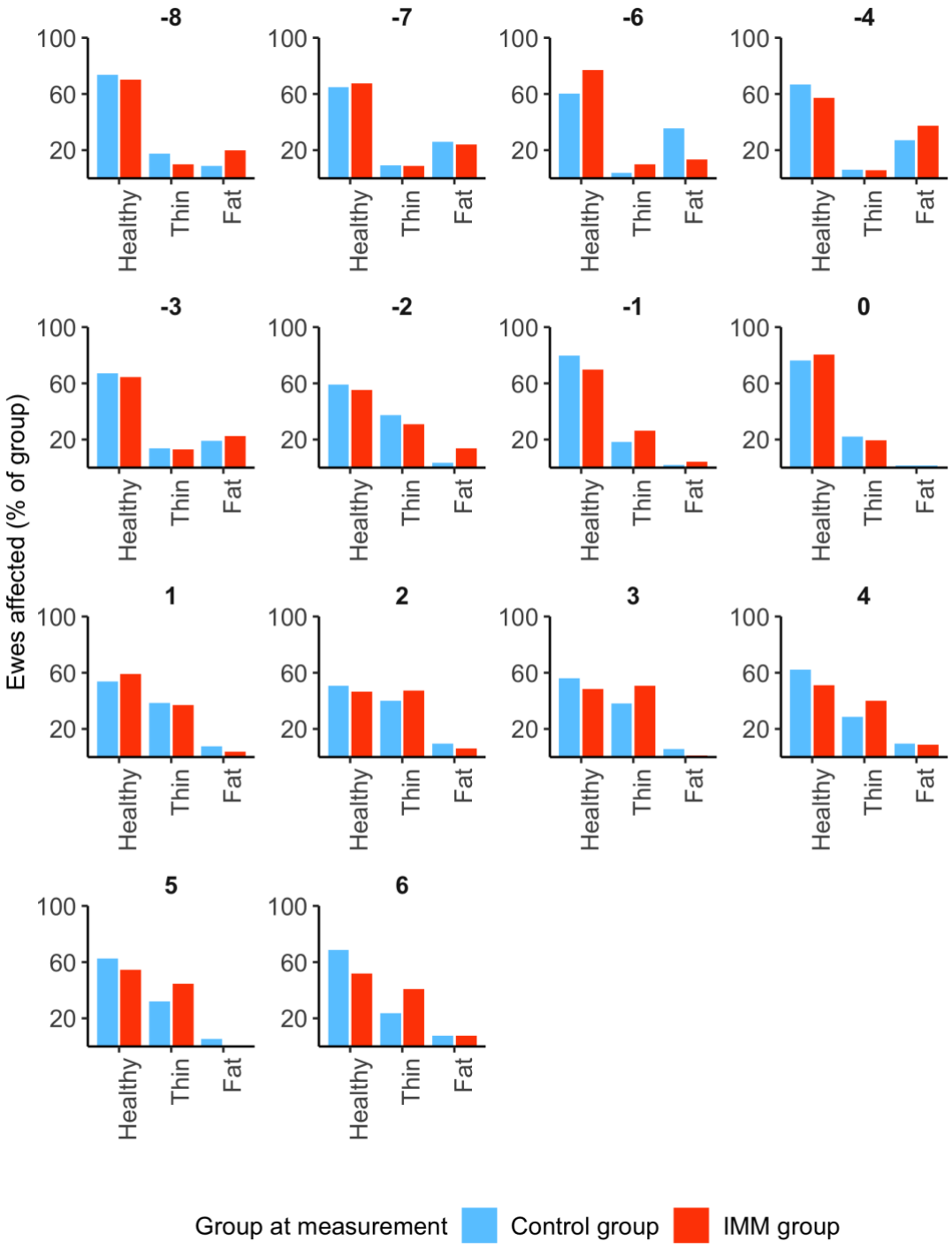


Figure 2-2: Body condition score over the study by group at examination centred by lambing date. At each examination, ewes were given a body condition score (BCS) 0-5. BCS <2.5 : Thin; BCS between 2.5 - 3.5 : Healthy; >3.5 : Fat. Numbers above each graph represent the month in relation to lambing date

2.3.2. Chronic intramammary mass results

2.3.2.1. Number of ewes in the control group and in the IMM group

In the first examination month 98 ewes (18.4 %) had an IMM and were placed into the separate IMM group. The second examination had the second highest number of ewes (47) being moved into the IMM group. The three months prior to lambing had low numbers of ewes with IMM being detected for the first time, where between 0 - 5 ewes were moved. The examinations after lambing in May, June and August had nearly 5 % ewes moving into the isolated IMM group (21 - 24 ewes). There were 7 ewes with an IMM detected for the first time in July and 10 in August. Total number of ewes decreased due to ewe deaths and culling (Table 2-5).

Table 2-5: Number of ewes in the control group and in the IMM group

Month of examination	Control Group	IMM detected for the first time	IMM group	Total number ewes examined
Sep 18	436 (81.6%)	98 (18.4%)	0 (0.0%)	534
Oct 18	408 (74.2%)	47 (8.5%)	95 (17.3%)	550
Dec 18	383 (69.1%)	29 (5.2%)	142 (25.6%)	554
Jan 19	382 (68.7%)	5 (0.9%)	169 (30.4%)	556
Feb 19	372 (67.8%)	2 (0.4%)	175 (31.9%)	549
Mar 19	386 (68.0%)	0 (0.0%)	182 (32.0%)	568
May 19	335 (63.4%)	22 (4.2%)	171 (32.4%)	528
Jun 19	287 (56.9%)	21 (4.2%)	196 (38.9%)	504
Jul 19	276 (55.1%)	7 (1.4%)	218 (43.5%)	501
Aug 19	247 (50.3%)	24 (4.9%)	220 (44.8%)	491
Sep 19	210 (49.5%)	10 (2.4%)	204 (48.1%)	424

IMM: intramammary mass
Gives the numbers and proportions of ewes in each of the groups for each month of examination. For example: in October 2018 there were 550 ewes examined. Of these, 408 (74.2%) had no IMM and remained in the control group, 47 (8.5%) were moved into the IMM group due to detection of an IMM, and 95 (17.3%) were already in the IMM group. Once ewes had been moved into the IMM group they did not return to the control group regardless of their future IMM status.

2.3.2.2. Incidence of intramammary masses

The majority of ewes (92%) were first examined 7 months prior to their lambing date. The only definite new IMM at 7 months were in 4/44 ewes that were first examined 8 months prior to their lambing date. For all other ewes, 7 months before lambing was the first examination and therefore it is impossible to tell if the IMM was new or existing in that month. All ewes were examined 6 months before lambing, and new cases of IMM were detected in 43/432 (10%) of the control flock. The number of new cases of IMM decreased as pregnancy progressed, until no new

cases were detected the same month as lambing. New cases increased again after lambing; ranging between 3.3% 4 months after lambing, where there was a small dip in new cases, and 8.4% 5 months after lambing (Figure 2-3).

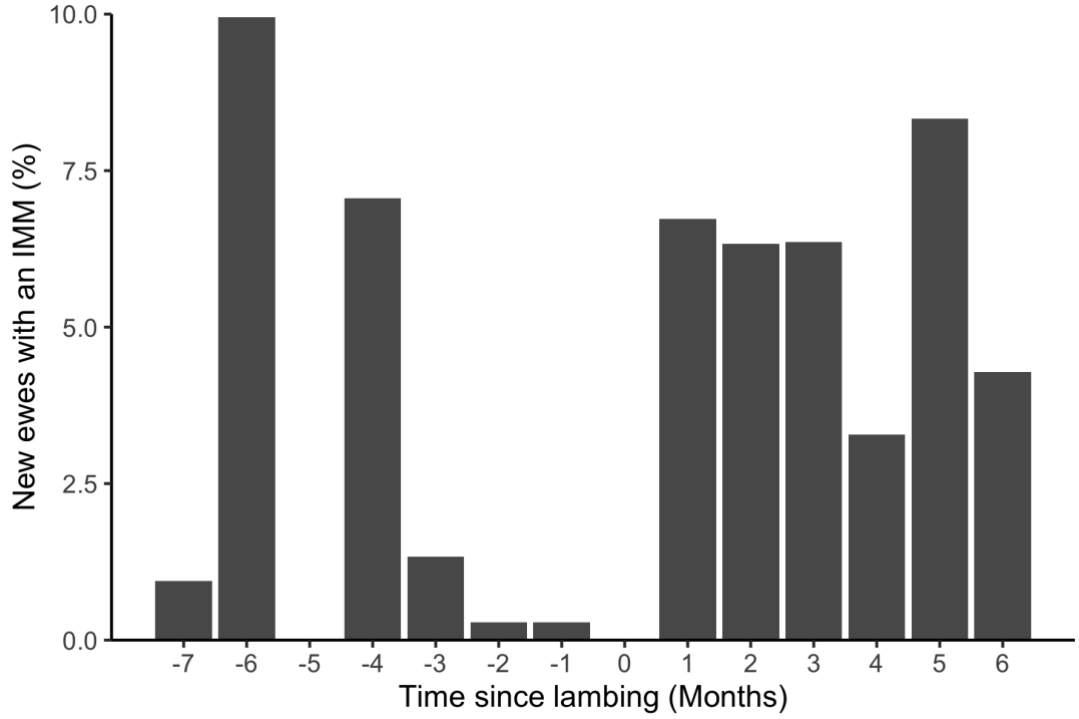


Figure 2-3: IMM incidence centred by lambing date.

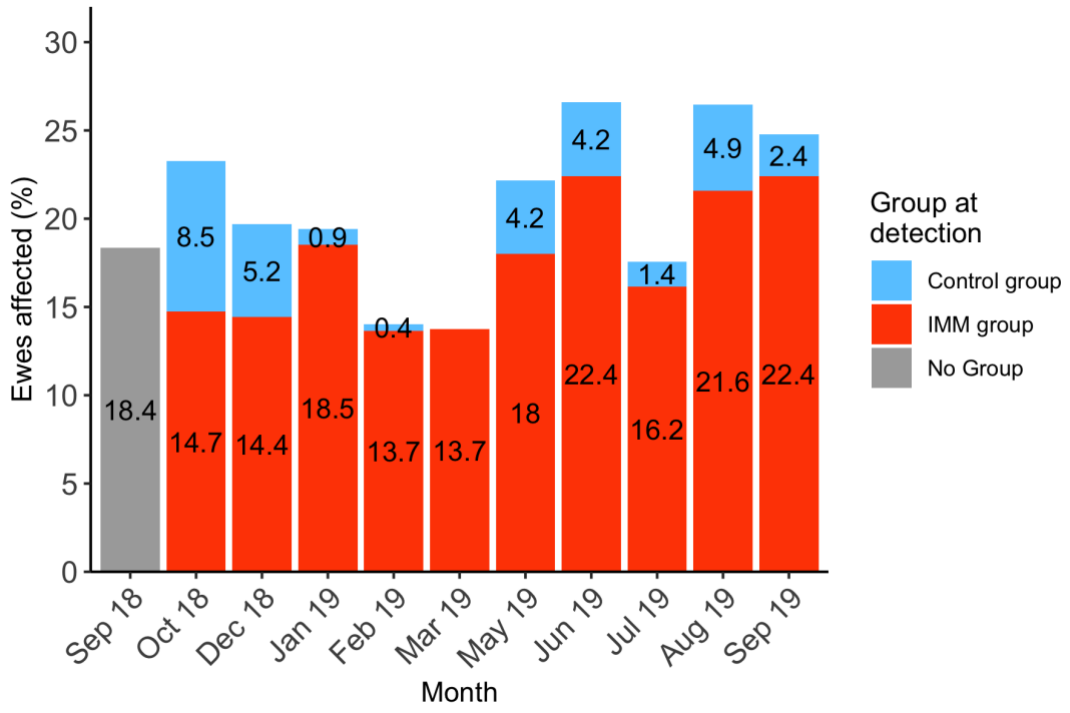


Figure 2-4: Proportion of ewes with IMM by group at detection. All ewes entered the study with no group (grey), and subsequently were moved into the IMM group if an IMM was detected.

2.3.2.3. Prevalence of intramammary masses during the study year

The period prevalence of intramammary masses (IMM), where a ewe had at least one detected IMM over the study (September 2018 - September 2019), was 48%. IMM prevalence during lactation was 35%.

Monthly prevalence of IMM ranged between 14% and 26%, with the highest proportion in June and August and the lowest in February and March (Figure 2-4). Ten sheep (1.75%) always had an IMM and 298 sheep (52%) never had an IMM, and of those with a detected IMM, only having an IMM for one observation was the most common category (Table 2-6). Details of IMM occurrence by individual ewe over the course of the study are shown in Appendix Figure A-1 – Figure A-5.

Table 2-6: Number of observations recorded IMM positive over the study period

Number of observations IMM positive	N Sheep	%
0	298	52.30
1	60	10.50
2	30	5.26
3	29	5.09
4	32	5.61
5 - 10 observations	111	19.50
All observations (9 - 11)	10	1.75

Total number of observations ranged between 1 - 11 for each ewe as not every ewe was examined every occasion; 5 - 10 observations category does not include sheep IMM positive in all observations

The prevalence of IMM was lowest in the same month as lambing (9.7% ewes examined in the month of lambing had a detectable IMM), and the two months prior to lambing (14% in both). The month after lambing has the highest prevalence of IMM, peaking at 28%, including 4% of the flock that had an IMM detected for the first time. The prevalence of IMM remains high for the remainder of the study, between 18% and 25% of the flock were affected from 2 months to 6 months after their lambing date (Figure 2-5).

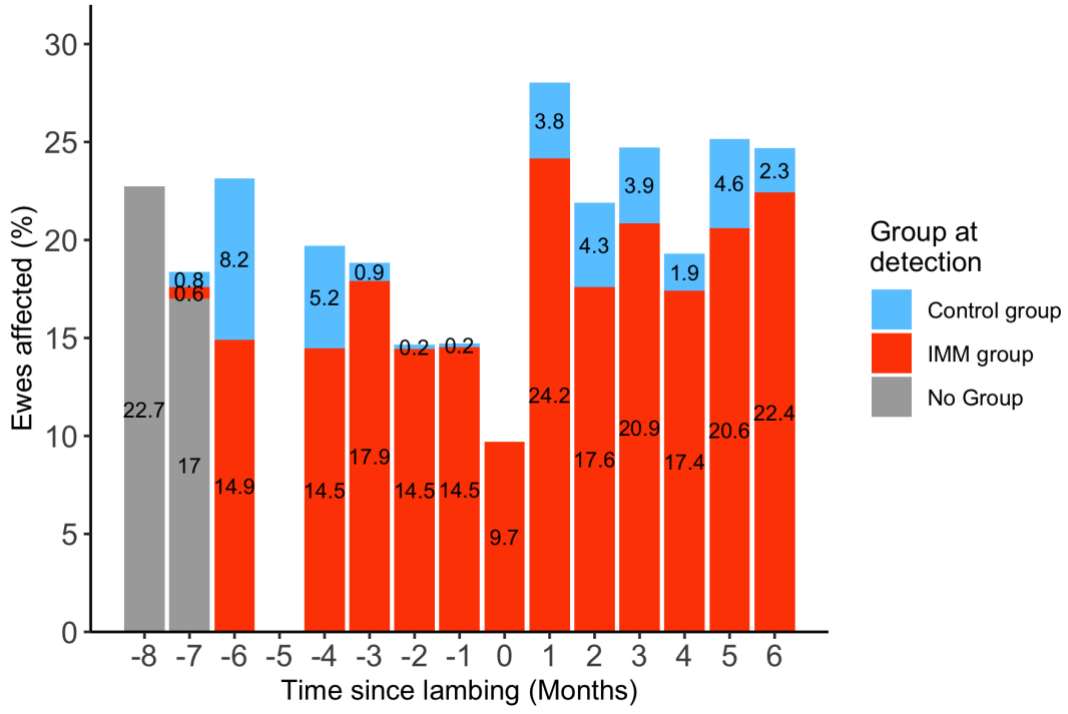


Figure 2-5: Proportion of ewes with an IMM at the monthly examination centred by lambing date.

2.3.2.4. Intramammary mass classification

At each observation IMM were classified based on size using a fruit scale. Where a ewe had an IMM in both udder halves, the larger IMM was counted. Every month the majority of ewes had a small IMM, recorded as ‘grape’ or ‘plum’ size. From 8 months before lambing to 2 months before lambing, over 75% of ewes had an IMM of one of these two sizes. This decreased the month before lambing to 65%; where there was an increase in the proportion of ewes with an IMM classified as ‘kiwi’ or ‘apple’, and a smaller proportion classified ‘grape’. The largest IMM on the scale; ‘bramley’ and ‘melon’ are uncommon throughout, accounting for between 1% - 5% of recorded IMM. (Figure 2-6)

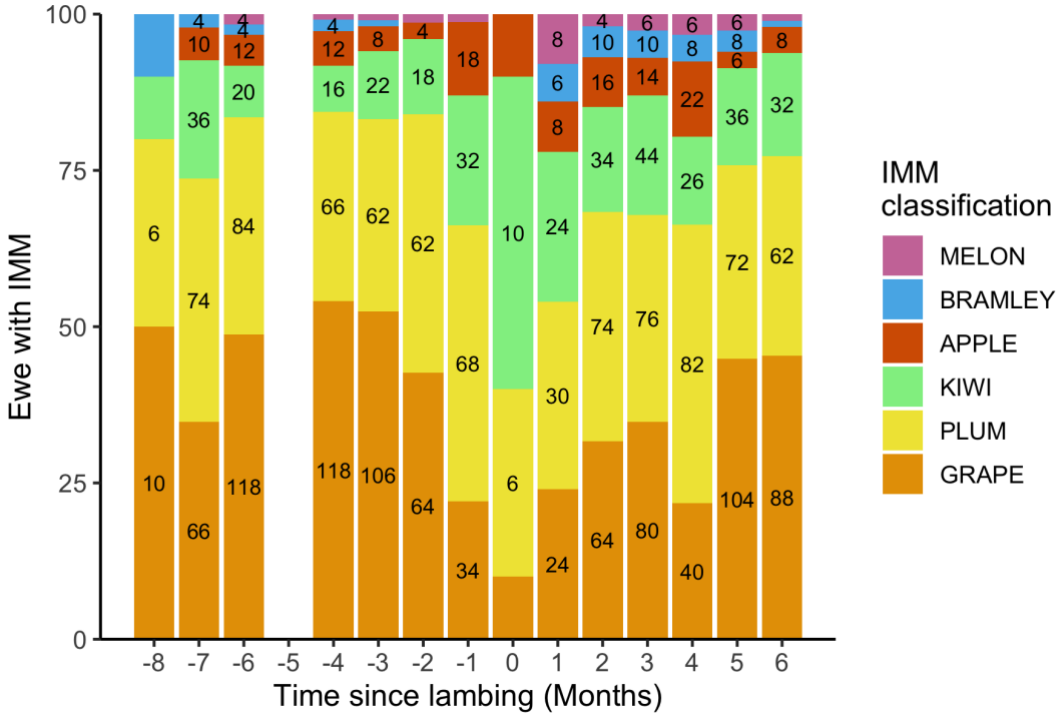


Figure 2-6: Relative proportion of IMM by size, with number of ewes in each group labelled within bars. Counts under 3 not printed

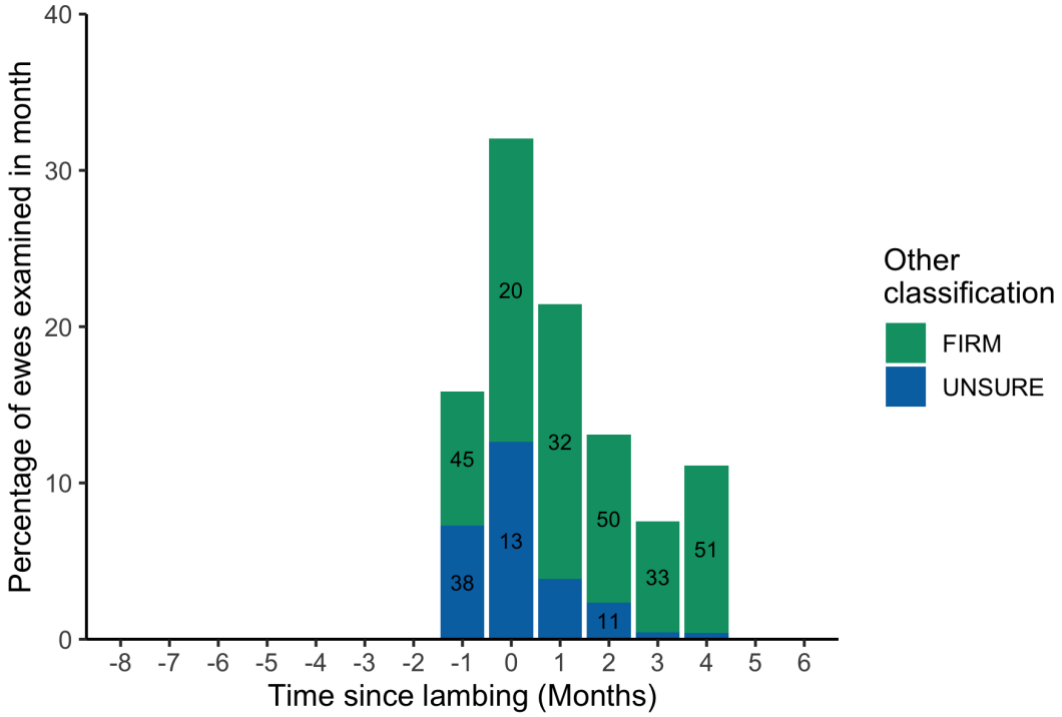


Figure 2-7: Percentage of ewes with one or both udder halves classified as firm or unsure, with number of ewes in each group labelled within bars

Of those ewes without a detectable IMM, the month before lambing until 4 months after lambing there were a number of ewes where the udder was ‘firm’, when the mammary gland was too large to detect if a IMM was present, presumed due to lactation. This affected between 7% to 20% of ewes. Another classification, ‘unsure’ was also used when the researcher was not sufficiently sure that the udder contained an IMM. These measurements accounted for 3% of all observations over this period. (Figure 2-7)

2.3.2.5. Associations and correlations between explanatory variables

Associations between variables are shown in Table 2-7. The udder conformation variables teat position, teat angle, teat length, and teat width, udder drop, degree of udder half separation and udder symmetry were all significantly associated. Udder lesions were associated with traumatic teat lesions, and traumatic teat lesions were associated with non-traumatic teat lesions. Age was significantly associated with all other variables.

Table 2-7: Associations between explanatory variables in the IMM data set

	IMM	Age	AM	IMM previous month	IMM size previous month	BCS	BCS month before	Teat position	Teat angle	Udder Drop	Separation	Teat length	Teat width	Symmetry	Udder lesion	Traumatic teat lesion	Non-traumatic teat lesion
IMM		*	*	*	*	*	*		*	*		*	*	*	*	*	*
Age			*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
AM				*	*			*	*	*	*		*	*		*	*
IMM previous month					*				*	*		*	*	*	*	*	*
IMM size previous month							*	*	*	*	*	*	*	*	*	*	*
BCS							*		*	*	*	*				*	*
BCS month before									*	*		*				*	*
Teat position									*	*	*	*	*	*	*	*	*
Teat angle										*	*	*	*	*	*	*	*
Udder Drop											*	*	*	*	*	*	
Separation												*	*	*	*	*	*
Teat length													*	*	*	*	*
Teat width														*	*	*	*
Symmetry																*	*
Udder lesion																*	
Traumatic teat lesion																	*
Non-traumatic teat lesion																	

All associations were tested using Fisher's Exact Test for Count Data with simulated p-value (based on 2000 replicates); * indicates a significant test result.

2.3.2.6. Binomial logistic mixed effects model of factors associated with intramammary mass over the study period.

Results of the univariable binomial mixed effects models are shown in Table 2-8. Interaction terms were also tested and no significance found (results not shown).

Table 2-8: Univariable binomial logistic mixed effect model of factors associated with intramammary masses over the study period in 570 ewes

Variable	Category	Total N (%)	OR	95% CI
Month - centred on month of lambing	-8	44 (0.8)	Ref	
	-7	517 (9.0)	0.93	0.34 - 2.55
	-6	523 (9.1)	1.53	0.56 - 4.21
	-4	553 (9.6)	1.06	0.39 - 2.9
	-3	536 (9.3)	0.99	0.36 - 2.71
	-2	512 (8.9)	0.54	0.2 - 1.5
	-1	523 (9.1)	0.58	0.21 - 1.6
	0	103 (1.8)	0.29	0.08 - 1.05
	1	182 (3.2)	1.51	0.52 - 4.33
	2	466 (8.1)	1.37	0.5 - 3.75
	3	465 (8.1)	1.61	0.59 - 4.41
	4	477 (8.3)	0.88	0.32 - 2.43
	5	461 (8.0)	1.66	0.6 - 4.55
	6	397 (6.9)	1.63	0.59 - 4.53
BCS	Healthy	3554 (61.7)	Ref	
	Thin	1421 (24.7)	1.22	0.96 - 1.56
	Fat	784 (13.6)	0.85	0.63 - 1.14
AM	0	5179 (89.9)	Ref	
	1	**580 (10.1)**	**7.05**	**3.07 - 16.2**
Age	1-3	968 (16.8)	Ref	
	4-6	3540 (61.5)	1.66	0.79 - 3.48
	7+	**1251 (21.7)**	**5.99**	**2.52 - 14.2**
Previous month IMM	0	4161 (80.2)	Ref	
	1	**1028 (19.8)**	**4.09**	**3.2 - 5.24**
Previous month IMM size	None	4161 (80.2)	Ref	
	Small	**770 (14.8)**	**3.83**	**2.96 - 4.96**
	Medium	**214 (4.1)**	**5.2**	**3.4 - 7.95**
	Large	**42 (0.8)**	**18.44**	**5.56 - 61.14**
Previous month BCS	Fat	736 (14.2)	Ref	
	Healthy	3227 (62.2)	0.92	0.68 - 1.24
	Thin	1226 (23.6)	1	0.68 - 1.48
Teat position	3	1081 (38.6)	Ref	
	1-2	891 (31.8)	1	0.54 - 1.86
	4-7	830 (29.6)	0.96	0.51 - 1.81
Teat angle	5	427 (15.2)	Ref	
	1-3	505 (18.0)	1.54	0.63 - 3.76

Variable	Category	Total N (%)	OR	95% CI
	4	459 (16.4)	0.68	0.27 - 1.71
	6	478 (17.1)	0.8	0.32 - 2
	7-9	933 (33.3)	1.73	0.78 - 3.84
Udder Drop	5-6	1748 (62.4)	Ref	
	2-4	194 (6.9)	0.94	0.33 - 2.69
	7-8	860 (30.7)	0.73	0.41 - 1.3
Separation	4-6	863 (31.0)	Ref	
	1-3	1870 (67.2)	1.16	0.66 - 2.04
	7-9	48 (1.7)	2.7	0.35 - 20.86
Teat length	1	627 (22.5)	Ref	
	2	1504 (53.9)	1.54	0.81 - 2.96
	3	472 (16.9)	1.74	0.76 - 3.97
	4	**188 (6.7)**	**6.9**	**2.21 - 21.56**
Teat width	1	1039 (37.5)	Ref	
	2	1512 (54.6)	1.17	0.67 - 2.03
	3	**218 (7.9)**	**3.19**	**1.15 - 8.8**
Symmetry	Symmetrical	3982 (73.8)	Ref	
	Asymmetric	**1411 (26.2)**	**6.99**	**3.8 - 12.84**
Udder lesion	No	2728 (97.4)	Ref	
	Yes	74 (2.6)	2.56	0.51 - 12.74
Traumatic Teat Lesion	No	5044 (93.5)	Ref	
	Yes	349 (6.5)	2.94	0.98 - 8.8
Non-traumatic Teat Lesion	No	4188 (77.7)	Ref	
	Yes	**1205 (22.3)**	**2.16**	**1.11 - 4.19**

OR: Odds ratio; CI: confidence intervals; N: number of observations; Wald estimates used for CI; Month: centred on lambing date; AM: acute mastitis; IMM: intramammary mass; BCS: body condition score

Following forward model selection, five variables were retained in the final multivariable mixed effect model (Table 2-9). The odds of IMM increased if a ewe had a case of acute mastitis during the study (OR 3.13 95% CI 1.66-5.9). The odds of IMM also increased if an IMM was detected in the previous month, and odds increased with increasing IMM size score. There were 2-fold, 3-fold and 11-fold greater odds of IMM when a ewe had a small, medium or large IMM respectively in the previous month compared to no IMM. Ewes aged 4-6 and over 7 years old both had greater than 2-fold greater odds of IMM Additionally, ewes with long teats (4 cm) or asymmetrical udders also had increased odds of IMM (OR 2.9 95% CI 1.27-6.65; OR 2.8 95% CI 1.86 - 4.22 respectively).

Table 2-9: Multivariable binomial logistic mixed effect model of factors associated with intramammary masses over the study period in 570 ewes

Variable	Category	Total N (%)	OR	95% CI
Intercept			**0.06**	
AM	No	5179 (89.9)	Ref	
	Yes	**580 (10.1)**	**3.13**	**1.66 - 5.9**
Age	1-3	968 (16.8)	Ref	
	4-6	**3540 (61.5)**	**2.15**	**1.27 - 3.63**
	7+	**1251 (21.7)**	**2.87**	**1.59 - 5.21**
Previous month IMM size	None	4161 (80.2)	Ref	
	Small	**770 (14.8)**	**2.66**	**2.02 - 3.5**
	Medium	**214 (4.1)**	**3.39**	**2.17 - 5.3**
	Large	**42 (0.8)**	**11.77**	**3.52 - 39.39**
Teat Length	1	627 (22.5)	Ref	
	2	1504 (53.9)	1.47	0.92 - 2.37
	3	472 (16.9)	1.2	0.65 - 2.2
	4	**188 (6.7)**	**2.9**	**1.27 - 6.65**
Symmetry	Symmetrical	3982 (73.8)	Ref	
	Asymmetric	**1411 (26.2)**	**2.8**	**1.86 - 4.22**
Random effects	*Variance*			
Ewe	1.4502			
Observation	0.1977			

OR: Odds ratio; CI: confidence intervals; N: number of observations; Wald estimates used for CI; AM: acute mastitis; IMM: intramammary mass; Udder conformation variables described in methods

2.3.2.7. Binomial logistic mixed effects model of factors associated with intramammary mass after lambing

A second binomial model was constructed with the outcome IMM after lambing. This uses the same data as the model described in section 2.3.2.6 but was restricted to examinations carried out after lambing. The results of the univariable analysis are in Table 2-10. Interaction terms were also tested and no significance found (results not shown).

Table 2-10: Univariable binomial logistic mixed effect model of factors associated with intramammary masses detected after lambing in 570 ewes

Variable	Category	Total N (%)	OR	95% CI
Month after lambing	1	182 (7.4)	Ref	
	2	466 (19.0)	0.79	0.46 - 1.36
	3	465 (19.0)	1.01	0.59 - 1.73
	4	**477 (19.5)**	**0.57**	**0.33 - 0.98**
	5	461 (18.8)	1.04	0.61 - 1.77
	6	397 (16.2)	1.06	0.61 - 1.83
BCS	Healthy	1366 (55.8)	Ref	
	Thin	925 (37.8)	1.35	0.97 - 1.88
	Fat	**157 (6.4)**	**0.4**	**0.2 - 0.82**
AM	No	2207 (90.2)	Ref	
	Yes	**241 (9.8)**	**6.1**	**2.83 - 13.12**
Age	1-3	420 (17.2)	Ref	
	4-6	1478 (60.4)	1.73	0.87 - 3.42
	7+	**550 (22.5)**	**3.93**	**1.78 - 8.68**
Previous month IMM	No	1938 (79.2)	Ref	
	Yes	**508 (20.8)**	**3.67**	**2.4 - 5.62**
Previous IMM month size	None	1938 (79.3)	Ref	
	Small	**343 (14.0)**	**3.33**	**2.15 - 5.14**
	Medium	**134 (5.5)**	**5.19**	**2.86 - 9.4**
	Large	**29 (1.2)**	**25.21**	**6.99 - 90.86**
Previous month BCS	Healthy	1482 (60.6)	Ref	
	Thin	**844 (34.5)**	**1.36**	**0.98 - 1.89**
	Fat	**120 (4.9)**	**1.19**	**0.6 - 2.38**
Biggest size before	**No**	**1600 (65.6)**	**Ref**	
	Small	528 (21.6)	18.49	11.1 - 30.79
	Medium	288 (11.8)	42.23	22.42 - 79.55
	Large	23 (0.9)	56.64	8.82 - 363.76
Teat position	3	437 (37.7)	Ref	
	1-2	363 (31.3)	0.9	0.52 - 1.55
	4-7	359 (31.0)	0.67	0.38 - 1.16
Teat angle	5	177 (15.3)	Ref	
	1-3	199 (17.2)	1.71	0.78 - 3.78
	4	186 (16.0)	0.86	0.38 - 1.93
	6	193 (16.7)	0.72	0.32 - 1.63
	7-9	404 (34.9)	1.26	0.63 - 2.54
Udder Drop	5-6	713 (61.5)	Ref	
	2-4	80 (6.9)	1.26	0.51 - 3.13

Variable	Category	Total N (%)	OR	95% CI
	7-8	366 (31.6)	0.75	0.75 - 0.75
Separation	4-6	365 (31.7)	Ref	
	1-3	765 (66.5)	1.2	0.73 - 1.97
	7-9	20 (1.7)	4.28	0.7 - 26.33
Teat length	1	241 (20.9)	Ref	
	2	632 (54.8)	1.15	0.63 - 2.07
	3	198 (17.2)	1.52	0.72 - 3.21
	4	83 (7.2)	2.65	0.97 - 7.26
Teat width	1	433 (37.8)	Ref	
	2	**618 (54.0)**	**1.26**	**1.26 - 1.26**
	3	93 (8.1)	2.02	0.89 - 4.57
Symmetry	Symmetrical	1694 (73.1)	Ref	
	Asymmetric	**623 (26.9)**	**4.22**	**2.42 - 7.37**
Udder lesion	No	1124 (97.0)	Ref	
	Yes	35 (3.0)	3.06	0.77 - 12.16
Traumatic Teat Lesion	No	2168 (93.6)	Ref	
	Yes	**149 (6.4)**	**2.89**	**1.08 - 7.72**
Non-traumatic Teat Lesion	No	1798 (77.6)	Ref	
	Yes	**519 (22.4)**	**1.92**	**1.06 - 3.48**

OR: Odds ratio; CI: confidence intervals; N: number of observations; Wald estimates used for CI; AM: acute mastitis; IMM: intramammary mass; BCS: body condition score; Biggest size before: maximum size recorded before lambing

Five variables were retained in the final model (Table 2-11). Similar to the model of IMM throughout the study, this model found increased odds of IMM if a ewe has a case of acute mastitis and if a ewe has a detected IMM in the previous month. Ewes with a BCS over 3.5 had decreased odds of an IMM in the same month. Ewes with an IMM recorded before lambing, and therefore in the IMM group at lambing, had greater than 10-fold odds of IMM after lambing compared to ewes with no IMM prior to lambing.

Table 2-11: Multivariable binomial logistic mixed effect model of factors associated with intramammary masses detected after lambing in 570 ewes

Variable	Category	Total N (%)	OR	95% CI
Intercept			**0.06**	
Month after lambing	1	182 (7.4)	Ref	
	2	466 (19.0)	0.83	0.49 - 1.41
	3	465 (19.0)	0.89	0.53 - 1.5
	4	**477 (19.5)**	**0.5**	**0.29 - 0.84**
	5	461 (18.8)	0.96	0.57 - 1.61
	6	397 (16.2)	1	0.59 - 1.71
BCS	Healthy	1366 (55.8)	Ref	
	Thin	925 (37.8)	1.1	0.82 - 1.48
	Fat	**157 (6.4)**	**0.5**	**0.26 - 0.99**
AM	0	2207 (90.2)	Ref	
	1	**241 (9.8)**	**2.74**	**1.6 - 4.69**
Previous IMM month size	None	1938 (79.3)	Ref	
	Small	**343 (14.0)**	**1.91**	**1.3 - 2.81**
	Medium	**134 (5.5)**	**2.49**	**1.43 - 4.32**
	Large	**29 (1.2)**	**9.05**	**2.38 - 34.44**
Biggest size before	No	1600 (65.6)	Ref	
	Small	**528 (21.6)**	**10.94**	**6.69 - 17.87**
	Medium	**288 (11.8)**	**17.36**	**9.24 - 32.59**
	Large	**23 (0.9)**	**17**	**3.16 - 91.45**
Random effects	*Variance*			
Ewe	1.4659			
Month	0			

OR: Odds ratio; CI: confidence intervals; N: number of observations; Wald estimates used for CI; AM: acute mastitis; BCS: body condition score; Biggest size before: maximum size recorded before lambing; IMM: intramammary mass

2.3.3. Acute mastitis results

2.3.3.1. Prevalence of clinical acute mastitis cases

During lactation, acute mastitis was observed in 58 ewes (10.3%). There were significantly more ewes with acute mastitis in the IMM group when spilt by group at lambing, 31 / 387 ewes (8 %) had a case of acute mastitis in the control group and 27 / 183 ewes (14.8 %) were in the IMM group (Chi squared test p = 0.019). Between the start of lactation and the recorded case of acute mastitis, 6 ewes moved from the control group into the IMM group.

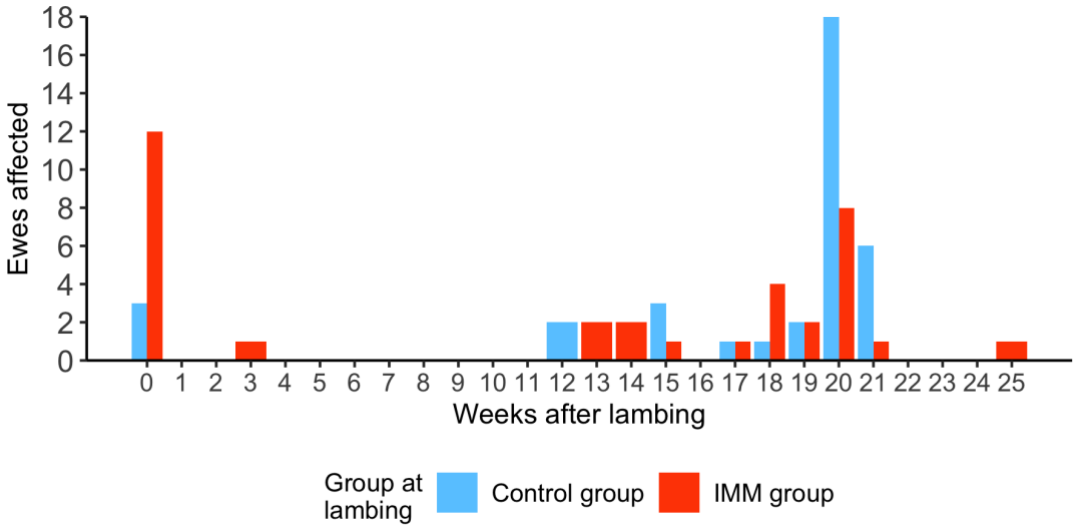


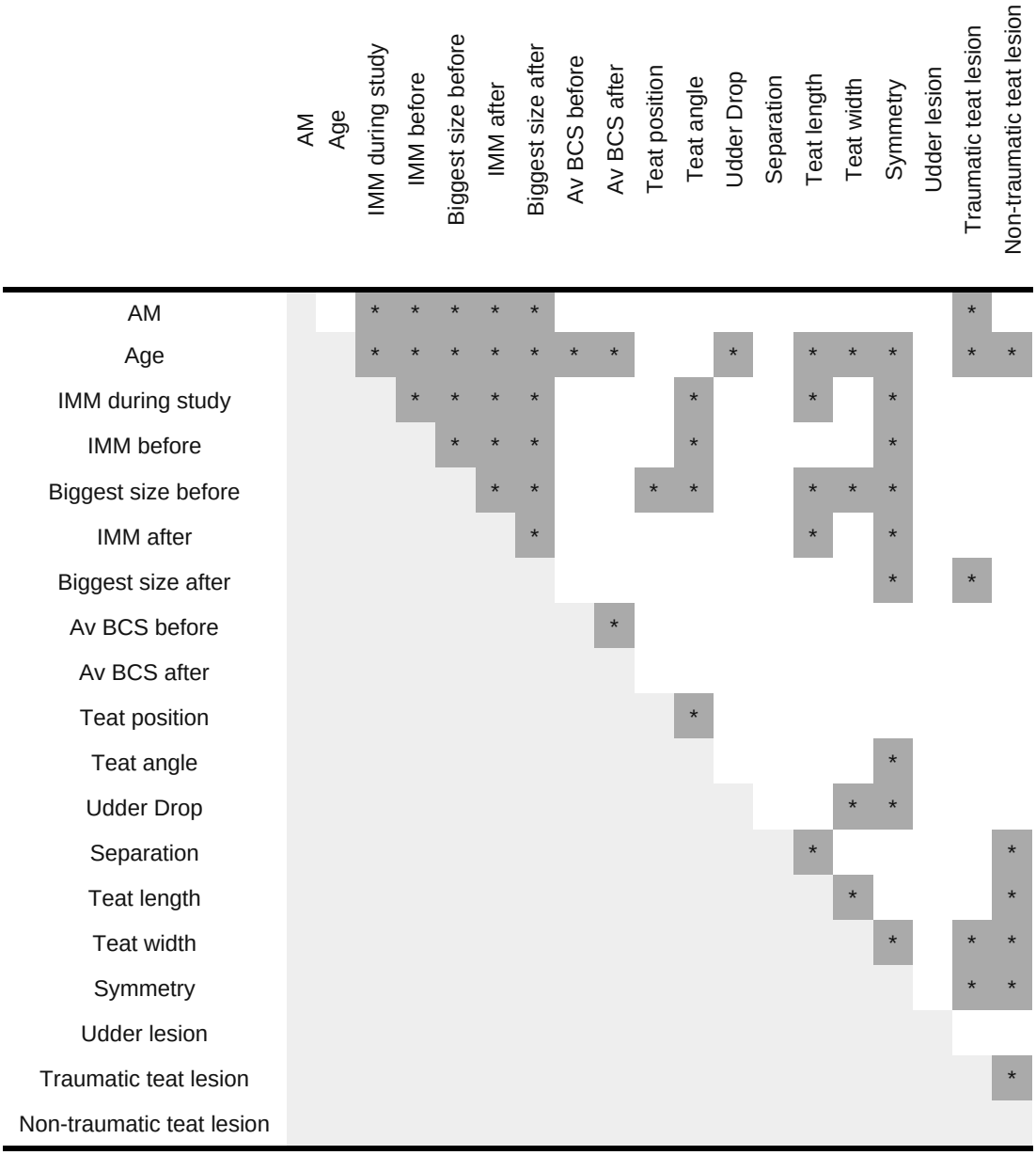
Figure 2-8: Number of ewes with acute mastitis by week following lambing. The size of the peak between 18 - 20 weeks may be inflated as an examination occurred when most ewes were in their 19th and 20th week of lactation

Cases of acute mastitis peak in week 0 and week 20 for both groups, following a biphasic pattern. There are no recorded cases of acute mastitis between weeks 4 and 11 (Figure 2-8). 8% of the control group and 15% of the IMM group at lambing have a case of acute mastitis during lactation.

2.3.3.2. Associations and correlations between explanatory variables

Associations between variables are shown in Table 2-12.

Table 2-12: Variables associated with acute mastitis



All associations were tested using Fisher's Exact Test for Count Data with simulated p-value (based on 2000 replicates); * indicates a significant test result.

2.3.3.3. Binomial logistic model of factors associated with acute mastitis

The results of the univariable analysis are shown in Table 2-13. In univariable models, a ewe had increased odds of acute mastitis if they had a IMM before lambing (OR 1.79 95% CI 1.01-3.16), if they had an IMM after lambing (OR 2.14 95% CI 1.21-3.86) and if they had an IMM at any point in the study (OR 2.92 95% CI 1.64-5.41). These variables are correlated (Table 2-12), and therefore cause multicollinearity in the multivariable model.

Table 2-13: Univariable binomial logistic model of factors associated with acute mastitis in 570 ewes

Variable	Category	Total N (%)	OR	95% CI
Age	1-3	96 (17.1)	Ref	
	4-6	347 (61.7)	1.84	0.81 - 4.98
	7+	119 (21.2)	2	0.77 - 5.84
IMM during study	No	293 (52.1)	Ref	
	Yes	**269 (47.9)**	**2.92**	**1.64 - 5.41**
Average BCS before	Healthy	414 (73.9)	Ref	
	Thin	70 (12.5)	0.66	0.22 - 1.59
	Fat	76 (13.6)	1.31	0.6 - 2.63
Average BCS after	Healthy	414 (73.9)	Ref	
	Thin	70 (12.5)	0.66	0.22 - 1.59
	Fat	76 (13.6)	1.31	0.6 - 2.63
IMM before	No	380 (67.9)	Ref	
	Yes	**180 (32.1)**	**1.99**	**1.14 - 3.44**
IMM after	No	314 (55.9)	Ref	
	Yes	**248 (44.1)**	**2.45**	**1.4 - 4.37**
Biggest size before	No	380 (67.9)	Ref	
	Small	113 (20.2)	1.34	0.64 - 2.64
	Medium	**62 (11.1)**	**2.99**	**1.42 - 5.99**
	Large	**5 (0.9)**	**7.51**	**0.96 - 46.94**
Biggest size after	No	314 (56.2)	Ref	
	Small	141 (25.2)	0.62	0.22 - 1.48
	Medium	**79 (14.1)**	**3.27**	**1.58 - 6.66**
	Large	**25 (4.5)**	**20.93**	**8.52 - 53.86**
Teat position	3	109 (38.7)	Ref	
	1-2	91 (32.3)	1.69	0.68 - 4.33
	4-7	82 (29.1)	0.88	0.28 - 2.54
Teat angle	5	42 (14.9)	Ref	
	1-3	**52 (18.4)**	**7.45**	**1.29 - 141.38**
	4	48 (17.0)	5.86	0.94 - 113.19
	6	48 (17.0)	5.86	0.94 - 113.19
	7-9	92 (32.6)	2.86	0.47 - 54.96
Udder Drop	5-6	178 (63.1)	Ref	
	2-4	**19 (6.7)**	**3.88**	**1.13 - 11.8**
	7-8	85 (30.1)	0.98	0.36 - 2.41
Separation	4-6	88 (31.4)	Ref	
	1-3	187 (66.8)	1.23	0.51 - 3.28
	7-9	**5 (1.8)**	**7.71**	**0.91 - 54.89**
Teat length	1	65 (23.1)	Ref	

Variable	Category	Total N (%)	OR	95% CI
	2	150 (53.4)	1.14	0.41 - 3.68
	3	48 (17.1)	1.71	0.49 - 6.3
	4	18 (6.4)	1.5	0.2 - 7.7
Teat width	1	104 (37.3)	Ref	
	2	153 (54.8)	2.47	0.94 - 7.73
	3	**22 (7.9)**	**4.4**	**1.01 - 18.23**
Symmetry	Symmetrical	393 (74.4)	Ref	
	Asymmetric	**135 (25.6)**	**1.85**	**0.99 - 3.36**
Udder lesion	No	275 (97.5)	Ref	
	Yes	7 (2.5)	1.6	0.08 - 9.85
Traumatic Teat Lesion	No	494 (93.6)	Ref	
	Yes	**34 (6.4)**	**4.6**	**1.98 - 10.06**
Non-traumatic Teat Lesion	No	410 (77.7)	Ref	
	Yes	118 (22.3)	1.36	0.69 - 2.55

SD: standard deviation; OR: odds ratio; CI: confidence intervals; N: number of observations; Wald estimates used for CI; AM: acute mastitis; IMM: intramammary mass; Before: presence before lambing; After: presence after lambing; Biggest size: maximum size recorded in time period; BCS: body condition score;

In the final model four variables were retained (Table 2-14). Of the five IMM variables (IMM during the study, IMM before lambing, IMM after lambing, biggest size before lambing, biggest size after lambing), IMM after lambing was retained. All were significant in the model individually, but not in combination due to multicollinearity. IMM before lambing gave the lowest AIC when combined with the other three retained explanatory variables.

Ewes with IMM before lambing had greater than 3-fold odds of AM. The udder conformation traits udder drop and degree of separation were also significantly associated with AM. Ewes with low hanging udders (score 2 - 4) had greater than 5-fold odds of AM, and ewes with a greater degree of separation between udder halves had increased odds of AM. Finally, ewes with traumatic teat lesions, for example lamb bites, had increased odds of acute mastitis compared to ewes with no traumatic teat lesions.

Table 2-14: Multivariable binomial logistic model of factors associated with acute mastitis in 570 ewes

Variable	Category	Total N (%)	OR	95% CI
Intercept			**0.02**	
IMM before lambing	No	380 (67.9)	Ref	
	Yes	**180 (32.1)**	**3.18**	**1.14 - 10.79**
Udder drop score	5-6	178 (63.1)	Ref	
	2-4	**19 (6.7)**	**5.5**	**1.43 - 19.61**
	7-8	85 (30.1)	1.21	0.4 - 3.39
Separation of udder score	4-6	88 (31.4)	Ref	
	1-3	187 (66.8)	0.99	0.36 - 2.94
	7-9	**5 (1.8)**	**16.75**	**1.78 - 141.3**
Traumatic teat lesion	No	494 (93.6)	Ref	
	Yes	**34 (6.4)**	**5.35**	**1.58 - 16.68**

OR: Odds ratio; CI: confidence intervals; N: number of observations; Wald estimates used for CI; AM: acute mastitis; Udder drop score: distance from abdominal wall to upper cleft with values between 1 (below the hock) - 9 (level with abdominal wall); Separation of udder score: degree of separation of two udder halves between 1 (no separation) - 9 (clearly separated); Traumatic teat lesions: lesions on the teat where there was visible broken skin

2.3.4. Somatic cell counts

2.3.4.1. Summary of somatic cell counts

Somatic cell counts (SCC) were taken from a sample of ewes in mid April 2019, when they were in their 3rd - 6th week of lactation. Samples were collected from 372 udder halves, 18 had no milk or insufficient milk to carry out the measurement, giving results from 354 samples from 181 ewes. Raw SCCs ranged from 11x10^3 cells/ml to 7873x10^3 cells/ml and the common logarithm (log_{10}) SCC ranged from 4.01 - 6.89. The mean log_{10} was 5.44 and the geometric mean SCC was 279,000 cells/ml. (Table 2-15)

Table 2-15: Summary of log10 SCC measurements

Group at collection	N	Min	Max	Mean (95% CI)	SD	Geometric mean x10^3 (95% CI)
Control	212	4.04	6.75	5.36 (5.29 - 5.43)	0.50	228 (195 - 266)
IMM	142	4.57	6.90	5.6 (5.52 - 5.67)	0.45	395 (333 - 469)
Total	354	4.04	6.90	5.45 (5.4 - 5.51)	0.50	284 (252 - 320)

N = number of udder halves; Control group: no detected intramammary mass any time before lambing; IMM group: at least one detected intramammary mass at any point before lambing; 95% CI: 95% confidence intervals; SD: standard deviation

2.3.4.2. Linear mixed model of log_{10} somatic cell counts in

A two level linear regression mixed model was fit with ewe as a random effect to account for clustering within udder halves. The results of the univariable analysis are shown in Table 2-16.

Table 2-16: Univariable linear mixed effect model of log10 somatic cell count in 354 udder halves

Variable	Category	N (%)	Mean (SD)	ß	95% CI
Age	1-3	51 (14.4)	5.2 (0.5)	Ref	
	4-6	**231 (65.3)**	**5.5 (0.5)**	**0.26**	**0.07 - 0.51**
	7+	**72 (20.3)**	**5.5 (0.5)**	**0.29**	**0.07 - 0.44**
Litter size	>2	289 (81.6)	5.5 (0.5)	Ref	
	1	65 (18.4)	5.4 (0.4)	-0.02	-0.19 - 0.14
Future IMM (udder)	No	227 (64.1)	5.3 (0.5)	Ref	
	Yes	**127 (35.9)**	**5.6 (0.5)**	**0.22**	**0.11 - 0.32**
Previous IMM (udder)	No	241 (68.1)	5.4 (0.5)	Ref	
	Yes	113 (31.9)	5.6 (0.5)	0.26	0.15 - 0.37
AM (udder)	No	336 (94.9)	5.4 (0.5)	Ref	
	Yes	**18 (5.1)**	**5.8 (0.7)**	**0.28**	**0.07 - 0.49**
AM (ewe)	No	321 (90.7)	5.4 (0.5)	Ref	
	Yes	33 (9.3)	5.6 (0.6)	0.15	-0.09 - 0.39
Group at lambing	Control	212 (59.9)	5.4 (0.5)	Ref	
	IMM	**142 (40.1)**	**5.6 (0.5)**	**0.22**	**0.09 - 0.35**
Group in Sept 19	Control	173 (48.9)	5.3 (0.5)	Ref	
	IMM	**181 (51.1)**	**5.6 (0.5)**	**0.26**	**0.13 - 0.38**
Lactation week	3	59 (16.7)	5.5 (0.6)	Ref	
	4	130 (36.7)	5.5 (0.5)	0.03	-0.69 - 0.37
	5	159 (44.9)	5.4 (0.4)	-0.04	-0.23 - 0.15
	6	6 (1.7)	5.3 (0.3)	-0.16	-0.17 - 0.22

SD: standard deviation; ß: coefficients; CI: confidence intervals; N: number of observations; Wald estimates used for CI; AM: acute mastitis; IMM: intramammary mass; udder: variable at udder half level; ewe: variable at ewe level; Group in Sept 19: group membership at the end of the study

The final model retained four significant variables: ewe age, IMM in the same udder half in the future, IMM in the same udder half in the past, and a case of acute mastitis in the udder half at any point in the study (Table 2-17). Estimated marginal means (EMMs) were calculated for the disease variables using the same linear mixed effect model, averaged over the age variable (Table 2-18). The geometric EMM for uninfected udders was 186 $x10^3$. A case of IMM before milk collection

raises the geometric mean to 288 x10^3 without a case of AM, and 471 x10^3 if the ewe also has a case of acute mastitis in the same half. A future case of IMM where no IMM has previously been detected also raises the geometric EMM compared to uninfected udders, but the geometric EMM more than doubles in udders where IMM has been detected both before and after milk collection.

Table 2-17: Multivariable linear mixed effect model of log10 somatic cell count in 354 udder halves

Variable	Category	N (%)	Mean (SD)	ß	95% CI
Intercept				**5.13**	
Age	1-3	51 (14.4)	5.2 (0.5)	Ref	
	4-6	**231 (65.3)**	**5.5 (0.5)**	**0.24**	**0.06 - 0.42**
	7+	72 (20.3)	5.5 (0.5)	0.16	-0.05 - 0.38
Future IMM (udder)	No	227 (64.1)	5.3 (0.5)	Ref	
	Yes	**127 (35.9)**	**5.6 (0.5)**	**0.15**	**0.04 - 0.26**
Previous IMM (udder)	No	241 (68.1)	5.4 (0.5)	Ref	
	Yes	**113 (31.9)**	**5.6 (0.5)**	**0.19**	**0.07 - 0.31**
AM (udder)	No	336 (94.9)	5.4 (0.5)	Ref	
	Yes	**18 (5.1)**	**5.8 (0.7)**	**0.21**	**0.01 - 0.42**
Random effects	*Variance*				
Ewe	0.1298				

SD: standard deviation; ß: coefficients; CI: confidence intervals; N: number of observations; Wald estimates used for CI; AM: acute mastitis; IMM: intramammary mass; udder: variable at udder half level; ewe: variable at ewe level

Comparing the EMM across the three disease variables in the model shows that regardless of the ewe's IMM status, acute mastitis increases the EMM. Previous or future cases of IMM also increase the EMM compared to healthy ewes and having both previous and future IMM increases the EMM further. (Table 2-18)

Table 2-18: Estimated marginal means of log10 somatic cell count and estimated marginal geometric means

Variable				Log_{10} SCC		SCC ($x10^3$)	
Previous IMM (udder)	Future IMM (udder)	AM	N	Mean (SE)	95% CI	Mean (SE)[a]	95% CI
No	No	No	199	5.27 (0.05)	5.18 - 5.36	186 (19.5)	151 - 228
No	Yes	No	38	5.42 (0.06)	5.30 - 5.47	261 (36.1)	198 - 342
Yes	No	No	27	5.46 (0.06)	5.33 - 5.59	288 (42.5)	216 - 385
Yes	Yes	No	88	5.61 (0.06)	5.49 - 5.72	404 (53.4)	311 - 524
No	No	Yes	6	5.48 (0.11)	5.26 - 5.71	304 (79.2)	182 - 507
No	Yes	Yes	2	5.63 (0.12)	5.47 - 5.70	426 (115)	250 - 726
Yes	No	Yes	2	5.67 (0.12)	5.44 - 5.9	471 (128)	276 - 803
Yes	Yes	Yes	10	5.82 (0.11)	5.6 - 6.04	661 (171)	398 - 1100

a: geometric mean;
N: number of udder halves; SE: standard error; CI: confidence intervals; SCC: somatic cell count; IMM: intramammary mass detected in udder half; AM: acute mastitis case recorded during lactation

2.4. Discussion

This study separated ewes with IMM at the start of the study and then after each monthly inspection over a 12 month period to investigate if ewes with IMM act as a reservoir of infection to other ewes in the flock. By examining IMM over a year, this study has been able to investigate the risk of IMM based on previous observations. Additionally, udder conformation and SCC were investigated as they have previously been associated with acute mastitis and IMM (Huntley et al., 2012; Grant et al., 2016).

Each month all ewes were examined for IMM, and ewes with new IMM were moved into the isolated group of ewes with at least one IMM detected previously (IMM group). An IMM was not always detected in subsequent examinations of all ewes in the IMM group, and only 1.75% ewes had a detectable IMM in all examinations (Table 2-6). This explains why the prevalence of IMM throughout the year did not continuously increase, despite new ewes being added to the IMM group in all months except the month of lambing.

Grant et al. (2016) previously reported the variability of IMM over a two year period, hypothesising that this was due to a rupture-reform abscess cycle (Cheng et al., 2011). In the current study a large number of ewes only had IMM at a few observations (e.g. 60/570 ewes had one single positive IMM detection). Some of these ewes may have developed IMM late in the study and had the study continued, subsequent positive IMM observations may have occurred.

Despite the variability of IMM presence, ewes with an IMM were significantly more likely to have an IMM detected at the subsequent observation. Another finding was that larger IMM resulted in higher odds of detection in the next month. A large IMM indicates that the mammary abscess is larger with a higher volume of pathogenic bacteria than ewes with a small IMM. The rupture-reform cycle may occur less regularly in large abscesses, increasing the number of occasions the IMM is detected. The number of ewes with a ‘large’ IMM detected was low, reducing the confidence in this result, and larger IMM are easier to detect than small IMM that may have escaped detection. Nevertheless, any size IMM previously detected gave an increased likelihood of an IMM compared to no previous IMM, showing the reoccurring and persistent nature of IMM (Table 2-9).

A key result was that despite separating ewes with IMM, the incidence of IMM (to be precise ewes in the control group with their first recorded IMM) continued following

lambing. It has been hypothesised that lambs are a transmission vector of pathogens causing acute mastitis and IMM (Bergonier and Berthelot, 2003). The current study removed this transmission vector: lambs of dams with IMM could not come into contact with ewes without an IMM, yet ewes in the control group had new IMM detected in the months following lambing (Figure 2-3). In the month following lambing, control ewes had not been in contact with any ewes with an IMM for at least a month, as no new IMM were detected in the observation preceding lambing. These newly detected IMM may have been reoccurring IMM from before start of the study, or new IMM that have taken time to form following contagious pathogen transmission whilst ewes with IMM were still mixing with healthy ewes. However, control ewes that had their first detected IMM following lambing could be evidence of an environmental reservoir of pathogens causing IMM formation rather than contagious transmission from ewes with IMM. It was still the case after lambing that ewes in the IMM group were more likely to have a positive IMM observation in the months after lambing than ewes in the control group, again showing the importance of reoccurrence and persistence of IMM (Table 2-11).

Acute mastitis risk was associated with IMM and vice versa (Table 2-9 and Table 2-14). Grant et al. (2016) reported 12-fold greater odds of IMM in lactation when a ewe had an episode of acute mastitis, which they indicated could be evidence of causality of IMM as a result of acute mastitis. This study also reports increased odds of IMM in lactation following acute mastitis and reports 3-fold odds of acute mastitis when a ewe had IMM. Acute mastitis and IMM are highly associated, with each increasing the risk of the other, but it is impossible to distinguish which starts the cycle of mammary disease.

A number of udder conformation scores outside of the optimum (1, Teat angle: 5, Teat position: 4, udder drop: 5, symmetrical, with no lesions) were associated with IMM and acute mastitis (Table 2-9 and Table 2-14). Poor udder conformation has been previously associated with acute mastitis and IMM, in some cases as causal associations, for example where low pendulous udders are more exposed to environmental pathogens (Casu et al., 2006; Huntley et al., 2012; Grant et al., 2016). The current study reported pendulous udders and presence of traumatic teat lesions increased the odds of acute mastitis. Traumatic teat lesions, which were mostly cases where lambs had bitten the teat (KB personal observation), were also associated with acute mastitis. This association could be where ewes with acute mastitis receive bites from hungry lambs. Grant et al. (2016) reported udder conformation changes in ewes with IMM or after a severe case of acute mastitis. In

the current study, asymmetric udders were significantly associated with IMM. It may be that in some cases asymmetric udders are a consequence of IMM, as the IMM changes the conformation of the udder half it affects.

This is the first study to measure the somatic cell counts (SCC) of ewes with IMM. SCC give an indication of whether an immune response has begun by measuring inflammatory cells in milk samples (Conington et al., 2008). Increased SCC in milk samples are important: they have been associated with reduced milk production, lower lamb weights and decreased microbial diversity (Fthenakis and Jones, 1990b; Huntley et al., 2012; Esteban-Blanco et al., 2019). The current study has reported higher somatic cell counts in ewes with IMM and with future IMM. The estimated marginal means for SCC indicates ewes with IMM can also reach the suggested threshold for subclinical mastitis of 400,000 cells/ml (Huntley et al. 2012; Esteban-Blanco et al., 2019). Ewes where IMM was detected both before and after the measurement of SCC and ewes with at least one IMM and a case of acute mastitis had mean SCC over this threshold. This highlights the importance of IMM on the overall health of the udder, these masses and abscesses do not simply just exist within the udder, but also act to increase the immune response in a similar manner to subclinical mastitis.

2.4.1. Strengths and limitations of the study

This study regularly isolated ewes and their lambs when an IMM was detected, enabling a thorough investigation into the potential transmission pathways of chronic mastitis within a flock. Additionally, the regular examinations improved our understanding of the stability of these abscesses.

A potential weakness of this study was that that it was carried out on a single commercial farm in the UK in one year and may not be representative of other farms. Grazing quality was not measured and so the potential confounding effects of nutrition could not be included in this analysis. Both groups of ewes were managed in the same way and provided concentrates and additional forage as determined by the farmer but were grazed in separate areas of the farm. Dietary levels of protein and energy have been shown to have a significant impact on IMM and AM (Grant et al., 2016) and so to fully understand the causes of IMM and AM future studies should measure or control ewe nutrition.

Once an udder contains a pathogen or community of pathogens capable of forming an IMM it is not understood how long it takes for an IMM to form or if pathogen

colonisation always leads to IMM formation. Therefore, it is possible that in this study, ewes in the control group still carried pathogens capable of causing IMM and transmission pathways via lambs were still effective.

This study has not been able to recommend culling ewes with IMM to control IMM in a flock as isolation of affected ewes did not stop new ewes with IMM being detected throughout this study, suggesting that culling ewes with IMM would not control IMM prevalence in a flock. The IMM status of ewes before the study start was unknown, and there may have been ewes within the control group that had reoccurring IMM from previous years later in this study, enabling continued contagious transmission. Alternatively, pathogens causing IMM may have an environmental reservoir, reducing the impact of isolating ewes with IMM. A study carried out over several years with bacteriological analysis would go some way to verify the results of the current study, monitoring ewes over a longer period and analysing bacteria from milk samples and the environment.

Additionally, measuring IMM is a subjective examination and relies on manual detection and the use of a commercial farm restricted measurement and adjustment of confounders such as grazing quality and quantity. Therefore, a study carried out in controlled settings with more regular udder examinations and accurate measurement of IMM, for example use of ultrasound technology, would improve our confidence in the results outlined in this chapter.

2.4.2. Conclusions from Chapter 2

This study has described that IMM are unstable and detection in one month does not guarantee future detection, although once an IMM has formed, future detection is more likely than in ewes where no IMM has been detected. This short term isolation study was not effective in reducing IMM in the flock, indicating that transmission of IMM pathogens may be via an environmental reservoir or happens far before IMM formation.

IMM and acute mastitis are highly associated mammary gland conditions, with one often following the other. Both cause an increase in SCC and are associated with poor udder condition. We conclude that although isolating ewes with IMM does not appear effective, the negative impacts of the condition, including the influence on lamb weights (Chapter 3 of this thesis), make it sensible for farmers to avoid breeding from ewes with IMM.

Chapter 3 A longitudinal study of the effect of mastitis on the live weight of lambs

3.1. Introduction

Much of the income of sheep farmers in the UK depends on the sale of lambs, and therefore lamb weight is an important income for the sheep industry (Redman and Jerman, 2019). McLaren et al. (2018) estimated that the smaller weight of a lamb born to a dam with mastitis resulted in losses of up to £5.87 per lamb in the GB market compared to lambs with healthy mothers.

Acute, subclinical and chronic mastitis have all been associated with lower lamb growth rates, hypothesizing that this is because of a reduction in milk production (Arsenault et al., 2008; Huntley et al., 2012; Grant et al., 2016). Griffiths et al. (2019) found that detection of intramammary masses (IMM) during pregnancy, lactation and at weaning had an effect on lamb growth to weaning, and Grant et al. (2016) associated lower daily lamb weight gain with IMM during lactation.

Lambs with dams with mastitis consume more supplemental feed (Fthenakis and Jones, 1990b) and Keisler et al. (1992) found subclinical mastitis had no influence on lamb growth when they had access to supplemental feed. Once lambs have been weaned and are no longer dependent on milk, they have the opportunity to carry out compensatory growth. However, it is unknown what effect intramammary infections have on the growth rates of lambs after weaning. Moreover, lambs are known to supplement milk intake via 'robbing' from ewes other than their dam (Bergonier and Berthelot, 2003), which would obscure the full impact of intramammary infections on lamb growth rate if lambs take milk from ewes with IMIs.

The aim of the study in this chapter was to determine the impact of acute mastitis and intramammary masses on lamb growth rates up to weaning and slaughter, using monthly longitudinal data on two groups of ewes one with and one without intramammary masses.

3.2. Methods

3.2.1. Study farm and data collection

The same indoor lambing and outdoor rearing flock in North Yorkshire that was used for the study investigating the role of chronic intramammary masses (Chapter 2) was simultaneously used for this chapter.

The methods for data collection and management are outlined in Chapter 2, section 2.2.3.

3.2.2. Data analysis

All statistics were carried out in R studio version 1.2.5 using R version 4.0.2 (2020-06-22). Data were explored using descriptive statistics as described in Chapter 2, section 2.2.5.

3.2.2.1. Data preparation

Prior to detailed analysis data were examined using base R functions to investigate descriptive statistics, including measures of central tendency, spread and distribution.

Lamb weights followed a linear growth curve for approximately the first 90 days of life until weaning (Figure 3-1). After this the growth did not fit a linear regression curve. The data were split into a data set with only measurements before weaning, and a data set including all lamb weights. At weaning, lambs were 73 - 101 days old (mean = 90 days). All 919 lambs were included, each with 1 - 6 weights, excluding birth weights.

The full, unfiltered dataset also included data from all 919 lambs. Lambs had between 1 to 16 measurements over the study length, from birth until the last lamb went to market (total observations = 6475).

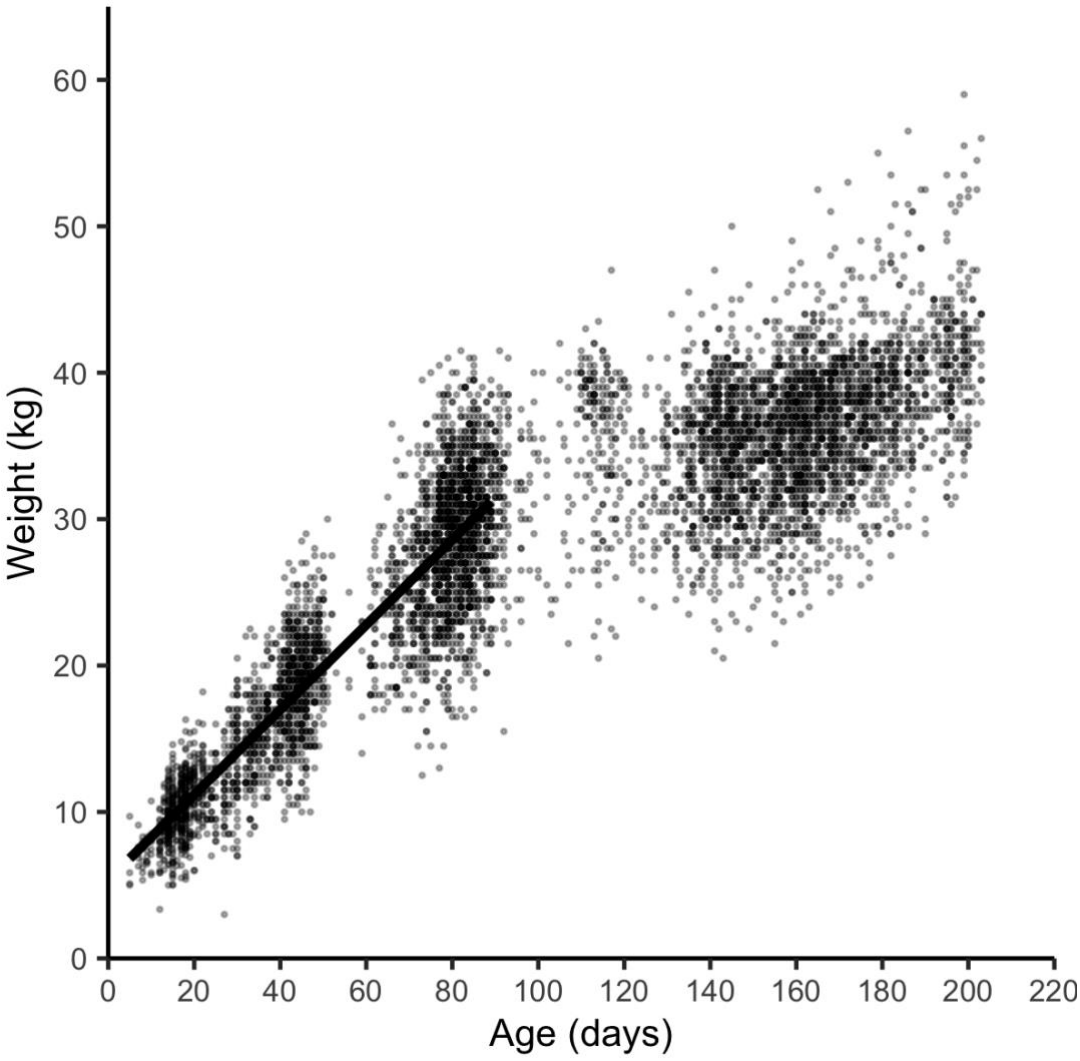


Figure 3-1: Lamb weight up to 220 days, with a line of best fit showing linear growth for the first 90 days

3.2.2.2. Associations in the data set

Associations between explanatory variables in the full data set were tested using Pearson's correlation coefficient and Chi squared tests.

3.2.2.3. Linear mixed regression model of lamb weight up to weaning (approximately 90 days old)

As discussed above, the full dataset of lamb weights did not follow a linear regression curve. The filtered dataset was used to construct a linear mixed effects regression model to investigate factors associated with lamb weight up to weaning, with lamb weight (kg) as the continuous outcome variable. Ewe (lamb within litter), lamb (repeated weights) and observation were set as random effects to account for

repeated measures in the data. Lamb age in days was included as a polynomial term for time (days + days^2 + days^3). All fixed effect explanatory variables were tested in univariable models and variables significant at p<0.1 were taken forward for model selection. The final multivariable model was selected using a manual forward selection process (Dohoo et al., 2003). Variables were selected based on significance of p<0.05 and a lower akaike information criterion (AIC). Where variables were highly correlated, the variable with the most biological plausibility was retained. All non-significant variables were retested in the final model to investigate residual confounding (Cox and Wermuth, 1996). Models were constructed using the 'lmer' function from the package lme4 (Bates et al., 2015). Model fit was tested by examination of residual plots. The model took the form:

$$y_{ijk} = \beta_0 + \beta x_k + \beta x_{jk} + \beta x_{ijk} + b_{0jk} + b_{jk}x_{jk} + v_k + u_{kj} + e_{ijk}$$

where y_{ijk} was the weight (kg) of the $j\text{-}th$ lamb born to ewe i at time index k, β_0 was the intercept, and βx was a series of vectors of fixed effects varying at i (ewe), j (lamb) and k (obervation). b_{0j} and $b_{jk}x_{jk}$ are the random intercept and random time slope effects for the $j\text{-}th$ lamb, where the random slope and random intercept are assumed to be uncorrelated. Residual variance estimates were included at (v_k), (u_{ij}), and (e_{ijk}).

3.2.2.4. Generalised additive mixed model of lamb weight for the study length

A multivariable generalised additive mixed model (GAMM) was fitted using the R package mgcv (Wood, 2011). Generalised additive models (GAMs) are an extension of generalised linear models where complex non-linear relationships exist and are expressed as a reduced rank smoothing spline. The model build was carried out as follows:

1. introduction of random effects for lamb, ewe and lamb age;

2. fit of a smoothing terms for continuous variables lamb age, ewe age and birthweight, selection using lowest AIC;

3. addition of fixed effects as univariable models, including ewe age, birth weight, lamb gender, number of lambs suckled, management group, one month lagged BCS, one month lagged IMM and acute mastitis at different times in the study;

4. stepwise addition of variables with a p value <0.1 in the univariable models using AIC and variable significance to produce a final model.

The model took the form:

$$y_{ijk} = \beta_0 + \beta x_k + \beta x_{jk} + \beta x_{ijk} + f(age_{ijk}) + b_{0jk} + b_{jk}x_{jk} + v_k + u_{jk} + e_{ijk}$$

which is as above, with the addition of $f(age_{ijk})$ as a smoothing function on lamb age (days since birth).

Linear mixed models were tested as described in section 3.2.2.3 for the full study length. Lamb age (days) was tested as a linear variable and as a quadratic variable. The two linear models and the generalised additive models were compared using AIC. All non-significant variables were retested in the final model to investigate residual confounding (Cox and Wermuth, 1996).

3.2.2.5. Linear mixed effect regression model of age at slaughter

In order to better represent the impact of mastitis; a linear regression model was constructed using the continuous outcome variable age at slaughter (days) for a proxy for farm profit. Data was not available for the whole flock on accurate price, value and deadweight from the abattoir and so age at slaughter was used in this model for a proxy estimate of farm income. Ewe was included as a random effect to account for multiple lambs with the same mother. All explanatory variables were tested as univariable models, and then variables significant to $p < 0.05$ were tested in a forward model selection. Variables were considered significant when 95% confidence intervals did not cross 0. Where highly correlated variables resulted in poor model fit; the most biologically plausible variable was retained.

The model took the form:

$$y_{ij} = \beta_0 + \beta x_{ij} + u_j + e_{ij}$$

where y_{ij} was the continuous outcome variable of age at slaughter (days), β_0 was the intercept, and βx_{ij} was a series of fixed effect variables that varied at ij (observation) and j (ewe). u_j and e_{ij} are the residual variance estimates.

3.3. Results

3.3.1. Descriptive analysis

There were 570 ewes that gave birth to a total of 1089 live lambs. Of these, 919 lambs from 550 ewes remained with their mothers and were weighed at least once after birth. All but three lambs in this group survived to weaning. These ewes and lambs were in the dataset used for analysis. The 170 lambs excluded either died before the first weight measurement or were artificially reared.

The mean lamb birth weight was 4.7kg, ranging from 1.4kg to 9.7kg. Weights taken after birth ranged from 3kg to 59kg, when lambs were between 5 days and 203 days old.

Detailed results for intramammary masses (IMM) and acute mastitis (AM) can be found in Chapter 2. The period prevalence of IMM, where a ewe had at least one detected IMM over the study (September 2018 - 2019), was 48%. The period prevalence during lactation was 35%. Cases of acute mastitis were lower, with a period prevalence of 9.7%. There were 58 ewes with mastitis during the study, but five do not rear their lambs and so are excluded from the lamb weight analysis. (Table 3-1)

Table 3-1: Summary statistics for categorical ewe variables

Variable		Number of Ewes (%)
AM during study	Yes	53 (9.6)
	No	497 (90.4)
AM during lactation	Yes	14 (2.5)
	No	536 (97.5)
IMM during study	Yes	262 (47.6)
	No	288 (52.4)
IMM during pregnancy	Yes	175 (31.8)
	No	375 (68.2)
IMM during lactation	Yes	190 (34.5)
	No	360 (65.5)
Total N		550

AM: acute mastitis; IMM: intramammary mass
Yes/No categories are not synonymous with the control or IMM group. For example, a sheep recording ‘No’ for IMM during lactation could be in the IMM group due to a detected IMM prior to lactation.

3.3.1.1. Associations and correlations between explanatory variables in the models.

Associations between categorical explanatory variables used in the lamb weight and age at slaughter models are shown in Table 3-2, and associations between continuous and categorical variables are shown in Table 3-3.

Table 3-2: Associations between categorical variables

	Lamb:male	Litter size ≥ 2	AM at time	AM during lactation	AM during study	IMM at time	IMM during pregnancy	IMM during lactation	IMM during study	IMM month before	Age	BCS	Group at Lambing
Lamb:male				*	*		*		*		*	*	*
Litter size ≥ 2				*	*	*		*		*	*	*	
AM at time				*	*	*				*		*	
AM during lactation					*	*	*	*	*	*	*		*
AM during study						*	*	*	*	*	*		*
IMM at time							*	*	*	*	*	*	*
IMM during pregnancy								*	*	*	*	*	*
IMM during lactation									*	*	*	*	*
IMM during study										*	*	*	*
IMM month before											*	*	*
Age												*	*
BCS													*
Group at Lambing													

All associations were tested using a Chi Squared Test; * indicates a significant test result.

Table 3-3: Associations between categorical variables and lamb birth weight and lamb age at slaughter using Chi-squared tests

	Birth weight (kg)	Lamb age at slaughter (days)
Sex		
Female	4.5 (0.9)	199.4 (49.6)
Male	4.7 (1.0)	181.6 (50.1)
	*	*
Litter size		
1	5.3 (1.1)	152.0 (51.1)
≥ 2	4.5 (0.9)	195.4 (48.5)
	*	*
AM at time		
No	4.6 (0.9)	190.4 (50.9)
Yes	4.5 (0.9)	199.2 (36.5)
AM during lactation		
No	4.6 (0.9)	190.0 (50.5)
Yes	4.2 (1.0)	216.3 (51.2)
	*	*
AM during study		
No	4.6 (0.9)	189.7 (50.9)
Yes	4.5 (1.0)	199.6 (46.6)
	*	*
IMM at time		
No	4.6 (0.9)	187.3 (50.9)
Yes	4.7 (0.9)	199.1 (49.0)
	*	*
IMM during pregnancy		
No	4.6 (0.9)	186.7 (50.0)
Yes	4.6 (1.0)	195.9 (51.0)
	*	*
IMM during lactation		
No	4.6 (0.9)	184.9 (49.1)
Yes	4.7 (1.0)	198.1 (51.7)
	*	*
IMM during study		
No	4.5 (0.9)	187.4 (49.4)
Yes	4.7 (1.0)	193.1 (51.5)
	*	*
IMM month before		
No	4.6 (0.9)	186.8 (50.5)
Yes	4.7 (0.9)	202.6 (49.3)
	*	*

	Birth weight (kg)	Lamb age at slaughter (days)
Age		
1-3	4.2 (0.9)	204.2 (47.4)
4-6	4.7 (0.9)	185.3 (50.1)
7+	4.6 (1.0)	195.3 (52.2)
	*	*
BCS		
Fat	4.6 (1.1)	188.1 (50.8)
Healthy	4.6 (0.9)	189.5 (51.7)
Thin	4.6 (0.9)	192.2 (49.3)
	*	
Group at lambing		
Control	4.6 (0.9)	186.7 (50.1)
IMM	4.7 (1.0)	195.9 (51.0)
	*	*

Pearson's correlations are given for significant correlations between continuous variables. For correlations between continuous and binary variables the mean and standard deviation is given for each category and an analysis of variance test carried out; * indicates a significant test result.

3.3.2. Linear mixed effect model of lamb weight up to weaning

For approximately the first 90 days of life, lamb growth was linear. There was an increase in lamb variation in weight with age, that can be seen by the ‘cone’ shape of the data in Figure 3-2. This heteroskedasticity was explained in part using a covariance structure; and adding a random slope term for lamb age in the model. A comparison of the residual plots for the final model, with and without this correction, show that model fit is better when taking account of this change in variance (Figure 3-3).

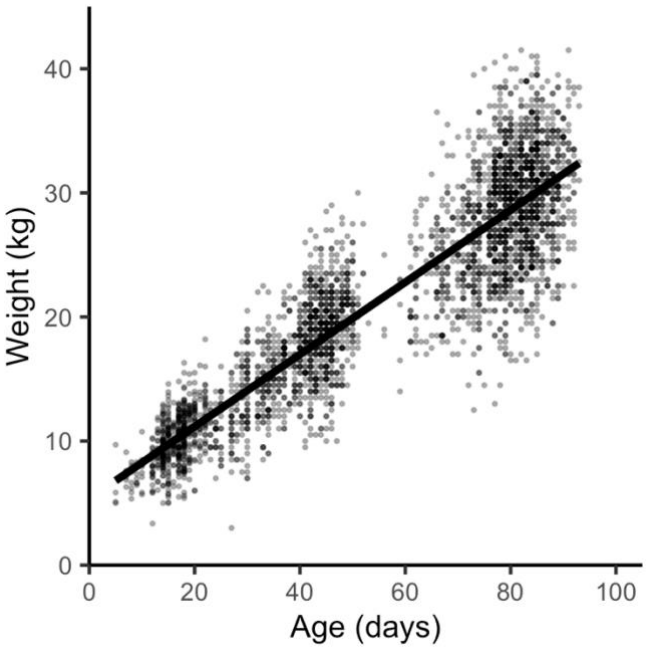


Figure 3-2: Lamb weight from birth to weaning

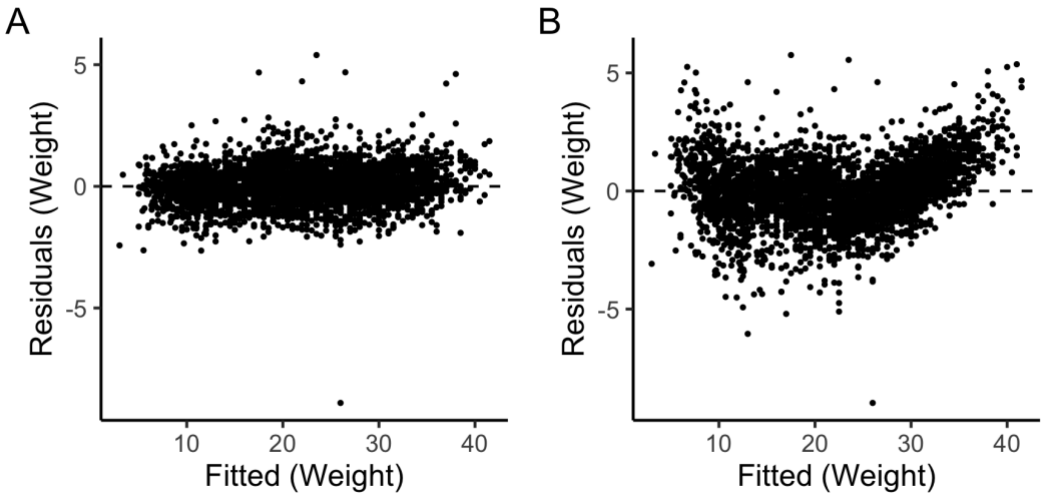


Figure 3-3: Residual plots for linear mixed regression model of lamb weight with (a) a fixed variance structure and (b) no fixed variance structure

The results of the univariable linear mixed effect regression models are shown in Table A- 1 in the appendix.

Five variables ($p < 0.05$) were retained in the final linear model (Table 3-4). An increase in lamb weight was explained by age and birth weight (1.53kg heavier for

each kg at birth). Lambs were 0.62kg lighter when their mothers had an IMM detected during pregnancy. Presence of an IMM in the month prior to the lamb weight recording resulted in lambs 0.36kg lighter. Although IMM detected during lactation was not retained in the final model, it is strongly associated with all other IMM variables, including IMM in the month prior and IMM detected during pregnancy (Table 3-2). Ewes with acute mastitis during lactation had significantly lighter lambs than ewes with no recorded acute mastitis symptoms (0.98kg).

Table 3-4: Multivariable linear mixed effect model of lamb weight up to weaning

Variable	Category [Range]	N (%)	Mean (SD)	ß	95% CI
Intercept				15.13	14.59 - 15.66
Lamb age (days)	**[5.0,93.0]**	**3281 (100.0)**	**22.0 (8.3)**	**423.02**	**418.11 - 427.94**
Lamb age (days)^2				**-7.3**	**-9.53 - -5.08**
Lamb age (days)^3				2.03	-0.37 - 4.42
Birth Weight (kg)	**[1.4,9.7]**	**3281 (100.0)**	**22.0 (8.3)**	**1.53**	**1.43 - 1.64**
Group at Lambing	Control	1835 (55.9)	23.6 (8.1)	Ref	
	IMM	**1446 (44.1)**	**20.0 (8.2)**	**-0.62**	**-0.86 - -0.38**
AM during lactation	No	3217 (98.0)	22.1 (8.3)	Ref	
	Yes	**64 (2.0)**	**16.5 (9.2)**	**-0.98**	**-1.72 - -0.25**
IMM previous month	No	2532 (77.2)	22.4 (8.4)	Ref	
	Not Recorded	5 (0.2)	19.3 (12.9)	-0.36	-1.98 - 1.27
	Yes	**744 (22.7)**	**20.9 (8.2)**	**-0.35**	**-0.55 - -0.15**
Random effects	*Variance*				
Lamb:age(days) covariance	0.0019				
Lamb	0				
Ewe	0.726				

SD: standard deviation; ß: coefficients; CI: confidence intervals; N: number of observations; Wald estimates used for CI; AM: acute mastitis; IMM: intramammary mass;

3.3.3. Generalised additive mixed effect model (GAMM) of lamb weight over the whole study

The GAMM model fit the data significantly better than using linear terms alone or using quadratic terms (Figure 3-4). Maintaining the same random effects and linear variables, a quadratic term on lamb age was a significant improvement on the linear only model (change in AIC = 3232). The GAMM further improved the model (change in AIC = 3376 with 17 degrees of freedom) (Table 3-5). The results of each regression model are not shown.

Table 3-5: AIC comparison of three model types

Model	df	AIC
GAMM	18	26350
Quadratic	18	29726
Linear	17	32958

df: degrees of freedom; AIC: akaike information criterion

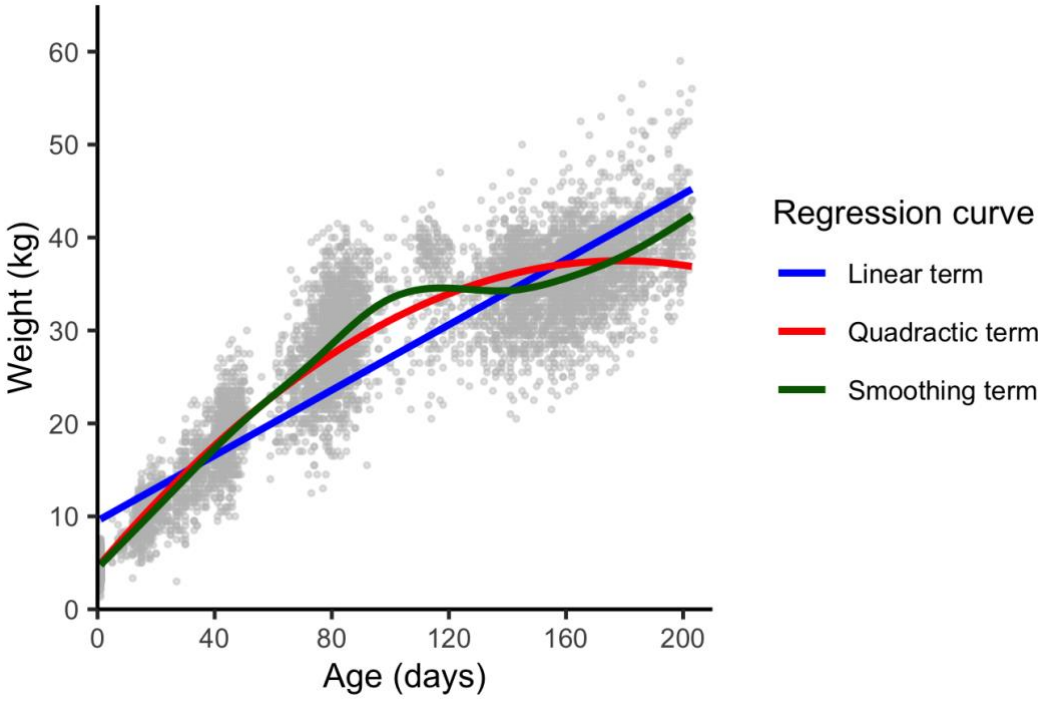


Figure 3-4: Fitting different regression lines to the lamb growth curve

The results of the GAMM univariable analysis are shown in Table A- 2 in the appendix.

In the final model the residuals showed a mostly normal distribution, with a slight left skew (Figure 3-5). This is a result of heteroscedasticity within the data where there is an increase in the variance of weights as the lambs get older. Including a random slope effect in the model accounts for some of this between-lamb variation and the residuals are close to a normal distribution. The estimates will not be affected by the heteroskedastic nature of the data; but the standard deviations may be too big, which means the model may be more conservative than necessary.

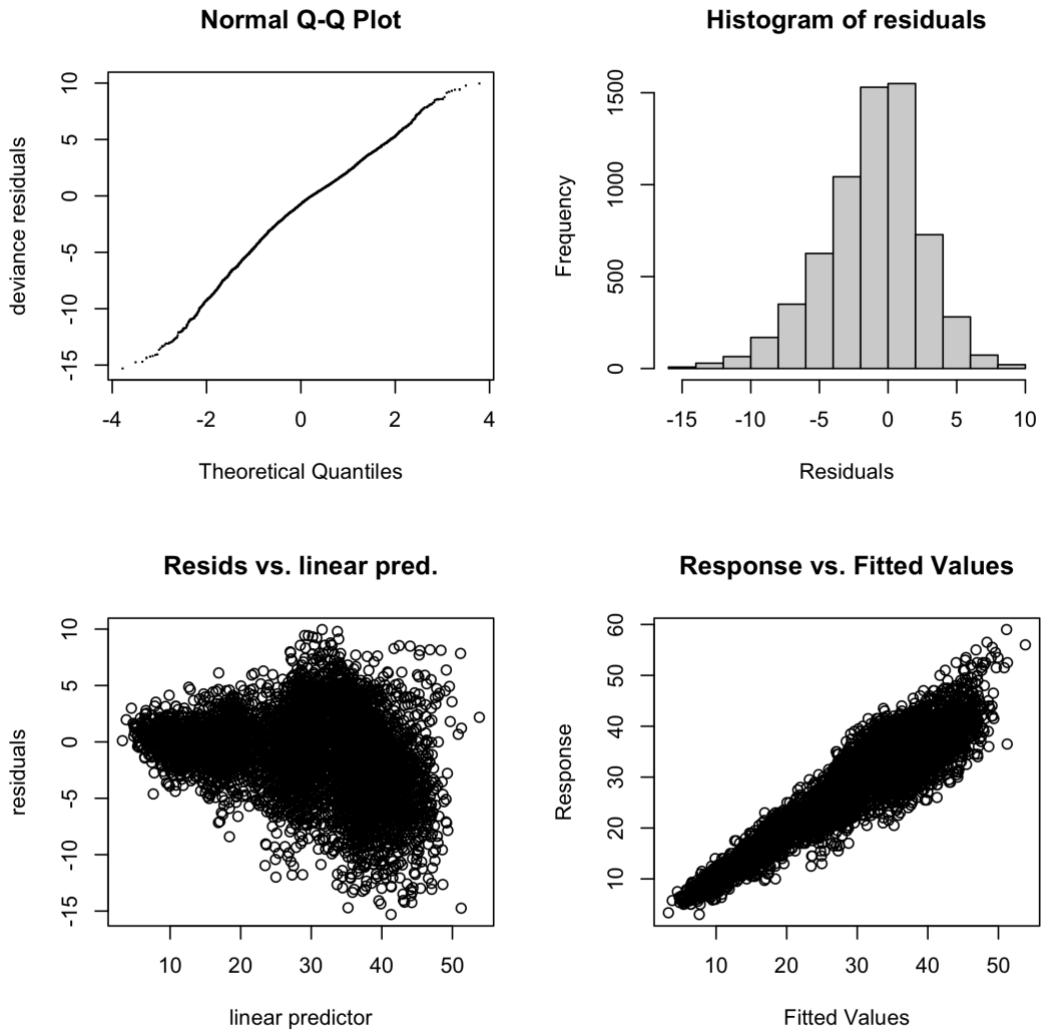


Figure 3-5: Fitted GAMM final model residual statistics

All variables with a p value < 0.05 in the univariable analysis were tested in the final model, and seven variables were retained (Table 3-6). Lambs with a higher birth weight maintained a higher weight (1.89kg), and lambs reared in pairs were lighter (-1.56kg). Aberfield X lambs were heavier than the reference breed, Texel X (0.82kg, 0.34kg, -1.31kg). Ewes that had a case of acute mastitis during the study, an IMM in the same month or the previous month as the weight measurement or a case of IMM during pregnancy had lighter lambs (-0.6kg, -0.28kg, -0.36kg and -1.07kg respectively). Figure 3-6 shows the regression curves for each disease variable kept in the final model.

Table 3-6: Multivariable generalised additive mixed model

Variable	Category [Range]	N (%)	Mean (SD)	ß	95% CI
Intercept				24.69	23.45 - 25.93
Birth weight (kg)	**[1.4,9.7]**	**6475 (100.0)**	**28.9 (9.8)**	**1.89**	**1.74 - 2.04**
Lambs suckled	1	727 (11.2)	30.0 (10.2)	Ref	Ref
	2	**5748 (88.8)**	**28.8 (9.7)**	**-1.47**	**-1.9 - -1.05**
Breed	Texel X	3905 (60.3)	28.5 (9.4)	Ref	Ref
	Aberfield X	**817 (12.6)**	**32.0 (11.0)**	**0.82**	**0.34 - 1.31**
	Abermax X	1082 (16.7)	29.8 (9.6)	-0.32	-0.74 - 0.1
	Rouge X	671 (10.4)	26.4 (9.9)	0.34	-0.25 - 0.93
AM during study	No	5879 (90.8)	29.0 (9.8)	Ref	Ref
	Yes	**596 (9.2)**	**27.8 (10.1)**	**-0.6**	**-1.14 - -0.06**
IMM at time	No	4741 (73.2)	29.2 (9.9)	Ref	Ref
	Yes	**1732 (26.8)**	**28.2 (9.6)**	**-0.27**	**-0.41 - -0.14**
Group at lambing	Control	3872 (59.8)	30.4 (9.3)	Ref	Ref
	IMM	**2603 (40.2)**	**26.7 (10.2)**	**-1.13**	**-1.52 - -0.74**
IMM previous month	No	4960 (76.6)	29.2 (9.8)	Ref	Ref
	Not Recorded	5 (0.1)	19.3 (12.9)	4.54	2.41 - 6.67
	Yes	**1510 (23.3)**	**28.3 (9.8)**	**-0.35**	**-0.5 - -0.21**
Random effects	*Variance*				
Ewe	1.84				
Lamb	0.06				
Lamb:age	1.32				
	edf	*F*	*p*		
smooth term (age (days))	20.24	1041.35	<0.01		

SD: standard deviation; ß: coefficients; CI: confidence intervals; N: number of observations; Wald estimates used for CI; AM: acute mastitis; IMM: intramammary mass; Rouge X: Rouge de l'ouest; edf: estimated degrees of freedom; F: F statistic; p: p value for smooth

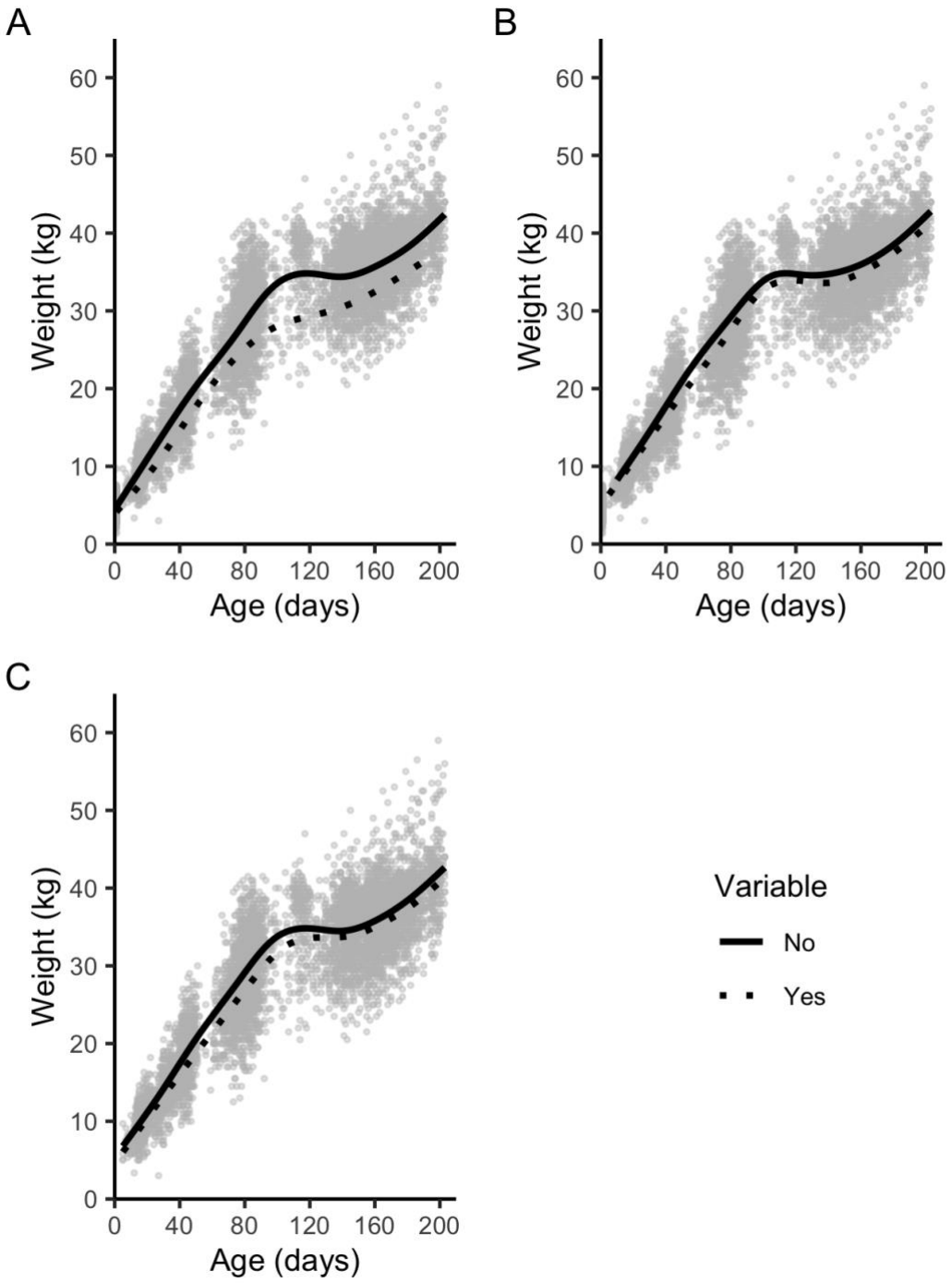


Figure 3-6: Lamb weight over the whole study when ewes had A) acute mastitis during study, B) IMM group at lambing, C) IMM month before

3.3.4. Linear regression model of age at slaughter

A linear regression mixed model of age at slaughter was fit with ewe as a random effect to account for clustering of lambs born to the same ewe. The results of the univariable analysis are shown in Table A- 3 in the appendix. Interactions between variables were not significant and were not included in the table.

Four variables were retained in the final multivariable model for age at slaughter. Birth weight, male sex and single lambs all reduced the age to slaughter (Table 3-7). Lambs born into the IMM group were 16 days older at slaughter than lambs born into the control group (95% CI 8.44 - 23.25).

Table 3-7: Multivariable linear mixed effect model of lamb age at slaughter (days)

Variable	Category [Range]	N (%)	Mean (SD)	ß	95% CI
Intercept				239.93	211.67 - 268.2
Birth weight (kg)	[1.6,9.7]	802 (100.0)	174.1 (53.9)	-23.09	-26.58 - -19.6
Lamb sex	Female	380 (47.4)	184.9 (53.6)	Ref	
	Castrated male	**422 (52.6)**	**164.4 (52.4)**	**-11.23**	**-17.39 - -5.06**
Lambs suckled	1	111 (13.8)	134.7 (48.7)	Ref	
	2	**691 (86.2)**	**180.5 (52.0)**	**23.73**	**13.94 - 33.52**
Group at lambing	Control	545 (68.0)	169.2 (53.1)	Ref	
	IMM	**257 (32.0)**	**184.6 (54.1)**	**15.84**	**8.44 - 23.25**
Random effects	*Variance*				
Ewe	678.5557				

SD: standard deviation; ß: coefficients; CI: confidence intervals; N: number of observations; Wald estimates used for CI; AM: acute mastitis; IMM: intramammary mass

3.4. Discussion

The longitudinal study described in this chapter investigated the effect of acute mastitis and intramammary masses (IMM) on the weight of lambs. IMM presence was examined each month and detected in 48% of ewes at least once, and acute mastitis was recorded when detected, affecting 9% of ewes during the study. Linear mixed effects models and generalised additive models were used to investigate the variables associated with a reduction of lamb weight and increased age at slaughter. These support previous findings that both IMM and acute mastitis have a significant impact on lamb growth rates (Grant et al., 2016; Griffiths et al., 2019). A key finding is that lambs of dams in the IMM group at lambing were lighter at weaning and at slaughter than lambs born into the control group and took longer to finish.

There was linear growth up to weaning, and then a reduction in growth rate at about 90 days. This is in line with other studies which have similarly reported low growth rates following weaning, in part due to stress and the removal of milk (Cañeque et al., 2001; Velasco et al., 2004). After a short period of reduced growth, lamb growth rates then returned up until slaughter (Figure 3-6A).

Acute mastitis had the biggest impact on lamb growth up to weaning (Table 3-4), in line with previous studies (Arsenault et al., 2008; Grant et al., 2016). Keisler et al. (1992) found that supplemental feed can prevent the impact of subclinical mastitis on lambs. Despite this, in the current study, lambs remain significantly lighter at slaughter when dams had AM, even if the case of acute mastitis occurred after weaning. Reduction in milk consumption has been positively correlated with increased grass intake, but not sufficiently to compensate for the energy lost from a lower milk supply (Penning and Gibb, 1979; Doney et al., 1984), explaining why lambs were unable to counteract the influence of a reduced milk intake. The finding of reduced lamb weight when acute mastitis occurred after weaning indicates mastitis was already affecting the lamb, either as subclinical mastitis or an IMM. Potential explanations for reduced milk intake are subclinical mastitis, an IMM or a teat lesion, and the ewe preventing a lamb from sucking, which might arise in each situation.

In the current study there was a significant reduction in lamb weight when ewes had an IMM during pregnancy in both the linear model up to weaning and the generalised additive model up to slaughter, placing the ewe in the IMM group at

lambing (Table 3-4 and Table 3-6). In the current study ewes were examined monthly rather than less periodically as in previous studies (Grant et al., 2016; Griffiths et al., 2019), and the variable 'IMM previous month' was included in the model. This was associated with a lower lamb weight in both models and would be related to either the month before birth or a month during lactation. IMM in lactation was therefore closely associated to IMM in the previous month (Table 3-2). Similarly, Grant et al. (2016) reported a significant reduction in lamb growth rate when ewes had an IMM during lactation and Griffiths et al. (2019) reported any ewe with a 'lump' score after mating was associated with a lower lamb weight than ewes with a healthy udder score. IMM presence is associated with a higher risk of acute mastitis (Grant et al. (2016); Chapter 2 in this thesis), which itself is associated with lower lamb growth rates. As IMM are abscesses (Smith et al., 2015), they will occupy space within the mammary gland and might reduce the mammary mass producing milk.

Many other factors have been previously associated with a reduced lamb growth rate, including sex, breed, litter size, ewe body condition score (BCS) and lamb birth weight (Arsenault et al., 2008; Grant et al., 2016). Breed and litter size were only associated with lamb weight in the more complex generalised additive model, and lamb sex was not significant in either model. As birth weight was included in the model, this probably explained difference by gender, breed, or litter size. Ewe BCS was not significant in either model, although it was negatively associated with all IMM disease variables (Table 3-2).

Finally, a simple linear mixed model of lamb age at slaughter was constructed to closer investigate the impact of IMM and acute mastitis on farm income. Lambs that take longer to finish cost more in time and feed. Lambs were selected by the farmer for slaughter based on weight and fat cover, and so it would be expected that lambs with slower growth rates would be older at slaughter than faster growing lambs. Lambs born to dams in the IMM group at lambing were significantly older at slaughter. Ewes in the IMM group had at least one IMM detected prior to lambing and were more likely to have acute mastitis. Furthermore, this group was isolated from the control group, reducing the availability of healthy ewes for 'milk robber' lambs to supplement milk intake. Acute mastitis was not significantly associated with increased age at slaughter, potentially due to the lower number of observations in this model.

3.4.1. Strengths and limitations of the study

The current study is the first to isolate ewes with IMM and their lambs from the rest of the flock, enabling a clear indication of the association between IMM and lamb growth rates. Additionally, regular examinations of ewe udder health mean it is likely the majority of IMM were recorded.

A weakness of this study is that it was carried out on a single commercial farm in the UK in one year and may not be representative of other farms. A future study should investigate lamb growth across several commercial farms or across groups within a controlled study farm. The farm was convenience-selected, with a self-reported high acute mastitis prevalence. Acute mastitis prevalence in the study year was 9 %, which is on the upper end of most acute mastitis prevalence estimations (Cooper et al., 2016; Grant et al., 2016), although this is unlikely to affect the subsequent impact on lambs. Another weakness is that grazing quality was not measured (discussed in section 2.4.1) which could have a confounding effect on both the risk of IMM and acute mastitis and on the growth of the lamb. Manual measurement of IMM by researchers could have resulted in overlooking some ewes with IMM, developing a less subjective measurement of IMM and examining for IMM more regularly would improve this study.

3.4.2. Conclusions from Chapter 3

Acute mastitis and IMM have a negative impact on lamb weight even past weaning. IMM presence outside of lactation can influence lamb weight, and acute mastitis cases after weaning are also associated with reduced lamb weight. IMM presence results in lambs taking longer to reach slaughter weight, costing the farmer in increased foliage intake and delayed income.

Chapter 4 Epidemiological analysis of mastitis-associated pathogens in sheep flocks using MALDI-ToF MS and Whole Genome Sequencing

4.1. Introduction

Mastitis is primarily a disease caused by bacterial infection. In order to investigate the dynamics of the disease within sheep flocks, it is important to understand the associated species, the potential reservoirs and the capacity to transmit between sheep or to persist within the mammary tissue. Identification of strains is essential to discover where true persistence has occurred and to identify probable transmission events.

Persistent mastitis is where the same infectious agent is detected in the milk of an udder half repeatedly over a time period. This can include persistence in the non-lactating period between pregnancies (Vanderhaeghen et al., 2014). It is important to identify strains rather than rely on species identification, as the same species, but different strain, can cause recurrent infections (Wente et al., 2020). Furthermore, strains within a species can have different transmission routes and pathogenicity, and therefore assumptions based on species identification may lead to inappropriate conclusions being drawn.

Recent developments in sequencing technologies has made whole genome sequencing (WGS) of bacterial genomes quicker and more affordable. WGS is highly discriminatory and is considered the gold-standard for detecting transmission events and outbreaks (Croucher and Didelot, 2015). However, MALDI-ToF MS is a faster and cheaper method of bacterial identification, if it also differentiates strains it would be a desirable tool in epidemiological investigation of transmission and persistence.

MALDI-TOF MS is high-throughput and cost-effective, so it's use as a strain typing method is an attractive alternative to traditional methods such as PFGE. Strain typing is carried out by comparison of mass spectra to give the likelihood of two isolates being the same strain (Smith et al., 2015; Archer et al., 2017). In cattle, Archer et al. (2017) used MALDI-TOF MS to detect strains of *Streptococcus uberis* and in sheep Smith et al. (2015) compared strains extracted from milk and abscesses within the same udder.

MALDI-ToF MS as a strain-typing method is controversial, with several studies reporting a range of reliability and discriminatory power, generally attributed to the species. *Serratia marcesens.*, *E. coli*, and *Listeria* have all been strain-typed by MALDI-ToF MS (Barbuddhe et al., 2008; Veenemans et al., 2016; Rödel et al., 2019), whereas there has been insufficient discriminatory power reported for *K. pneumoniae*, *S. aureus* and *E. faecium* (Sandrin et al., 2012; Lasch et al., 2014; Rodrigues et al., 2016). Some studies have reported successful discrimination of *S. aureus* strains, particularly in characterising methicillin-resistant and sensitive strains (Bernardo et al., 2002; Shah et al., 2011). It has been suggested that this range of reliability is due to differences in sample preparation and data processing (Sandrin et al., 2012).

This study isolated and identified bacterial species from milk samples collected from ewes with acute and chronic mastitis and from ewes with no detectable mammary disease. MALDI-ToF MS and WGS were used to investigate persistent strains and the possible transmission pathways within flocks for *S. aureus*. Finally, the aim was to validate the use of MALDI-ToF MS as a strain-typing method by comparing *S. aureus* MALDI-ToD results with WGS, the gold-standard for strain-typing, in order to investigate transmission and persistence across all species detected in the study.

4.2. Methods

4.2.1. Study farm and milk collection for bacteriological analysis

As previously described (Grant et al., 2016), between November 2012 and July 2014 researchers visited 10 flocks twice each year, when ewes were in late pregnancy and when ewes were in mid-late lactation. Of the ten flocks taking part in the study, three farmers only participated in year 1 and one farmer only participated in year 2. Six flocks were studied for both years, and only ewes within these flocks were chosen for bacteriological analysis.

Milk samples were collected from both udder halves by farmers in early lactation and by researchers in mid-late lactation (during research visit 2 and 4) each year. Data was collected on acute mastitis, intramammary masses (IMM), body condition score (BCS: 0-5 in 0.5 increments; Defra PB1875), nutrition, udder conformation, litter size and lamb weight (all described in Grant et al., 2016). Intramammary masses were defined as a physically detectable mass of abnormal consistency compared to the rest of the glandular tissue.

Farmers were trained to collect milk samples aseptically and provided with sampling kits. Farmers were asked to take samples in the first week after lambing during early lactation from both udder halves from sheep which had had an IMM (cases) detected at late pregnancy and from the same number of age-matched ewes which had not had an IMM detected during pregnancy (controls, selected by the researchers). Farmers were also asked to take milk samples from both udder halves of all ewes that developed clinical mastitis during lactation.

At the second examination by researchers during late lactation, researchers collected second milk samples from the case-control selected ewes and from the ewes with IMM detected for the first time during lactation. Where possible ewes sampled in year 1 were sampled again in year 2 and any new cases, with new age-matched controls, were sampled as necessary.

The study described in this chapter uses milk samples and data collected during the Grant et al. (2016) study and is not a continuation of the study described in Chapters 2 and 3. Methods that follow are novel and were not carried out during the original study. All samples were coded with a unique barcode. Samples were frozen at -20˚C once collected and transported and stored at -20˚C until 2016 when a proportion were thawed for bacteriological analysis.

In total, 1971 milk samples were taken from the six flocks. Due to time and resource constraints, selection criteria were applied to select milk samples for bacteriological analysis. Ewes that had data on IMM presence for all four researcher visits plus at least four milk samples over both years were selected. Bacteriological analysis was therefore carried out on 624 milk samples from 89 ewes (Table 4-1).

Table 4-1: Milk samples used in bacteriological study

Farm	Main Breed	Lambing time	Lambing location	N examined for IMM at all 4 visits	N examined with >4 milk samples	N with milk samples in both years	Total milk samples[a]
A	Charollais	Dec	Indoor	66	22	18	135
B	Charollais	Dec/Jan	Indoor	25	11	8	58
C	Charollais	Dec/Jan	Indoor	38	20	11	76
D	Lleyn	Mar/Apr	Indoor	923	172	20	120
E	Texel	Apr/May	Outdoor	37	22	16	131
F	Lleyn	Apr/May	Outdoor	210	37	16	104
Total				**1299**	**531**	**89**	**624**

N: number of ewes; IMM: intramammary mass; a: L & R udder half at a sampling count as 2 samples

Each milk sample was coded by sample type and visit number based on the date the sample was taken (Table 4-2). Individual sheep data were then linked to the time code.

Table 4-2: Sampling time points in the study

Sample Type	Planned samples	Clinical samples	Clinical sample type definition	Code for samples in year 1	Code for samples in year 2
Early Lactation (EL)	Collected from cases and age matched controls by researchers during visit	Collected from ewes with AM by trained farmers	First date of EL sample to first date of LL sample	1	3
Late Lactation (LL)	Collected from cases and controls by trained farmers	Collected from ewes with AM by trained farmers	Any sample in the same year on or after the first LL sample	2	4

EL: Early lactation; LL: Late lactation; AM: acute mastitis

4.2.2. Growth and selection of bacterial isolates

Milk sample barcodes were randomised within flocks using a random number generator in R (R Core Team) and grouped into batches of 10 for culture. Samples

were thawed at room temperature in batches and each sample mixed well. 10 µl of milk were spread across a sheep blood agar (SBA) plate with sterile inoculation loops and incubated inverted at 37˚C for 48 hours, as shown in Figure 4-1A.

Where selection of a pure isolate was difficult due to overgrowth on the agar plate, individual colonies were sub-cultured onto one quadrant of an SBA plate to ensure a pure isolate could be chosen (Figure 4-1B). Sub-culturing onto one quadrant was also carried out on colonies smaller than 1mm. Ethanol-isolate solutions were then stored at -20˚C until MALDI-ToF analysis.

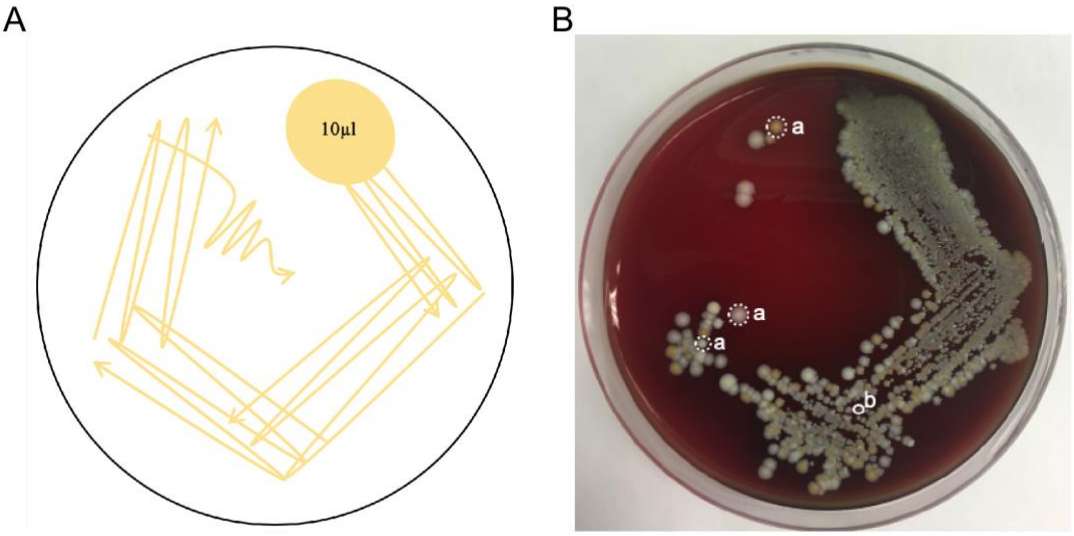


Figure 4-1: Preparation of cultures by streaking (A) schematic of method, (B) example. Each plate was carefully labelled with the unique barcode of the sample and 10 µl milk spread on the agar plate. A) For every sample milk was spread following the same pattern. A new inoculation loop was used for each section of the plate. B) In this sample three colonies were selected for direct suspension in 75% ethanol (white-dotted circles; labelled a) and one colony was sub-cultured onto quarter of a SBA plate before suspension (white circle); labelled b.

Morphologically unique isolates were defined by differences in appearance in the colony form, size, elevation, colour, margin and halo. At least one colony of each morphologically unique isolate was selected and given a unique id number. A loop of bacterial material was suspended in 300 µl water in a pre-labelled Eppendorf tube and mixed thoroughly for each identified isolate. 900 µl ethanol (Fisher

Chemical) was added to create a 75% ethanol solution in preparation for processing for MALDI-ToF analysis.

4.2.3. Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-ToF) mass spectrometry

4.2.3.1. Protein extraction from bacterial isolates for biotyper preparation

Formic acid protein extraction was carried out on isolates to produce clear spectra with low background noise and strong peak intensity (Alatoom et al., 2011). Ethanol-isolate solutions were thawed at room temperature and pelleted at 18,000 x g for 2 minutes and the supernatant discarded. Centrifugation was repeated, residual ethanol removed and the pellet left to air dry for 30 minutes. The pellet was resuspended in equal volumes of 70% formic acid and acetonitrile (equal to pellet size: either 5 µl, 10 µl, 20 µl, 30 µl or 40 µl) and thoroughly pipette-mixed. Finally, the sample was centrifuged at 18,000 x g for 2 minutes. 1 µl of supernatant was placed onto a steel target plate (Bruker UK Ltd., Coventry, UK) and air dried for 5 - 20 minutes before being overlaid with 1 µl HCCA matrix solution (α-Cyano-4-hydroxycinnamic acid, Bruker UK Ltd.) and left to air dry.

4.2.3.2. Spectra production and species identification

Target plates were transported in a dark container for MALDI-TOF MS analysis at Quality Milk Management Services Ltd (QMMS), Somerset. All plates were processed within 24 hours of preparation. Target plates were placed into a Microflex LT instrument (Bruker UK Ltd.), and protein mass spectra obtained three times for each target sample using FlexControl software (Bruker UK Ltd.) with default settings.

MALDI Biotyper 3 OC compared each spectra to an updated mastitis database of known species and returned a species identification and a confidence score for each spectra produced. All raw spectra and species identifications were saved. Where a confidence score fell below 1.70, the species identification was automatically allocated to ‘not reliable’ (Table 4-3). Where no spectra was produced, the species identification was allocated to ‘no peaks’.

There were three spectra and therefore three identifications for each isolate. To give a single identification for each isolate, the following rules were applied:

1. Where all three identifications give the same species, keep identification
2. Where at least two identifications give the same species, keep identification
3. Where only one spectra produced a species, if the Bruker score value above 2.00, keep this identification. If below 2.00, rename as ‘not reliable identification’
4. Where all three spectra give different species, rename as ‘not reliable identification’
5. Where all three ‘not reliable identification’ and/or ‘no peaks’, rename as ‘not reliable identification’

Table 4-3: Bruker Daltonik MALDI Biotyper given meanings for score values, taken from Bruker classification results

Range	Description	Colour
2.30 - 3.00	highly probable species identification	Green
2.00 - 2.29	secure genus identification, probable species identification	Green
1.70 - 1.99	probable genus identification	Orange
0.00 - 1.69	not reliable identification	Red

4.2.4. Data storage and management

Ewe meta data and associated milk samples were recorded throughout the study and stored into an Excel spreadsheet (2010; Microsoft Corp., Redmond, WA). Following isolate selection, each milk sample was associated with the isolate id given.

Each isolate and species identification were associated with the milk sample barcode. Using the R package ‘dplyr’ (Wickham et al., 2020) a presence/absence table was created, with milk samples as rows and each possible species identification as columns. Ewe metadata was joined to the table with the ‘left_join’ function. Ewe metadata included presence/absence of acute mastitis and IMM at any time on either udder half during the study, only in the same year as the sample on either udder half, at any time during the study on the same udder half as the sample, and only in the same year as the sample on the same udder half as the sample.

4.2.5. Data analysis of bacterial species isolated from milk

All statistical analyses were carried out using the R (v3.6.1) statistical environment (R Development Core Team, 2008) with the R studio user interface (v1.2.5001).

4.2.5.1. Summary statistics and distribution determination

Prior to detailed analysis data were examined using base R functions to investigate descriptive statistics, including measures of central tendency, spread and distribution.

When exploring the species identified in all milk samples, species denominator was the number of samples they were positive for that species, except for species occurring in less than 7 samples, which were combined into an 'others' group. Species were also described by their genus and the percentage of udder halves and ewes that a species belonging to that genus was isolated from at least once was calculated. Coagulase negative staphylococcus and *S. aureus* were kept separate in this analysis due to the biological relevance of *S. aureus* associated with mammary disease.

4.2.5.2. Association between disease status and species present

Generalised binomial mixed effect regression models were used to investigate associations between disease status and species. Individual species where the total number of positive observations were under ten were combined into groups based on genus. Where the total number of positive observations once grouped remained under ten, these groups were combined into a group named 'other'. For each ewe, samples were combined across udder halves and time points to leave one result for each year.

The outcome variable was presence/absence of acute mastitis or an intramammary mass in the year the sample was taken. Flock, ewe and year were included as random effects. Tested fixed effects were the presence or absence of species or species groups, year and flock. Univariable models were created for each explanatory variable and then multivariable models created in a forward stepwise selection process (Dohoo et al., 2003). The models took the form:

$$Logit(\pi_{ijk}) = \beta_0 + \beta x_{ijk} + v_k + u_{jk} + e_{ijk}$$

where $Logit(\pi_{ijk})$ is the log odds of the probability that a ewe had acute mastitis or an intramammary mass,β_0 is the intercept, and βx_{ijk} are the explanatory variables. The residual variance estimates at flock v_k, ewe u_{jk} and observation e_{ijk}.

The function 'glmer' from the R package lme4 (Bates et al., 2015) was used to construct models. The "bobyqa" optimizer with 1 x 10^5 as the maximum number of function evaluations was used to enhance model convergence. Associations between bacteria presence and disease status were considered significant when 95% confidence intervals (Wald estimates) of the odds ratios did not include 1. All non-significant variables were retested in the final model to investigate residual confounding (Cox and Wermuth, 1996).

4.2.6. Strain typing using MALDI-ToF for investigation of transmission and persistence

4.2.6.1. Mass spectra processing

Raw spectra were read into R and open source packages MALDIquant, readBrukerFlexData, and MALDIrppa used for quality control and processing (Gibb and Strimmer, 2012; Palarea-Albaladejo et al., 2017) (Figure 4-2). Peak data (a peak list) was successfully extracted for 1563 isolates, 136 isolates returned no spectrum or were removed at the quality control stage.

4.2.6.2. Unsupervised hierarchical clustering

Peak lists generated from processed spectra were clustered based on similarity. A Euclidean distance matrix and a hierarchical tree was generated using the R package 'stats' (R Core Team).

4.2.6.3. Setting a threshold Euclidean distance to differentiate strains

18 isolates of the most common genera and species (*Arthrobacter spp.*, *Enterococcus faecium*, *S. equorum*, *S. aureus*, *S. lentus*, *S. xylosus*, *Bacillus licheniformis*, *S. sciuri*, *Wickerhamomyces anomalus*) were chosen across the six farms. Biological replicates and technical replicates were taken for MALDI-ToF MS analysis and processed as in section 4.2.3.2 above.

Pearson correlation was calculated on spectra peak lists of replicates using 'cor' (R base stats) and squared to give percentage similarity.

The hierarchical clusters for these replicates were used to determine a threshold Euclidean distance. Isolates that clustered under this distance were considered very similar and therefore the same strain. Each strain was given a unique identification number.

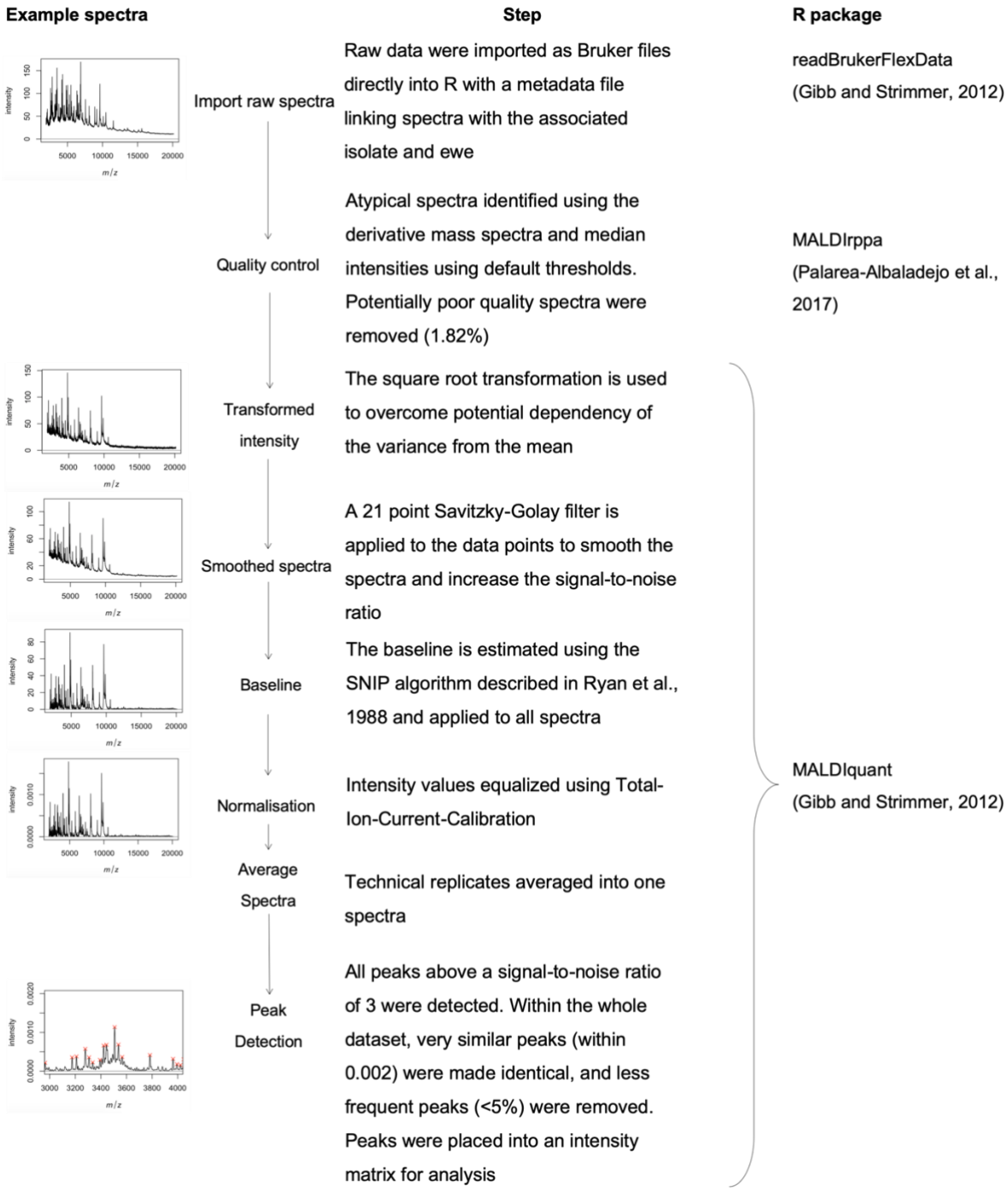


Figure 4-2: Pipeline for processing mass spectra to generate peak lists. 4689 spectra from 1563 isolates were processed using R, using the entire dataset to improve quality control. All raw spectra were passed through each step. Spectra from technical replicates

4.2.7. Whole Genome Sequencing of *Staphylococcus aureus* isolates

4.2.7.1. Selection of isolates for sequencing and analysis

After identification of all isolates, 89 *Staphylococcus aureus* isolates were chosen for whole genome sequencing (WGS). Where no original isolate duplicate was available, milk samples were cultured as in section 4.2.2 and isolates with matching morphological characteristics selected by eye using notes on colony form, size, elevation, colour, margin and halo produced in the first culture. Isolates were then analysed using MALDI-ToF MS as above (section 4.2.3) and sent for WGS.

4.2.7.2. Sequencing and quality control

Genome sequencing was provided by MicrobesNG (**http://www.microbesng.uk**). DNA extraction (agar method), sequencing, quality control and bioinformatics, including genome assembly and annotation, were all carried out by MicrobesNG.

There were 18 sequenced isolates that had been incorrectly selected after the second bacterial culture due to the inaccurate process of selecting of isolates by eye (section 4.2.7.1) and were not *S. aureus*. The remaining 71 isolates were uploaded to the web-based portal Pathogenwatch (**https://pathogen.watch/**) where a phylogenetic tree was produced and visualised using Phylocancas (**http://phylocanvas.org**). Duplicate sequences were selected for removal if they belonged to the same milk sample and existed on the same clade on the phylogenetic tree. There were 26 sequences removed as duplicates and 1 sequence removed due to loss of meta data.

The remaining 44 sequences were uploaded to the web-based portal Pathogenwatch in a separate collection with their metadata in a ‘csv’ file. The phylogenetic tree was exported as a ‘Newick’ file.

4.2.7.3. Phylogenetic tree visualisation

The phylogenetic tree was loaded into R as a ‘Newick’ file using the package ‘ape’ (version 5.4.1) (Paradis and Schliep, 2019). Visualisation was processed using the package ‘ggtree’ (Yu, 2020).

4.2.8. Defining persistence and transmission

For MALDI-ToF MS spectra, clusters were defined as closely related spectra using the Euclidean distance selected using technical and biological replicates as described above in section 4.2.6.2.

For WGS, clusters were defined using core genome MLST (cgMLST) clustering on the web-based portal Pathogenwatch (**https://pathogen.watch/**), using the schemes from cgmlst.org (Leopold et al., 2014), as described on the webpage documentation (CGPS, 2020). In brief, cgMLST profiles are clustered using Single Linkage Clustering based on calculated distances between different loci for a given scheme. (CGPS, 2020)

Potential transmission events (PTE) were defined as isolates in the same cluster within the same flock but in different ewes at any time during the study. Potential persistence events (PPE) were defined as isolates in the same cluster within the same ewe and udder half at more than one sampling time. Finally, isolates in the same cluster from both udder halves of a ewe at the same sampling time were defined as 'across mammary gland' events.

4.2.9. Validating MALDI -ToF MS strain typing

Technical and biological replicates of isolates were taken from 18 isolates to calculate reproducibility. Technical replicate samples were taken from the same colony and then underwent separate protein extraction (section 4.2.3.1). Biological replicate samples were created by regrowing pure isolates on spread plates and selecting individual colonies for protein extraction. Following spectra production (as described in section 4.2.3.1) reproducibility was measured by calculating the similarity of each spectra using Pearsons correlation coefficient.

MALDI-ToF MS strain clusters were compared to WGS cgMLST clusters for each *S. aureus* isolate. Cluster groups were mapped onto unrooted trees to visualise the differences in cluster membership. PPEs, strains across the mammary gland and PTEs were calculated for both cluster types as described in section 4.2.8 in order to investigate performance of MALDI-ToF as a method to determine transmission and persistence. The persistence and transmission identified by clusters designated using cgMLST were considered the gold standard and the results using MALDI-ToF MS were directly compared to estimate specificity and sensitivity.

4.3. Results

4.3.1. Identification of bacterial species in milk samples from healthy ewes, ewes with chronic mastitis, and ewes with acute mastitis

4.3.1.1. Bacterial culture of milk samples

In total, 625 milk samples from 178 udder halves (89 ewes) and 6 flocks were cultured. Occasionally, 2 milk samples were taken at the same time point from the same ewe. The results of these samples were combined, leaving 605 unique samples in total (Table 4-4).

There were 60 samples with no growth and therefore no isolates. From the rest of the samples, 1562 isolates were selected for MALDI-ToF MS analysis (median: 2 isolates per sample).

Table 4-4: Summary of milk samples and isolates detected by flocks and sampling times

				Isolates per sample				
Time	Ewes N	Milk samples N	Isolates N	Range (min - max)	Mean	SD	Median	IQR
Flock: A								
1	13	26	96	1 - 10	3.69	2.11	3.50	2.75
2	18	36	151	1 - 14	4.19	2.47	4.00	2.00
3	18	36	93	0 - 8	2.58	1.81	2.00	3.00
4	18	37	115	1 - 14	3.11	2.62	2.00	1.00
Total	18	135	455	0 - 14	3.37	2.35	3.00	2.00
Flock: B								
1	5	10	42	2 - 9	4.20	2.39	3.50	3.25
2	8	16	52	1 - 8	3.25	1.84	2.50	2.00
3	8	16	50	0 - 6	3.12	1.63	3.00	2.25
4	8	16	43	0 - 5	2.69	1.20	3.00	1.00
Total	8	58	187	0 - 9	3.22	1.77	3.00	2.00
Flock: C								
1	6	12	25	0 - 4	2.08	1.08	2.00	1.25
2	11	22	26	0 - 4	1.18	1.14	1.00	2.00
3	11	22	63	0 - 5	2.86	1.42	3.00	2.00
4	10	20	52	1 - 7	2.74	1.63	2.00	1.00
Total	11	76	166	0 - 7	2.21	1.51	2.00	2.00
Flock: D								
1	16	32	91	0 - 8	2.84	1.69	3.00	2.00
2	17	34	60	0 - 7	1.76	1.58	1.00	1.00
3	7	16	40	0 - 8	2.50	3.03	1.00	3.50
4	19	38	80	0 - 6	2.11	1.45	2.00	2.00
Total	20	120	271	0 - 8	2.26	1.85	2.00	2.00
Flock: E								
1	12	33	69	0 - 4	2.03	1.34	2.00	2.00
2	14	28	47	0 - 4	1.68	0.98	1.00	1.25
3	16	40	127	1 - 6	3.17	1.28	3.00	2.00
4	15	30	83	1 - 5	2.77	1.57	2.50	3.00
Total	16	131	326	0 - 6	2.47	1.43	2.00	2.00

				Isolates per sample				
Time	Ewes N	Milk samples N	Isolates N	Range (min - max)	Mean	SD	Median	IQR
Flock: F								
1	12	24	38	0 - 5	1.81	1.63	1.00	2.00
2	16	32	28	0 - 7	1.17	1.58	1.00	2.00
3	9	18	27	0 - 4	1.59	1.23	2.00	1.00
4	15	30	64	0 - 6	2.78	1.57	3.00	2.00
Total	16	104	157	0 - 7	1.85	1.62	2.00	2.00
	89	**624**	**1562**	**0 - 14**	**2.58**	**1.89**	**2.00**	**3.00**

SD: standard deviation; N: number of observations; Time 1: Year 1 early lactation; Time 2: Year 2 early lactation; Time 3: Year 3 early lactation; Time 4: Year 4 early lactation; Note: not all ewes had samples taken in all sampling times

4.3.1.2. Ewes used for bacterial culture of milk samples

All ewes where milk samples were selected were examined in all four examination points. Across all 6 flocks, between 19 - 39 (21.3 % - 42.7 %) ewes had an IMM in at least one udder half at each examination point. Acute mastitis was only recorded during lactation and affected 25 (7 %) ewes across the whole study. This ranged from 0 % - 69 % within flocks. (Table 4-5)

Table 4-5: Summary of ewe disease data over the study period

Exam number	Ewes N	IMM N (%)	AM N (%)
Flock: A			
1	18	7 (38.9)	
2	18	6 (33.3)	0
3	18	2 (11.1)	
4	18	7 (38.9)	5 (27.8)
Flock: B			
1	8	1 (12.5)	
2	8	6 (75.0)	1 (12.5)
3	8	1 (12.5)	
4	8	5 (62.5)	0
Flock: C			
1	11	2 (18.2)	
2	11	8 (72.7)	2 (18.2)
3	11	1 (9.1)	
4	11	4 (36.4)	0
Flock: D			
1	20	9 (45.0)	
2	20	7 (35.0)	2 (10.0)
3	20	8 (40.0)	
4	20	8 (40.0)	1 (5.0)
Flock: E			
1	16	5 (31.2)	
2	16	7 (43.8)	11 (68.8)
3	16	1 (6.2)	
4	16	11 (68.8)	2 (12.5)
Flock: F			
1	16	6 (37.5)	
2	16	4 (25.0)	0
3	16	6 (37.5)	
4	16	8 (50.0)	1 (6.2)
Flock: All Flocks			
1	89	30 (33.7)	
2	89	38 (42.7)	16 (18.0)
3	89	19 (21.3)	
4	89	43 (48.3)	9 (10.1)

N: number of observations; IMM: intramammary mass; AM: acute mastitis; Note: Exam times 1 & 3 are not equivalent to sampling times 1 & 3. Exam numbers = 1: year 1 late pregnancy; 2: year 1 late lactation; 3: year 2 late pregnancy; 4: year 2 late lactation

4.3.1.3. Species in each milk sample

In total, 80 species were identified by MALDI-ToF MS. Of these, 23 occurred in 7 (1%) or more samples (Figure 4-3). The genus *Staphylococcus* accounted for 10 of the top 23 species.

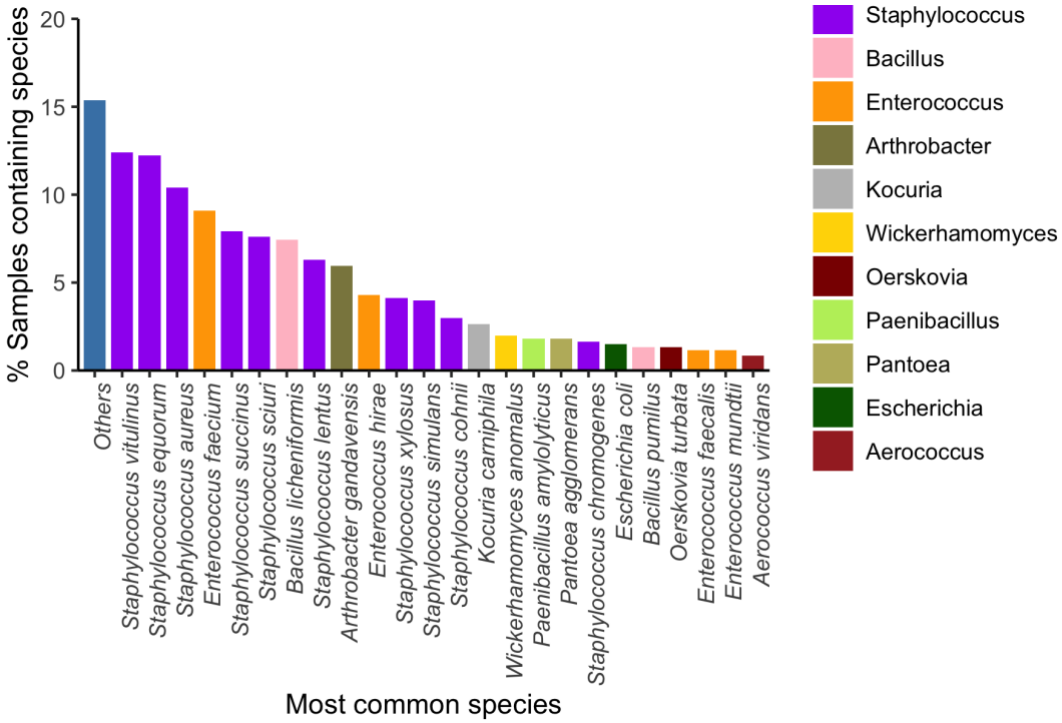


Figure 4-3: Species detected in milk samples ordered by percentage. Species occurring in less than 7 samples were put into a separate "Others" category.

The 4 most common species were *Staphylococcus vitulinus* (75 samples, 12.4%), *Staphylococcus equorum* (74 samples, 12.2%), *Staphylococcus aureus* (63 samples, 10.4%) and *Enterococcus faecium* (55 samples, 9%).

Coagulase Negative *Staphylococcus* (CNS) and *Enterococcus* spp. were isolated at least once in over 40% of udder halves and ewes, and *Bacillus* spp. was isolated at least once in over 50% of ewes (Table 4-6).

Table 4-6: Genus of bacteria isolated from udder halves and ewes over the study

	Udder halves (n = 178)		Ewes (n = 89)	
Genus	N	%	N	%
CNS	136	76.40	76	85.39
Enterococcus	80	44.94	54	60.67
Bacillus	56	31.46	46	51.69
S. aureus	46	25.84	32	35.96
Arthrobacter	32	17.98	29	32.58
Kocuria	16	8.99	14	15.73
Paenibacillus	12	6.74	12	13.48
Wickerhamomyces	11	6.18	9	10.11

N: number of cases where genus observed at least once; Staphylococcus spp. has been split into CNS and *S. aureus*

4.3.1.4. Association between disease status and bacterial species presence

Each species or group of species was tested in two univariable mixed effect binomial models, one with IMM as the outcome variable and one with acute mastitis as the outcome variable. Results of the univariable analysis are in Table A-4 and Table A-5 in the appendix. There were two species significantly associated with IMM (Table 4-7). There was an increased likelihood of a ewe having an IMM if S. aureus was isolated from a milk sample (OR: 2.78, 95 % CI: 1.20 - 6.42), and S. sciuri had increased odds of being isolated from healthy ewes compared to ewes with an IMM (OR: 0.38, 95 % CI: 0.17 - 0.88).

Where a case of acute mastitis was the outcome, four species *(S. aureus*, *E. faecium*, *S. succinus*, *S. cohnii*) were significant in univariable models, and *S. aureus* and *E. faecium* were retained in the final multivariable model. Ewes with a case of acute mastitis had over 9-fold increased odds of having an isolate of *S. aureus* in samples from the same year (95 % CI: 2.26 - 40.08). There were increased odds of isolating *E. faecium* in an healthy ewe than a ewe with acute mastitis (OR: 0.03, 95% CI: 0.01 - 0.47). (Table 4-8)

Table 4-7: Multivariable binomial mixed effects regression model of presence of an IMM in the same year as the sample

Variable	Category	Not Affected N(%)	Affected N(%)	Total N (%)	OR	95% CI
Intercept					1.63	1.07 - 2.48
Staphylococcus aureus	Absent	58 (86.6)	76 (68.5)	134 (75.3)	Ref	
	Present	**9 (13.4)**	**35 (31.5)**	**44 (24.7)**	**2.78**	**1.20 - 6.42**
Staphylococcus sciuri	Absent	48 (71.6)	97 (87.4)	145 (81.5)	Ref	
	Present	**19 (28.4)**	**14 (12.6)**	**33 (18.5)**	**0.38**	**0.17 - 0.88**
Random effects	*Variance*					
Ewe	0.1113					
Flock	0.0000					
Year	0.0000					

OR: Odds ratio; CI: confidence intervals; N: number of observations; Wald estimates used for CI;

Table 4-8: Multivariable binomial mixed effects regression model of acute mastitis in the same year as the sample

Variable	Category	Not Affected N(%)	Affected N(%)	Total N (%)	OR	95% CI
Intercept					0.07	0.01 - 0.32
Staphylococcus aureus	Absent	122 (79.7)	12 (48.0)	134 (75.3)	Ref	
	Present	**31 (20.3)**	**13 (52.0)**	**44 (24.7)**	**9.51**	**2.26 - 40.08**
Enterococcus faecium	Absent	114 (74.5)	23 (92.0)	137 (77.0)	Ref	
	Present	**39 (25.5)**	**2 (8.0)**	**41 (23.0)**	**0.03**	**<0.01 - 0.47**
Random effects	*Variance*					
Ewe	0.1994					
Flock	1.7244					
Year	0.0000					

OR: Odds ratio; CI: confidence intervals; N: number of observations; Wald estimates used for CI

4.3.2. Investigation into transmission and persistence of *Staphylococcus aureus* using Whole Genome Sequences

4.3.2.1. Descriptive statistics for sequenced isolates

There were 25 ewes with a *Staphylococcus aureus* isolate with 1 - 7 positive ewes per flock with 2 - 13 isolates per flock (Table 4-9). Ewes had 1 - 4 samples with a *S. aureus* isolate. Table 4-10 gives the individual disease status of each ewe over the 2 year longitudinal study. There were 13 ewes with both at least one case of IMM and of acute mastitis, 11 ewes with at least one case of IMM, and a single ewe with no recorded mammary disease.

Table 4-9: Distribution of *S. aureus* isolates by flock, ewe and udder

Flock	Ewes	Number of isolates
A	3	5
B	1	2
C	3	5
D	7	13
E	6	10
F	5	9

Table 4-10 Disease status of ewes used in this study

Flock	Ewe	Isolates	Disease
A	E1	1	IMM and AM
A	E7	3	IMM and AM
A	E9	1	IMM
B	E21	2	IMM
C	E27	2	No disease
C	E30	2	IMM and AM
C	E32	1	IMM and AM
D	E37	3	IMM
D	E41	2	IMM and AM
D	E42	3	IMM and AM
D	E45	1	IMM and AM
D	E46	2	IMM
D	E52	1	IMM
D	E55	1	IMM
E	E57	1	IMM and AM
E	E58	1	IMM and AM
E	E59	4	IMM and AM

Flock	Ewe	Isolates	Disease
E	E64	1	IMM
E	E66	2	IMM and AM
E	E71	1	IMM and AM
F	E79	1	IMM
F	E81	2	IMM
F	E82	1	IMM
F	E84	4	IMM and AM
F	E85	1	IMM

IMM: intramammary mass; AM: acute mastitis

4.3.2.2. Whole genome sequencing strain typing

There were 4 sequence types (ST) in the 44 isolates, 34 (77%) were ST 133 and the others were novel STs (Table 4-11). Novel STs “7269*”, and “cdc1*” were phenotypically similar to ST 133, but ST “1ff9*” was separate (Figure 4-4A red shading). For ease of visualisation, the 4 ST “1ff9*” isolates from flock D were removed from Figure 4-4B).

Table 4-11: MLST profiles for each sequence type as given on pathogen.watch

Sequence Type	MLST profile							N
	arcC	aroE	glpF	gmk	pta	tpi	yqiL	
133	6	66	46	2	7	50	18	34
cdc1*	6	66	46	novel	7	novel	18	5
1ff9*	6	57	45	2	7	novel	52	4
7269*	novel	66	46	2	7	50	18	1

4.3.2.3. Transmission and persistence of *Staphylococcus aureus* strains based on whole genome sequencing

Strains were found in both udder halves of 6 ewes at the same time point. Four of these can be seen in Figure 4-4B labelled as ‘a’.

Based on cgMLST clustering alone, 7 ewes were identified as having at least one potential persistence event (PPE), where the same strain was identified across sampling times. Figure 4-4B shows 6 examples of cases where the isolates from the same ewe have clustered closely together on the phylogenetic tree, labelled as ‘b’.

There were 9 ewes in flocks E and F with isolates defined as potential transmission events (PTE), where the same cgMLST strain-type was identified in different ewes

in the same flock (labelled at ‘c’). Although flocks A, C and D do not have any PTEs when based on cgMLST clustering, isolates from these flocks are genetically similar, with evidence of tight clustering on the phylogenetic tree (Figure 4-4B).

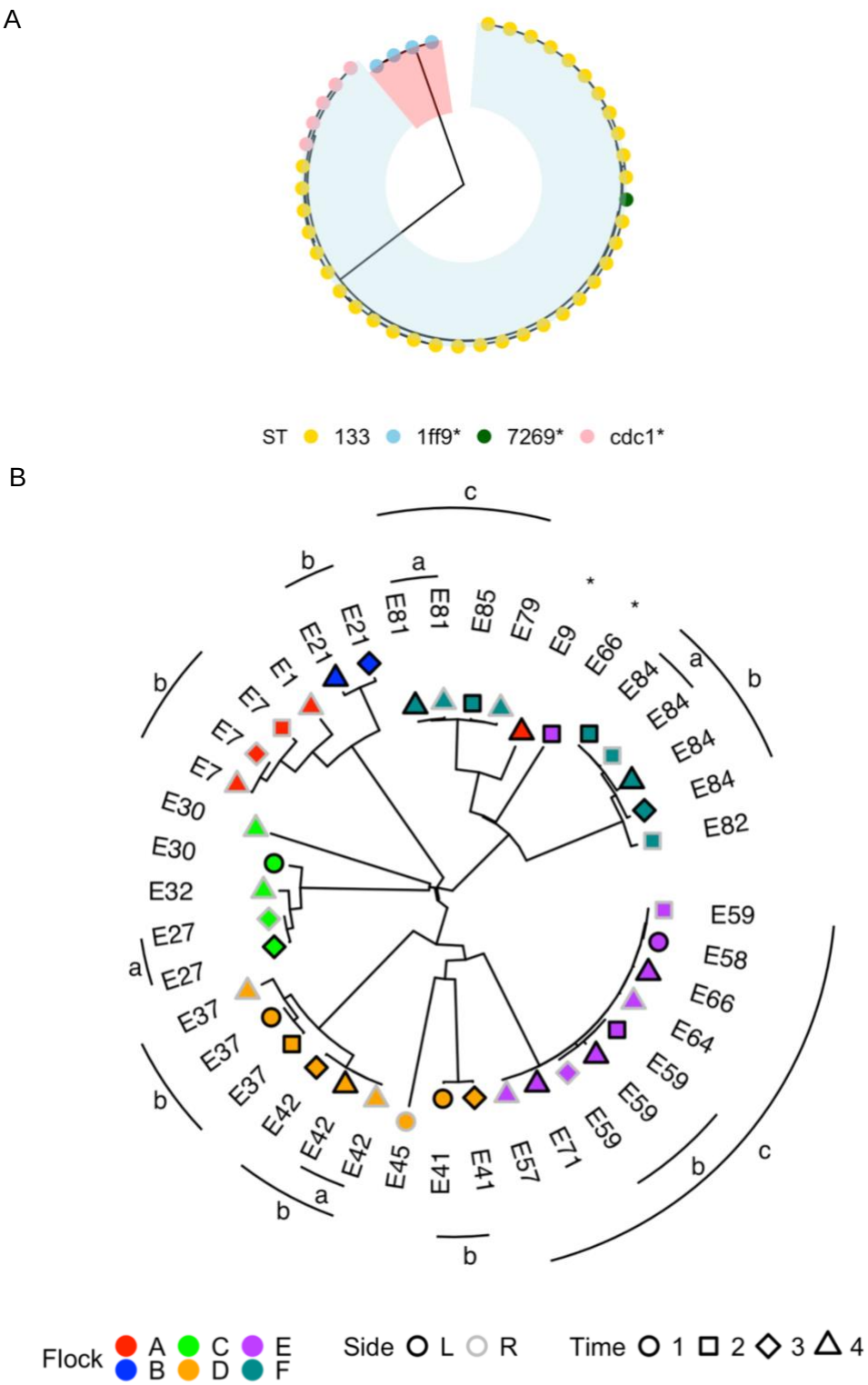


Figure 4-4: Staphylococcus aureus isolates are concentrated into flock lineages. A**,** Phylogenetic tree of 44 *S. aureus* isolates analysed in this study, demonstrates division into two major clades, represented by blue and red shading; B, Detailed phylogenetic tree of the 40 isolates represented by blue shading in A. Clade labelling shows examples of a: isolates taken from a ewe at the same time point from each udder half, b: isolates taken from a ewe at different time points, c: strains of the same cgMLST cluster across more than one ewe., *: isolates not belonging to a cluster. Tip labels represent unique ewe identifiers. Flocks are represented by different tip colours, time of sample collection by tip shape and udder half by tip outline.

4.3.3. Validation of MALDI-ToF MS strain typing

4.3.3.1. Percentage correlation of spectra from biological and technical replicates

Technical replicates were taken from the same isolate but underwent a separate protein extraction. All species had average reproducibility above 95% for technical replicates, and only *Wickerhamomyces anomalus* had a spectra below 90% similarity (Figure 4-5A).

Biological replicates were taken from pure isolates regrown on spread plates. There is a wide range in reproducibility between species, and a lower average reproducibility for all samples compared to technical replicates (Figure 4-5B).

Clusters of biological replicates were used to create the threshold Euclidean distance for strain-typing using MALDI-ToF MS.

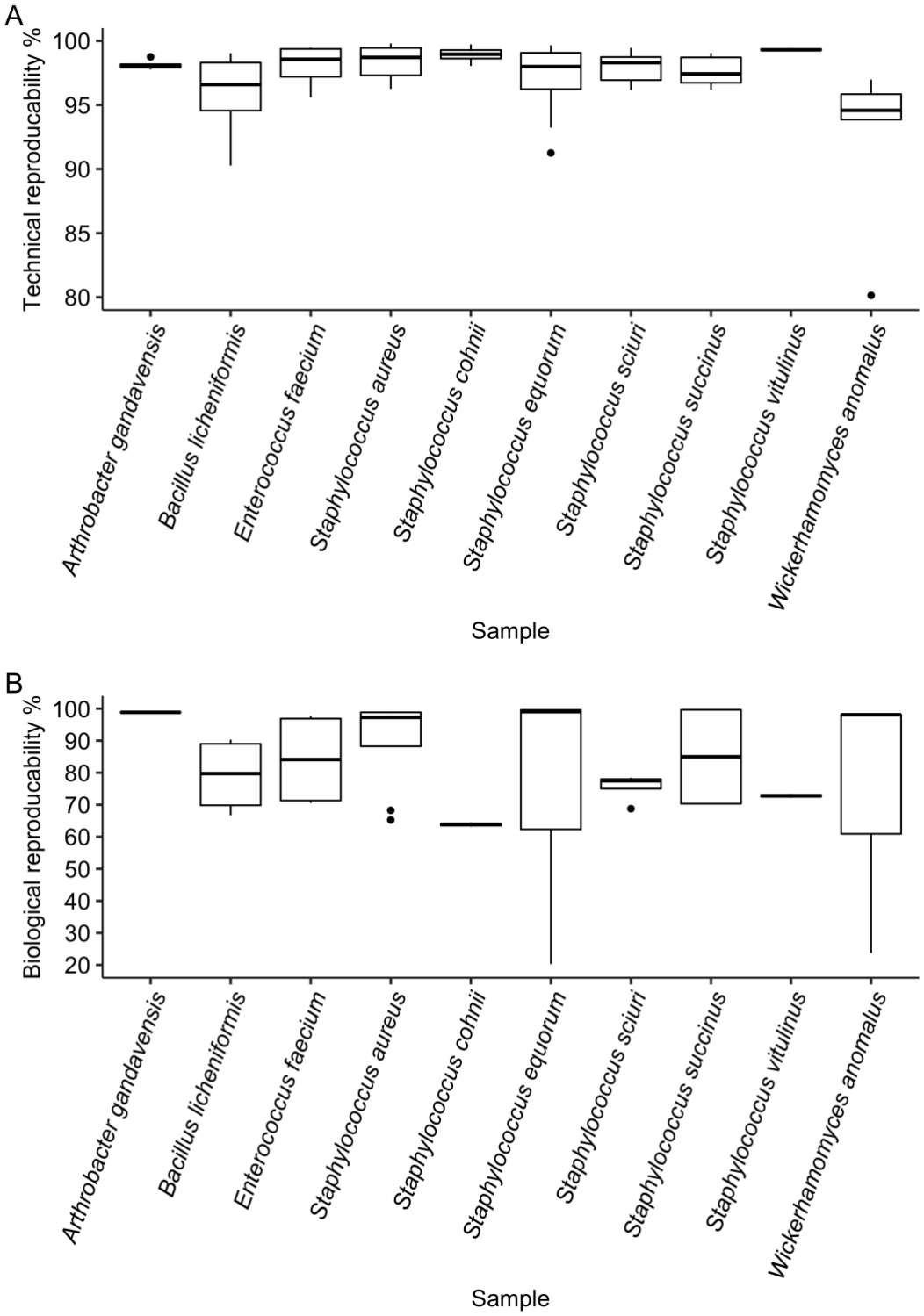


Figure 4-5: Percentage reproducibility of A: Technical samples, B: Biological samples. 18 isolates of the most common genera and species were chosen for A) technical and B) biological replicates. Spectra were processed into peak lists and then compared.

4.3.3.2. Comparison of strain typing using whole genome sequencing and using MALDI-ToF MS for *Staphylococcus aureus* isolates

The MALDI-ToF MS clustering method produced 7 *Staphylococcus aureus* clusters with between 1 - 17 isolates. The cgMLST clustering identified 22 clusters, 12 of which had a single isolate, and the rest between 2 - 9 isolates. In no case did a group of cgMLST isolates match directly onto a single MALDI-ToF MS cluster. (Figure 4-6)

Figure 4-7 compares unrooted trees of equal branch length for the MALDI-ToF MS Euclidean tree and the WGS phylogenetic tree. There are two groups of 3 isolates or more where part of the MALDI-ToF MS cluster has been retained as a cluster in the phylogenetic tree (ID31:ID30 Farm E and ID37:ID35 Farm F). The other parts of these two groups and all other MALDI-ToF MS clusters are spread around the phylogenetic tree.

PPEs, strains across the mammary gland, and PTEs were calculated for MALDI-ToF MS clusters and cgMLST clusters as described in section 4.2.8. MALDI-ToF MS clusters did not predict any positive PPEs that weren't detected using the cgMLST method (false positive), although it failed to predict 7 PPEs that were identified using the cgMLST method (false negative) (sensitivity = 59%, specificity = 100%). Similarly, there were no false positive strains across the mammary gland, but there were 6 isolates that had a strain pair identified by cgMLST that weren't detected using MALDI-ToF MS (sensitivity = 50%, specificity = 100%). In contrast, when predicting PTE within a flock, the large clusters created by MALDI-ToF MS meant that there were 24 isolates identified as strains which weren't identified using cgMLST. Only one isolate was identified using cgMLST and not by MALDI-ToF MS (sensitivity = 92%, specificity = 23%). (Table 4-12)

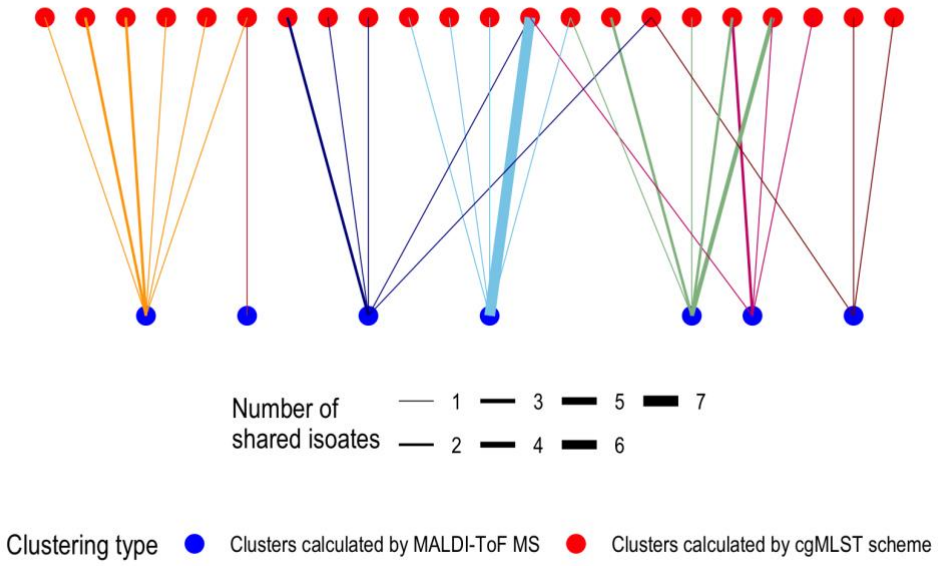


Figure 4-6: Comparison of isolate clusters calculated by MALDI-ToF MS spectra and by cgMLST profiles using WGS. Points at the top are 25 cgMLST clusters calculated by pathogen.watch. Points at the bottom are 7 clusters calculated by using a Euclidean distance matrix on MALDI-ToF MS spectra (as described in section 4.2.6.2). Line colours are MALDI-ToF MS clusters and line weight is the number of shared isolates between the two cluster types.

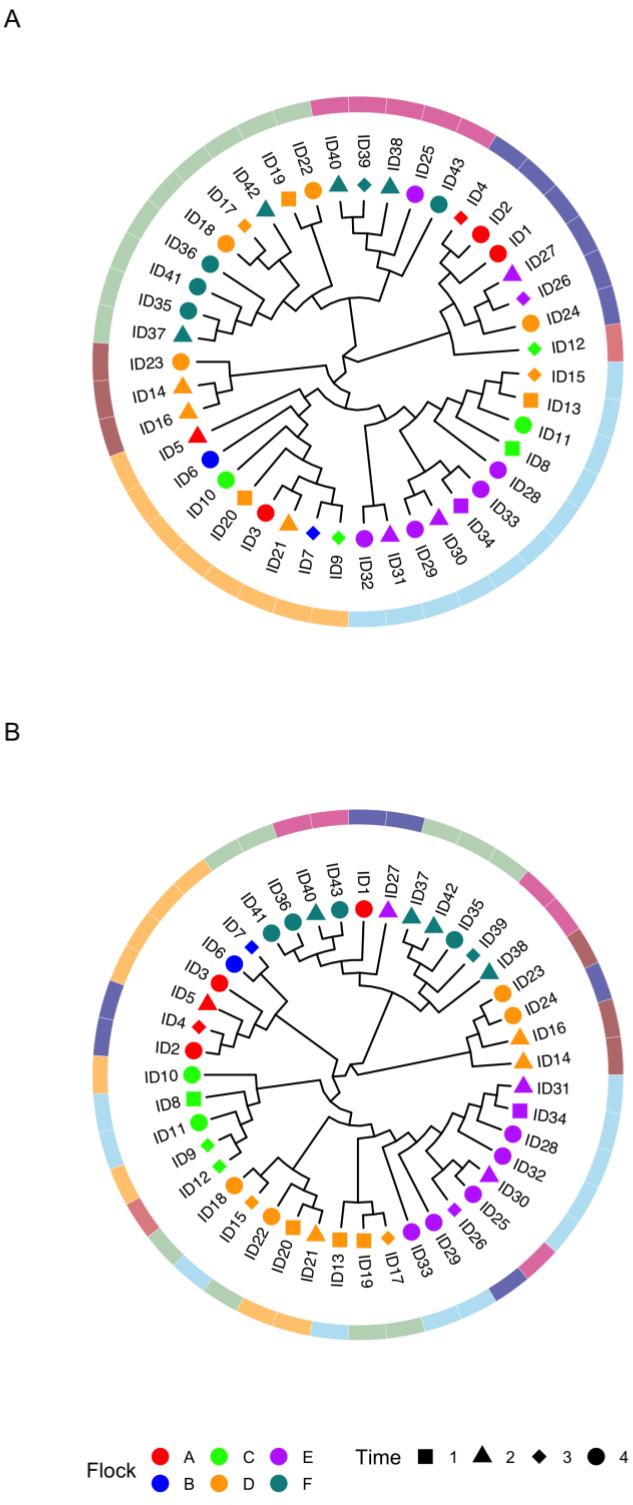


Figure 4-7: MALDI-ToF MS calculated clusters mapped onto unrooted trees with fixed branch length. Tip point colours are flocks and tip point shapes are time of sampling. The outer ring colours isolates by MALDI-ToF MS cluster membership in both trees A; Euclidean distance tree calculated using MALDI-ToF MS spectra peak lists B; phylogenetic tree of *Staphylococcus aureus* isolates analysed in this study using whole genome data.

Table 4-12: MALDI-ToF MS clustering compared to cgMLST as a gold standard

Variable		Number of isolates (%)
PPE	True positive	10 (22.7)
	True negative	27 (61.4)
	False positive	0 (0.0)
	False negative	7 (15.9)
Across gland	True positive	6 (13.6)
	True negative	32 (72.7)
	False positive	0 (0.0)
	False negative	6 (13.6)
PTE	True positive	12 (27.3)
	True negative	7 (15.9)
	False positive	24 (54.5)
	False negative	1 (2.3)

PPE: potential persistence event (strain identified in same ewe at different sampling time); Across gland: across mammary gland (strain identified in both udder halves of same ewe at same sampling time); PTE: potential transmission event (strain identified in different ewe within the same farm)

4.4. Discussion

This study has looked at culturable bacteria from suckler sheep milk samples associated with healthy and diseased mammary glands.

4.4.1. Bacterial species presence

Bacteria were cultured from both healthy and diseased milk samples. Although traditionally, cultured bacteria in healthy samples were considered an indication of contamination or subacute disease, this is now understood to be incorrect (Angelopoulou et al., 2018). Many culture-dependent and culture-independent studies have shown the healthy udder to contain a diverse bacterial community (Bergonier et al., 2003; Mørk et al., 2007; Contreras and Rodríguez, 2011; Jiménez et al., 2015; Smith et al., 2015).

Culture dependent studies are limited in that only culturable bacteria can be identified and one cannot be sure that all of these grow in all samples. In addition, calculations of abundance are much more complex. Nevertheless, this study found 80 species belonging to over 10 genera, showing the diversity of species in sheep milk.

This study found coagulase-negative staphylococci (CNS) and enterococci were the predominant genera in all sheep regardless of disease state, with isolates detected in 76% and 45% of udder halves respectively (Table 4-6). Although studies into suckler sheep milk are limited, CNS species have been associated with subclinical mastitis in dairy ewes and have been isolated from the teat skin of ewes (Burriel, 1998; Leitner et al., 2019). Similarly, *S. aureus* has been cultured from healthy teat skin and is a normal skin commensal in sheep (Bergonier et al., 2003).

The current study found that sheep with acute mastitis were significantly more likely to have *S. aureus* isolated from their milk. A study by Kvist et al. (2008) investigated the culturable bacterial flora of human milk from healthy women and women with mastitis, finding that many healthy women had bacteria considered pathogenic in their milk. CNS was not linked to clinical disease, but *S. aureus* was more frequently isolated from women with clinical signs of mastitis (Kvist et al., 2008).

There was a significant association between presence of intramammary mass in the udder and isolation of *S. aureus*. Smith et al. (2015) used sectioned udders from abattoirs to investigate the bacterial species present within abscesses and found that abscesses ranged from small pus-filled sacs to large intramammary voids.

Their study found a predominance of *S. aureus* in the abscesses, which were closely related to strains in milk from the same gland, although no comparison was made to healthy udders. The association between *S. aureus* and IMM in the current study suggests that this species is important in pathogenicity. Smith et al. (2015) found related isolates within the milk and abscesses, which suggests it is likely that those strains isolated from milk in the current study would also be similar to *S. aureus* in any abscesses (IMM) in these udders. Although Smith et al (2015) used MALDI-ToF MS to calculate relatedness between strains, strain typing within an udder was found to be highly specific in the current study, giving confidence that the related strains were not falsely associated.

There was also a significant association with species *Enterococcus faecium* and *S. sciuri* with milk samples from healthy ewes (25% and 28% of healthy samples respectively). A study into the inhibitory activity of isolates in human breast milk found commensal bacteria that interfere with the growth of *S. aureus*, including several CNS and *E. faecium* (Heikkilä and Saris, 2003). It is therefore a possibility that these species have an important role in protecting against pathogens within the udder.

4.4.2. Persistence of *Staphylococcus aureus* within ewes

Despite the small sample size, there were several examples of where the same strain of *S. aureus* was isolated from the same ewe in each udder half. As the two udder halves are anatomically separated, with two glands, the milk from each half does not usually have an opportunity to mix within the udder tissue. This suggests that *S. aureus* infected both glands separately. This may have occurred from the same source e.g. sheep skin, or subsequent to the initial infection, for example by a lamb mouth carrying the strain from one half to the other.

There was also evidence of persistence of *S. aureus* strains from one examination to the next, including across lactations. This is important as it indicates continued infection, a sustained reservoir of pathogens, and potentially a failure of treatment. The likely mechanism for persistence is through the formation of a *S. aureus* abscess (Smith et al., 2015), however there was only one ewe in the *S. aureus* subset without a detected IMM, and so a bigger sample size of healthy ewes would have to be included to confirm that persistent *S. aureus* is only found in the milk of ewes with IMM.

S. aureus was significantly associated with presence of IMM, but it was not isolated from 67% of IMM cases. Although in cattle studies *S. aureus* has been recorded as causing the highest frequency of reoccurring infections (Wente et al., 2020), and Smith et al. (2015) found it in 10/14 sheep udder abscesses, it is likely that *S. aureus* is not the only pathogen capable of causing IMM formation. Species previously associated with chronic mastitis and udder abscesses such as *Streptococcus* species (Onnasch et al., 2002, Smith et al., 2005) are probably also able to trigger IMM development.

4.4.3. Transmission of *Staphylococcus aureus* within flocks

Molecular epidemiological studies on dairy cattle have classified *S. aureus* as a contagious pathogen (Zadoks et al., 2000; Smith et al., 2005; Tenhagen et al., 2007), where the detection of the same strain-type in multiple cows represents cow-cow transmission. Sommerhäuser et al. (2003) reported that in some herds there was evidence of environmental *S. aureus* due to the presence of multiple strains within the herd. These strains appeared only sporadically and showed little affinity to spread. In this study, *S. aureus* samples from 25 ewes across six flocks were sequenced to investigate transmission within flocks. Of the five flocks with isolates from multiple ewes, all showed tight clustering within flocks. This supports the hypothesis that *S. aureus* is a contagious pathogen capable of spreading from ewe to ewe. All 6 ewes with samples in flock E had isolates of *S. aureus* that belonged to the same cgMLST cluster, which is evidence for contagious ewe-ewe transmission or a common external source. Flock E also has the highest prevalence of acute mastitis, 5 of the 6 ewes had a case of acute mastitis and a case of IMM, and the remaining ewe had a recorded IMM and a contagious strain of *S. aureus* (Table 4-10). Flock F also had a cgMLST cluster shared between ewes, again providing strong evidence for contagious mastitis in this case. Flocks A, B and C had samples from fewer than 4 ewes, so had the sample size been larger, strains shared between ewes may have been detected. Regardless, strains between ewes were genetically closely related in these flocks, which indicates contagious transmission in the past.

Flock D had the largest number of samples, but none were strains shared between ewes. Each ewe had their own strain according to cgMLST clustering, and in some cases were genetically distant, for example the isolates that had a different ST (Figure 4-4A). The *S. aureus* in this flock may have been a strain with an

environmental reservoir, as has been occasionally reported in cattle herds (Sommerhäuser et al., 2003; Zadoks et al., 2011).

There were two isolates that did not cluster within their flock clades and were not clustered with any other isolates (labelled * in Figure 4-4B). These strains match the epidemiology of environmental strains, suggesting that environmental strains of *S. aureus* are present in sheep flocks as they are in cattle herds (Sommerhäuser et al., 2003).

Bringing in control measures such as postmilking teat and milking cluster disinfectant has been successful in reducing *Staphylococcus aureus* mastitis in cattle herds (Zadoks et al., 2002; Zecconi et al., 2003). The evidence of contagious *S. aureus* in sheep flocks reported in the current study leads to the possibility of reducing mastitis in sheep via similar control measures.

4.4.4. Validation of MALDI-ToF MS as a strain-typing method for *Staphylococcus aureus* isolates

Although technical replicates were very similar, many biological replicates showed a wider range of similarity. Despite this, a study by Oberle et al. (2016) also found a wide range of variability in biological replicates but concluded that they were similar enough to group species into distinguished clusters if spectra were sufficiently high quality. They note that some species and strains will have MALDI profiles that are too similar to separate, but that in many cases MALDI poses a potential opportunity to investigate outbreak strains.

MALDI-ToF strain-types were identified using a similarity matrix comparing MALDI-ToF mass spectra. Choosing a threshold value to determine what was sufficiently similar to be considered a strain was complex, with replicates between species showing different levels of reproducibility.

Compared to cgMLST, the MALDI-ToF MS strain-typing method was less discriminatory, with 7 strain-types rather than 22. Furthermore, these strain-types did not nest inside each other, rather cgMLST clusters are split into several of the larger MALDI-ToF clusters. Where the phylogenetic distance tree created using WGS produces clear flock-based clades, the MALDI-ToF hierarchical cluster analysis breaks flocks up (Figure 4-7). Despite the large clusters created by MADLI-ToF clusters, the method has a low sensitivity and fails to identify 7 cases of PPE which were identified by the much smaller cgMLST clusters. Unsurprisingly, the

large clusters did identify a large number of PTE, but with low specificity, as 24 of these were additional to the PTE selected using cgMLST.

This is not the first study where MALDI-ToF MS failed to sufficiently discriminate *S. aureus* strains to the same power as existing strain-typing tools (Lasch et al., 2014). As in Lasch et al. (2014), the current study prepared samples using the trifluoroacetic acid method (Lasch et al., 2008), which produces high quality spectra which was able to successfully discriminate samples on a species-level if not a strain level. The current study also ensured that culture conditions (temperature, medium and culture time) were kept constant, as it is thought these can affect the spectral profiles (Sandrin et al., 2012). As bacterial isolation took place over several months there is a possibility that some culture conditions changed over time and so it cannot be discounted the culture conditions played some role in limiting strain-level discrimination, although if true this highlights a disadvantage of using MALDI-ToF MS for strain-typing.

Based on the comparison of WGS typing with MALDI-ToF, the use of MALDI-ToF MS strain-typing for outbreak investigation of *S. aureus* appears ineffective. It is not discriminative enough for investigation of transmission within flocks but is also unsuccessful in clustering isolates from the same ewe together, so failing to detect persistence events that were found by WGS.

4.4.5. Strength and limitations of the study

A major strength of this study is that milk of healthy ewes was collected and cultured, which allowed for the investigation into species associated with disease.

Culture dependent studies are limited to isolating only culturable bacteria and this study only isolated using a single medium, further limiting the potential culturable bacteria. However, by culturing pure isolates, it is possible to investigate outbreaks and true persistence of strains, which gives us an understanding of the dynamics of the disease. The milk samples were frozen for up to four years, which would have resulted in some loss of sensitivity of bacterial cultures (Smith et al. 2011)

Due to cost limitations, only *S. aureus* was used for WGS and successfully investigated for transmission and persistence. Had the MALDI-ToF MS clustering method described in this study been proved as an equivalent strain-typing method, the investigation into transmission and persistence could have been expanded into many other species. Species have different capabilities to persist and transmit as

contagious pathogens (Zadoks et al., 2011; Wente et al., 2020), and so strain-typing all isolated bacteria would have provided a more complete understanding of the differences between species and mammary health. Nevertheless, WGS is considered the gold standard for investigating disease outbreaks, so even limited data is valuable (Croucher and Didelot, 2015).

4.4.6. Conclusions from Chapter 4

A large number of species were isolated from the milk of healthy ewes and ewes with mammary disease, supporting the hypothesis that a mammary microbiota exists regardless of infection status. However, *Staphylococcus aureus* was significantly associated with both cases of IMM and cases of acute mastitis.

This is the first study to investigate the transmission and persistence of *S. aureus* in sheep flocks using WGS. Both transmission and persistence were clearly demonstrated using WGS despite the small sample size, and there was one distinct case of contagious *S. aureus* in one flock.

Chapter 5 General discussion

5.1. Key findings

1. Separating ewes with IMM does not affect IMM in a flock.
2. *Staphylococcus aureus* presence is associated with mammary disease, and transmission and persistence of the same strain can be evidenced.
3. Mammary glands contain a large number of species of bacteria regardless of disease status.
4. The MALDI-ToF MS strain-typing method described in this study is not suitable for strain typing *S. aureus* isolates from sheep milk.
5. IMM and acute mastitis are associated with an increase in SCC.
6. IMM and acute mastitis result in significantly lower lamb weights and therefore affect farm income.

5.2. Discussion of key findings

The aim of the current study was to investigate the extent to which bacteria associated with IMM and acute mastitis can transmit in flocks and persist in the mammary gland, and to understand the effect of IMM on lamb weights.

A key finding of this study was that separating ewes with IMM from those without in a flock with reasonably high prevalence (48%) did not reduce the number of new IMM found, indicating that removal of these ewes via culling or separate management is not a useful tool for reducing mastitis in a flock. Studies on mastitis in dairy herds conclude that where control measures are unsuccessful, there is an environmental reservoir rather than contagious transmission (Sommerhäuser et al., 2003; Anderson and Lyman, 2006). Therefore, one explanation is that pathogens causing IMM can transmit through an environmental reservoir, and the failure of this study to improve mastitis prevalence was because control measures removed only contagious pathways.

However, limitations of the farm trial in Chapter 2 hinder the ability to make definitive conclusions. Ewes were only examined for 12 months, and their disease status before and after the trial is unknown. IMM are unstable and can form, burst and reform in a matter of months (Section 2.3.2). It is possible therefore that ewes with pathogens capable of forming IMM remained in the control group throughout the study and remained as a reservoir of infection. Furthermore, the latency time

between infection and abscess formation is unknown, potentially allowing ewes to act as carriers of pathogens while IMM form within the mammary gland.

Chapter 4 used a case-control trial to investigate bacteria isolated from milk of healthy ewes, ewes with IMM and ewes with acute mastitis. A key finding was that *S. aureus* was positively associated with both types of mastitis (Section 4.3.1.4). Importantly, Chapter 4 used WGS to investigate the epidemiological links in a subset of *S. aureus* isolates and found evidence of both transmission within flocks and persistence within ewes (Section 4.3.2). This is evidence that pathogens associated with disease can transmit contagiously in a flock. This suggests that together the findings in Chapter 2 and 4 present evidence for both contagious and environmental transmission, although the limitations of both studies prevent certainty in this evidence.

MALDI-ToF MS was used to identify bacterial species. The potential use of MALDI-ToF MS a strain-typing method was examined by comparing the results with WGS. MALDI-ToF MS was not as discriminatory as WGS, and clustered isolates together differently, demonstrating that it is not a suitable method to detect transmission or persistence events of *S. aureus*. Therefore, only the isolates with genome sequences were used for persistence and transmission analysis, reducing the sample size and restricting the analysis to a single species.

The WGS in Chapter 4 demonstrated that strains could persist in the mammary gland. Even though the sample size in this study was small, there were several examples of the same *S. aureus* strain identified in more than one examination including across lactations. In order to calculate the proportion of strains causing persistent infection, a larger sample size would be required, with the inclusion of samples from healthy ewes to ensure all persistent strains are associated with disease.

Ewes with IMM had lambs that took nearly 16 days longer than lambs of healthy dams to reach slaughter weight (Section 3.3.4). Lambs of dams with IMM and acute mastitis were consistently lighter throughout their growth phase (Chapter 3). The only other two studies on IMM and lamb weight also reported lambs of dams with IMM were lighter than lambs of healthy ewes, but neither weighed lambs after weaning (Grant et al., 2016; Griffiths et al., 2019). Chapter 3 finds that lambs born to ewes with IMM or acute mastitis remain lighter up until slaughter and did not reach a similar weight at slaughter to their counterparts through compensatory growth after weaning. This study was also better able to elucidate the difference between lambs

of healthy ewes and lambs of ewes with IMM as they were kept separately until weaning. This prevented lambs from being 'milk robbers' as they were with other ewes with IMM.

Ewes with IMM in an udder half before or after a milk sample was taken had a significantly higher SCC compared to ewes with no clinical signs (Section 2.3.4). SCC above 400,000 cells/ml has been associated with low lamb weight, poor udder conformation and decreased microbial diversity, emphasising the effect of IMM on the overall health of the udder and the consequences for lambs (Huntley et al., 2012; Esteban-Blanco et al., 2019).

Grant et al. (2016) was the first to highlight the association between IMM and acute mastitis, and Chapter 2 provided more evidence for this association. There were over 3-fold odds of acute mastitis when ewes had IMM and vice versa (Sections 2.3.2 and 2.3.3). This further emphasises the impact of IMM on farm income. Acute mastitis has a negative economic impact through treatment, replacement of ewes and loss of productivity (Conington et al., 2008). The combined impact of increased odds of acute mastitis and the direct influence on lamb growth rates highlights the importance of IMM for farm income.

5.3. Limitations and future work

The farm trial was time limited, so presence of IMM and the outcome of control measures were only investigated over one lactation. A longer study continuously removing ewes with IMM may have reduced the proportion of carriers within the flock. A study over several years separating ewes with IMM or keeping ewe lambs separate would confirm if removing IMM from a flock can reduce IMM incidence.

The 12-month farm trial was carried out on a single farm, and although findings from this study may be representative of other farms, care should be taken before generalising results. Zadoks et al. (2011) reviewed molecular epidemiology of mastitis pathogens in dairy cattle, reporting many different transmission pathways and pathogens linked to different herds and management systems. Therefore, carrying out this trial over a number of different flocks may give different conclusions and would enable comparisons.

No bacteriological samples were examined from the 12-month farm trial. It is not known which bacteria were associated with the IMM in Chapters 2 and 3, although the significant association of *S. aureus* with IMM and acute mastitis reported in

Chapter 4, means we could speculate that *S. aureus* is also a causative agent in this trial. Regardless, we do not know how or when ewes were infected. Samples taken from milk, lamb mouths and the environment with bacteriological analysis and strain typing may be able to produce more compelling evidence as to the transmission pathways within the flock. The bacteriological samples investigated in Chapter 4 went some way to examining potential transmission pathways but were limited to milk samples. Although these samples were taken aseptically, contamination in the field may have occurred, and bacteria on the udder skin and within the teat apex may also have been detected in the samples. Samples taken from the environment and udder skin would enable a more comprehensive analysis of the microbial communities.

Finally, only *S. aureus* isolates were sequenced for epidemiological analysis. *S. aureus* was significantly associated with disease in Chapter 4 and therefore was chosen for WGS. This study aimed to develop a suitable strain-typing methodology using MALDI-ToF MS in order to strain-type all detected species within the milk samples. This would have given a more comprehensive understanding of persistence and transmission of strains in milk samples, and whether this was associated with mammary disease. The success of WGS for identifying epidemiological links in the *S. aureus* isolates indicates a larger study sequencing all isolates detected in milk would be effective in finding transmission and persistence of other species.

5.4. Conclusions of thesis

This study positively associates *S. aureus* with acute mastitis and IMM. In addition, evidence for transmission and persistence of *S. aureus* strains in sheep milk is presented, proving that a contagious transmission pathway is possible. Removal of ewes with IMM did not reduce the prevalence of mastitis within a flock, which suggests pathogens can be transmitted through an environmental reservoir or that ewes can be carriers of mastitis-associated pathogens without persistently showing signs of disease. This may be due to the time taken for IMM to form, or because not all ewes infected with mastitis-associated pathogens go on to develop disease. This suggests that culling ewes with IMM will never be a successful control measure for managing transmission of mastitis-associated pathogens. This study is the first to show IMM in ewes result in lighter lambs past weaning and up to slaughter and to show IMM significantly increase SCC. These highlight the importance of IMM for the

sheep industry. Further work on strains of mastitis-associated pathogens in milk and in the environment over a longer period of time would give a more complete understanding of transmission routes and persistence mechanisms.

5.5. Conclusions for industry

This thesis has furthered our understanding of the impact of both acute mastitis and IMM on ewes and their lambs. IMM are important to ewe health, as they increased risk of acute mastitis and increased SCC compared to ewes without IMM, and are also important for lambs, as they adversely affect lamb growth. Although ewes with IMM may provide a reservoir of mastitis-associated pathogens, it seems unlikely that culling these ewes will have an effect on reducing risk of IMM in other ewes in the flock. This is because not all ewes carrying mastitis-associated pathogens will continuously show signs of chronic mastitis through the presence of an IMM. The reoccurring nature of IMM mean it is difficult to detect at any one point, leaving many ewes in a flock with potential to develop IMM in the future.

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Appendix

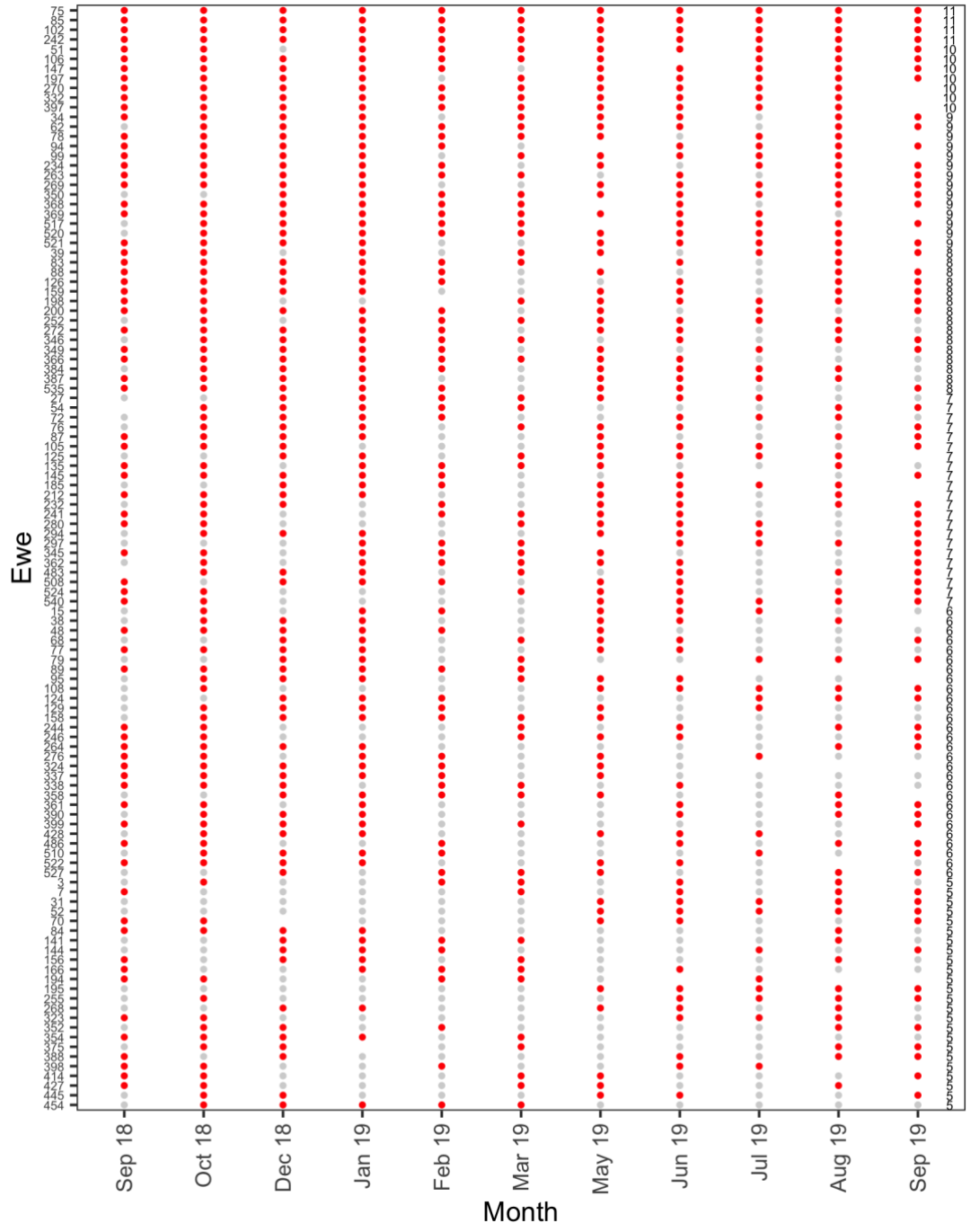

Figure A- 1: IMM occurrence by ewe part 1.
Grey dots represent measurements with no IMM, red dots are occurrences of IMM, blank spaces are missing data. Numbers on right show the total number of IMM occurrences for each ewe

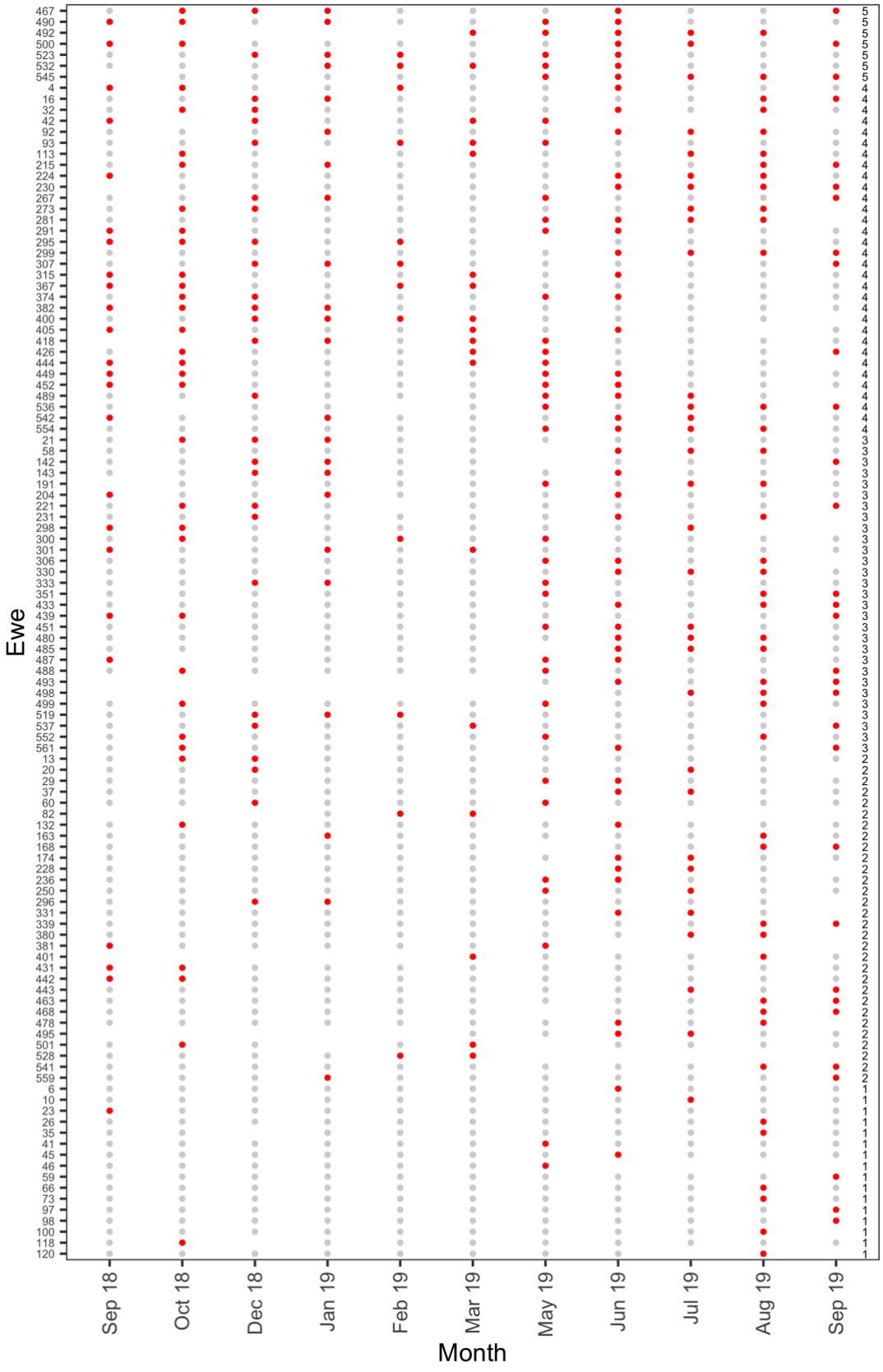

Figure A- 2: IMM occurrence by ewe part 2. Grey dots represent measurements with no IMM, red dots are occurrences of IMM, blank spaces are missing data. Numbers on right show the total number of IMM occurrences for each ewe.

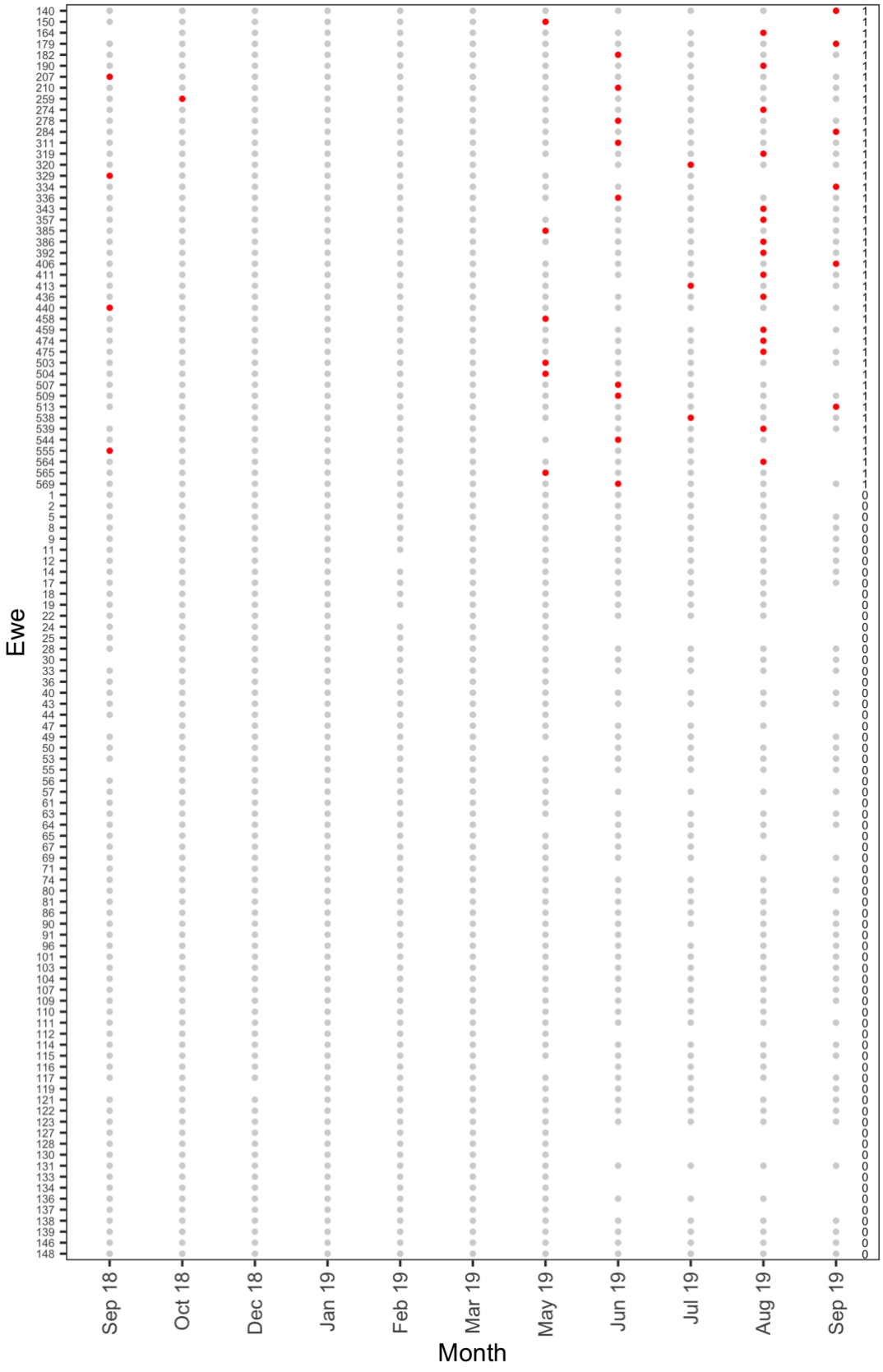

Figure A- 3: IMM occurrence by ewe part 3. Grey dots represent measurements with no IMM, red dots are occurrences of IMM, blank spaces are missing data. Numbers on right show the total number of IMM occurrences for each ewe.

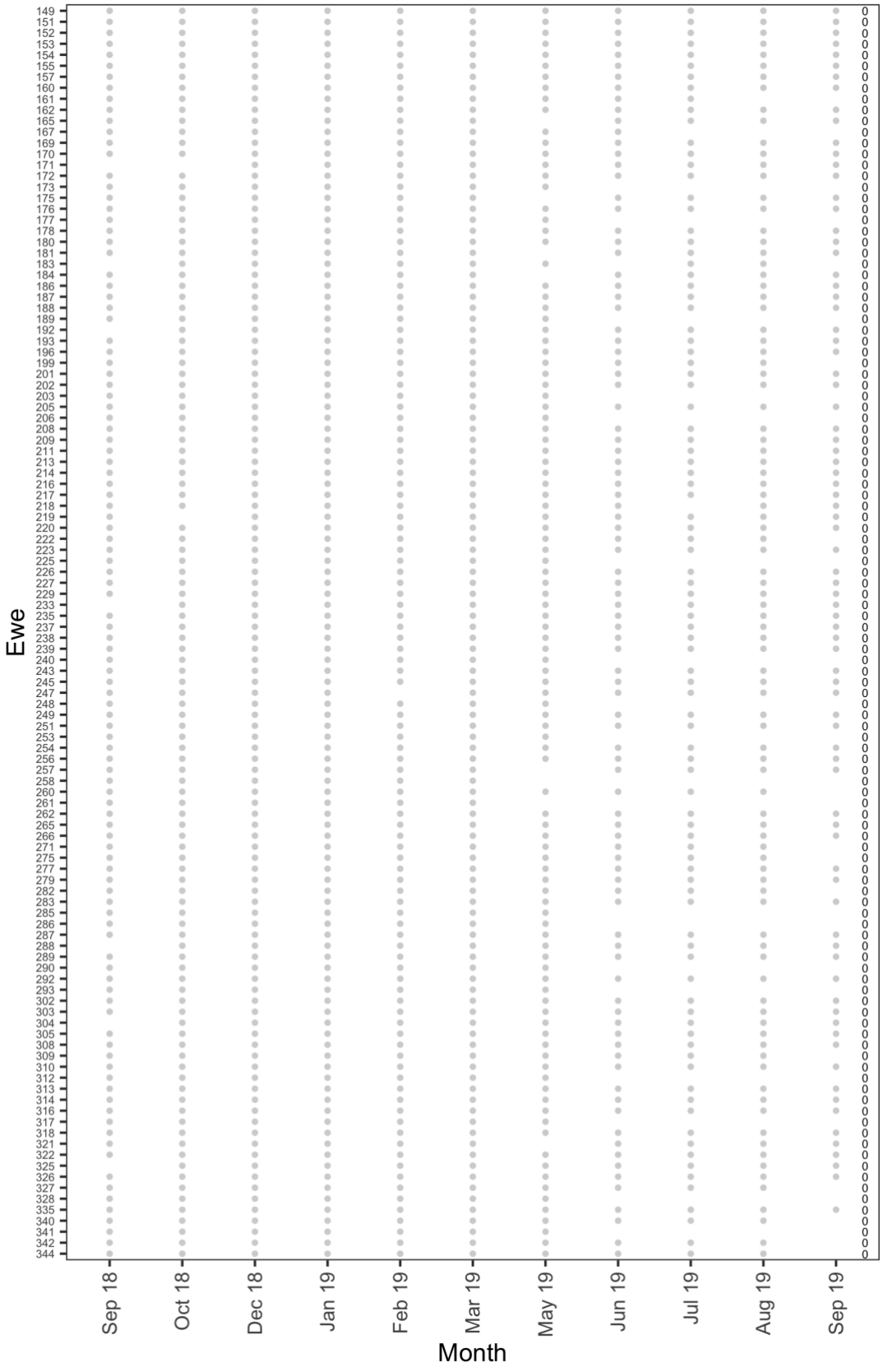

Figure A- 4: IMM occurrence by ewe part 4. Grey dots represent measurements with no IMM, red dots are occurrences of IMM, blank spaces are missing data. Numbers on right show the total number of IMM occurrences for each ewe.

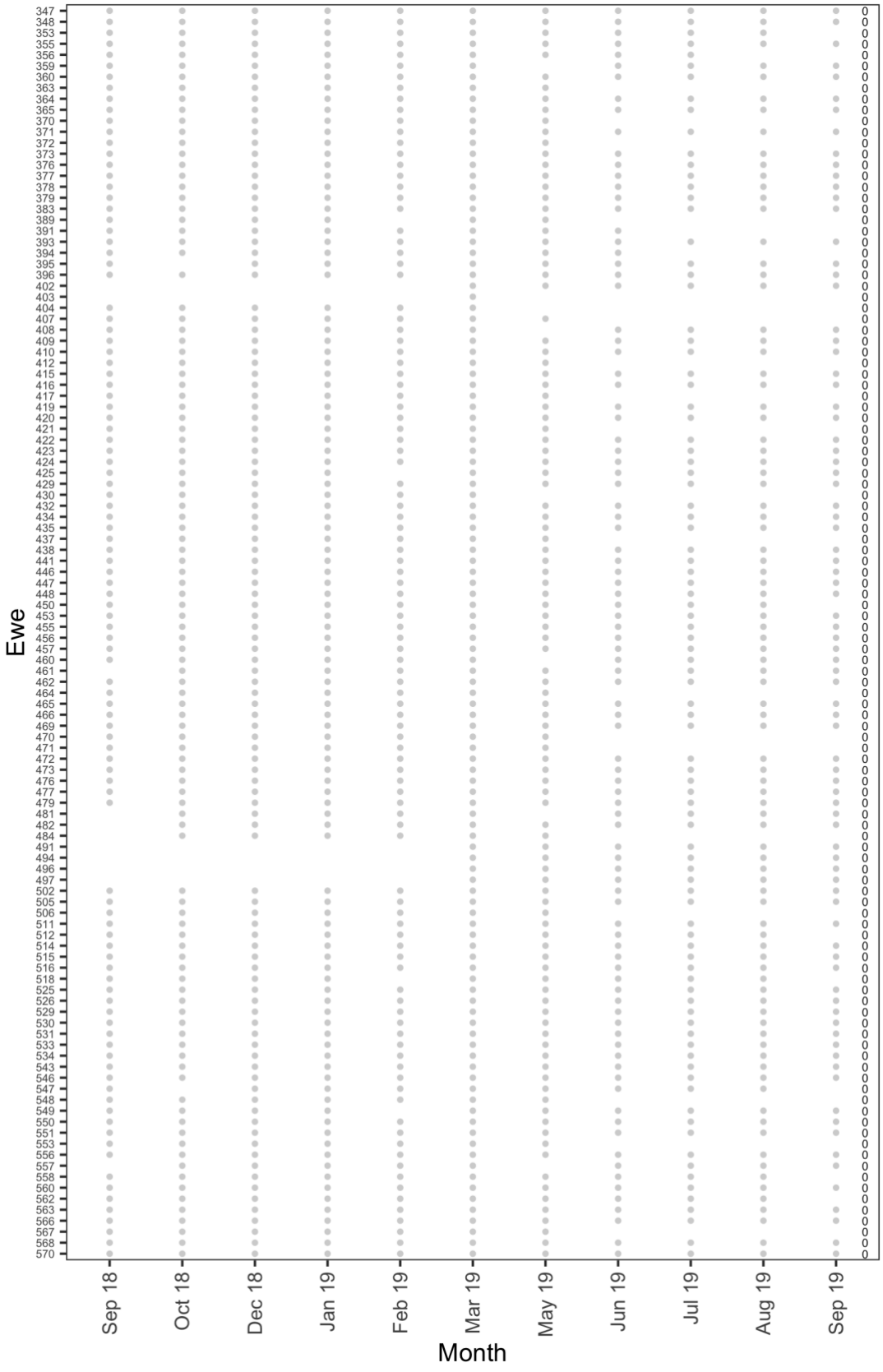

Figure A- 5: IMM occurrence by ewe part 5 Grey dots represent measurements with no IMM, red dots are occurrences of IMM, blank spaces are missing data. Numbers on right show the total number of IMM occurrences for each ewe.

Table A- 1: Univariable linear mixed effect model of lamb weight up to weaning

Variable	Category [Range]	N (%)	Mean (SD)	ß	95% CI
Lamb age (days)	[5.0,93.0]	3281 (100.0)	22.0 (8.3)	0.29	0.28 - 0.29
Lamb age (days)^2				-8.17	-10.39 - -5.94
Lamb age (days)^3				2.79	0.38 - 5.19
Birth weight (kg)	[1.4,9.7]	3281 (100.0)	22.0 (8.3)	1.26	1.14 - 1.37
Ewe age (years)	1-3	454 (13.8)	20.9 (7.9)	Ref	
	4-6	2099 (64.0)	22.5 (8.3)	0.88	-0.18 - 0.92
	7+	728 (22.2)	21.4 (8.6)	0.37	0.41 - 1.35
Lamb sex	Female	1681 (51.2)	21.5 (8.2)	Ref	
	Castrated male	1600 (48.8)	22.5 (8.5)	0.17	-0.09 - 0.44
Lamb breed	Rouge X	385 (11.7)	23.5 (8.6)	Ref	
	Abermax X	576 (17.6)	23.7 (8.8)	-0.8	-1.16 - -0.12
	Rouge de l Quest X	393 (12.0)	20.4 (8.2)	-0.84	-1.53 - -0.15
	Texel X	1927 (58.7)	21.5 (8.1)	-0.64	-1.41 - -0.19
Lambs suckled	1	457 (13.9)	25.2 (9.0)	Ref	
	2	2824 (86.1)	21.5 (8.1)	-1.7	-2.11 - -1.29
AM in same month	No	3271 (99.7)	22.0 (8.3)	Ref	
	Yes	10 (0.3)	13.4 (12.8)	-1.37	-2.78 - 0.04
AM during lactation	No	3217 (98.0)	22.1 (8.3)	Ref	
	Yes	64 (2.0)	16.5 (9.2)	-1.32	-2.44 - -0.21
AM during study	No	2985 (91.0)	22.2 (8.3)	Ref	
	Yes	296 (9.0)	20.6 (8.5)	-0.38	-0.96 - 0.2
IMM in same month	No	2404 (73.3)	22.2 (8.4)	Ref	
	Yes	875 (26.7)	21.5 (8.2)	-0.19	-0.38 - -0.01
IMM during pregnancy	No	1832 (55.8)	23.5 (8.2)	Ref	
	Yes	1449 (44.2)	20.1 (8.2)	-0.65	-0.99 - -0.32
IMM during lactation	No	1817 (55.4)	23.2 (8.2)	Ref	
	Yes	1464 (44.6)	20.5 (8.3)	-0.22	-0.56 - 0.12

Variable	Category [Range]	N (%)	Mean (SD)	ß	95% CI
IMM previous month	No	2532 (77.2)	22.4 (8.4)	Ref	
	Not Recorded	5 (0.2)	19.3 (12.9)	-0.19	-0.77 - -0.25
	Yes	744 (22.7)	20.9 (8.2)	-0.51	-2.56 - 2.19
Lambing Group	Control	1835 (55.9)	23.6 (8.1)	Ref	
	IMM	1446 (44.1)	20.0 (8.2)	-0.68	-1.01 - -0.34
IMM during study	No	1388 (42.3)	23.3 (8.2)	Ref	
	Yes	1893 (57.7)	21.1 (8.3)	-0.19	-0.52 - 0.15
BCS in month	Healthy	1810 (55.2)	20.8 (8.8)	Ref	
	Fat	95 (2.9)	21.1 (7.3)	0.66	0.05 - 0.38
	Thin	1376 (41.9)	23.7 (7.5)	0.22	0.3 - 1.02

SD: standard deviation; ß: coefficients; CI: confidence intervals; N: number of observations; Wald estimates used for CI; AM: acute mastitis; IMM: intramammary mass; BCS: body condition score; Rouge X: Rouge de l'ouest X; Fat: BCS > 3.5, Healthy: BCS between 2.5-3.5, Thin: BCS < 2.5

Table A- 2: Univariable generalised additive mixed model

Variable	Category [Range]	N (%)	Mean (SD)	ß	95% CI
Birth weight (kg)	[1.4,9.7]	6475 (100.0)	28.9 (9.8)	2.01	1.86 - 2.16
Ewe age (years)	1-3	988 (15.3)	28.3 (9.3)	Ref	
	4-6	4084 (63.1)	29.3 (9.8)	1.12	0.48 - 1.77
	7+	1403 (21.7)	28.3 (10.0)	0.23	-0.54 - 1
BCS	Healthy	3562 (55.0)	28.3 (10.4)	Ref	
	Fat	275 (4.2)	32.1 (10.6)	-0.04	-0.33 - 0.25
	Thin	2638 (40.7)	29.5 (8.7)	-0.09	-0.21 - 0.04
Lamb sex	Female	3525 (54.4)	29.1 (10.0)	Ref	
	Castrated male	2950 (45.6)	28.8 (9.6)	0.67	0.34 - 0.99
Breed	Texel X	3905 (60.3)	28.5 (9.4)	Ref	
	Aberfield X	817 (12.6)	32.0 (11.0)	1.33	0.61 - 2.06
	Abermax X	1082 (16.7)	29.8 (9.6)	0.21	-0.4 - 0.82
	Rouge X	671 (10.4)	26.4 (9.9)	-0.42	-1.26 - 0.42
Lambs suckled	1	727 (11.2)	30.0 (10.2)	Ref	
	2	5748 (88.8)	28.8 (9.7)	-3.11	-3.66 - -2.55
Group at Lambing	Control	3872 (59.8)	30.4 (9.3)	Ref	
	IMM	2603 (40.2)	26.7 (10.2)	-1.22	-1.71 - -0.72
AM at time[a]	No	6354 (98.1)	28.9 (9.8)	Ref	
	Yes	121 (1.9)	32.6 (8.2)	-0.16	-0.5 - 0.17
AM during lactation	No	6349 (98.1)	29.0 (9.8)	Ref	
	Yes	126 (1.9)	23.8 (10.5)	-1.94	-3.5 - -0.38
AM during study	No	5879 (90.8)	29.0 (9.8)	Ref	
	Yes	596 (9.2)	27.8 (10.1)	-1.03	-1.85 - -0.2
IMM at time[a]	No	4741 (73.2)	29.2 (9.9)	Ref	
	Yes	1732 (26.8)	28.2 (9.6)	-0.3	-0.43 - -0.16
IMM during pregnancy	No	3861 (59.6)	30.4 (9.3)	Ref	
	Yes	2614 (40.4)	26.8 (10.1)	-1.19	-1.68 - -0.69

Variable	Category [Range]	N (%)	Mean (SD)	ß	95% CI
IMM during lactation	No	3761 (58.1)	30.2 (9.5)	Ref	
	Yes	2714 (41.9)	27.2 (10.0)	-0.84	-1.33 - -0.35
IMM previous month	No	4960 (76.6)	29.2 (9.8)	Ref	
	Not Recorded	5 (0.1)	19.3 (12.9)	2.58	0.62 - 4.54
	Yes	1510 (23.3)	28.3 (9.8)	-0.37	-0.51 - -0.23
IMM during study	No	2994 (46.2)	30.5 (9.4)	Ref	
	Yes	3481 (53.8)	27.6 (9.9)	-0.65	-1.13 - -0.18

SD: standard deviation; ß: coefficients; CI: confidence intervals; N: number of observations; Wald estimates used for CI; AM: acute mastitis; IMM: intramammary mass; [a]: time of measurement; BCS: body condition score; Rouge X: Rouge de l'ouest X; Fat: BCS > 3.5, Healthy: BCS between 2.5-3.5, Thin: BCS < 2.5

Table A- 3: Univariable linear mixed effect model of lamb age at slaughter (days)

Variable	Category [Range]	N (%)	Mean (SD)	ß	95% CI
Birth Weight (kg)	[1.6,9.7]	802 (100.0)	174.1 (53.9)	-26.9	-30.17 - -23.63
Ewe age (years)	1-3	122 (15.2)	189.3 (52.7)	Ref	
	4-6	514 (64.1)	169.1 (52.7)	-20.2	-23.46 - 1.56
	7+	166 (20.7)	178.4 (56.3)	-10.95	-30.77 - -9.64
Sex	Female	380 (47.4)	184.9 (53.6)	Ref	
	Castrated male	422 (52.6)	164.4 (52.4)	-20.46	-27.81 - -13.11
Breed	Aberfield X	63 (7.9)	138.0 (46.3)	Ref	
	Abermax X	174 (21.7)	160.7 (51.0)	22.66	31.16 - 58.58
	Rouge X	72 (9.0)	178.3 (55.0)	40.26	22.59 - 57.94
	Texel X	493 (61.5)	182.9 (52.9)	44.87	7.59 - 37.72
Lambs suckled	1	111 (13.8)	134.7 (48.7)	Ref	
	2	691 (86.2)	180.5 (52.0)	45.81	35.47 - 56.16
AM during lactation	No	784 (97.8)	173.8 (53.7)	Ref	
	Yes	18 (2.2)	189.1 (61.0)	15.27	-9.95 - 40.48
AM during study	No	729 (90.9)	173.2 (53.9)	Ref	
	Yes	73 (9.1)	183.0 (53.2)	9.79	-3.19 - 22.77
IMM during pregnancy	No	544 (67.8)	169.3 (53.1)	Ref	
	Yes	258 (32.2)	184.3 (54.4)	14.93	6.99 - 22.86
IMM during lactation	No	523 (65.2)	168.7 (51.6)	Ref	
	Yes	279 (34.8)	184.3 (56.6)	15.55	7.78 - 23.33
IMM during study	No	418 (52.1)	170.4 (52.4)	Ref	
	Yes	384 (47.9)	178.2 (55.2)	7.87	0.41 - 15.33
Lambing Group	Control	545 (68.0)	169.2 (53.1)	Ref	

Variable	Category [Range]	N (%)	Mean (SD)	ß	95% CI
	IMM	257 (32.0)	184.6 (54.1)	15.35	7.41 - 23.29

SD: standard deviation; ß: coefficients; CI: confidence intervals; N: number of observations; Wald estimates used for CI; AM: acute mastitis; IMM: intramammary mass; Rouge X: Rouge de l'ouest X

Table A- 4: Univariable binomial mixed effects regression model of presence of an IMM in the same year as the sample

Variable	Category	Not Affected N(%)	Affected N(%)	OR	95% CI	p
Flock	A	17 (25.4)	19 (17.1)	Ref		
	B	5 (7.5)	11 (9.9)	2.06	0.53 - 8.09	ns
	C	9 (13.4)	13 (11.7)	1.32	0.40 - 4.29	ns
	D	15 (22.4)	25 (22.5)	1.53	0.56 - 4.21	ns
	E	10 (14.9)	22 (19.8)	2.06	0.69 - 6.18	ns
	F	11 (16.4)	21 (18.9)	1.78	0.60 - 5.24	ns
Year	1	29 (43.3)	60 (54.1)	Ref		
	2	38 (56.7)	51 (45.9)	0.62	0.33 - 1.19	ns
Staphylococcus equorum	Absent	46 (68.7)	77 (69.4)	Ref		
	Present	21 (31.3)	34 (30.6)	0.97	0.46 - 2.06	ns
Staphylococcus vitulinus	Absent	42 (62.7)	77 (69.4)	Ref		
	Present	25 (37.3)	34 (30.6)	0.71	0.35 - 1.43	ns
Staphylococcus succinus	Absent	48 (71.6)	91 (82.0)	Ref		
	Present	19 (28.4)	20 (18.0)	0.53	0.24 - 1.16	ns
Bacillus	Absent	58 (86.6)	99 (89.2)	Ref		
	Present	9 (13.4)	12 (10.8)	0.77	0.28 - 2.07	ns
Staphylococcus lentus	Absent	57 (85.1)	92 (82.9)	Ref		
	Present	10 (14.9)	19 (17.1)	1.23	0.49 - 3.09	ns
Enterococcus faecium	Absent	50 (74.6)	87 (78.4)	Ref		
	Present	17 (25.4)	24 (21.6)	0.77	0.35 - 1.67	ns
Wickerhamomyces anomalus	Absent	64 (95.5)	105 (94.6)	Ref		
	Present	3 (4.5)	6 (5.4)	1.31	0.28 - 6.01	ns
Staphylococcus aureus	Absent	58 (86.6)	76 (68.5)	Ref		
	Present	**9 (13.4)**	**35 (31.5)**	**2.98**	**1.31 - 6.8**	**0.009**
Staphylococcus xylosus	Absent	57 (85.1)	103 (92.8)	Ref		
	Present	10 (14.9)	8 (7.2)	0.38	0.12 - 1.18	ns
Staphylococcus cohnii	Absent	61 (91.0)	103 (92.8)	Ref		
	Present	6 (9.0)	8 (7.2)	0.79	0.24 - 2.59	ns
Other	Absent	45 (67.2)	80 (72.1)	Ref		
	Present	22 (32.8)	31 (27.9)	0.78	0.38 - 1.58	ns
Bacillus licheniformis	Absent	54 (80.6)	87 (78.4)	Ref		

Variable	Category	Not Affected N(%)	Affected N(%)	OR	95% CI	p
	Present	13 (19.4)	24 (21.6)	1.21	0.53 - 2.74	ns
Enterococcus	Absent	59 (88.1)	102 (91.9)	Ref		
	Present	8 (11.9)	9 (8.1)	0.67	0.23 - 1.96	ns
Staphylococcus sciuri	Absent	48 (71.6)	97 (87.4)	Ref		
	Present	**19 (28.4)**	**14 (12.6)**	**0.33**	**0.14 - 0.8**	**0.014**
Staphylococcus simulans	Absent	58 (86.6)	99 (89.2)	Ref		
	Present	9 (13.4)	12 (10.8)	0.76	0.28 - 2.08	ns
Paenibacillus amylolyticus	Absent	62 (92.5)	105 (94.6)	Ref		
	Present	5 (7.5)	6 (5.4)	0.69	0.17 - 2.69	ns
Staphylococcus	Absent	60 (89.6)	100 (90.1)	Ref		
	Present	7 (10.4)	11 (9.9)	0.91	0.31 - 2.68	ns
Enterococcus hirae	Absent	58 (86.6)	98 (88.3)	Ref		
	Present	9 (13.4)	13 (11.7)	0.85	0.32 - 2.26	ns
Arthrobacter gandavensis	Absent	52 (77.6)	92 (82.9)	Ref		
	Present	15 (22.4)	19 (17.1)	0.7	0.31 - 1.6	ns
Pantoea agglomerans	Absent	64 (95.5)	105 (94.6)	Ref		
	Present	3 (4.5)	6 (5.4)	1.22	0.27 - 5.61	ns
Streptococcus	Absent	63 (94.0)	107 (96.4)	Ref		
	Present	4 (6.0)	4 (3.6)	0.51	0.11 - 2.48	ns
Kocuria carniphila	Absent	61 (91.0)	102 (91.9)	Ref		
	Present	6 (9.0)	9 (8.1)	0.88	0.27 - 2.82	ns

OR: Odds ratio; CI: confidence intervals; N: number of observations; Wald estimates used for CI; ns: not significant to $p < 0.05$; Genus only variables do not include cases where the species has been retained as a variable

Table A- 5: Univariable binomial mixed effects regression model of acute mastitis in the same year as the sample

Variable	Category	Not Affected N(%)	Affected N(%)	OR	95% CI	p
Flock	A	31 (20.3)	5 (20.0)	Ref		
	B	15 (9.8)	1 (4.0)	0.41	0.04 - 3.85	ns
	C	20 (13.1)	2 (8.0)	0.62	0.11 - 3.51	ns
	D	37 (24.2)	3 (12.0)	0.50	0.11 - 2.27	ns
	E	**19 (12.4)**	**13 (52.0)**	**4.28**	**1.31 - 14.00**	**0.016**
	F	31 (20.3)	1 (4.0)	0.20	0.02 - 1.81	ns
Year	1	73 (47.7)	16 (64.0)	Ref		
	2	80 (52.3)	9 (36.0)	0.48	0.19 - 1.18	ns
Staphylococcus equorum	Absent	105 (68.6)	18 (72.0)	Ref		
	Present	48 (31.4)	7 (28.0)	0.83	0.28 - 2.45	ns
Staphylococcus vitulinus	Absent	101 (66.0)	18 (72.0)	Ref		
	Present	52 (34.0)	7 (28.0)	0.63	0.23 - 1.73	ns
Staphylococcus succinus	Absent	116 (75.8)	23 (92.0)	Ref		
	Present	**37 (24.2)**	**2 (8.0)**	**0.15**	**0.03 - 0.69**	**0.016**
Bacillus	Absent	133 (86.9)	24 (96.0)	Ref		
	Present	20 (13.1)	1 (4.0)	0.17	0.02 - 1.37	ns
Staphylococcus lentus	Absent	129 (84.3)	20 (80.0)	Ref		
	Present	24 (15.7)	5 (20.0)	1.64	0.45 - 5.95	ns
Enterococcus faecium	Absent	114 (74.5)	23 (92.0)	Ref		
	Present	**39 (25.5)**	**2 (8.0)**	**0.13**	**0.02 - 0.81**	**0.029**
Wickerhamomyces anomalus	Absent	145 (94.8)	24 (96.0)	Ref		
	Present	8 (5.2)	1 (4.0)	0.81	0.09 - 7.5	ns
Staphylococcus aureus	Absent	122 (79.7)	12 (48.0)	Ref		
	Present	**31 (20.3)**	**13 (52.0)**	**4.15**	**1.62 - 10.66**	**0.003**
Staphylococcus xylosus	Absent	136 (88.9)	24 (96.0)	Ref		
	Present	17 (11.1)	1 (4.0)	0.18	0.02 - 1.52	ns
Staphylococcus cohnii	Absent	143 (93.5)	21 (84.0)	Ref		

Variable	Category	Not Affected N(%)	Affected N(%)	OR	95% CI	p
	Present	**10 (6.5)**	**4 (16.0)**	**4.49**	**1.01 - 19.89**	**0.048**
Other	Absent	106 (69.3)	19 (76.0)	Ref		
	Present	47 (30.7)	6 (24.0)	0.78	0.28 - 2.14	ns
Bacillus licheniformis	Absent	119 (77.8)	22 (88.0)	Ref		
	Present	34 (22.2)	3 (12.0)	0.41	0.11 - 1.57	ns
Enterococcus	Absent	137 (89.5)	24 (96.0)	Ref		
	Present	16 (10.5)	1 (4.0)	0.44	0.05 - 3.52	ns
Staphylococcus sciuri	Absent	126 (82.4)	19 (76.0)	Ref		
	Present	27 (17.6)	6 (24.0)	3.18	0.76 - 13.31	ns
Staphylococcus simulans	Absent	132 (86.3)	25 (100.0)	Ref		
	Present	21 (13.7)	0 (0.0)	0	0 - 9.7e+181	ns
Paenibacillus amylolyticus	Absent	146 (95.4)	21 (84.0)	Ref		
	Present	7 (4.6)	4 (16.0)	3.4	0.75 - 15.35	ns
Staphylococcus	Absent	138 (90.2)	22 (88.0)	Ref		
	Present	15 (9.8)	3 (12.0)	0.58	0.13 - 2.64	ns
Enterococcus hirae	Absent	131 (85.6)	25 (100.0)	Ref		
	Present	22 (14.4)	0 (0.0)	0	0 - 4.1e+186	ns
Arthrobacter gandavensis	Absent	124 (81.0)	20 (80.0)	Ref		
	Present	29 (19.0)	5 (20.0)	1.14	0.36 - 3.63	ns
Pantoea agglomerans	Absent	147 (96.1)	22 (88.0)	Ref		
	Present	6 (3.9)	3 (12.0)	1.56	0.3 - 8.08	ns
Streptococcus	Absent	146 (95.4)	24 (96.0)	Ref		
	Present	7 (4.6)	1 (4.0)	1.16	0.12 - 10.85	ns
Kocuria carniphila	Absent	140 (91.5)	23 (92.0)	Ref		
	Present	13 (8.5)	2 (8.0)	0.64	0.12 - 3.51	ns

OR: odds ratio; CI: confidence intervals; N: number of observations; Wald estimates used for CI; ns: not significant; Genus only variables do not include cases where the species has been retained as a variable