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Microbial ecology of the sheep mammary gland

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CONTENTS

1. Abstract

Improving the understanding of animal-associated microbial communities (microbiomes) is essential in identifying strategies to maximise health and generate novel approaches to management of persistent bacterial infections. Intramammary infections (IMI) in sheep present as a range of scenarios from acute severe systemic clinical mastitis to subclinical infection detectable by a raised somatic cell count (SCC) (which is indicative of an immune response to infection). Intramammary infections (IMI) in sheep have a major economic impact reduced milk production, premature culling and even death of ewes. Over 130 species of bacteria have been associated with IMI in cattle and there is no reason to consider that a similar number of species cannot infect the sheep mammary gland (MG). Given the inevitability of IMI, this study hypothesizes that the sheep MG could host a microbiome with certain members affecting SCC.

Previous studies have been cross-sectional i.e. at one time point, with only one sample per subject and none have been conducted in sheep. This limits understanding causality; that is, how infection develops and what triggers development of disease. This study was therefore longitudinal, monitoring 30 sheep, each with two mammary gland halves, collecting milk samples over 8 weeks, providing 379 milk samples and data on sheep parity and milk SCC. DNA was extracted from milk samples and processed using a bacterial 16S rRNA gene targeted PCR. Bacterial community diversity was visualised using denaturing gradient gel electrophoresis (DGGE).

DGGE banding patterns were analysed in a model to identify associations between individual bacterial species and changes in SCC. Those bands associated with SCC were sequenced. *Corynebacterium efficiens*, *Psychrobacter maritimus*, *Streptococcus uberis*, *Burkholderia cepacia*, *Fusobacterium necrophorum*, *Trueperella pyogenes*, *Pseudomonas chlororaphis* and *Psychrobacter faecalis* were significantly associated with a higher SCC. *Achromobacter xylosoxidans*, *Nocardia globerula* or *Rhodococcus qingshengii*, *Atopostipes suicloacalis*, *Mannheimia haemolytica*, *Jeotgalicoccus psychrophilus* and *Sharpea azabuensis* were significantly associated with a lower SCC.

A protocol to analyse all study samples using DNA sequencing was developed. The DGGE and sequencing results show a persistent community has been detected over time, with similarities and differences by mammary gland half, lactation and age in sheep with no clinical signs of disease. Associations between individual bacterial species and SCC were identified through modelling. This study highlights the importance of further research to improve the understanding of what changes in a bacterial community lead to disease to advise farmers of management strategies to minimise IMIs. Analysis of all 379 samples by DNA sequencing and modelling will be used to directly test the study hypotheses in future work.

2. Introduction

2.1. The study of microbiomes

Microbial communities are defined as multi-species assemblages in which organisms live and interact in a shared environment. Such communities form from populations of bacteria conducting interdependent physiological processes (Davey and O'Toole, 2000). These communities have evolved to form an essential part of the host genetic composition which is vital in maintaining host health (Costello *et al.*, 2009; Turnbaugh *et al.*, 2007). The term 'microbiome' refers to the totality of microbes, their genetic information and the milieu in which they interact. Microbiomes typically consist of environmental or biological niches containing complex communities of microbes (Cho and Blaser, 2012). Most host-associated microbes are difficult to culture because laboratory conditions are not always optimal. However, advances in DNA sequencing techniques that do not require culture, have made large-scale studies on host-microbe interactions possible (Proctor, 2011). For example, the Human Microbiome Project generated a 16S RNA metagenomic data set of over 35 billion DNA sequence reads from 690 samples collected from over 200 human subjects from up to 18 body sites (Methe *et al.*, 2012; Turnbaugh *et al.*, 2007).

Hence, a key example of the important role that microbial communities play in host health is the human microbiota. The term 'microbiota' refers to the microbial organisms that constitute the microbiome. The microbiota can vary according to the host and environmental site (Cho and Blaser, 2012). Membership of the microbiota is diverse; one survey of the skin microbiota from 10 volunteers identified 19 bacterial phyla and 205 bacterial genera (Grice *et al.*, 2008). A second study of human breast milk found between 100-600 bacterial species per subject from three milk samples collected over a four-week period (Hunt *et al.*, 2011). Stability and composition of the microbiota varied according to subject, but a 'core' microbiome of 9 bacterial species were present in every milk sample taken as part of the study (Hunt *et al.*, 2011). This contrasts with microbiome studies of other anatomical sites such as the gut, where no highly abundant bacterial species have been found to be shared between individuals (Turnbaugh *et al.*, 2007).

Microbiomes at different anatomical sites are not isolated, but rather a network of inter-related communities that experience change. Costello *et al.,* (2009) analysed microbiota samples from several human body sites and found each site to have its own characteristic microbiota as well as a relatively stable set of abundant bacterial species across individuals over time. It is therefore likely that communities in animals such as cows and sheep are no different, with exposure and interaction with other microbial populations and environmental influences resulting in complex communities that change over time.

Several studies have investigated the milk microbiome of dairy cattle using high-throughput DNA sequencing techniques (Bhatt *et al.*, 2012; Kuehn *et al.*, 2013; Oikonomou *et al.*, 2014; Oikonomou *et al.*, 2012).

Kuehn *et al.,* (2013) identified significant differences in milk microbiota composition between healthy and diseased MG quarters. Higher abundances of *Brevundimonas*, *Burkholderia*, *Sphingomonas* and *Stenotrophomonas* were found in diseased samples. In healthy MG quarters, *Pseudomonas*, *Psychrobacter* and *Ralstonia* were most prevalent. Oikonomou *et al.,* (2014; 2012) also found differences in microbial community composition between healthy and diseased milk samples from dairy cattle. The difference in microbiota according to disease state could suggest the introduction of specific bacterial species into the community from other animals and/or the environment leading to disease.

Despite this, the microbiota in healthy and diseased milk samples were not entirely separate. For example, Kuehn *et al.,* (2013) found *Staphylococcus* and *Corynebacterium* in both healthy and diseased milk samples and Oikonomou *et al.,* (2012) found *Streptococcus* spp. to be prevalent in all milk samples as did Hunt *et al.,* (2011) in human breast milk. Kuehn *et al.,* (2013) also reported the presence of large numbers of bacterial species with no evidence of any inflammatory response. The bacterial species found most commonly in healthy milk in several studies have not been linked to causing IMI (Kuehn *et al.*, 2013; Oikonomou *et al.*, 2014). It is possible that bacterial species associated with disease can persist as part of a commensal microbiota or play a stabilising and/or protective role in the mammary gland, with changes in abundance or interaction with other constituents of the microbiota leading to disease. However, the Oikonomou *et al.,* (2014; 2012) and Kuehn *et al.,* (2013) studies were cross-sectional i.e. all milk samples were taken at one time point, meaning it could not be determined what specific changes in the microbiota were occurring to result in healthy or diseased MGs.

2.2. Intramammary infections (IMI)

IMI result from inflammation of the mammary gland, usually due to a bacterial infection. The term ‘mastitis’ refers to when disease occurs as a result of infection (Harmon, 1994). Mastitis is often caused by one species of bacteria that could be part of a normal microbial community in the mammary gland.

2.2.1. Presentations of mastitis

Severity, clinical signs or type of bacterial infection can define mastitis presentation. When severity is used to define mastitis, subclinical and clinical are commonly used terms. When duration is used, acute (rapid) and chronic (long lasting) are used.

Subclinical mastitis has no visible signs of disease and does not lead to changes in milk or udder appearance (Harmon, 1994). It is identified through changes in milk composition, causative bacterial species in the milk or SCC (Albenzio *et al.*, 2002; Keisler *et al.*, 1992; Watkins *et al.*, 1991). It can remain undetected for long periods due to its lack of visible symptoms and can also occur because farmers do not detect clinical mastitis. The point at which clinical and subclinical mastitis are defined is therefore highly subjective.

Clinical mastitis is diagnosed visually through changes in the udder, milk or animal behaviour (Mavrogenis *et al.*, 1996). Clinical manifestations are categorized into three grades in cattle (Green *et al.*, 1997). Type one is an acute local infection, type two is a systemic acute infection and type three is a toxic infection. These distinctions are also present in sheep, although depend on farmer observation to detect them.

It has been proposed that subclinical and clinical mastitis are different stages in the progression of a single disease in sheep (Watson and Buswell, 1984). Watkins (1991) reported 38.5% of 26 mammary glands that developed clinical mastitis had a subclinical infection caused by the same bacterial species identified in the milk before the onset of clinical signs. This is supported by research in dairy cattle where two-thirds of enterobacterial mastitis occurred in quarters infected with the same species of pathogen for up to 100 days (Bradley and Green, 2001). This suggests that bacterial species persist in the mammary gland for long periods of time in subclinical states, possibly recrudescing to cause disease.

2.2.2. Causative agents of mastitis

Many species of bacteria cause mastitis in sheep. Watts (1988) states that more than 130 organisms have been reported to infect the mammary gland of dairy cattle and it is likely that this is a similar number of sheep. The range of bacterial species detected in sheep milk during microbiological and molecular analysis (Smith *et al.*, 2011) suggests a mixed community of bacteria are present in the mammary gland.

The major bacterial pathogens in sheep associated with mastitis are; *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Escherichia coli* and *Mannheimia haemolytica* (Green *et al.*, 2005; Heras *et al.*, 2002; Mavrogenis *et al.*, 1996; Omaleki *et al.*, 2011; Rowe *et al.*, 2001; Watkins *et al.*, 1991). Coagulase-negative staphylococci (CNS) are considered the predominant minor pathogens because they are usually associated with milk and/or subclinical mastitis. The ability of CNS to cause mastitis is thought to be variable depending on the pathogenicity of the individual bacterial species (Contreras *et al.*, 2007; Fthenakis and Jones, 1990; Pengov, 2001; Supré *et al.*, 2011).

2.3. Somatic cell count (SCC)

The SCC is the number of somatic cells, typically leukocytes, present per millimetre of milk (Lafi, 2006). The SCC is used as a method of monitoring milk health over time. It is used as an indicator of infection; a rise in SCC is indicative of a polymorphonuclear cell immune response to a bacterial intramammary infection (Mavrogenis *et al.*, 1996).

The SCC has not been studied extensively in suckler sheep, so an accepted 'normal' value has not been determined. In dairy sheep, SCCs of 600,000-800,000 cells per ml are considered indicative of infection, with up to 30% of new infections within a year associated with values in this range (Billion and Decremoux, 1998). However, this set level of SCC determines whether sheep milk can be sold for human consumption and there is no reason to believe that SCC would be this high. In a recent study at the University of Warwick, SCC of sheep milk was analysed and found to be similar to dairy cow milk.

In dairy cattle, an SCC of greater than 200,000 cells/ml was found to significantly increase the risk of IMI and SCCs below 100,000 cells/ml were indicative of a healthy mammary gland (Green *et al.*, 2006). However, lower SCCs (<150,000 cells/ml) in dairy cattle herds have been associated with a higher incidence of mastitis (Peeler *et al.*, 2002). This could suggest that dairy cattle selected for breeding based on low SCCs may be more susceptible to infection. Also, if organisms form part of a commensal microbial community in the MGs, the immune system may be less equipped to recognise an infection and raise an immune response that would result in a rise in SCC. Some bacterial strains may have evolved resistance to treatment or the ability to evade the immune system and recrudesce to cause disease when there is a community change (Peeler *et al.*, 2002).

An association between increasing age and SCC has been found (Biffa *et al.*, 2005; Green *et al.*, 2002; Peeler *et al.*, 2002). Conformational changes and/or damage to the mammary glands over time could increase the risk of disease (Green *et al.*, 2002). Alternatively, intramammary defence mechanisms may deteriorate with age (Green *et al.*, 2005; Reneau, 1986). The increasing risk of disease with age could also indicate compositional changes in the community acting as a trigger for infection.

Research on the persistence of bacterial pathogens in the sheep mammary gland is very limited. Hence, the investigation of the bacterial genera present over time in relation to the SCC may provide insights into how disease develops, which could help determine novel approaches to disease management.

2.4. Methods to investigate milk microbiomes

2.4.1. Culture-independent methods

The approach chosen in this study was culture-independent. The gold standard to diagnose mastitis is the culture of bacteria from 2 samples of milk (Hogan *et al.*, 1999) although this is hotly disputed because a number of factors can affect results of a bacteriological analysis. These include; inappropriate media/culturing conditions, transient infections and intermittent shedding of organisms (Bishop *et al.*, 2010). Hence, culture-independent processes provide a more rapid and reproducible way to view complex microbial ecosystems. The culture-independent approaches used in this study have been used previously to profile microbial communities (Braem *et al.*, 2012; Ercolini, 2004; Hunt *et al.*, 2011; Oikonomou *et al.*, 2014).

2.5. Conclusions from current knowledge and study objectives

Previous studies on milk microbiomes in humans and dairy cattle have shown the presence of a community of organisms that is not merely the result of skin contamination. Diverse and complex communities with unique and shared organisms have been identified, as have differences between milk samples from animals in healthy and diseased states. Similarities and differences in milk microbiota over lactation, between quarters and between animals as well as correlations between the presence/absence of certain bacterial species across different anatomical sites have been identified.

However, no studies to date have been done on the mammary gland microbiome of suckler sheep. Published studies generally have not used many samples over time to monitor changes longitudinally. Longitudinal studies have the potential to identify bacterial species correlated to protective/stabilising roles and altering the risks of infection and disease. This in turn could improve our understanding of the complex, dynamic microbiology of the mammary gland to determine how to manage IMIs more effectively.

The study objectives were to test the following hypotheses:

1. A natural microbial community (microbiome) forms in the suckler sheep mammary gland.
2. Changes in the community results in disease (measured by a change in SCC).
3. With increasing numbers of lactations, the number and species of bacteria colonising the mammary gland increases.
4. Differences in microbial community composition occur between mammary gland halves.
5. Colonisation of the mammary gland is inevitable.

A further objective was to:

1. Develop effective molecular-based whole community approaches that were culture-independent. These included: DNA extraction, Polymerase Chain Reaction (PCR), Denaturing Gradient Gel Electrophoresis (DGGE) and high-throughput DNA sequencing.

3. Materials and methods

3.1. Longitudinal study samples

Milk samples were collected using standard techniques (Hogan *et al.*, 1999) from each mammary gland half of 30 sheep on one farm for up to 8 consecutive weeks in January and February 2010. The sheep were part of a flock of 220 mules on a commercial meat farm in Wolverhampton, UK. Milk samples were placed on ice immediately and transported to the laboratory and frozen at -20^{o}C until used. Each sample was split before freezing into three aliquots. Two aliquots were submitted to a commercial laboratory QMMS Ltd (Easton Hill, Wells, Somerset, UK). One aliquot was for somatic cell count (SCC) analysis using the Fossomatic method (Delta CombiScope – Model FTIR 400, Drachten, Netherlands) and the second for microbiological culture. The third aliquot was retained for culture-independent molecular analysis.

The sheep in this study were part of a larger longitudinal study (Huntley *et al.*, 2012). They were free from clinical signs of mastitis during the sampling period. Milk samples from sheep that were part of the larger study were used during method optimisation to ensure protocols were sufficient for the specific sample type. In total, 379 milk samples were processed as part of this project. Information on sample identification, the number of milk samples per sheep, SCC data and results from bacteriological culture of each set of milk samples are in Appendix 1.

3.2. DNA extraction

The DNA extraction method was performed as stated in Purdy (2005). Briefly, the protocol uses a physical (bead-beating), detergent (Sodium dodecyl sulphate) and chemical (phenol) based lysis followed by nucleic acid separation from cell debris and sample substrate and purification using hydroxyapaptite (HTP) and Sephadex columns respectively. DNA is precipitated using ethanol and re-suspended in sterile water. Extracted DNA was aliquoted into 10µl aliquots and stored at -20^{o}C. DNA extractions were completed in batches of 8 or 16 samples with a *Staphylococcus aureus* spiked positive control and nuclease-free water negative control per batch.

3.3. Polymerase chain reaction (PCR)

The bacterial DNA from each milk sample was amplified in a 16S rRNA bacterial PCR. All PCR reactions were prepared in a UV cabinet that was UV sterilised for 30 minutes prior to each use. A DNA extraction and no template PCR negative control were included in each PCR. All reactions were carried out under standard conditions on an Eppendorf master cycler in a 50µl final reaction volume. Table 1 and

Table 2 detail the PCR primer information and PCR master mix components respectively.

Table 3 provides the PCR program. All PCR products were assessed by ethidium bromide-stained agarose gel electrophoresis in 1% agarose gels made with 1X TAE and run in the same buffer at 100 volts for 40 minutes. PCR products were stored at 4^{o}C until further use.

Table 1: Details of primers used to amplify bacterial DNA from sheep milk samples

Primer name	Primer sequence (5'-3')	Target region	Amplicon size (bp)	Reference
27F	AGA GTT TGA TCC TGG CTC AG	V1-V2	300	(Hunt *et al.*, 2011)
338R*	TGC TGC CTC CCG TAG GAG T			

GC clamp (for DGGE): CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G

Table 2: Components of PCR master mix per 50µl reaction

Master mix component	Working concentration	Final concentration	Volume (µl)
27F	20 µM	0.2 µM	0.5
338R	20 µM	0.2 µM	0.5
Bioline MyTaq mix	2X	-	25
DNA	-	-	1
Water	-	-	23
Total (µl)	-	-	50

Table 3: PCR program for 16S bacterial PCR

Primers	PCR program	Reference
27F/338R	94^{o}C for 5 min followed by 35 cycles of 94^{o}C for 1 minute, 55^{o}C for 1 minute and 72^{o}C for 2 minutes with a final extension of 72^{o}C for 2 minutes.	(Hunt *et al.*, 2011)

3.4. Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed as described in Muyzer and Schäfer (2001). Pre-DGGE, the DNA concentration for each PCR product was estimated by comparison of PCR product intensity to known DNA concentrations on a reference DNA ladder (Hyperladder 1kb, Bioline, UK) on 1% agarose gels. The DNA concentration was then used to determine the volume of PCR product required to load 300ng/µl for each PCR amplicon. Any samples that required more than the maximum volume of PCR product were not analyzed by DGGE, as any bands produced would have been too weak to visualize.

DGGE was performed using a DCode universal mutation detection system (BioRad, USA). Polyacrylamide gels (20cm x 20cm x 1mm) consisted of 6% (v/v) polyacrylamide (37:5:1,

acrylamide/bisacrylamide) in 1X TAE buffer with a linear 20-80% denaturing gradient (100% denaturant contains 40% (v/v) formamide and 7M urea). Electrophoresis was performed at 60^{o}C for 18 hours at 100 volts. Each gel contained a custom reference ladder consisting of 7 reference bands produced via excision of bands from an initial DGGE gel of sheep milk DNA samples using the protocol described by Muyzer and Schäfer (2001). The excised bands underwent PCR and purified amplicons were mixed in equal amounts to produce the reference ladder. Each DGGE gel was stained with SYBR Gold (Invitrogen) and visualised on a UV GelDoc (Geneflow, UK).

To identify the bacterial species present in DGGE bands associated with a change in SCC, selected DGGE bands were excised using the protocol in stated above. Samples were cloned using the TOPO-TA cloning kit (Life Technologies, Paisley, UK) according to the providers' instructions and submitted for Sanger sequencing (GATC, Cologne, Germany). All cloned DNA sequences were edited using the DNAstar SeqMan II sequence analysis package (Lasergene Inc). DGGE band identity was determined using the National Centre for Biotechnology Innovation (NCBI) standard nucleotide blast function with the 16S ribosomal RNA sequences (Bacteria and Archea) database (Altschul *et al.*, 1990).

3.4.1. Analysis of DGGE images

DGGE images were edited for brightness and contrast using Adobe Photoshop CS6 (Adobe, UK) to reduce background noise that interfered with subsequent DGGE banding pattern recognition. DGGE banding patterns were analysed using the software package Gel Compar II version 5.1 (Applied Maths, Belgium) according to the instructions of the software provider. Briefly, each DGGE image was cropped and individual lanes defined and normalised. Normalisation minimized migration differences between gels by alignment of the reference ladder at either end of each DGGE gel. Optimal parameters for each DGGE lane were then adjusted to maximise the use of the automatic band recognition function to assign bands. DGGE profiles were then compared using a ranked Pearson-Product moment correlation coefficient and Unweighted-Pair Group Method with Arithmetic average (UPGMA).

3.4.2. Modelling DGGE data

Data from the Gel Compar II analysis of DGGE images were imported into the software package MLwiN version 2.27 (Rasbash *et al.*, 2009). Data imported included sheep identification, parity, week of sampling, mammary gland half, milk sample identification and logSCC. The Gel Compar II analysis categorized each DGGE band into 1 of 35 band positions. Binary data on the presence or absence of a band for each milk sample and numerical data on the band intensity were analysed for the week of sampling and week prior to sampling in the model.

The model was a mixed effects regression model with logSCC as the dependent variable and sheep, mammary gland half and week as random variables. Parity was added as a fixed effect. A

forward stepwise backward elimination approach with retention criteria P <0.05 in the final model was used to determine the DGGE bands associated with a significant change in SCC.

The model took the form:

$$\text{Log(SCC)}_{ijk} = \beta_0 + \sum \beta x_{ijk} + \sum \beta x_{jk} + \sum \beta x_k + f_k + v_{jk} + u_{ijk}$$

where Log(SCC)_{ijk} = the log(SCC) on occasion ijk, where *i* is week, *j* is mammary gland half and *k* is sheep. β_0 = intercept, βx is a vector of fixed effects varying at level 1 (*ijk*), level 2 (*jk*), and level 3 (*k*), f_k, v_{jk} and u_{ijk} are the level 3, 2 and 1 residual variances, respectively.

3.5. Sequencing library preparation

Five libraries of milk samples (84 samples in each) were prepared for Illumina MiSeq paired-end sequencing using a two-step PCR protocol. Briefly, the first PCR to attach the required read sequences (to allow the MiSeq to read indexes added to samples in the second PCR) was performed using primers 27F-rd1/338R-rd2. For some milk samples, no DNA and/or milk was present post PCR-DGGE analysis. Hence, to enable a fair comparison between samples and reduce the potential for PCR inhibition, 1µl of a 1:10 dilution of PCR product from the 27F/338R PCR detailed in Section 3.3 was used as the starting template for the 27F-rd1/338R-rd2 PCR. Primer sequences, PCR master mix and PCR program details for the 27F-rd1/338R-rd2 PCR are presented in Table 4: Details of primers used for 27F-rd1/338R-rd2 PCRTable 4,

Table 5 and Table 6 respectively.

Table 4: Details of primers used for 27F-rd1/338R-rd2 PCR

Primer	Sequence 5'-3'	Annealing temp (ºC)	Product size (bp)
27F-rd1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG AGAGTTTGATCCTGGCTCAG		
338R-rd2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACA GTGCTGCCTCCCGTAGGAGT	61	406

Table 5: Components of 27F-rd1/338R-rd2 PCR master mix

Master mix component	Working concentration	Final concentration	Volume (µl)
27F-rd1	10 µM	0.1 µM	0.5
338R-rd2	10 µM	0.1 µM	0.5
Bioline MyTaq red Hot-start mix[1]	2X	-	25
DNA (1:10 PCR product)	-	-	1
Water	-	-	23
Total (µl)	-	-	50

Table 6: 27F-rd1/338R-rd2 PCR program

Primers	PCR program
27F-rd1 338R-rd2	95°C for 1 minute (hot-start), 94°C for 5 min, followed by 8 cycles of 94°C for 1 minute, 61°C for 30 seconds, 72°C for 2 minutes, with a final extension at 72°C for 2 minutes.

PCR products from the read sequence PCR were purified using the Agencourt AMPure XP purification system (Beckman-Coulter, High Wycombe, UK) as per the manufacturer's instructions and 1µl of purified PCR product used as the DNA template in the second indexing PCR. The primers used in the indexing PCR are detailed in Table 7. The PCR master mix was produced as previously detailed in

[1] The hot-start version of the MyTaq red mix was used from the read sequence PCR onwards to minimize non-specific amplification due to excessive PCR cycling.

Table 5. The PCR program for the indexing PCR is presented in Table 8.

Table 7: PCR primers used in the indexing PCR for MiSeq sample preparation

Primer	Sequence 5'-3'	Annealing temp. (°C)	Product size (bp)
N501-rd1	AATGATACGGCGACCACCGAGATCTACACGCGATCT ATCGTCGGCAGCGTCAGATGT	55	516
N502-rd1	AATGATACGGCGACCACCGAGATCTACACATAGAGA GTCGTCGGCAGCGTCAGATGT		
N503-rd1	AATGATACGGCGACCACCGAGATCTACACAGAGGAT ATCGTCGGCAGCGTCAGATGT		
N504-rd1	AATGATACGGCGACCACCGAGATCTACACTCTACTC TTCGTCGGCAGCGTCAGATGT		
N505-rd1	AATGATACGGCGACCACCGAGATCTACACCTCCTTA CTCGTCGGCAGCGTCAGATGT		
N506-rd1	AATGATACGGCGACCACCGAGATCTACACTATGCAG TTCGTCGGCAGCGTCAGATGT		
N507-rd1	AATGATACGGCGACCACCGAGATCTACACTACTCCT TTCGTCGGCAGCGTCAGATGT		
N508-rd1	AATGATACGGCGACCACCGAGATCTACACAGGCTTA GTCGTCGGCAGCGTCAGATGT		
N701-rd2	CAAGCAGAAGACGGCATACGAGATTAAGGCGAGTCT CGTGGGCTCGGAGATGT		
N702-rd2	CAAGCAGAAGACGGCATACGAGATCGTACTAGGTCT CGTGGGCTCGGAGATGT		
N703-rd2	CAAGCAGAAGACGGCATACGAGATAGGCAGAAGTC TCGTGGGCTCGGAGATGT		
N704-rd2	CAAGCAGAAGACGGCATACGAGATTCCTGAGCGTCT CGTGGGCTCGGAGATGT		
N705-rd2	CAAGCAGAAGACGGCATACGAGATGGACTCCTGTCT CGTGGGCTCGGAGATGT		
N706-rd2	CAAGCAGAAGACGGCATACGAGATTAGGCATGGTCT CGTGGGCTCGGAGATGT		
N707-rd2	CAAGCAGAAGACGGCATACGAGATCTCTCTACGTCT CGTGGGCTCGGAGATGT		
N708-rd2	CAAGCAGAAGACGGCATACGAGATCAGAGAGGGTC TCGTGGGCTCGGAGATGT		
N709-rd2	CAAGCAGAAGACGGCATACGAGATGCTACGCTGTCT		

	CGTGGGCTCGGAGATGT		
N710-rd2	CAAGCAGAAGACGGCATACGAGATCGAGGCTGGTC TCGTGGGCTCGGAGATGT		
N711-rd2	CAAGCAGAAGACGGCATACGAGATAAGAGGCAGTC TCGTGGGCTCGGAGATGT		
N712-rd2	CAAGCAGAAGACGGCATACGAGATGTAGAGGAGTC TCGTGGGCTCGGAGATGT		

Table 8: The PCR program for the indexing PCR used for MiSeq sample preparation

Primers	PCR program	Reference
N501-8-rd1 /N701-12-rd2	95°C for 1 minute (hot-start), 72°C for 3 minutes, 95°C for 30 seconds followed by 8 cycles of 95°C for 10 seconds, 55°C for 30 seconds, 72°C for 30 seconds, with a final extension at 72°C for 5 minutes.	Nextera –XT sample preparation guide (Illumina)

Before a second PCR purification, approximately 10% of samples in each library were randomly selected for a PCR to check that the indexes added were in the correct orientation. The primer sequences and PCR program used in the confirmatory PCR are detailed in Table 9 and Table 10 respectively. The combinations of primers tested in the index check PCR and the expected amplification result are detailed in Table 11.

Table 9: Primers used in PCR to check indexes added for MiSeq sequencing

Primer	Sequence 5'-3'	Annealing temp (°C)	Product size (bp)
P5 check	AATGATACGGCGACCACCGAGATCTACAC	55	569
P7 check	CAAGCAGAAGACGGCATACGAGAT		

Table 10: PCR program for index check PCR

Primers	PCR program	Reference
27F/338R	94°C for 5 min followed by 10 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes with a final extension of 72°C for 2 minutes.	(Hunt *et al.*, 2011)

Table 11: Expected amplification results from index check PCR if adapters added in correct orientation

Primer combination	Expected result
P5/27F	✗No product
P5/338R	✓~ 400bp product
P7/27F	✓~ 400bp product
P7/338R	✗No product

3.5.1. Model community control for sequencing data

Each library of samples analysed using the Illumina MiSeq sequencing technology contained a model community in triplicate. The model community consisted of 5 bacterial species commonly associated with IMI in sheep; *Escherichia coli*, *Staphylococcus hyicus*, *Mannheimia haemolytica*, *Staphylococcus aureus* and *Streptococcus uberis*. DNA from each bacterial species was extracted from a pure culture using the DNeasy Blood and Tissue kit (Qiagen, UK). DNA concentration was measured using the NanoDrop spectrometer (Thermo Fisher Scientific, Loughborough, UK) and normalised to 25ng/µl. Each bacterial species was processed in the bacterial PCR previously detailed in Section 3.3. PCR product for each bacterial species was purified using the QIAquick PCR purification kit (Qiagen, UK) and quantified and normalised to 25ng/µl again. Normalised PCR product for each bacterial species was combined in equal volumes to produce the model community stock.

3.5.2. Sequencing data analysis pipeline

Raw sequence data was edited using a combination of custom Perl and shell scripts and the software packages USEARCH (Edgar, 2010), UPARSE (Edgar, 2013) and QIIME (Caporaso *et al.*, 2010). Briefly, the forward and reverse read sequences for each sample were merged in USEARCH with 1 mismatch allowed and forward and reverse reads truncated at the first quality score <=3 to remove low quality tails. A custom Perl script re-labelled sequence headers in merged files which were then quality-filtered in USEARCH.

In the quality-filtering process, sequence read length was analysed using the program Read Length Incremental Clustering (ReLIC). ReLIC determines whether sequences of specific lengths around the median read length lead to large changes in the number of OTUs detected (Williams and Purdy, submitted). This analysis selected $\underline{\leq}$312bp as the minimum acceptable read length for this dataset. In addition, a maximum error rate of 0.065 was used to determine whether sequences were of sufficient quality. Representative sequences for each sample were identified using dereplication scripts in USEARCH. Dereplicated files for individual samples were clustered into OTUs (Operational Taxonomic Units i.e. bacterial species), re-labelled and chimera checked in

UPARSE. A confidence limit for a minimum OTU size of 0.43% of total reads was determined using analysis of the dereplicated model community samples across the 5 libraries. OTUs below this size cut-off were removed from each sample before reads were mapped back to each sample from the quality-filtered file using the UPARSE pipeline to produce OTU tables. To produce the initial analysis of data for the 5 sheep presented in this report, OTU tables for individual samples were merged using automated scripts in QIIME to produce an OTU table per sheep.

4. Results

4.1. Testing the DNA extraction protocol

The method developed by Purdy (2005) was previously used to extract DNA from sediment (Purdy *et al.*, 1997) but had not been used on other sample types. Two initial trials were conducted to confirm that DNA of a suitable quality for further molecular analysis could be consistently extracted from milk. The results of each trial were evaluated using a combination of nucleic acid purity and yield measurements determined by Nanodrop spectroscopy (Thermo Fisher Scientific, Loughborough, UK). Table 12 shows results from one of the trials, with similar DNA yields for replicate milk samples and a high yield in the spiked positive control. The 260/280 ratio is a measure of DNA purity. A ratio of >1.8 indicates pure DNA, so the ratios ranging from 1.5 -1.7 in Table 12 indicate the Purdy (2005) protocol produced good quality DNA.

Table 12: DNA concentrations (ng/µl), yields (µg) and 260/280 ratios for milk samples from trial of Purdy (2005) protocol

Sample ID	DNA concentration (ng/µl)	260/280 ratio	DNA yield (µg)
Milk 1	29	1.7	2.9
Milk 2	21	1.6	2.1
Staphylococcus aureus spiked positive control	554	1.5	55.4

4.1.1. Sensitivity testing the DNA extraction protocol

Sensitivity testing was conducted on the Purdy (2005) DNA extraction protocol to ensure that DNA could be extracted from milk samples with a low bacterial abundance. Sensitivity testing used pasteurized milk spiked with either *Escherichia coli* K12 or *Staphylococcus hyicus*. Briefly, a ten-fold dilution series to 10^7 was made of the two cultures. Subsequently, 5µl drops of each dilution were spotted on LB agar plates and grown overnight at 37°C, after which colonies were counted at a suitable dilution to determine the colony forming units per millilitre (cfu/ml) in the spiked pellet. DNA was extracted using the Purdy (2005) protocol and each sample analyzed using PCR. Table

13 shows the results for 5 sensitivity-testing trials. In the first three trials, *Escherichia coli* K12 was used and results were analyzed using primers PRBA338F/518R and 341F/907R (Muyzer and Schäfer, 2001; Ovreas *et al.*, 1997). PCR amplification was detected to 10^3 cfu/ml for PRBA338F/518R and 10^1 cfu/ml for 341F/907R. The same result was produced in trial 4 where *Staphylococcus hyicus* was used. After optimizing the PCR protocol, sensitivity testing was repeated a fifth time using primers 27F/338R (Hunt *et al.*, 2011) to ensure sensitivity remained high and consistent using the PCR protocol selected to process all sheep milk samples. PCR product was detected to 10^1 cfu/ml in trial 5 (Table 13), indicating a high level of sensitivity in the optimized PCR assay.

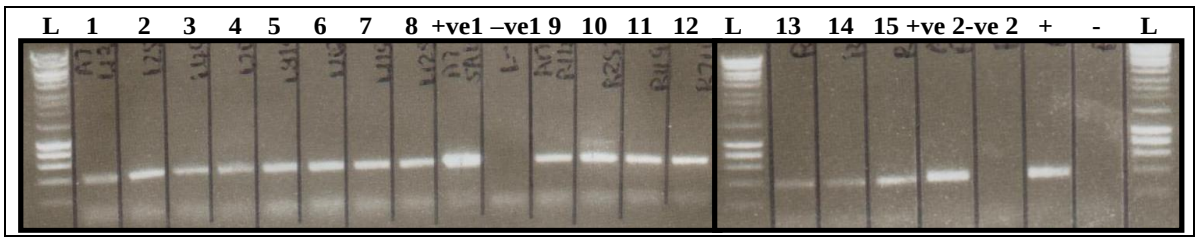
Table 13: Sensitivity testing results for Purdy (2005) DNA extraction protocol

Test No.	Bacterial species	Average colony number in 5µl	Dilution factor to determine cfu/ml	Cfu/ml in spiked pellet	PRBA F/R PCR result (cfu/ml)	341F/907R PCR result (cfu/ml)	27F/ 338R PCR result (cfu/ml)
1	*E.coli* K12	1	10^4	2 x10^6	10^3	10^1	N/A
2	*E.coli* K12	3	10^5	6 x10^7	10^3	10^1	N/A
3	*E.coli* K12	4	10^5	4 x10^7	10^3	10^1	N/A
4	*S. hyicus*	2	10^5	2 x10^7	10^3	10^1	N/A
5	*E.coli* K12	10	10^4	2 x10^7	N/A	N/A	10^1

4.2. Testing the PCR protocol

The PCR protocol detailed in Section 3.3 was tested with three sets of milk samples from three different sheep to confirm PCR amplification was successful and of an appropriate yield for use in further analysis. Figure 1 shows the PCR results for milk samples from one set of sheep, with visible PCR amplification in all milk samples and no amplification in DNA extraction or PCR negative controls. Repetition of the PCR protocol with other sets of milk samples from different sheep also produced consistent amplification.

Figure 1: PCR amplification results from one sheep for PCR protocol to amplify bacterial DNA from sheep milk DNA[2]



[2] In Figure 1, 'L' is the DNA ladder marker and numbers 1-15 are sheep milk samples. The '+ve1' and '+ve2' are DNA extractions positive controls for samples 1-8 and 9-18 respectively, with '-ve1' and '-ve2' the DNA extraction negative controls for samples 1-8 and 9-18 respectively. The PCR positive control is '+' and the negative '-'.

4.2.1. Results from PCR amplification of longitudinal study milk samples

Table 14 summarises the PCR results for milk samples from all 30 sheep in the longitudinal study.

Table 14: PCR amplification results for all milk samples in longitudinal study ordered by sheep parity[3]

Sheep	Left half (weeks)								Right half (weeks)							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
A20	NS	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	NS
A35	✓	✓	✓	✗✗	✓	✓	✓	NS	✓	✓	✓	✓	✗✓	NS	NS	NS
A50	✓	✓	✓	✓	✓	✓	✓	NS	✓	✓	✓	✗✗	✓	✓	✓	✓
A15	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✗✗	✗✓	✗✗
A21	✓	✓	✓	✓	✓	✓	NS	NS	✓	✓	✓	✓	✓	✓	NS	NS
A26	✓	✓	✓	✓	✓	✓	NS	NS	✗✓	✓	✓	✓	✓	✓	✓	NS
A27	✓	✓	✓	NS	✓	✓	NS	NS	✓	✓	✓	✗✗	✓	✗✗	NS	NS
A28	✓	✓	✓	✓	✓	NS	NS	NS	✓	✓	NS	✓	✓	✓	NS	NS
A39	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
A4	✓	✓	✓	✓	✓	✓	NS	NS	✓	✓	✗✓	✓	✓	✓	NS	NS
A41	✓	✓	✓	✓	✓	✓	NS	NS	✓	✓	✓	✗✓	✗✓	✗✓	NS	NS
A44	✓	✓	✗✓	✓	✓	✓	NS	NS	✓	✓	✓	✓	✓	✓	✗✓	NS
A45	✓	✓	✓	✗✗	✓	✓	✓	NS	✓	✓	✗✗	✓	✓	✓	NS	NS
A46	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	NS
A47	✓	✓	✓	✓	✓	✓	✗✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
A5	✓	✓	✓	✓	✓	✓	✓	NS	✓	✓	✓	✓	✗✓	✓	✓	NS
A9	✓	✓	✗✗	✓	✓	✓	✗✗	NS	✓	✓	✓	✓	✓	✗✗	✓	NS
A12	✓	✓	✓	NS	✓	✓	✓	NS	✓	✓	✓	✓	✓	✓	✓	NS
A2	✓	✓	✓	NS	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
A22	✓	✓	✓	✓	✓	✓	✓	NS	✓	✓	✓	✓	✓	✓	✓	NS
A25	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	NS	✓	✓	NS
A29	✓	✓	✓	✓	✓	✓	✓	NS	✓	✓	✓	✓	✓	✓	✓	NS
A3	✓	✓	✓	✓	✓	NS	NS	NS	✓	✓	✓	✓	✓	✓	NS	NS
A16	✓	✓	✓	✓	✓	X✓	✓	NS	✓	✓	✓	✓	✓	✓	✓	NS
A24	✓	✓	X✓	✓	✓	XX	NS	NS	✓	✓	✓	✓	NS	X✓	NS	NS
A40	✓	✓	✓	✓	✓	✓	NS	NS	✓	✓	✓	✓	✓	✓	✓	NS
A43	NS	✓	✓	✓	✓	✓	✓	✓	NS	✓	✓	✓	✓	✓	✓	NS
A49	✓	✓	✓	✓	✓	✓	✓	NS	✓	NS	NS	✓	✓	✓	✓	NS
A6	✓	✓	✓	✓	✓	✓	NS	NS	✓	✓	✓	✓	✓	✓	NS	NS
A23	✓	✓	✓	✓	✓	✓	NS	NS	✓	✓	✓	✓	✓	✓	NS	NS

[3] Successful amplification: ✓ and unsuccessful amplification ✗; successful amplification on a second PCR attempt: ✗✓; no successful amplification in two attempts (not analysed by DGGE): ✗✗; no sample for analysis: NS.

4.3. DGGE results

A DGGE image per sheep was produced using the protocol detailed in Section 3.4 resulting in 30 images. Exemplars of DGGE images from sheep of each parity are presented below.

Figure 2: DGGE image of PCR amplicons for parity one sheep A35 [4]

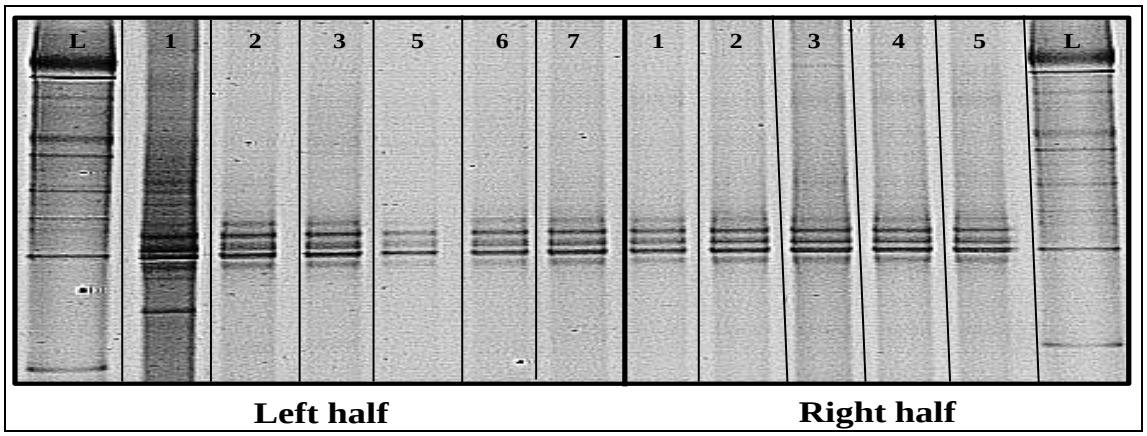


Figure 3: DGGE image of PCR amplicons for parity two sheep A41

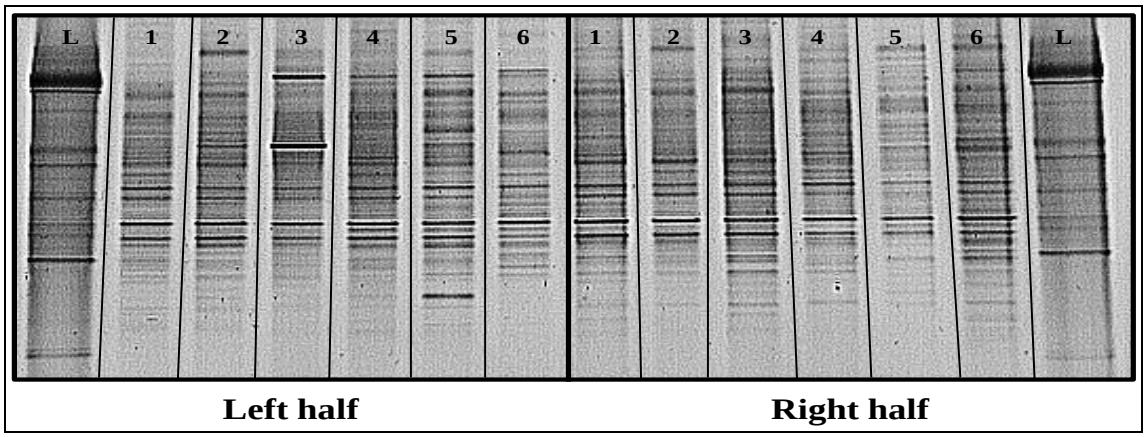
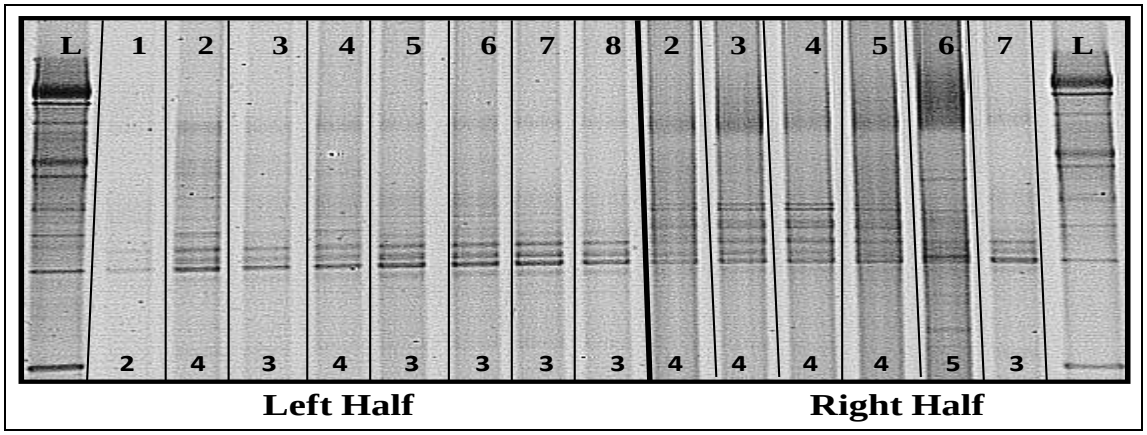


Figure 4: DGGE image of PCR amplicons for parity three sheep A25



[4] For all DGGE images, 'L' corresponds to the custom DGGE reference ladder; numbers 1-8 are milk samples from weeks 1-8, with the mammary gland half they originate from specified in each Figure.

Figure 5: DGGE image of PCR amplicons for parity four sheep A40

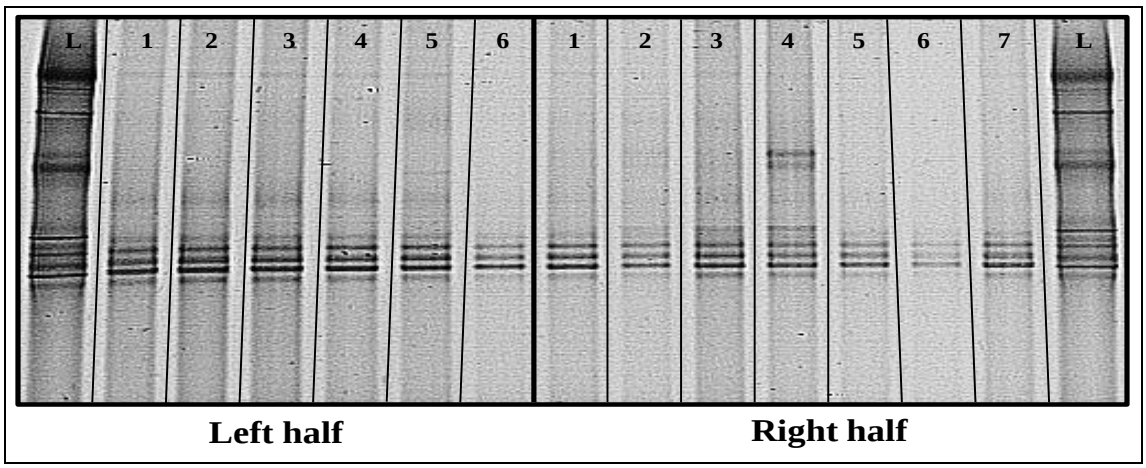
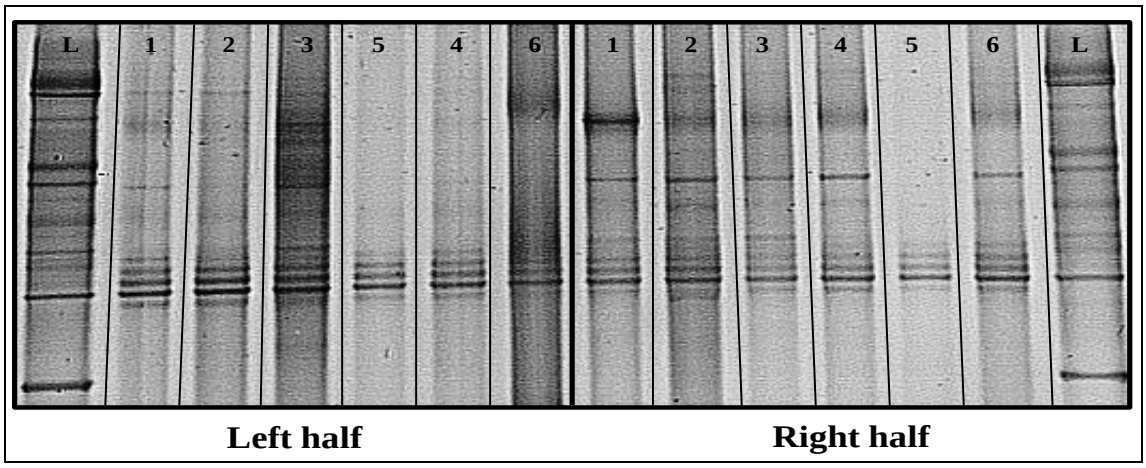


Figure 6: DGGE image of PCR amplicons for parity ten sheep A23



Visual inspection of the DGGE images shows both similarities and differences in DGGE banding patterns within and between the two mammary gland halves. There is a central region of approximately 5-6 DGGE bands that appear in the majority of the samples, which could suggest a stable community is present, with commensal organisms persisting over lactation. However, there were also differences in banding pattern for samples from different halves at the same time point e.g.

Figure 5, week 4 and between MG halves in general e.g. Figure 6. The DGGE image for the parity two sheep (Figure 3) shows the most complex DGGE banding pattern, with multiple bands with wide distributions in each sample. This could suggest differences in community composition between sheep of different parities, although the complex community could also be specific to the individual animal.

4.3.1. DGGE image analysis results

DGGE images were imported into the program Gel Compar II for further analysis as detailed in Section 3.4.1. Summary information on the number of DGGE bands grouped according to sheep, sheep parity, week in lactation and mammary gland half are provided in Table 15- Table 18.

Table 15: DGGE band count data per sheep ordered by sheep parity

Sheep	Parity	No. of milk samples	Total No. of DGGE bands	DGGE band count range per sample	Mean DGGE band count (SE)
A20	1	14	89	6-8	6.4 (0.20)
A35		11	59	4-10	5.4 (0.47)
A50		13	65	2-9	5.0 (0.62)
A15	2	14	46	3-10	4.2 (0.75)
A21		12	49	1-8	4.1 (0.58)
A26		13	60	3-14	4.6 (0.78)
A27		9	62	5-10	6.9 (0.66)
A28		10	69	4-11	6.9 (0.81)
A39		16	92	2-11	5.8 (0.70)
A4		10	100	2-11	6.7 (1.02)
A41		11	115	2-23	11.2 (0.59)
A44		13	105	2-16	8.1 (1.20)
A45		11	51	2-7	4.6 (0.56)
A46		15	83	3-8	5.5 (0.49)
A47		15	62	1-10	6.2 (0.80)
A5		14	85	2-14	6.1 (0.91)
A9		11	58	2-10	5.3 (0.93)
A12	3	13	52	1-13	4.0 (1.39)
A2		15	63	1-7	4.2 (0.48)
A22		14	74	4-8	5.3 (0.30)
A25		14	49	3-4	3.5 (0.20)
A29		14	74	4-7	5.3 (0.22)
A3		11	51	4-6	4.6 (0.20)
A16	4	14	59	1-8	4.2 (0.54)
A24		10	43	1-6	4.3 (0.58)
A40		13	65	4-7	5.0 (0.20)
A43		13	101	7-10	7.8 (0.26)
A49		12	46	2-5	3.8 (0.27)
A6	10	12	64	4-7	5.3 (0.33)
A23		12	77	3-9	6.4 (0.48)
Total	**-**	**379**	**2068**	**-**	**-**
Mean	**-**	**12.6**	**68.9**	**-**	**5.5**

Table 16: DGGE band count data grouped by sheep parity

Parity	No. of sheep	DGGE band range	Mean no. of DGGE bands
1	3	2-10	5.6 (0.27)
2	14	1-14	6.0 (0.23)
3	6	1-13	4.4 (0.20)
4	5	1-10	5.1 (0.25)
10	2	3-9	5.9 (0.31)
Total	**30**	**-**	**-**
Mean	-	**-**	**5.4**

Table 15 shows a mean of approximately thirteen milk samples per sheep, with the total number of DGGE bands per sheep ranging from 46-115 and the DGGE band count per samples from 1-23 bands. The variation in DGGE band count between and within sheep could suggest there are changes and/or fluctuations in community composition over time. In **Table 16**, parity 2 and 10 sheep had the highest mean number of DGGE bands, with parity 3 and 4 sheep having the lowest.

Table 17: DGGE band count data grouped according to week in lactation

Week	No. of milk samples	No. of DGGE bands	Mean no. of DGGE bands (SE)
1	56	390	7.0 (0.35)
2	59	376	6.4 (0.35)
3	56	308	5.5 (0.33)
4	53	290	5.5 (0.32)
5	56	249	4.5 (0.25)
6	52	237	4.6 (0.29)
7	35	160	4.6 (0.37)
8	12	58	4.8 (0.66)
Total	**379**	**2068**	**-**

Table 18: Mean number of DGGE bands grouped by MG half

Mammary gland half	Mean no. of DGGE bands (SE)
Left	5.6 (0.19)
Right	5.9 (0.17)

When the DGGE data was grouped by week of lactation (Table 17), the mean number of DGGE bands decreased from weeks 1-4 and then marginally increased from weeks 5-8. Hence, this also supports the idea of a potential fluctuation in the microbial community on weekly basis. When grouped by MG half (Table 18), the mean number of DGGE bands was similar. These results correlated with the SCC data on the milk samples (Appendix 1).

4.3.2. Investigating DGGE data clustering

The DGGE band data indicated a persistent bacterial community with at least 1 DGGE band detected in every sample. Some potential variation in DGGE band count was seen by sheep parity and week (Table 16 and Table 17 respectively) and very little variation between mammary gland half (Table 18). This suggested a consistent bacterial community was present, but with minor as opposed to major fluctuations in bacterial population occurring in the study period. This was further substantiated by additional analysis in Gel Compar II in which within DGGE gel (i.e. within sheep)

comparisons were made using a ranked Pearson Product-moment correlation coefficient and Unweighted Pair Group Method with Arithmetic mean (UPGMA). For each sheep in the study, a dendrogram and PCA plot were produced to visualise correlations between samples within sheep; examples of these for 2 sheep are given below with the associated DGGE images included for reference.

Figure 7: DGGE results for sheep A35 [5]

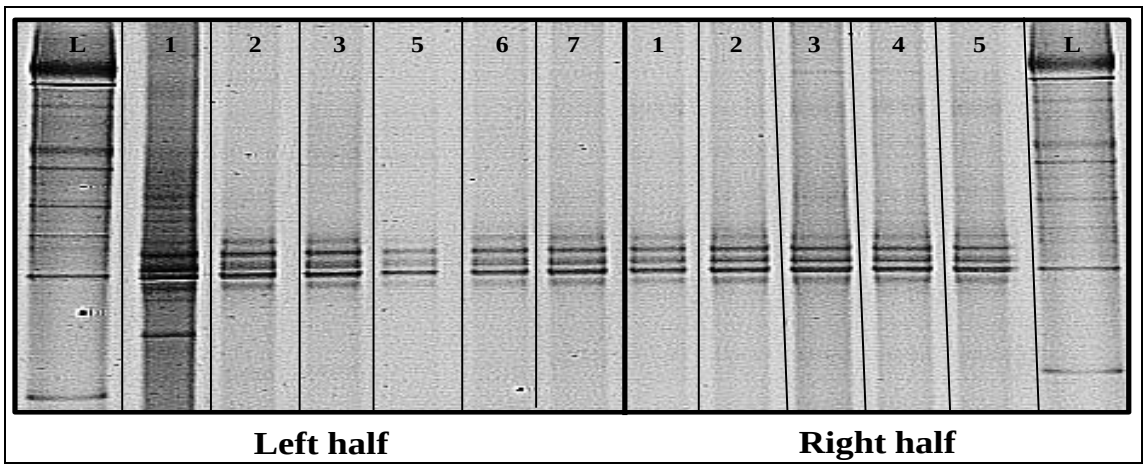


Figure 8: Dendrogram of sheep A35 milk samples

[5] In Figure 7-12, each milk sample is labelled according to mammary gland half i.e. 'L' for left and 'R' for right; the subsequent number is the week i.e. 1-8. Dendrogram axis is percentage similarity; the lowest percentage is the greatest similarity between the 2 most dissimilar samples. The axes on the PCA plots are the entry coordinates that are the coordinates of the entries in the first 2 components of the PCA.

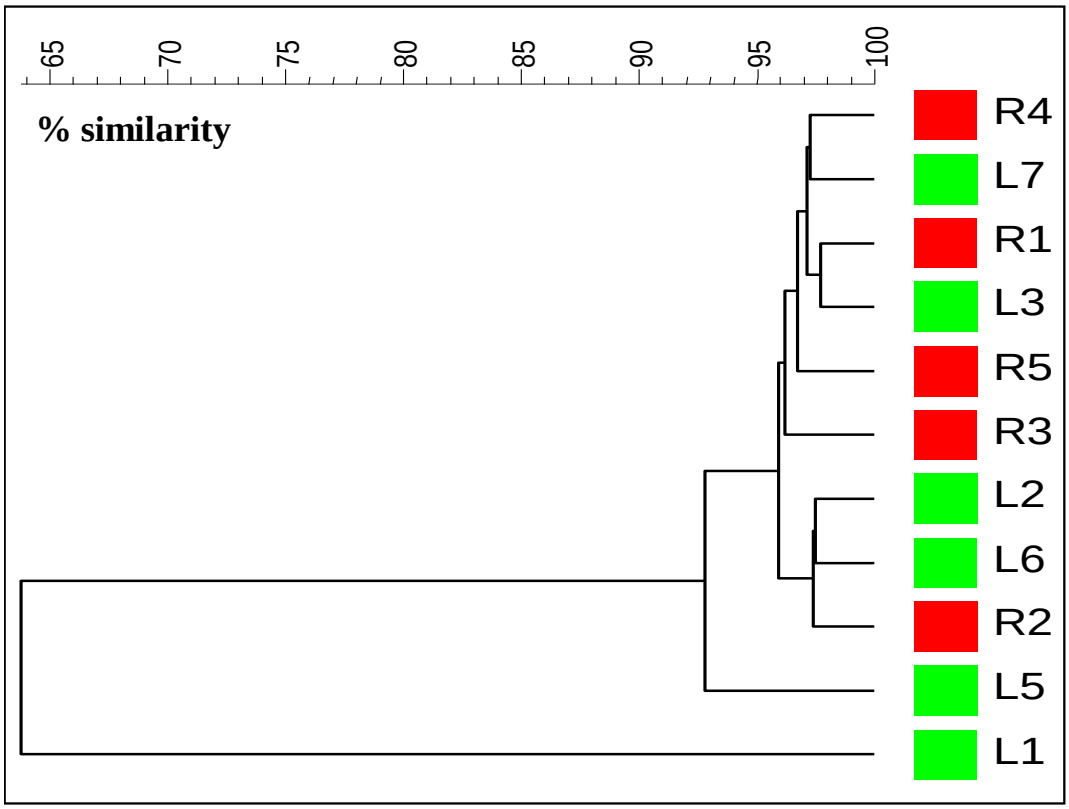

65
70
75
80
85
90
95
100
% similarity
R4
L7
R1
L3
R5
R3
L2
L6
R2
L5
L1

Figure 9: PCA plot of sheep A35 milk samples

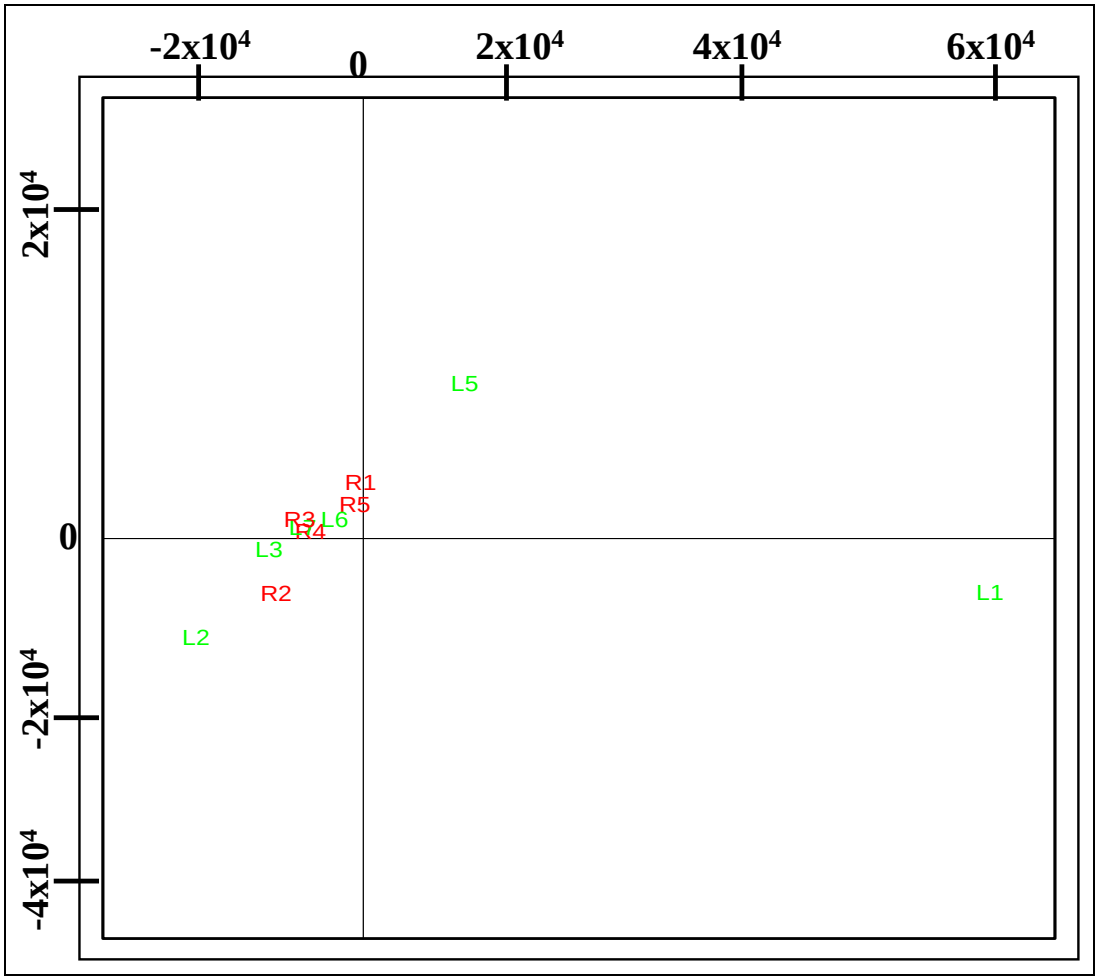


Figure 10: DGGE results for sheep A25

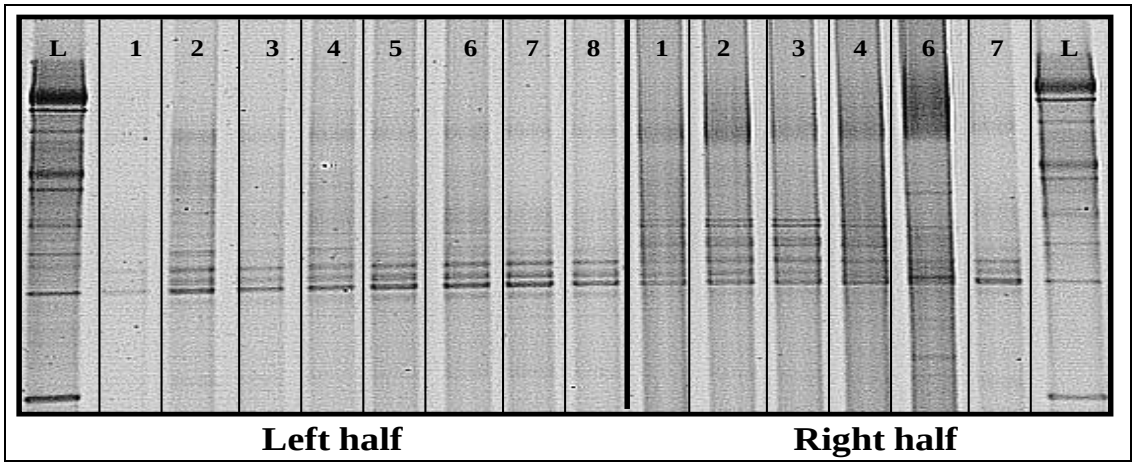


Figure 11: Dendrogram of sheep A25 milk samples

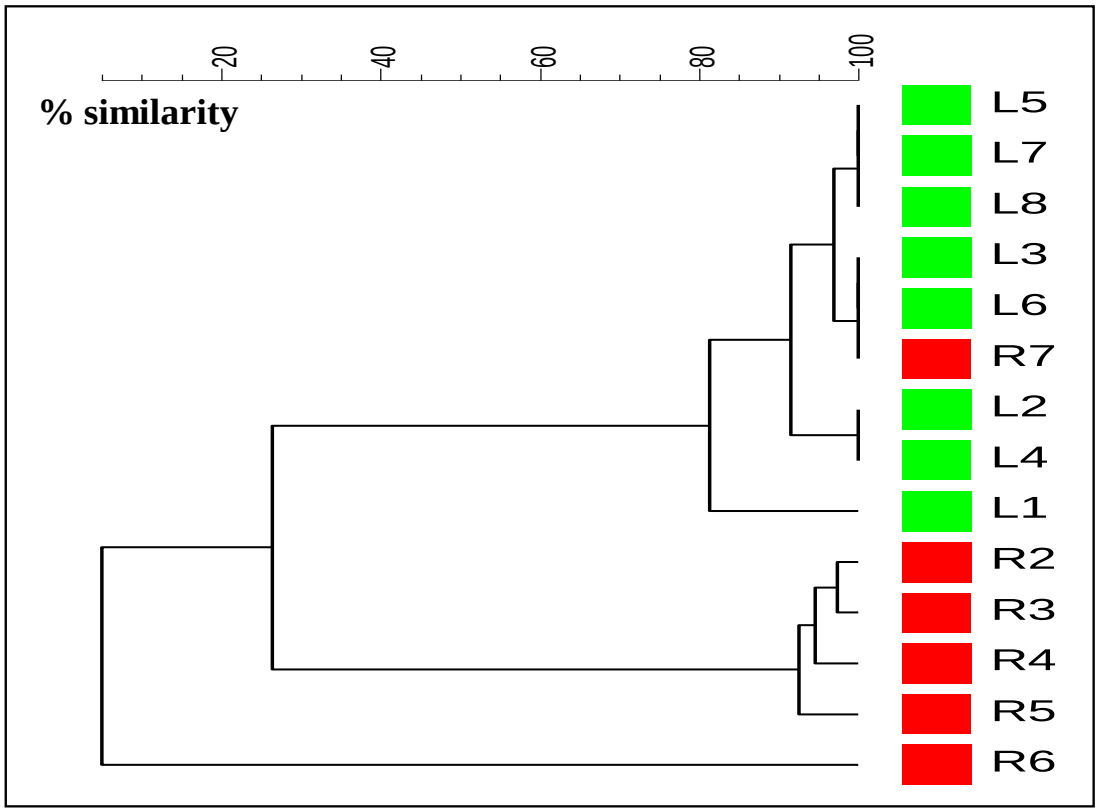


Figure 12: PCA plot of sheep A25 milk samples

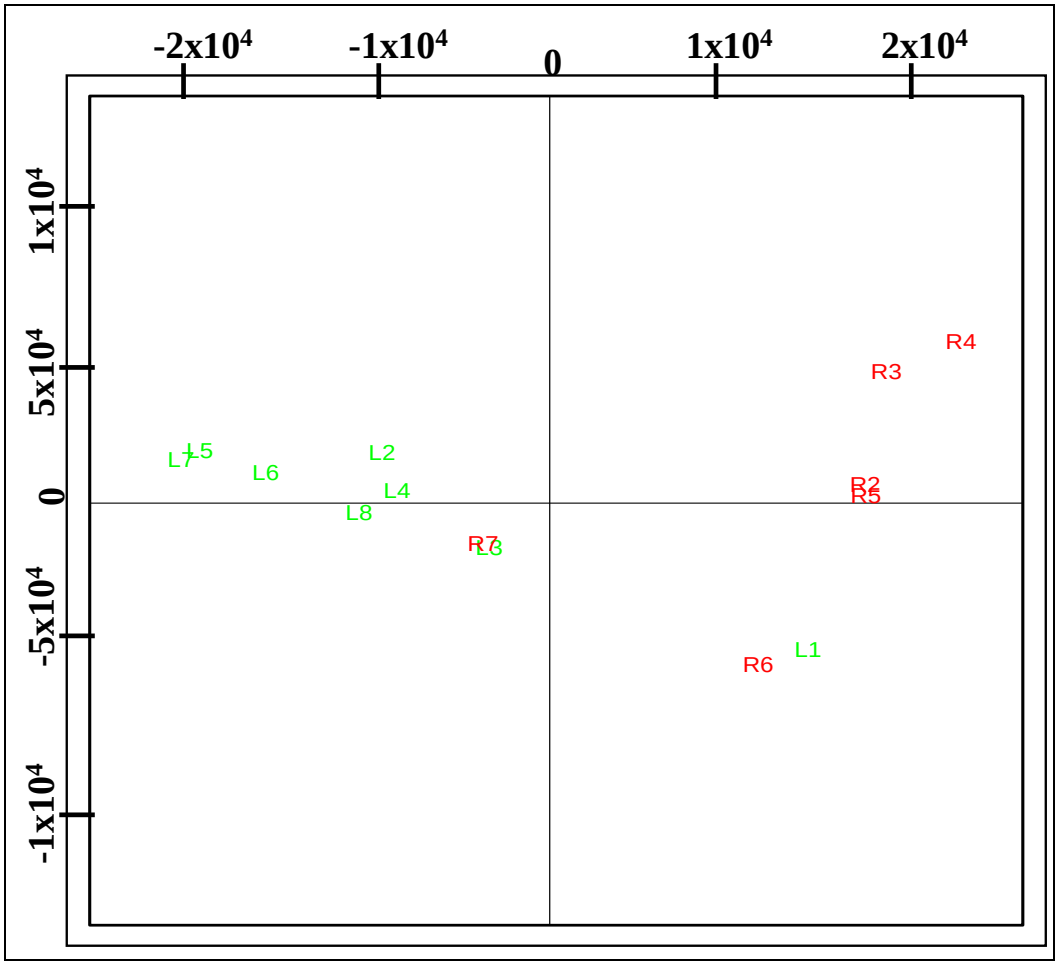


Figure 7- Figure 12 show the dendrogram and PCA plots for sheep A35 and A25. For sheep A35, there was no discernible pattern with all but two samples (L1 and L5) outliers of a tight cluster of the rest of the samples. This is also seen in the PCA plot for sheep A35 (Figure 9) where the majority of the samples are grouped in the same region, with no pattern according to mammary gland half.

The dendrogram for sheep A25 (Figure 11) shows more of a clustering pattern according to mammary gland half, with left and right half samples clustering separately (apart from R7 which clusters with left half samples). In Figure 12, the division of the majority of the samples according to mammary gland half for sheep A25 can also be seen.

The results from these two sheep are indicative of the results seen across sheep in the study i.e. despite some difference in DGGE banding pattern within and between sheep, samples within a sheep tended to either cluster together or by mammary gland half or a combination of both. Some similarity between halves could be expected as all of the sheep are from the same farm and the left and right halves are in close proximity to one another on each individual sheep. Differences would also be expected, as the halves are separate entities.

A similarity matrix of the bacterial communities in all milk samples was analysed using non-metric multidimensional scaling (non-metric MDS) and analysis of similarity (ANOSIM) in PRIMER, version 6, Primer-E (Clarke and Gorley, 2006). Data were clustered by sheep (Figure 13), parity (Figure 14) and week of lactation (Figure 15 and Figure 16) to investigate the effect of each factor on community similarity between individuals and over time.

There was no significant clustering of total number of DGGE bands by sheep, suggesting that there are differences in bacterial community diversity between sheep (Figure 13). Early (1 and 2) and late (3 and 4) parities formed two clusters, suggesting differences in bacterial community diversity according to sheep age (Figure 14). There were no clear patterns discernible when investigating all samples individually over time (Figure 15), however analysis of the mean band count by SCC per week (Figure 16) revealed differences in bacterial community diversity according to week. Earlier weeks clustered more closely to each other than later weeks, indicating a change in bacterial community diversity over the sampling time.

Figure 13: Non-metric MDS plot of all study data clustered by sheep

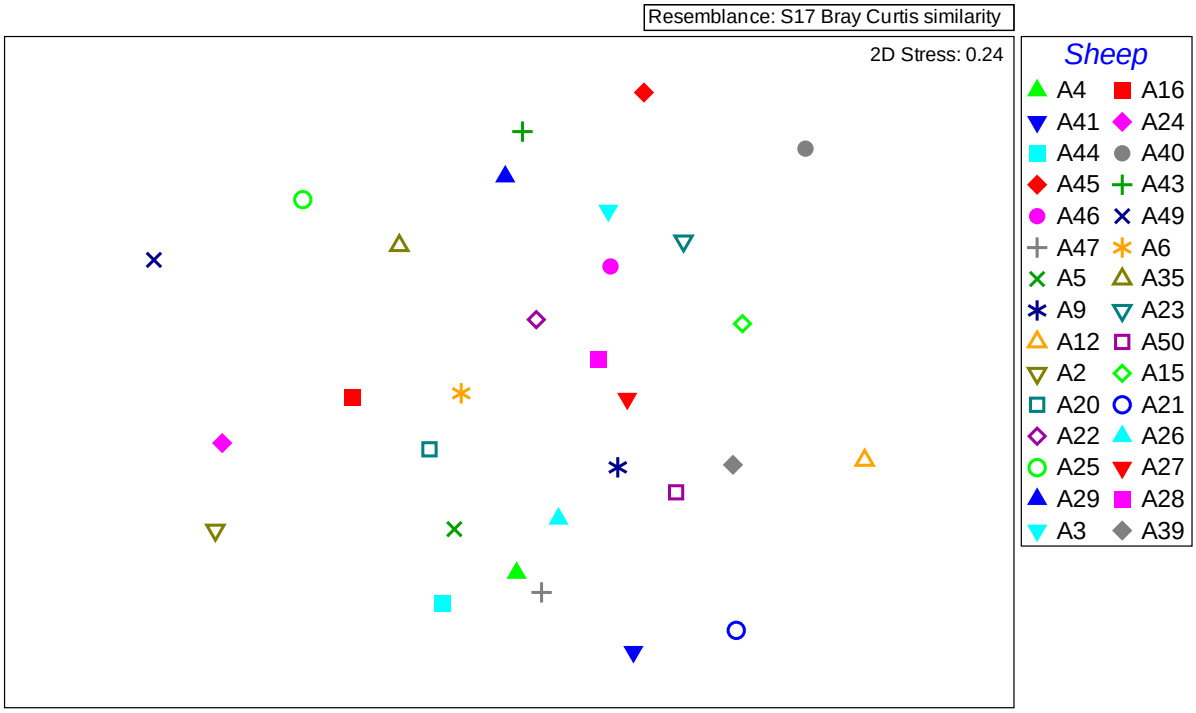


Figure 14: Non-metric MDS plot of all study data clustered by sheep parity (age)

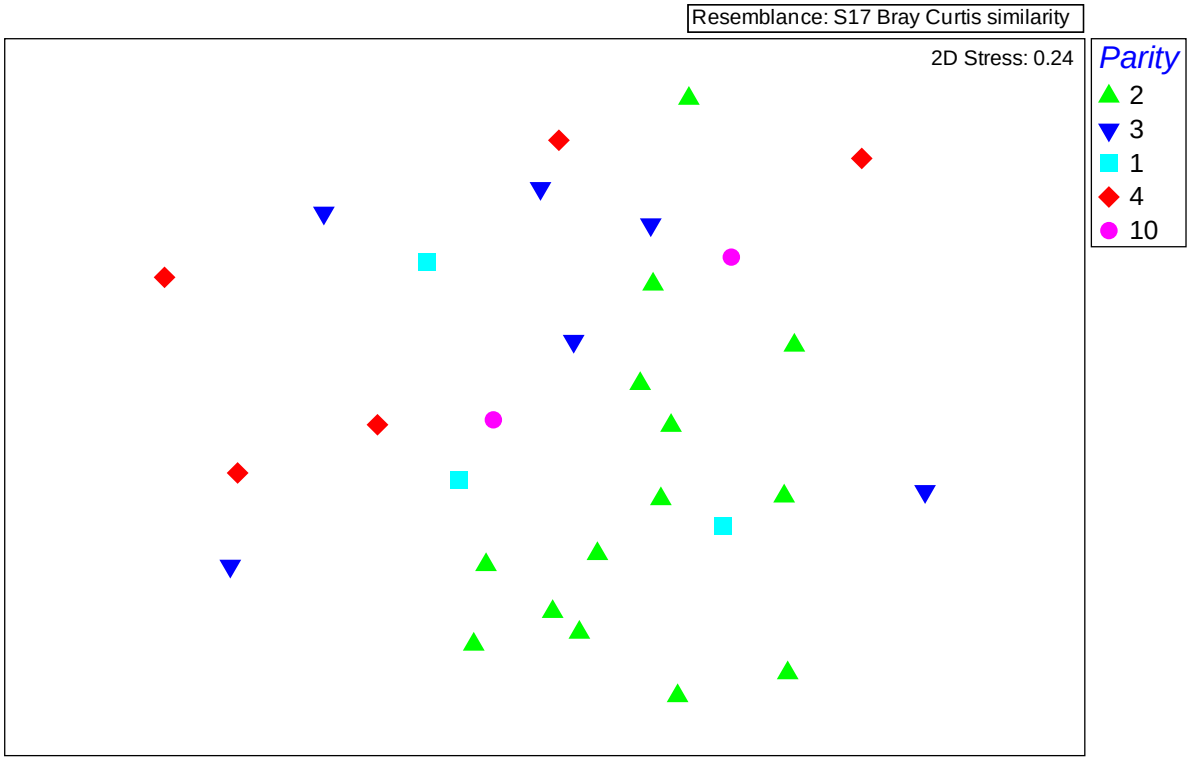


Figure 15: Non-metric MDS plot of all study data clustered by week of lactation (1-8)

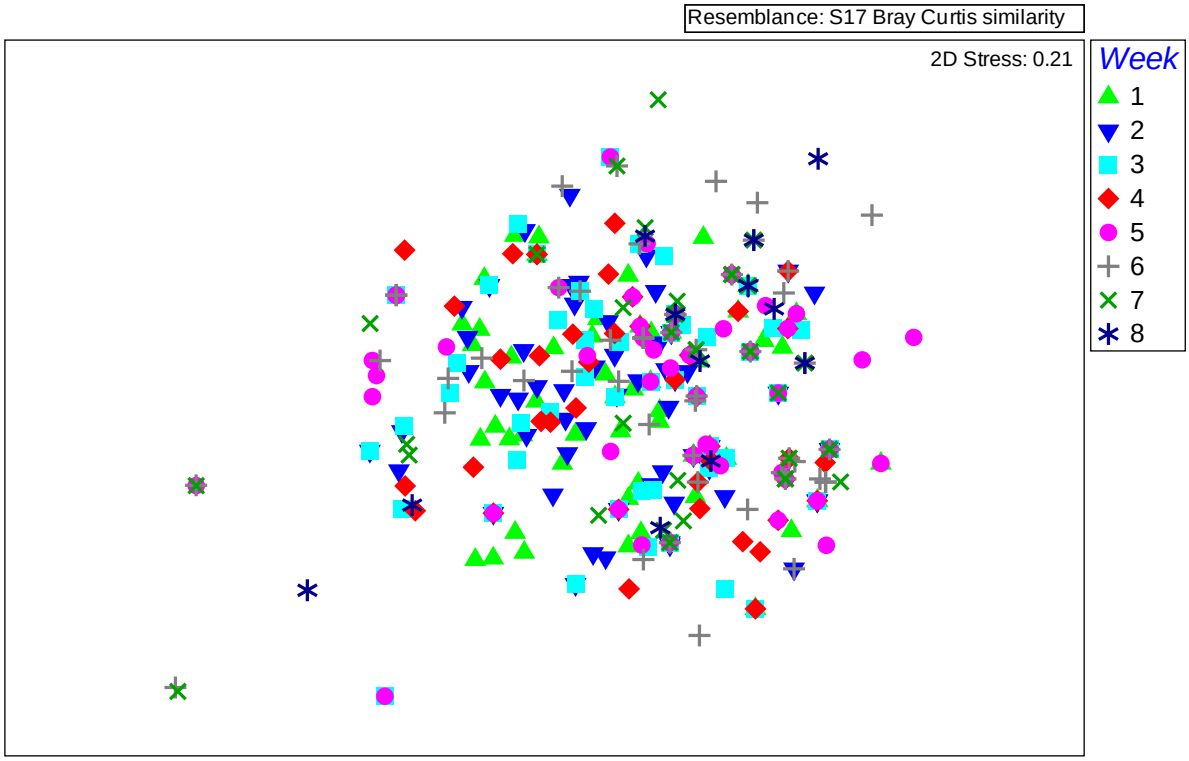
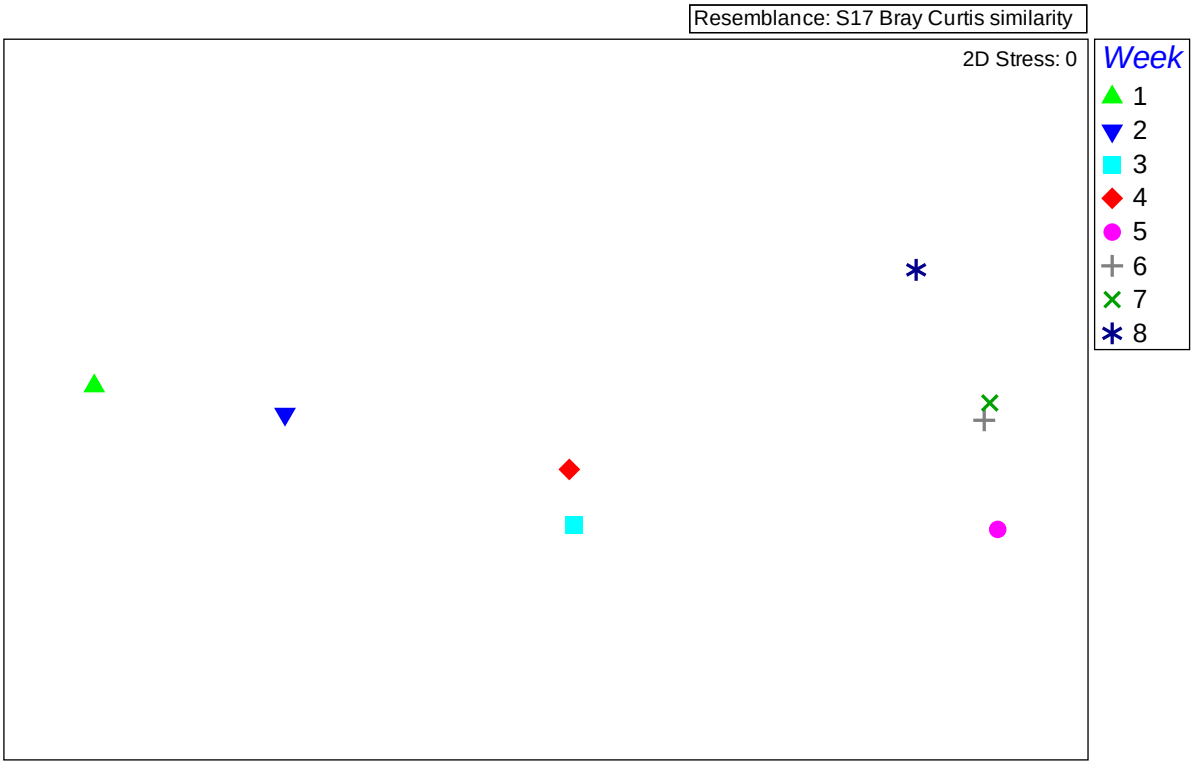


Figure 16: Non-metric MDS plot of all study data clustered by week of lactation (1-8) using the mean DGGE band count and LogSCC per week



The ANOSIM analysis of the study data supports the indications from the non-metric MDS plots, with sheep, parity and week of sampling shown to have a significant effect on the bacterial

community diversity (Table 19). The non-metric MDS plot grouping samples by mammary gland half indicated no clustering by half (not shown) and was supported by the ANOSIM (Table 19).

Table 19: ANOSIM results of study data analysed by sheep, parity, mammary gland half and week of lactation [6]

Factor	P-value	R-value
Sheep	**0.001**	0.661
Parity	**0.002**	0.081
Mammary gland half	0.093	0.005
Week	**0.002**	0.028

The non-metric MDS and ANOSIM results indicated that investigating community composition in individual sheep over time in further detail was required to identify specific bacterial species associated with a change in disease state (SCC).

4.3.3. Results from modelling DGGE data

Gel Compar II classified DGGE bands into thirty-five band categories in order to facilitate comparisons across samples within the longitudinal study. The band categories were then used in a mixed effects regression model (detailed in Section 3.4.2) to identify significant associations between community composition and changes in SCC.

A total of fifteen of the thirty-five DGGE band categories were significantly associated with change in SCC. To identify the bacterial species associated with each DGGE band (as in DGGE, different DGGE bands represent different bacterial species), a selection of milk samples from different sheep, mammary gland halves and weeks in lactation were re-analysed by DGGE. Significant bands were excised and either sequenced directly or cloned before sequencing. The DGGE band identifications are presented in Table 20.

Table 20 shows that 8 DGGE band categories were associated with a higher SCC and 6 associated with a lower SCC. DGGE band category 7.86 was also significantly associated with a lower SCC. However, no visible PCR product was produced post-excision from three repeat DGGE gels and so band 7.86 could not be identified.

[6] P-values showing significance of effects of factors by which the data was grouped (sheep, parity, mammary gland half and week). R-values close to zero indicate most similarity. Values in bold highlight significant differences (p = <0.05).

Table 20: Identifications for 15 DGGE bands that were shown using modelling to have a significant effect on SCC[7]

							Identified from:	
Band	**SCC effect**	**No. of milk samples present in**	**ID**	**Phyla**	**BLAST identity (%)**	**No. IDs**	**No. milk sample**	**No. sheep**
6.16	↑	260	*Corynebacterium efficiens*	A	94	5	4	2
2.54	↑	34	*Psychrobacter maritimus*	P	99	3	1	1
2.01	↑	16	*Streptococcus uberis*	F	99	1	1	1
8.91	↑	16	*Burkholderia cepacia*	P	98	2	1	1
2.19	↑	7	*Fusobacterium necrophorum*	Fu	98	9	2	2
3.84	↑	7	*Trueperella pyogenes*	A	99	3	1	1
7.72	↑	7	*Pseudomonas chlororaphis*	P	99	13	3	2
0.49	↑	2	*Psychrobacter faecalis*	P	98	2	2	2
6.54	↓	217	*Achromobacter xylosoxidans*	P	99	3	1	1
6.37	↓	135	*Rhodococcus qingshengii or Nocardia globerula*	A	98	15	10	5
3.01	↓	67	*Atopostipes suicloacalis*	F	94	4	2	2
4.69	↓	34	*Mannheimia haemolytica*	P	92	2	1	1
2.68	↓	7	*Jeotgalicoccus psychrophilus*	F	96	5	2	2
7.38	↓	3	*Sharpea azabuensis*	F	99	2	2	1

4.4. High-throughput DNA sequencing results

4.4.1. Optimisation of sequencing data analysis pipeline

The data analysis pipeline used to process the sequencing data for all study samples is detailed in Section 3.5.2. Each stage in the pipeline was tested with a range of parameters to ensure that high quality and reliable data was used in subsequent analyses. An additional method to assess the effectiveness of the quality filtering process was to process the model community positive control

[7] In 'Phyla' column, P: Proteobacteria; F: Firmicutes; Fu: Fusobacteria; A: Actinobacteria.

samples to ensure that the data analysis pipeline produced the expected results (as composition of the model community (MC) was known). Table 21 shows the results of the MC OTU analysis.

Table 21: OTU identity and distribution across the model communities (MC) in each sequencing library (L1-L5).

		MC1 L1	MC1 L2	MC1 L3	MC1 L4	MC1 L5	Total per OTU	% of total per OTU
OTU	**Bacterial ID**							
1	***Streptococcus uberis***	**12160**	**8695**	**6255**	**12219**	**10671**	**50000**	**24.11**
2	***Mannheimia haemolytica***	**9396**	**6560**	**4774**	**9068**	**8089**	**37887**	**18.27**
3	***Staphylococcus hyicus***	**9057**	**5524**	**4588**	**8054**	**7783**	**27223**	**13.13**
4	***Staphylococcus aureus***	**9401**	**6780**	**3022**	**9763**	**8462**	**37428**	**18.05**
5	***Escherichia coli***	**4223**	**2549**	**5386**	**4747**	**3654**	**20559**	**9.91**
6	*Escherichia coli/ Pseudomonas panacis*	8961	78	1525	68	29	10661	5.14
7	*Escherichia coli/ Pseudomonas panacis*	2	4	10	8275	20	8311	4.01
8	*Escherichia coli/ Pseudomonas panacis*	13	4725	0	5	6310	11053	5.33
9	*Escherichia coli/ Lacibacter spp./ Rhizobium spp.*	5	661	0	7	174	847	0.41
10	*Escherichia coli/ Limnobacter spp./ Burkholderia spp.*	2	2	0	1774	3	1781	0.86
11	*Escherichia coli/ Limnobacter spp./ Burkholderia spp.*	85	8	0	369	2	464	0.22
12	*Escherichia coli/* Rumen bacterium	0	0	0	0	2	2	0.00
13	*Alpha proteobacterium*	0	0	0	0	4	4	0.00
14	*Achromobacter xylosoxidans/ Escherichia coli*	0	0	0	0	1163	1163	0.56
Total	**-**	**53305**	**35586**	**25560**	**54349**	**46366**	**489913**	**-**

Table 21 shows that between seven and fourteen OTUs were identified per model community. This is greater than the five OTUs expected, as the model community was composed of five bacterial species (Section 3.5.1). The first five OTUs (highlighted in bold in Table 21) were the expected OTUs and account for approximately 83% of the total DNA sequence reads. However, there were an additional 9 OTUs produced, many of which had the same inconclusive bacterial ID suggesting the sequences in these OTUs could be chimeric sequences i.e. errors or PCR artefacts (from

excessive PCR cycling). This highlighted that the quality filtering of milk samples would also result in additional OTUs that did not represent biologically relevant diversity. The model community samples were therefore used to determine the OTU size that represented an unreliable cluster before remapping all sequences back onto the OTUs (Table 22).

Table 22: OTU sizes for model community (MC) for each library of sequenced samples[8]

MC ID	No. of OTUs	No. of inputs	OTU size distribution	% of largest unreliable cluster	Chimeras	No. of reads with no match
MC1	11	6847	3950, 3089, 2207, 1946, 1117, **4, 2(5)**	0.06	0	276
MC2	11	4182	2843, 2116, 1394, 1371, 745, **18, 3(2), 2(3)**	**0.43**	0	167
MC3	7	2751	1946, 1432, 893, **707, 528, 2(2)**	0.07	0	103
MC4	13	5812	4071, 3099, 2073, 2056, 1384, **18, 4, 2(7)**	0.31	1	348
MC5	14	6544	3169, 2575, 1744, 1621, 1016, **6, 4(2), 3, 2(5)**	0.09	1	279

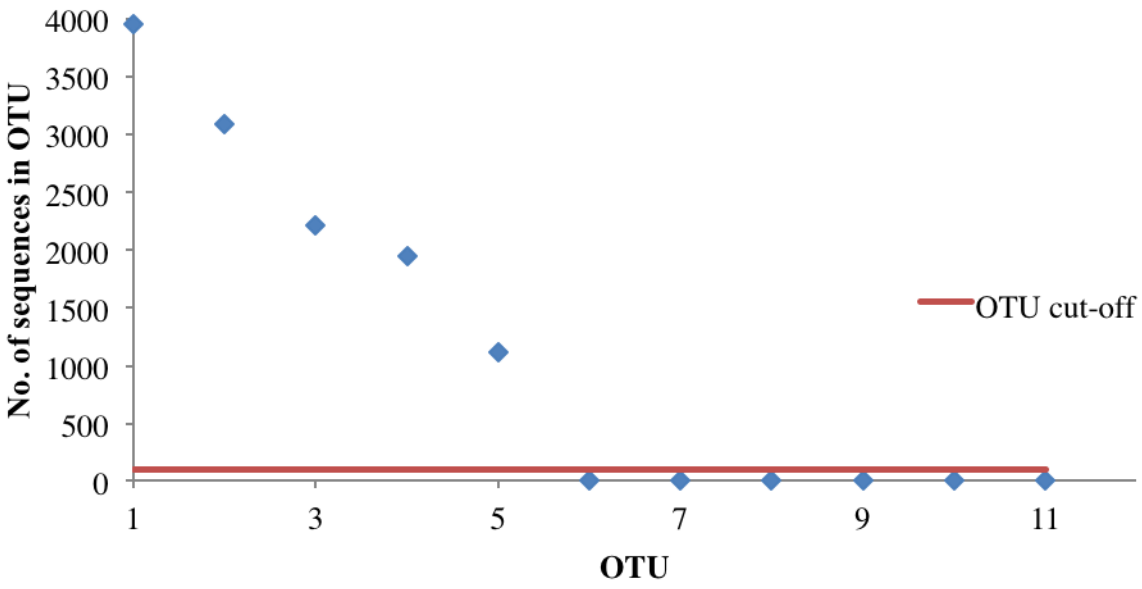
Table 22 shows that the maximum OTU size that represented the largest unreliable OTU (unreliable OTU sizes highlighted in bold in Table 22) was 0.43%. This OTU size was then used as an additional quality filter. OTUs that were equal to or lower than the determined confidence size were removed from each sample, excluding any data from the subsequent analysis that would have grouped into those OTUs. To check that the inclusion of the OTU size cut-off produced the expected results i.e. 5 OTUs for a model community sample, each OTU (x-axis) was plotted against the number of sequences assigned to that OTU (y-axis) for an example model community sample (Figure 17).

For the model community in Figure 17 there were 11 OTUs, with a large decline in OTU size between OTUs 5 and 6 i.e. between real and unreliable OTUs. The OTU size cut-off for the model community (based on the 0.43% size cut-off) was 86 (shown by the red line in Figure 17), resulting in only OTUs 1-5 proceeding through the OTU filtering process. As 5 bacterial species were present in the model community, this indicated that the OTU filtering process produced an accurate representation of community composition. The sharp decline in OTU size below the OTU

[8] 'MC ID' is model community identifier; 'No. of inputs' is the number of unique input sequences per MC used to complete the OTU clustering; in 'OTU size distribution' the size of each OTU is defined; the number in brackets after the size refers to the number of OTUs that were of that size if there were multiple OTUs of the same size; the '% of largest unreliable cluster' was determined by dividing the size of the largest OTU that did not represent 1 of the 5 known bacterial species in the model community by the number of input sequences x100; the 'No, of reads with no match' is the number of sequences that did not map back to any of the OTUs post-filtering.

confidence size cut-off provided evidence to support the use of this approach as an additional quality-filtering step in the sequencing data analysis pipeline.

Figure 17: OTU size distribution for a model community sample with OTU size cut-off shown in red



4.4.2. Sequencing results for five sheep

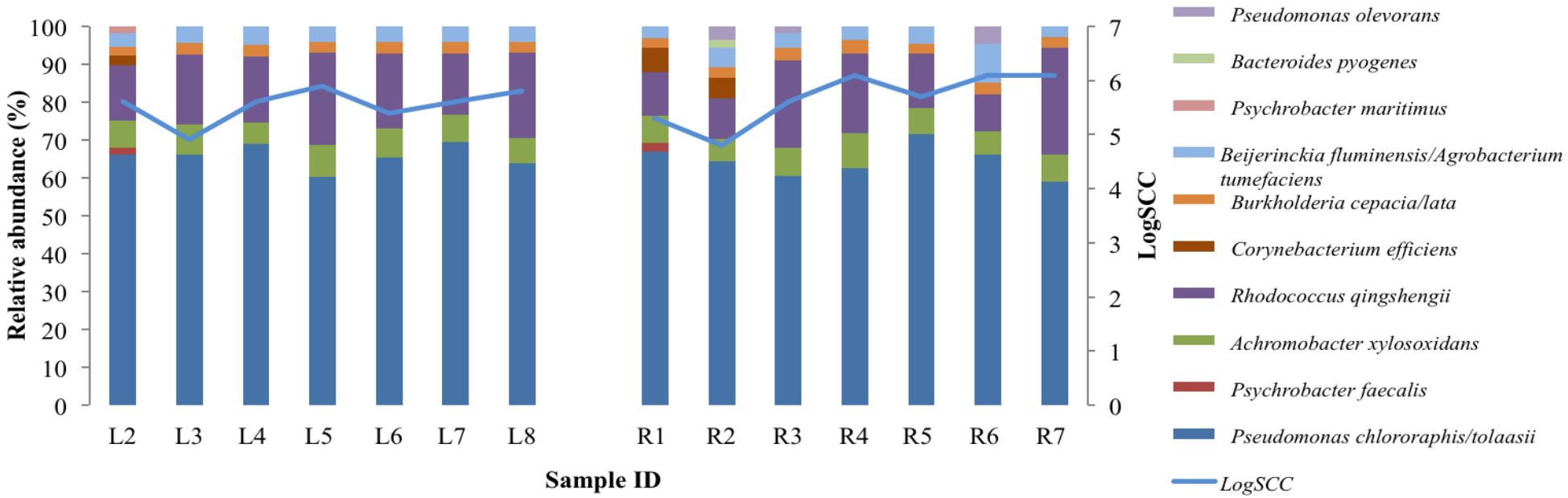
To illustrate the potential in the OTU filtered sequencing data set and to investigate the study hypotheses, the OTU identities and distributions were analysed for 5 sheep; one of each parity group. Figure 18 -

Figure 27 show the sequencing results for 5 sheep; (in increasing parity order); A20, A41, A25, A40 and A23. Each OTU distribution figure is shown with the corresponding DGGE image for reference. Figure 28 and Figure 29 are technical replicates for samples L8 and L2 from sheep A20 and A23 respectively.

For parity one sheep A20, 10 OTUs were identified, with *Pseudomonas* spp. dominating every milk sample. For parity two sheep A41, 34 OTUs were identified, with shifts in relative abundance of bacterial species from week to week within both halves, with *Corynebacterium efficiens* the most abundant in the majority of the milk samples. For parity three sheep A25, 10 OTUs were identified, with *Pseudomonas* spp. most abundant and a similar distribution of bacterial species across the weeks and mammary gland halves. Parity four sheep A40 had 6 OTUs with *Pseudomonas* spp. most abundant. For parity ten sheep A23, 9 OTUs were identified, with *Pseudomonas* spp. and *Rhodococcus qingshengii* most abundant. The technical replicates in Figure 28 and Figure 29

show good consistency between replicates, with both sets of samples consisting of the same OTUs in similar abundances.

Figure 18: OTU distribution for parity one sheep A20 milk samples



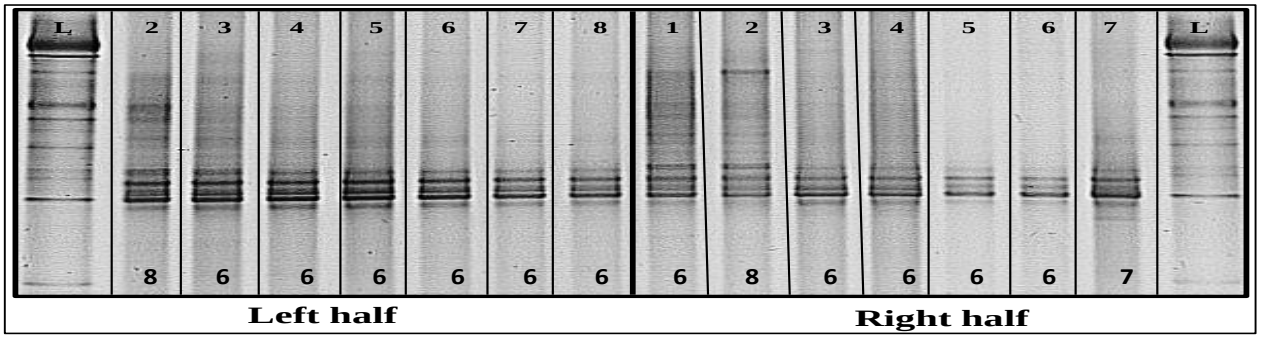
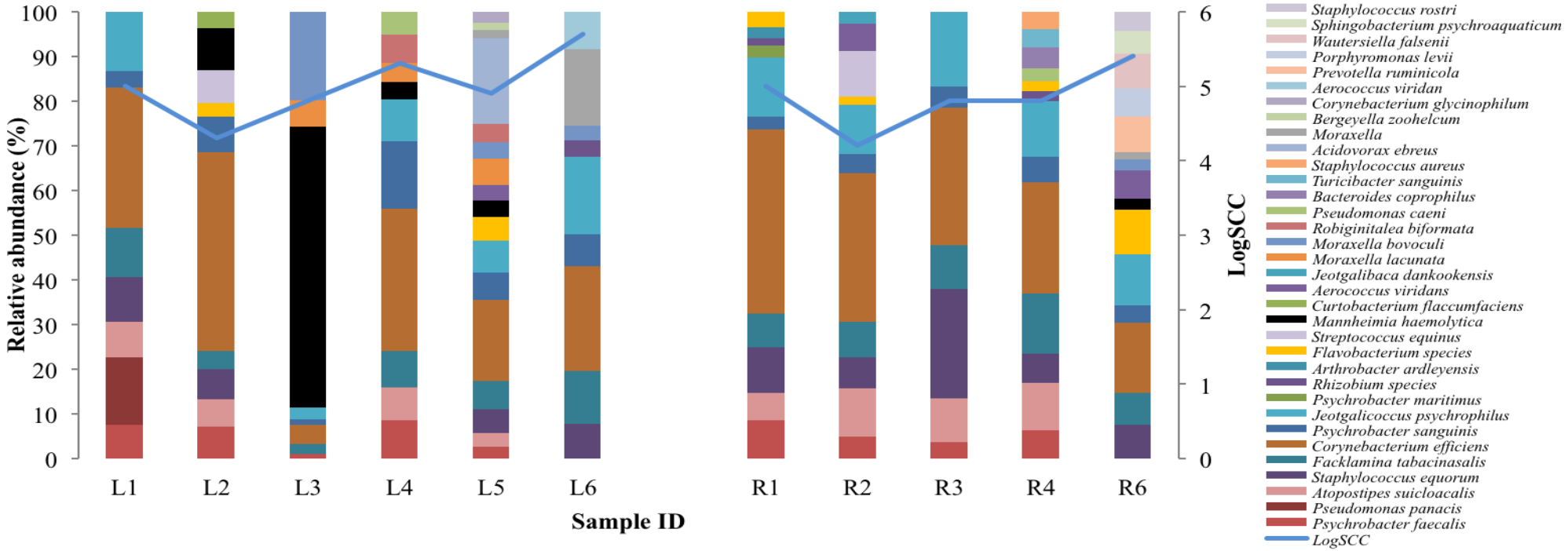


Figure 19: DGGE image of parity one sheep A20[9]

[9] For all DGGE images in this section; 'L' is the custom DGGE reference ladder; the number at the top of each lane is week in lactation and at the bottom is DGGE band count.

Figure 20: OTU distribution for parity two sheep A41 milk samples



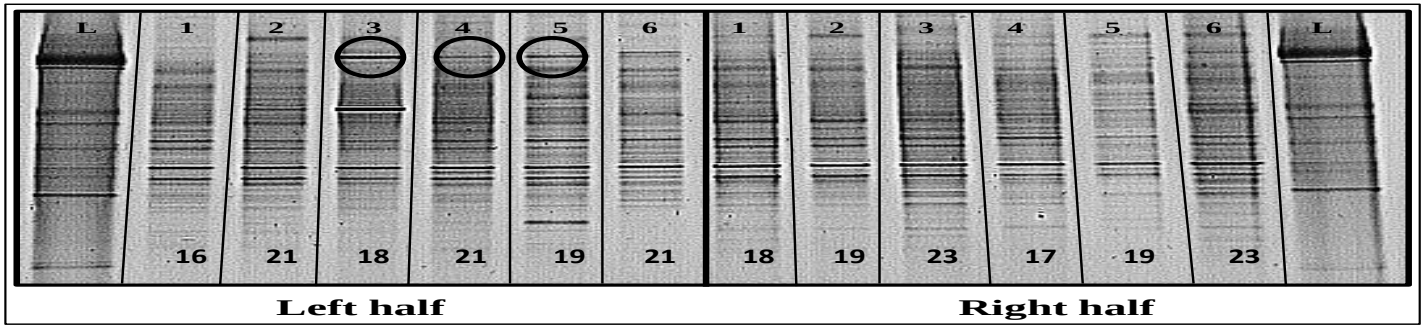
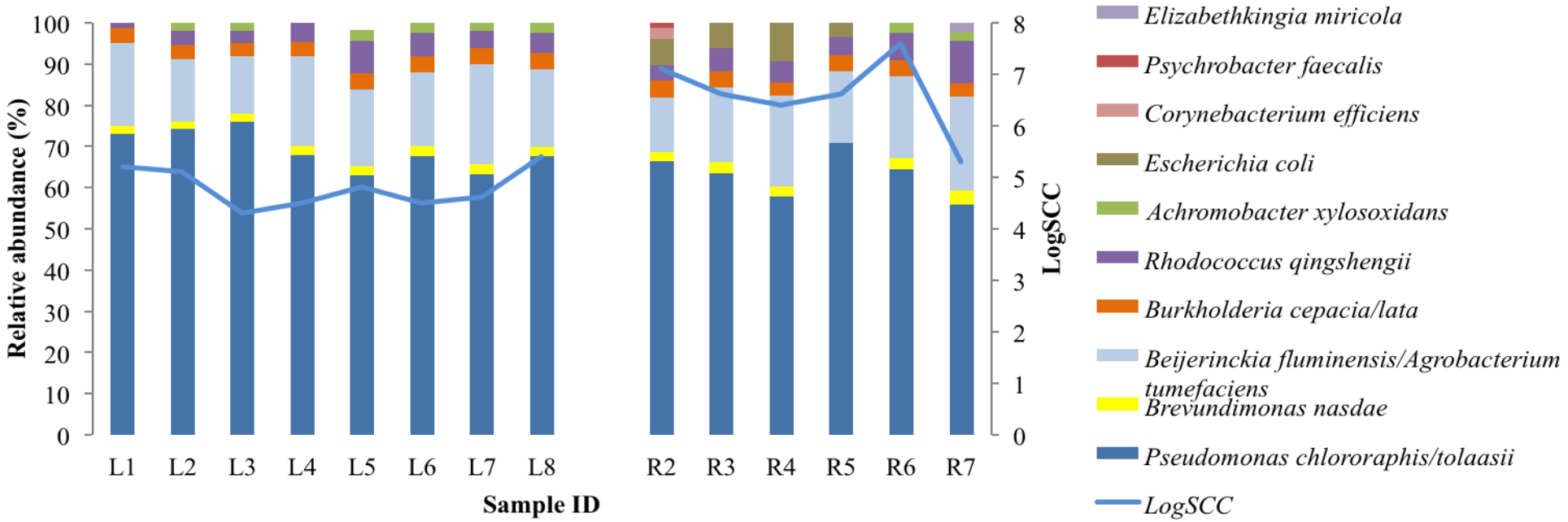


Figure 21: DGGE image for parity two sheep A41

Figure 22: OTU distribution for parity three sheep A25 milk samples



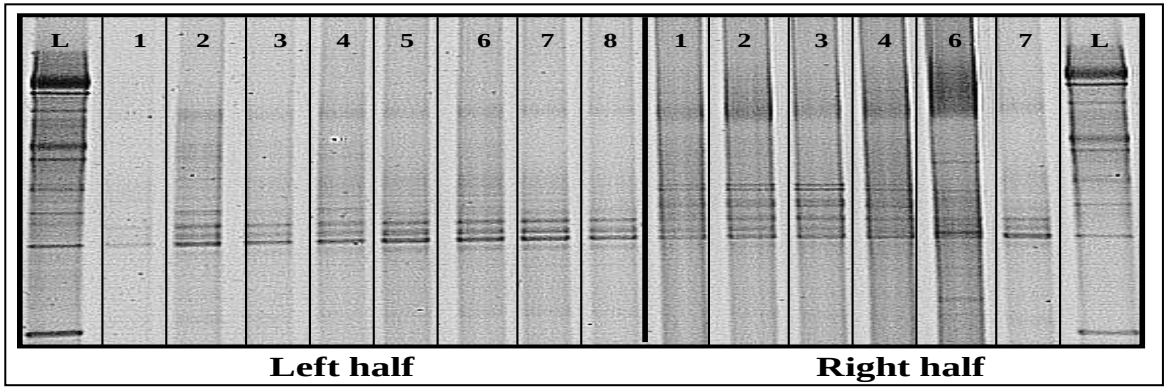
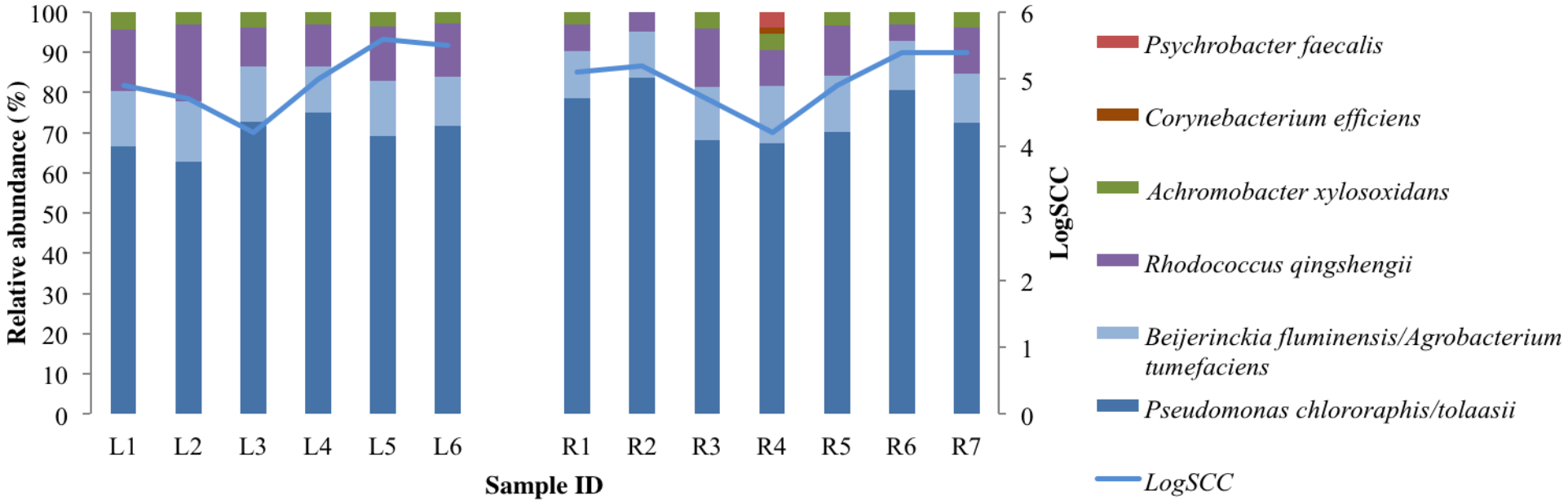


Figure 23: DGGE image for parity three sheep A25

Figure 24: OTU distribution for parity 4 sheep A40 milk samples



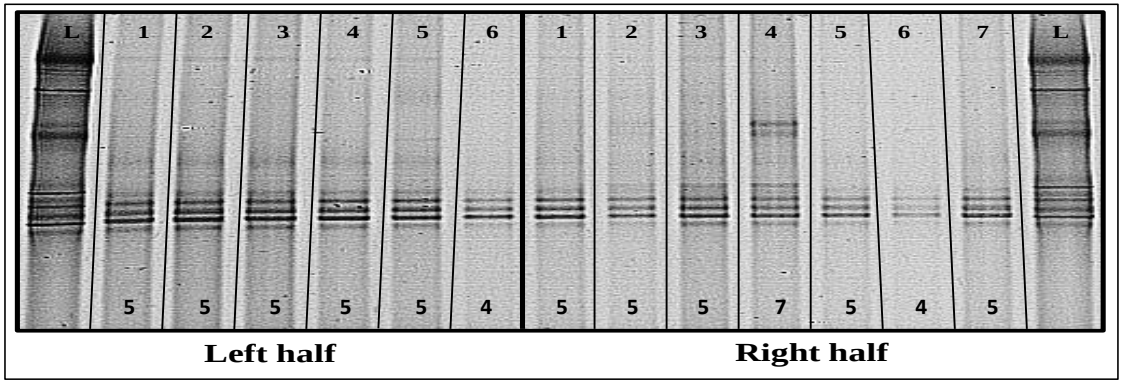
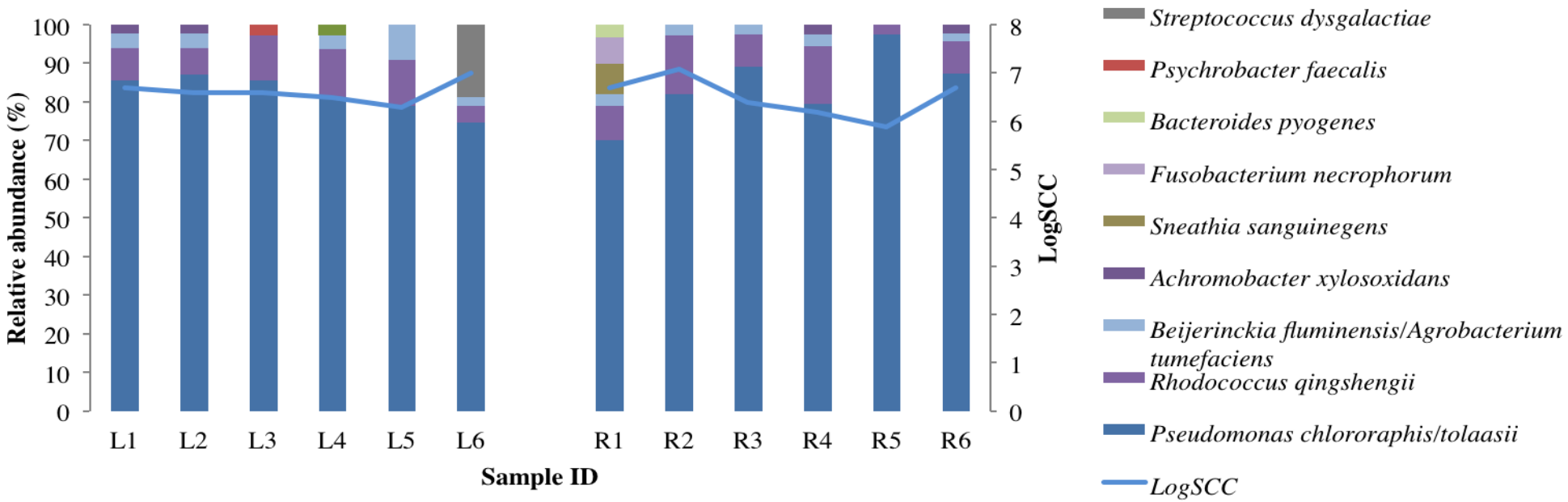


Figure 25: DGGE image of parity 4 sheep A40

Figure 26: OTU distribution for parity 10 sheep A23 milk samples



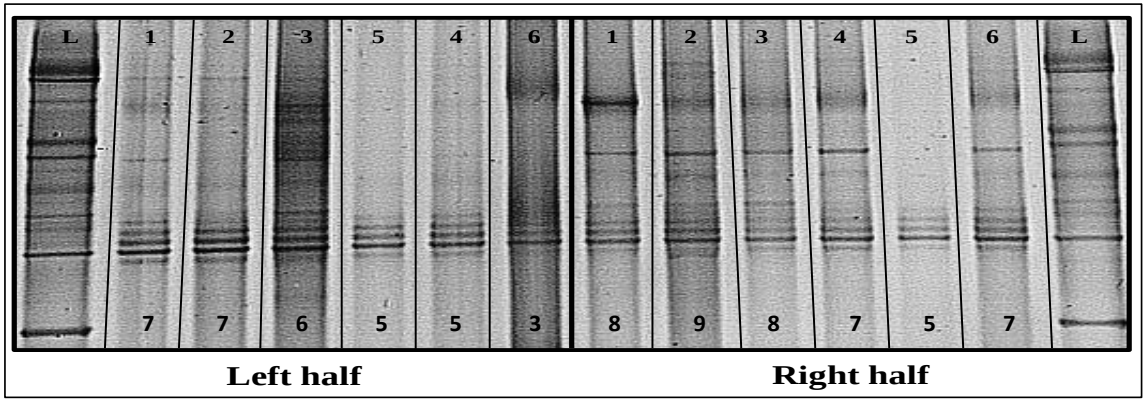


Figure 27: DGGE image for parity ten sheep A23

Figure 28: Technical replicate comparison for sample L8 from parity 1 sheep A20

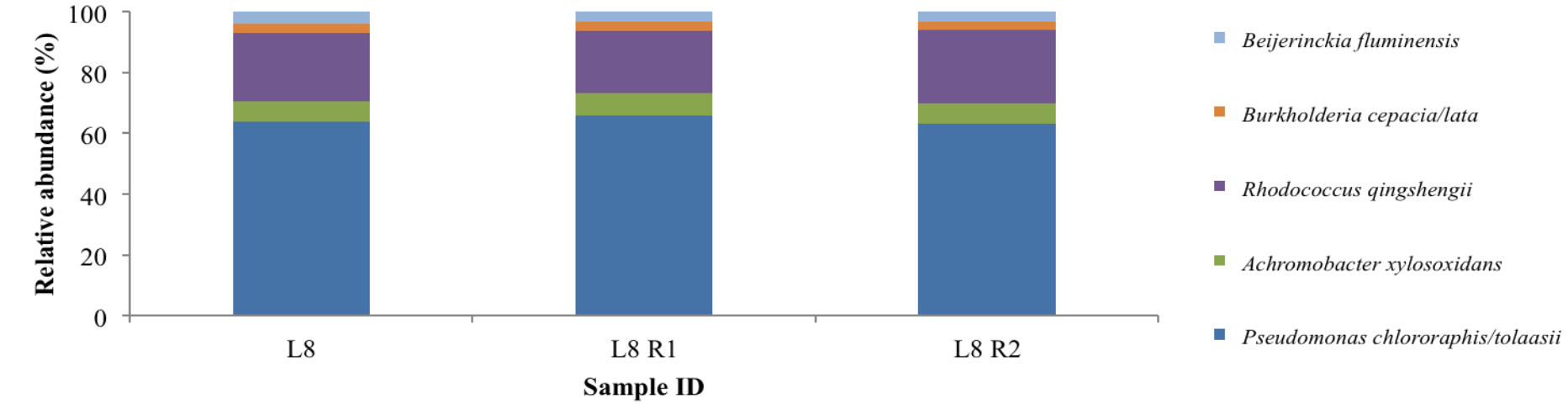
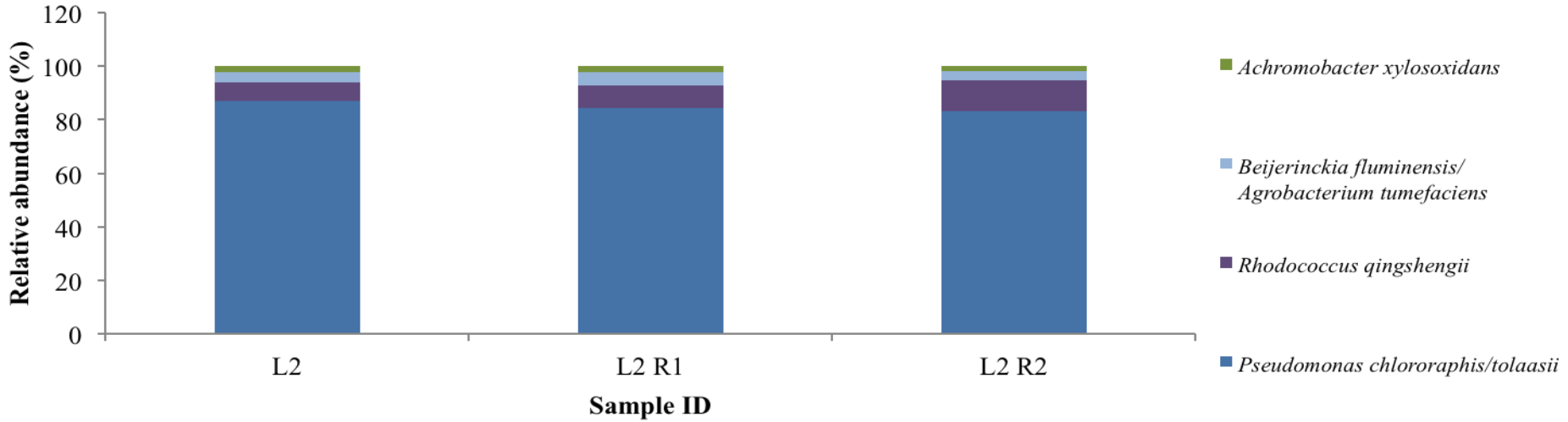


Figure 29: Technical replicate comparison for sample L2 of parity 10 sheep A23



5. Discussion

The overall aim of this study was to obtain an understanding of the bacterial genera present in the microbial community of the suckler sheep mammary gland using molecular-based whole community approaches that were culture-independent. Within this aim, there were several hypotheses established. Evidence to support and/or speculate on the validity of these hypotheses has been provided by this study.

5.1. Research findings and implications

5.1.1. DNA extraction

A culture-independent approach was chosen to assess the microbial community in suckler sheep mammary glands. The first step in using this approach was DNA extraction. The chosen protocol consistently extracted DNA from milk samples with a range of bacterial abundances without the detection of contamination. It is crucial in microbiome studies to extensively test the DNA extraction protocol, as even trace amounts of contaminants may produce significant numbers of DNA sequence reads in high-throughput analyses.

The ability of the Purdy method to consistently and sensitively extract DNA without contamination was the key factor in selecting this method for sample processing. However, the method does have some limitations. These include; preparation and extraction time, the use of phenol and a maximum of only 16 samples processed per batch of DNA extractions due to the protocol column design. Therefore, there is great potential for future studies in developing the DNA extraction protocol into a high-throughput method for processing samples on a large scale in both academic and commercial settings through the conversion of column design from falcon tubes to a 96 well format and testing of alternative chemicals to the highly toxic phenol currently used.

5.1.2. DGGE community analysis

A range of bacteria has been identified in milk using the PCR-DGGE approach. Braem *et al.,* (2012) identified four bacterial phyla encompassing 17 bacterial genera from 48 samples from dairy cow teat apices, the findings of which have been corroborated by other studies in different hosts (Costello *et al.*, 2009; Kuang *et al.*, 2009; Rasolofo *et al.*, 2010). For example, Costello *et al.,* (2009) found 92% of species were from the same four phyla when investigating samples from different sites of the human body. The results from this study support such findings, with four bacterial phyla found to have a significant effect on SCC (Table 20).

The DGGE data in Section 4.3 indicates from one to twenty-three bacterial species were detected per milk sample that produced a PCR product in the study. This provides evidence for the hypothesis that a microbial community is present in the sheep mammary gland and that there are both similarities and differences over lactation and between sheep and mammary gland halves. This also suggests that colonisation of the mammary gland is inevitable, although validation of this hypothesis would require further longitudinal studies to investigate how and when the mammary gland microbiome develops.

The DGGE analysis highlighted similarities and differences between MG halves, within halves, over lactation and between sheep (Figure 2 - Figure 6). For some sheep, complex DGGE profiles were present that comprised several bands corresponding to a wide variety within the bacterial population e.g. Figure 3, whilst for others, more consistent and simple profiles were seen e.g. Figure 2. With community diversity relatively stable in several sheep, it could be hypothesized that changes in interactions between members of the MG microbiota as opposed to diversity may have a role in disease development.

There was variation in the milk microbiota according to sheep parity and week of lactation. For parity, the highest mean SCC and second highest mean DGGE band count occurred in the oldest sheep in the study. Parity has been associated with an increased risk of severe clinical mastitis in dairy cattle (Biffa *et al.*, 2005; Green *et al.*, 2007; Green *et al.*, 2002; Peeler *et al.*, 2002). Increasing SCC without disease in dairy cattle and sheep (Green *et al.*, 2005; Huntley *et al.*, 2012) and conformational changes in the udder could predispose to infection (Huntley *et al.*, 2012). Infections in previous lactations may also alter/damage the mammary environment or reduce innate defence mechanisms (Green *et al.*, 2005). An increased risk of infection with increasing parity provides some evidence for a persistent bacterial community, with changes over time acting as triggers for infection.

There were also some changes in intensity of DGGE bands e.g. week 3 in Figure 3. Changes in intensity can be an indicator in DGGE of changes in the abundance of certain bacterial species. This could suggest that the community remains stable, but that there are fluctuations in the abundance of certain community members that could be linked to a shift towards a disease state. Such fluctuations could be represented by the changes in community diversity seen over the eight weeks of the study.

Despite visual differences, no significant difference in the number of DGGE bands was found between mammary gland halves. Some similarities between halves could be expected as transmission of the same bacterial strains between cow quarters (Phuektes *et al.*, 2001) and

sheep MG halves (Mork *et al.*, 2007) as well as persistence of the same bacterial pathogens before and after the development of clinical symptoms in dairy sheep have been reported (Fthenakis and Jones, 1990). However, each half is a separate entity so differences in bacterial community composition would be expected. Green *et al.,* (2002) found evidence of differing quarter susceptibility to IMI in dairy cattle, which does indicate the expectation of differences in community composition between MG halves in sheep. It is also possible that differences between MG halves are not clear from DGGE data, as it may be an underestimation of community diversity. DGGE can only visualise bacterial populations that are greater than 1% of the total community and dissimilar sequences can co-migrate to the same position in a DGGE gradient (Muyzer and Schäfer, 2001; Rossello-Mora *et al.*, 1999). Further analysis of the high-throughput sequencing data, which has the ability to identify less abundant community members, may provide a clearer understanding of the microbiota composition in MG halves of the same sheep.

The mixed effects regression model, with identification of DGGE bands significantly associated with a change in SCC, provides powerful evidence for the hypothesis regarding links between specific bacterial species causing and/or protecting against disease. This in turn provides some evidence that changes in the sheep mammary gland microbiota can predispose an animal to disease. Many of the bacterial species associated with a higher SCC have known associations with mastitis in both cows and sheep e.g. *Streptococcus uberis* and *Corynebacterium* species (Abureema *et al.*, 2014; Green *et al.*, 2002; Kumar *et al.*, 2013). Others such as *Fusobacterium necrophorum* and *Burkholderia cepacia* are opportunistic pathogens with reports of links to mastitis in the literature (Berriatua *et al.*, 2001; Jousimies-Somer *et al.*, 1996; Oikonomou *et al.*, 2012; Witcomb *et al.*, 2014). Bacteria found in a diverse range of environments but unknown as mastitis pathogens were associated with a low SCC e.g. *Atopostipes suicloacalis* (Watanabe et al., 2008) and *Jeotgalicoccus psychrophilus* (Delbes *et al.*, 2007) as were opportunistic pathogens such as *Achromobacter xylosoxidans* (De Baets et al., 2014). It could be hypothesized that these organisms are commensal, and persist in the mammary gland, stabilising the microbial community.

Future studies would benefit from identification of the bacterial species associated with all 35 DGGE band categories to facilitate a more extensive insight into bacterial community composition. Investigating the relationship between all bacterial species identified and SCC using modelling approaches would help identify commensal and pathogenic organisms. The addition of Q-PCR data would provide more accurate information on the abundance of difference bacterial species, which could be used to address the hypothesis that bacterial

abundance as opposed to presence/absence, may be involved in triggering disease development.

5.1.3. High-throughput sequencing

The Illumina MiSeq DNA sequencing results presented in Section 4.4 allow speculation on the accuracy of the study hypotheses. However, as these results only represent a small proportion of the sequencing data, the conclusions drawn are speculative and only provide inferences on the validity of the study hypotheses. Further analysis and exploration of the sequencing data is required to elucidate the accuracy of the proposed hypotheses.

Despite this, some inferences on the accuracy of the hypotheses suggested can be made as well as suggestions on new hypotheses for further investigation. For example, the sequencing results support the DGGE data and therefore the hypothesis that a microbial community forms in the suckler sheep mammary gland and that colonisation of the mammary gland may be inevitable, as a persistent community was detected in every sample from the 5 sheep presented (Section 4.4.2). Other studies have reported a mixture of bacterial species in the mammary glands of dairy cattle (Bhatt *et al.*, 2012; Oikonomou *et al.*, 2014). The data shown also indicates that there are differences in community composition according to mammary gland half, lactation and sheep age. However, similarities were also found, with OTUs shared by several sheep e.g. 3 OTUs were found in all milk samples from 4 of the 5 sheep presented, as well as similarities over time and between mammary gland halves of the same sheep. It could therefore be hypothesised that there would be similarities in mammary gland microbiota between halves and sheep, as bacterial species most adapted to the mammary gland environment are most likely to survive and persist.

Bacterial species previously associated with both healthy and clinical mastitis milk samples have been identified. For example, *Psychrobacter* spp. were found in all 5 sheep and this genera has been found in healthy milk samples from dairy cattle (Kuehn *et al.*, 2013). *Staphylococcus* spp. were found in sheep A41 and this genera has been identified in both healthy milk in humans (Hunt *et al.*, 2011) and both healthy and diseased milk samples in dairy cows (Oikonomou *et al.*, 2014). Furthermore, some bacterial genera associated with clinical disease in dairy cattle such as *Sphingomonas* spp. and *Stenotrophomonas* spp. (Kuehn *et al.*, 2013) have not been found in the 5 sheep investigated. Oikonomou *et al.,* (2012) reported a large abundance of anaerobic bacteria in mastitic milk from dairy cattle such as *Trueperella pyogenes*, a bacterial species not detected in the sheep analysed so far in this study. Therefore, the observations in this study could add support to previous findings

that healthy and diseased milk samples have differing microbiota profiles (Kuehn *et al.*, 2013; Oikonomou *et al.*, 2014). It must be noted however, that differences between this and other milk microbiome studies could be related to a combination of differences in host organism, sample processing, farm environment and management and/or sequencing method/data analysis pipeline.

Furthermore, bacterial pathogens with a known association with intramammary infection such as *Staphylococcus aureus* and *Escherichia coli* were identified in healthy sheep milk. (Figure 20 and Figure 22). It could be hypothesised that pathogenic bacteria can form part of a commensal microflora in the mammary gland and changes in the community can then predispose these bacterial species to cause infection. Also, genera such as *Pseudomonas* spp. and *Psychrobacter* spp. dominated in the majority of the healthy milk samples analysed in Section 4.4.2. Therefore, it could be proposed that these bacteria could dominate a healthy commensal bacterial community in the sheep mammary glands. However, all of the study data would need to be analysed to provide evidence to support this hypothesis and further longitudinal studies of sheep on different farms would be required to account for any farm effect on mammary gland microbiota composition.

Similarly, as the DGGE data identified associations between community composition and changes in SCC (Table 20) supported by the sequencing results (Section 4.4.2), it could be hypothesised that the interactions between community members are important in determining the predisposition to infection. Synergistic relationships between bacterial species have been suggested in published literature (Green *et al.*, 2005; Panciera *et al.*, 1989; Witcomb *et al.*, 2014) and could also play a role in determining what organisms persist in the mammary gland.

The sequencing data also suggests that the number and species of bacteria colonising the mammary gland does not increase with sheep age. However, as only 1 sheep per parity was analysed, this conclusion may not stand when further sheep of differing parities are incorporated into the analysis, although if the sequencing data reflects the DGGE results (which it does to date), this would continue to be the case. It is interesting to note however, that the parity 1, 3, 4 and 10 sheep seemed to have relatively stable communities, with similar OTU numbers (6-10) over time, whereas the parity 2 sheep had variable numbers of OTUs per sample (34 OTUs in total) and only 3 OTUs shared with milk samples from the other four sheep, with shifts in abundance over lactation and mammary gland half. It could be hypothesized that parity 3, 4 and 10 sheep have more stable communities as their microbiomes have reached a state of equilibrium, with parity 2 representing the transitional

period where bacteria are competing for dominance. However, as there is no clear pattern according to sheep parity from the data presented thus far, it is likely that community composition does not simply increase in complexity with age, as it is determined by several factors including those associated with individual animals, the bacteria, environment and management, making its development complex to elucidate and associate with age. It could also be that sheep have personalised microbiomes due to the complexity of factors that can affect microbiome development and composition. The concept of individual-specific milk microbiomes has already been raised in human studies (Costello *et al.*, 2009; Hunt *et al.*, 2011).

Whereas the DGGE data was unable to discern any significant differences between MG halves, the sequencing data does show differences between mammary gland halves of the same sheep. For example, parity three sheep had four OTUs that were specific only to the right mammary gland half (Figure 21). Also, differences in MG halves were often linked to specific time points. For example, week 4 of the right half for parity 4 sheep A40 (

Figure 24) had 2 otus not seen in any other milk samples for that sheep. As changes in community composition can be linked to both MG half and time, such changes could be random or specific to an environmental or management change at a certain time point or linked to factors specific to a mammary gland half such as udder conformation. However, the sequencing results also show a high degree of similarity in bacterial composition of milk samples over time across MG halves within sheep. This could suggest that the bacterial species most adapted to the mammary gland environment persist and so similarities in community will be detected across sheep on the same farm.

Furthermore, the hypotheses that the number and species of bacteria colonising the mammary gland will increase with time may be flawed. The risk of infection may increase over time as the immune system may deteriorate, or conformational changes in the udder make it easier for pathogens to invade at random and cause transient infections (Green *et al.*, 2005). However, this may not necessarily mean that the number of colonising bacterial species increases; changes in the community could result in commensal organisms causing infection or bacterial species may come and go at random as opposed to there being an association with time.

The identification of both similarities and differences over time, mammary gland half and sheep age could suggest that the composition of the mammary gland microbiome and what bacteria dominate could be determined at random, with different bacterial species appearing and disappearing over time by chance. Alternatively, further data analysis may suggest that

the species most adapted to exploit the unique niche of the mammary gland are those that survive and persist to form a stable commensal community.

In relation to this, if future studies investigate sheep on different farms, it may be that the organisms that dominate are different between farms, as the species that may enter the mammary gland could differ according to factors such as environment and management. In turn, this could mean those that dominate do so by chance. If certain species consistently dominate, it could mean that they are more adapted to the mammary gland environment and so are more likely to persist once they gain access to the mammary gland.

Previous studies have indicated differences in community composition according to disease state (Kuehn *et al.*, 2013; Oikonomou *et al.*, 2014). To elucidate further the changes in bacterial community when clinical infection develops, future longitudinal studies would benefit from sampling the transition from a healthy to clinically diseased state.

Future work can utilise the custom library preparation protocol and data analysis pipeline (Section 3.5) for further longitudinal studies. Future work includes the continued analysis of the sequencing data generated by this study. Clustering of all the data together as opposed to within individual milk samples would facilitate a more comprehensive analysis of the interactions between MG halves, time and sheep parity across all 30 sheep in the study. OTUs from the global clustering process could also then be incorporated into a mixed effects regression model to investigate the effect of specific bacterial species (OTUs) and different combinations of bacterial species on SCC. This process would not only enable community composition to be investigated further, but also the interactions between different bacterial species, which is essential in understanding how intramammary infections develop.

5.1.4. Study conclusions

This is the first longitudinal microbiome study of intramammary infections in a farm animal. The data presented in this report provides the first evidence of a microbial community in the sheep mammary gland. A persistent community was detected over time, with similarities and differences identified between sheep, mammary gland halves, over lactation and with sheep age. Associations between certain community members and mammary gland health have been identified through mixed effect modelling. Further analysis of the sequencing data produced in this project has the potential to improve our understanding of the role microbial community composition of the MG plays in IMI.

6. Industry messages

A persistent bacterial community was detected in the mammary glands of suckler ewes over a period of two months. The composition of this bacterial community changed between sheep, mammary gland halves, over time and with sheep parity. Improving our understanding of what bacteria are present, how they interact and what role they have in disease development, will facilitate the development of new strategies to more effectively manage intramammary infections to improve animal welfare and productivity.

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8. Appendix 1 – SCC and microbiological culture data

Parity 1 sheep [10]

Sheep A20 (No. of lambs reared = 2)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
279	L	2	396000	5.60	*Bacillus,* coliforms*, Staph*	S,S,S
567		3	94000	4.97	*Bacillus, Staph*	S,S
739		4	360000	5.56	*Bacillus,* G-ve*, Staph*	S,S,S
837		5	729000	5.86	coliforms, G-ve*, Staph*	S,S,S
1055		6	270000	5.43	*Bacillus, Staph*	S,S
1207		7	422000	5.63	*Bacillus, Staph*	S,S
1709		8	690000	5.84	*Bacillus, Staph*	S,S
280	R	1	216000	5.33	*Bacillus,* coliforms*, Staph*	S,S,S
568		2	76000	4.88	*Bacillus,* G-ve*, Staph*	S,S,S
740		3	404000	5.61	*Bacillus, Staph*	S,S
838		4	1130000	6.05	*Bacillus, Staph*	S,S
1056		5	450000	5.65	*Staph*	S
1208		6	1144000	6.06	*Bacillus, Staph*	S,S
1710		7	1072000	6.03	*Bacillus, Coryne, Staph*	S,S,S

Sheep A35 (No. of lambs reared = 2)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
67	L	1	140000	5.15	*Bacillus,* maybe *Nocardia, Staph*	S,S,M
331		2	52000	4.72	*Bacillus,* maybe *Nocardia*	S,S
591		3	164000	5.21	-	NG
759		4	92000	4.96	*Bacillus, Alpha colonies*	S,S
823		5	58000	4.76	*Bacillus, Staph*	S,S
1073		6	208000	5.32	*Bacillus, Staph*	S,S
1185		7	254000	5.41	*Coryne, Staph*	S,S
332	R	1	76000	4.88	*Bacillus*, coliforms*, Coryne, Staph*	S,S,S,S
760		2	486000	5.69	*Alpha colonies*	S
824		3	98000	4.99	*Bacillus, Staph*	S,S
1074		4	336000	5.53	*Bacillus, Staph*	S,S
1186		5	336000	5.53	*Bacillus,* coliforms, fungal	M,M,S

[10] In all summary tables for sheep data in Appendix 1: *Staph* = *Staphylococcus* spp. *Coryne* = *Corynebacterium* spp., *Bacillus* = *Bacillus* spp., maybe *Nocardia* = maybe *Nocardia* spp., G-ve = Gram negative bacteria, fungal = fungal organism.
S = small growth of bacteria, M = medium growth of bacteria, H = high growth of bacteria, NG = no growth of bacteria.

Sheep A50 (No. of lambs reared = 2)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
97	L	1	100000	5.00	*Bacillus,* G-ve*, Staph*	H,H,H
465		2	146000	5.16	*Bacillus,* G-ve, maybe *Nocardia, Staph*	S,M,S,H
681		3	48000	4.68	*Bacillus,* G-ve*, Staph*	S,S,H
777		4	76000	4.88	*Staph*	S
873		5	102000	5.01	*Bacillus, Staph*	S,S
1113		6	194000	5.29	*Bacillus,* coliforms*, Staph, Alpha colonies*	H,H,H,H
1235		7	90000	4.95	*Staph*	S
98	R	1	110000	5.04	*Bacillus, Staph*	H,H
224		2	160000	5.20	*Bacillus, Coryne, Staph*	S,S,S
466		3	948000	5.98	*Proteus*	H
874		6	138000	5.14	*Bacillus, Staph*	S,S
1114		7	1077000	6.03	*Staph*	S
1236		8	213000	5.33	*Bacillus, Coryne*	S,S

Parity 2 sheep

Sheep A15 (No. of lambs reared = 2)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
27	L	1	236000	5.37	*Bacillus,* maybe *Nocardia, Staph*	S,S,M
299		2	92000	4.96	*Bacillus*	M
553		3	132000	5.12	*Bacillus,* G-ve*, Staph*	S,S,S
717		4	28000	4.45	*Staph*	S
841		5	268000	5.43	*Bacillus*	S
1047		6	420000	5.62	*Staph*	S
1223		7	390000	5.59	*Bacillus, Staph*	S,S
1291		8	1544000	6.19	*Bacillus, Coryne*	S,S
28	R	1	436000	5.64	*Bacillus, Staph*	S,S
300		2	62000	4.79	*Bacillus,* coliforms*, Staph*	S,S,S
554		3	94000	4.97	*Bacillus, Staph*	S,S
718		4	30000	4.48	*Staph*	S
842		5	192000	5.28	-	NG
1224		7	76000	4.88	*Bacillus, Staph*	S,S

Sheep A21 (No. of lambs reared = 2)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
329	L	1	84000	4.92	*Bacillus,* coliforms*, Staph*	S,S,S
585		2	142000	5.15	*Bacillus,* maybe *Nocardia, Staph*	S,S,H
755		3	54000	4.73	*Coryne,* G-ve*, Staph*	S,S,S
819		4	148000	5.17	G-ve, *Staph*	S,S
1033		5	1642000	6.22	*Bacillus*	S
1159		6	116000	5.06	*Staph*	S
330	R	1	106000	5.03	*Bacillus,* coliforms*, Staph*	S,S,S
586		2	172000	5.24	*Bacillus, Coryne, Staph*	S,M,M
756		3	78000	4.89	*Bacillus*	S
820		4	156000	5.19	*Bacillus, Staph*	S,S
1034		5	1840000	6.27	*Bacillus, Staph*	S,S
1160		6	156000	5.19	*Bacillus, Staph*	S,S

Sheep A26 (No. of lambs reared = 3)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
225	L	1	60000	4.78	*Bacillus,* coliforms*, Staph*	S,S,S
457		2	93000	4.97	maybe *Nocardia, Staph*	S,S
669		3	26000	4.42	*Bacillus,* G-ve*, Staph*	S,S,S
775		4	54000	4.73	*Bacillus, Coryne*	S,S
875		5	172000	5.24	-	NG
1105		6	264000	5.42	*Coryne, Staph*	S,M
226	R	1	126000	5.10	*Bacillus, Coryne, Staph*	S,S,S
458		2	20000	4.30	*Bacillus, Staph*	M,H
670		3	70000	4.85	*Bacillus, Staph*	S,H
776		4	104000	5.02	*Bacillus,* maybe *Nocardia, Staph*	S,S,S
876		5	192000	5.28	G-ve	H
1106		6	160000	5.20	*Bacillus*	S
1238		7	450000	5.65	*Bacillus, Coryne, Staph*	S,S,S

Sheep A27 (No. of lambs reared = 3)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
455	L	1	206000	5.31	*Bacillus,* maybe *G-ve, Staph*	S,S,H
663		2	216000	5.33	maybe *Nocardia, Staph*	S,S
771		3	132000	5.12	*Bacillus, Staph*	S,S
1107		5	260000	5.42	*Bacillus, Staph, Alpha colonies*	S,S,S
1241		6	394000	5.60	*Bacillus, Staph*	S,S
456	R	1	276000	5.44	*Bacillus, Fungal, Staph*	S,M,M
664		2	108000	5.03	*Bacillus,* maybe *G-VE*	S,H
772		3	3000	3.48	*Bacillus, Coryne,* maybe G-ve, *Staph*	S,S,S,S
1108		5	566000	4.75	*Bacillus,* maybe *Nocardia, Staph*	S,S,S

Sheep A28 (No. of lambs reared = 3)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
589	L	1	176000	5.25	*Coryne, Staph*	S,S
761		2	126000	5.10	*Bacillus, Alpha colonies*	S,S
825		3	56000	4.75	*Staph*	S
1075		4	340000	5.53	-	NG
1183		5	152000	5.18	*Bacillus, Staph*	S,S
334	R	1	262000	5.42	*Bacillus,* maybe *Nocardia, Staph*	S,S,S
590		2	64000	4.81	*Bacillus, Coryne, Staph*	S,S,S
826		4	94000	4.97	G-ve, *Staph*	S,S
1076		5	291000	5.46	*Bacillus, Staph*	S,S
1184		6	110000	5.04	*Bacillus, Staph*	S,S

Sheep A39 (No. of lambs reared = 2)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
75	L	1	372000	5.57	*Bacillus, Staph*	M,M
239		2	164000	5.22	*Coryne, Staph*	S,S
487		3	118000	5.07	*Bacillus, Staph*	S,S
697		4	40000	4.60	*Staph*	S
811		5	22000	4.34	*Staph*	S
1007		6	104000	5.02	-	NG
1139		7	106000	5.03	*Staph*	S
1273		8	94000	4.97	*Bacillus*	S
76	R	1	150000	5.18	*Bacillus, Staph*	S,S
240		2	368000	5.57	*Bacillus, Coryne, Staph*	S,S,S
488		3	150000	5.18	*Bacillus, Staph*	M,M
698		4	50000	4.70	*Bacillus,* G-ve, *Staph*	S,S,H
812		5	30000	4.48	*Staph*	S
1008		6	106000	5.03	*Bacillus, Staph*	S,S
1140		7	26000	4.42	*Staph*	S
1274		8	106000	5.03	*Bacillus, Staph*	S,S

Sheep A4 (No. of lambs reared = unknown)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
265	L	1	302000	5.48	*Bacillus,* coliforms, *Staph*	S,S,S
501		2	166000	5.22	*Bacillus,* fungal, *Staph*	S,S,S
703		3	116000	5.06	G-ve, *Staph*	S,S

795		4	220000	5.34	*Staph*	S
1127		5	184000	5.27	*Bacillus, Staph*	S,H
1271		6	890000	5.95	*Bacillus, Staph*	S,S
266	R	1	417000	5.62	*Bacillus, Staph*	S,S
502		2	374000	5.57	*Bacillus,* G-ve*, Staph*	S,S,S
704		3	144000	5.16	*Bacillus, Staph*	S,H
796		4	170000	5.23	*Bacillus,* G-ve*, Staph*	H,H,H

Sheep A41 (No. of lambs reared = 3)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
471	L	1	106000	5.03	*Bacillus,* maybe *Nocardia, Staph*	S,S,S
671		2	22000	4.34	*Bacillus, Staph*	S,H
779		3	62000	4.79	*Bacillus*	S
881		4	186000	5.27	*Bacillus,* G-ve*, Staph*	S,S,S
1109		5	86000	4.93	*Staph*	S
1245		6	504000	5.70	*Bacillus*	S
472	R	1	94000	4.97	*Bacillus, Staph*	S,M
672		2	14000	4.15	*Bacillus,* G-ve*, Staph, Alpha colonies*	M,S,H,H
780		3	58000	4.76	*Staph*	S
882		4	56000	4.75	*Bacillus,* G-ve*, Staph*	S,S,S
1246		6	222000	5.35	*Bacillus, Staph*	S,S

Sheep A44 (No. of lambs reared = 2)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
559	L	1	68000	4.83	*Staph*	S
737		2	40000	4.60	*Staph*	S
863		3	48000	4.68	*Bacillus, Staph*	S,S
1067		4	254000	5.41	*Staph*	S
1201		5	94000	4.97	*Bacillus, Staph*	S,S
1287		6	284000	5.45	*Bacillus,* G-ve*, Staph*	S,H,H
282	R	1	274000	5.44	*Bacillus,* coliforms*, Coryne, Staph*	H,H,H,H
560		2	244000	5.39	*Bacillus,* G-ve	S,S
738		3	118000	5.07	*Staph*	S
864		4	318000	5.50	*Bacillus, Staph*	S,S
1068		5	212000	5.33	*Bacillus*	S
1202		6	112000	5.05	*Bacillus, Staph*	S,S
1288		7	634000	5.80	G-ve	S

Sheep A45 (No. of lambs reared = 2)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
87	L	1	108000	5.03	*Bacillus, Staph*	S,S
319		2	450000	5.65	*Bacillus, Coryne, Staph*	M,S,M
581		3	140000	5.15	*Bacillus*	H
847		5	94000	4.97	*Bacillus, Staph*	S,S
1049		6	2217000	6.35	*Staph*	S
1205		7	176000	5.25	*Bacillus, Coliforms,* G-ve*, Staph*	S,S,S,S
582	R	1	138000	5.14	*Bacillus*	H
728		2	84000	4.92	*Bacillus,* G-ve*, Staph*	S,S,S
1050		4	684000	5.84	-	NG
1206		5	66000	4.82	*Bacillus, Coliforms,* fungal*, Staph*	S,S,S,S
1712		6	140000	5.15	*Bacillus*	S

Sheep A46 (No. of lambs reared = 2)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
89	L	1	1146000	6.06	*Staph*	S
289		2	256000	5.41	*Bacillus,* fungal, *Staph*	M,S,S
575		3	80000	4.90	*Bacillus*	H
749		4	338000	5.53	*Staph*	H
853		5	78000	4.89	*Bacillus,* G-ve*, Staph*	S,S,S
1061		6	24000	4.38	*Bacillus, Staph*	S,S
1199		7	108000	5.03	*Bacillus,* fungal*, Staph*	S,S,M
1299	R	8	148000	5.17	*Staph*	H
90		1	1016000	6.01	*Bacillus,* maybe *Nocardia, Staph*	S,S,S
290		2	122000	5.09	*Bacillus,* maybe *Nocardia, Staph*	S,S,S
576		3	52000	4.72	*Bacillus*	H
750		4	84000	4.92	*Bacillus,* maybe *Nocardia, Staph*	S,S,H
854		5	58000	4.76	*Bacillus,* G-ve*, Staph*	S,S,S
1062		6	100000	5.00	*Bacillus, Coryne,* G-ve*, Staph*	M,M,M,M
1200		7	100000	5.00	*Bacillus, Staph*	S,S

Sheep A47 (No. of lambs reared = 2)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
91	L	1	230000	5.36	*Bacillus, Coryne,* maybe *Nocardia, Staph*	S,S,S,S
269		2	58000	4.76	*Bacillus,* coliforms*, Coryne, Staph*	M,M,M,M
569		3	54000	4.73	*Bacillus,* G-ve*, Staph*	S,S,S
725		4	134000	5.13	*Bacillus, Staph*	S,S
859		5	174000	5.24	*Bacillus, Staph*	S,S,
1065		6	188000	5.27	*Staph*	S
1721		8	412000	5.62	*Staph*	S
92	R	1	220000	5.34	*Coryne, Staph*	S,S
270		2	160000	5.20	*Bacillus,* coliforms, *Staph*	M,M,M
570		3	50000	4.70	*Bacillus,* G-ve*, Staph*	S,S,S
726		4	58000	4.76	*Staph*	S
860		5	140000	5.15	*Staph*	S
1066		6	118000	5.07	*Bacillus, Staph*	S,S
1214		7	112000	5.05	*Bacillus, Staph*	S,S
1722		8	236000	5.37	*Staph*	S

Sheep A5 (No. of lambs reared = unknown)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
9	L	1	974	5.99	*Coryne, Staph, Alpha colonies*	M,M,M
327		2	492	5.69	*Bacillus,* maybe *Staph, Alpha colonies*	S,S,S
587		3	492	5.69	*Bacillus*	S
757		4	182	5.26	-	NG
821		5	286	5.46	-	NG
1031		6	370	5.57	*Bacillus,* maybe G-ve*, Staph*	S,S,S
1181		7	234	5.37	*Bacillus, Staph*	S,S
10	R	1	612	5.78	*Bacillus, Coryne, Staph*	M,M,M
328		2	303	5.48	*Bacillus, Coliforms*	S,S
588		3	548	5.74	*Coryne, Maybe Nocardia, Staph*	S,S,S
758		4	320	5.51	*Bacillus, Coryne,* maybe G-ve, *Alpha colonies*	M,H,S,S
822		5	300	5.48	*Bacillus, Staph*	S,S
1032		6	272	5.44	*Bacillus*	S
1182		7	360	5.56	*Bacillus*	S

Sheep A9 (No. of lambs reared = 2)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
253	L	1	264000	5.42	*Bacillus, Coryne*	S,S
497		2	168000	5.23	*Bacillus*	S
807		4	46000	4.66	*Staph*	S
1009		5	164000	5.22	*Bacillus*	S
1137		6	120000	5.08	*Staph*	S
254	R	1	220000	5.34	-	NG
498		2	240000	5.38	*Bacillus, Staph*	S,S
694		3	52000	4.72	*Bacillus,* G-ve	S,H,H,
808		4	40000	4.60	*Staph*	S
1010		5	44000	4.64	*Bacillus, Staph*	S,S
1256		7	150000	5.18	*Staph*	S

Parity 3 sheep

Sheep A12 (No. of lambs reared = 2)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
271	L	1	240000	5.38	*Bacillus,* coliforms, fungal, *Staph*	M,S,S,M
571		2	52000	4.72	*Coryne*	S
743		3	182000	5.26	-	NG
1063		5	160000	5.20	*Bacillus*	S
1217		6	96000	4.98	*Coryne, Staph*	S,S
1295		7	760000	5.88	*Staph*	S
272	R	1	243000	5.39	*Bacillus, Coryne,* fungal, *Staph*	M,S,S,M
572		2	76000	4.88	*Staph*	M
744		3	198000	5.30	-	NG
844		4	405000	5.61	*Bacillus, Staph*	S,S
1064		5	124000	5.09	*Bacillus, Staph*	S,S
1218		6	106000	5.03	*Coryne, Staph*	S,S
1296		7	342000	5.53	-	NG

Sheep A2 (No. of lambs reared = unknown)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
3	L	1	170	5.23	*Bacillus, Coryne,* maybe *Nocardia, Staph*	S,S,S,S
263		2	294	5.47	*Bacillus,* G-ve*, Staph*	S,S,S
483		3	164	5.22	*Staph*	S
793		5	242	5.38	*Bacillus, Staph*	S,S
1001		6	180	5.26	*Staph*	S
1131		7	302	5.48	-	NG
1259		8	1094	6.04	-	NG
4	R	1	910	5.96	*Bacillus, Staph*	S,H
264		2	2202	6.34	*Bacillus,* coliforms*, Staph*	M,M,M
484		3	738	5.87	*Bacillus, Staph*	S,M
696		4	873	5.94	*Bacillus,* G-ve*, Staph*	S,S,S
794		5	590	5.77	*Staph*	S
1002		6	872	5.94	-	-
1132		7	1268	6.10	*Bacillus, Staph*	S,H
1260		8	3069	6.49	*Coryne, Staph*	S,S

Sheep A22 (No. of lambs reared = unknown)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
325	L	1	240000	4.38	*Bacillus,* maybe *Nocardia*	M,S
579		2	218000	4.34	*Bacillus, Staph*	S,S
745		3	336000	4.53	*Bacillus, Staph*	S,S
861		4	134000	4.13	*Bacillus, Staph*	S,S
1071		5	124000	4.09	*Coryne, Staph*	S,M
1221		6	164000	4.22	*Staph*	S
1719		7	112000	4.05	*Staph*	S
326	R	1	160000	4.20	*Bacillus,* coliforms	S
580		2	202000	4.31	*Bacillus*	H
746		3	258000	4.41	*Staph*	S
862		4	148000	4.17	*Bacillus, Staph*	S,S
1072		5	76000	3.88	*Bacillus,* G-ve*, Staph*	M,M,M
1222		6	78000	3.89	*Bacillus, Staph*	S,S
1720		7	96000	3.98	-	NG

Sheep A25 (No. of lambs reared = unknown)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
47	L	1	172000	5.24	-	NG
287		2	126000	5.10	*Bacillus,* coliforms*, Staph*	S,S,S
565		3	20000	4.30	*Bacillus, Staph*	S,S
721		4	30000	4.48	*Coryne, Staph*	S,S
827		5	56000	4.75	*Bacillus,* G-ve, *Staph*	S,S,S
1045		6	32000	4.51	*Bacillus, Staph*	S,S
1189		7	40000	4.60	*Bacillus*	S
1293		8	240000	5.38	*Coryne, Staph*	S,S
288	R	1	11436000	7.06	*Bacillus,* coliforms, fungal	S,S,S
566		2	3854000	6.59	coliforms, *Staph*	H,S
722		3	2346000	6.37	coliforms	H
828		4	3582000	6.55	coliforms, *Staph*	M,S
1190		6	35432000	7.55	coliforms, *Staph*	M,S
1294		7	176000	5.25	*Bacillus, Coryne,* maybe *Nocardia*	S,S,S

Sheep A29 (No. of lambs reared = 2)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
217	L	1	116000	5.06	*Bacillus, Proteus, Staph*	M,M,M
469		2	94000	4.97	-	NG
675		3	201000	5.30	*Bacillus, Staph*	S,H
773		4	66000	4.82	*Bacillus,* G-ve*, Staph, Alpha colonies*	M,M,M,M
877		5	146000	5.16	*Staph*	S
1101		6	94000	4.97	*Bacillus, Staph*	S,S
1231		7	684000	5.84	*Staph*	S
218	R	1	106000	5.03	*Bacillus, Staph*	M,M
470		2	42000	4.62	*Bacillus, Coryne,* maybe *Nocardia*	S,S,S
676		3	376000	5.58	*Staph*	S
774		4	52000	4.72	*Staph*	S
878		5	70000	4.85	-	NG
1102		6	144000	5.16	*Bacillus, Staph*	S,S
1232		7	748000	5.87	*Bacillus*	S

Sheep A3 (No. of lambs reared = unknown)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
261	L	1	160000	4.20	-	NG
803		2	30000	3.48	*Staph*	S
1019		3	92000	3.96	*Bacillus,* maybe *Nocardia*	S,S
1149		4	266000	4.43	*Staph*	S
1277		5	1032000	5.01	-	NG
544	R	1	88000	3.94	*Bacillus*	S
706		2	26000	3.43	*Staph*	S
804		3	24000	3.38	*Coryne,* G-VE*, Staph, Alpha colonies*	S,S,S,S
1020		4	72000	3.86	*Staph*	S
1150		5	292000	4.47	*Bacillus, Staph*	S,S
1278		6	1026000	5.01	*Bacillus,* maybe *Nocardia*	S,S

Parity 4 sheep

Sheep A16 (No. of lambs reared = unknown)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
317	L	1	172000	5.24	maybe *Nocardia*	S
557		2	14000	4.15	maybe *Nocardia, Staph*	S,S
715		3	26000	4.42	*Staph*	S
839		4	123000	5.09	*Bacillus*	S
1041		5	90000	4.95	*Bacillus*	S
1211		6	116000	5.06	coliforms, fungal, *Staph*	S,S,S
1297		7	446000	5.65	*Bacillus, Staph*	S,S
318	R	1	352000	5.55	*Bacillus, Coryne*, fungal*, Staph*	M,S,S,S
558		2	63000	4.80	*Staph*	S
716		3	58000	4.76	*Bacillus, Coryne,* G-ve*, Staph*	M,S,S,H
840		4	224000	5.35	*Bacillus, Staph*	H,H
1042		5	46000	4.66	*Bacillus, Staph*	S,S
1212		6	135000	5.13	*Bacillus, Staph*	S,S
1298		7	732000	5.87	*Bacillus*, G-ve*, Staph*	S,S,S

Sheep A24 (No. of lambs reared = 3)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
229	L	1	316000	5.50	*Bacillus,* maybe *Nocardia, Staph*	S,S,S
467		2	74000	4.87	*Bacillus, Staph*	S,S
677		3	192000	5.28	*Bacillus, Staph*	S,S
783		4	26000	4.42	*Bacillus*	S
885		5	124000	5.09	*Bacillus, Staph*	S,S
468	R	1	56000	4.75	*Bacillus,* G-ve, maybe *Nocardia, Staph*	S,S,S,H
678		2	206000	5.31	*Bacillus, Staph*	S,S
784		3	28000	4.45	*Bacillus, Coryne,* maybe *Nocardia, Staph*	S,S,S,S
886		4	78000	4.89	*Bacillus, Staph*	S,S
1244		6	256000	5.41	*Staph*	S

Sheep A40 (No. of lambs = 3)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
491	L	1	74000	4.87	*Bacillus, Staph*	S,S
691		2	54000	4.73	*Bacillus, Staph*	S,H
789		3	14000	4.15	*Bacillus,* maybe *Nocardia*	S,S
1003		4	104000	5.02	*Bacillus*	S
1133		5	432000	5.64	*Staph*	S
1265		6	294000	5.47	*Coryne*	S
250	R	1	120000	5.08	*Bacillus, Coryne*, maybe *Nocardia, Staph*	S,S,S,S
492		2	147000	5.17	*Bacillus,* G-ve*, Staph*	M,M,M
692		3	52000	4.72	*Staph*	S
790		4	18000	4.26	*Bacillus,* G-ve*, Staph*	H,H,H
1004		5	86000	4.93	-	NG
1134		6	270000	5.43	-	NG
1266		7	240000	5.38	*Coryne*	S

Sheep A43 (No. of lambs reared = 2)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
293	L	2	285000	5.46	*Bacillus, Staph*	S,S
547		3	207000	5.32	G-ve, *Staph*	S,S
735		4	82000	4.91	-	NG
829		5	98000	4.99	G-ve, *Staph, Alpha colonies*	S,S,S
1035		6	174000	5.24	*Staph*	S
1197		7	78000	4.89	*Bacillus,* fungal	S,S
1285		8	142000	5.15	G-ve, *Staph*	S,S
294	R	2	540000	5.73	*Bacillus,* maybe *Nocardia*	S,S
736		3	28000	4.45	-	NG
830		4	68000	4.83	*Bacillus,* G-ve, *Staph*	S,S,S
1036		5	192000	5.28	*Bacillus, Staph*	S,S
1198		6	46000	4.66	*Bacillus, Staph*	S,S
1286		7	180000	5.26	-	NG

Sheep A49 (No. of lambs reared = unknown)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
321	L	1	116	5.06	*Bacillus, Coryne*	S,S
555		2	60	4.78	*Bacillus,* coliforms, *Staph*	S,S,S
733		3	84	4.92	*Bacillus, Staph*	S,S
831		4	122	5.09	G-ve	S
1051		5	92	4.96	-	NG
1219		6	78	4.89	*Bacillus, Staph*	S,S
1713		7	204	5.31	*Bacillus, Coryne, Staph*	S,H,S
322	R	1	108	5.03	*Bacillus,* coliforms, *Staph*	S,S,S
832		4	148	5.17	*Bacillus, Staph*	S,S
1052		5	78	4.89	*Bacillus*	S
1220		6	68	4.83	*Staph*	S
1714		7	448	5.65	*Staph*	S

Parity 10 sheep

Sheep A6 (No. of lambs reared = unknown)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
11	L	1	628	5.80	*Bacillus, Staph*	S,S
699		2	26	4.42	*Bacillus, Staph*	S,H
787		3	32	4.51	*Bacillus, Staph*	S,S
1015		4	148	5.17	*Bacillus, Staph*	S,S
1135		5	76	4.88	*Staph*	S
1269		6	188	5.27	*Bacillus, Staph*	S,S
12	R	1	606	5.78	*Proteus*	H
700		2	104	5.02	*Bacillus, Staph*	S,S
788		3	48	4.68	*Bacillus, Staph*	S,S
1016		4	72	4.86	*Bacillus, Coryne, Staph*	S,S,S
1136		5	72	4.86	*Bacillus, Staph*	S,S
1270		6	147	5.17	*Bacillus, Coryne, Staph*	S,S,S

Sheep A23 (No. of lambs reared = 1)						
Sample No.	**Half**	**Week**	**SCC**	**Log SCC**	**Bacteria detected**	**Growth**
561	L	1	5358000	6.73	*Coryne, Staph*	S,S
751		2	3570000	6.55	*Bacillus*	S
855		3	3723000	6.57	*Bacillus,* G-ve*, Staph*	M,M,M
1043		4	3063000	6.49	*Bacillus*	S
1215		5	1956000	6.29	*Bacillus,* G-ve*, Staph*	S,S,S
1705		6	9084000	6.96	*Staph*	H
562	R	1	5256000	6.72	*Bacillus,* G-ve*, Staph*	S,S,S
752		2	12050000	7.08	-	NG
856		3	2372000	6.38	*Bacillus, Staph*	S,S
1044		4	1656000	6.22	*Bacillus, Staph*	S,S
1216		5	850000	5.93	-	-
1706		6	4578000	6.66	*Staph*	S