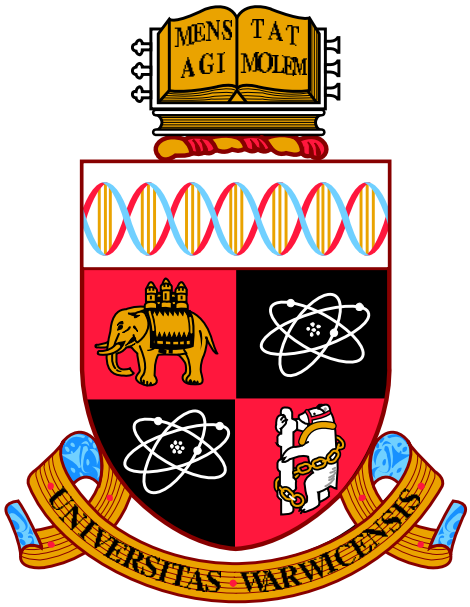
Investigating the impact of antibiotic treatment on the dairy cow mammary gland microbiome



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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

SCC	Somatic Cell Count
AB	Antibiotic treatment group (antibiotic and teat sealant)
Orb Only	Non-antibiotic treatment group (Orbeseal teat sealant)
D	Drying off time point
D0	Drying off time point
C0	Calving time point
PC1 – PC28	1 day …. 28 days post calving time points
Av_C0.PC1.PC3	Mean value for the time points C0, PC1 and PC3
LF	Left fore udder quarter
LH	Left hind udder quarter
RF	Right fore udder quarter
RH	Right hind udder quarter
DPL	Dry period length in days
OTU	Operational Taxonomic Unit
bp	Base pair
Gbp	Giga base pairs
DNA	Deoxyribonucleic acid
16S rRNA	16s Ribosomal RNA
dsDNA	Double stranded DNA
3’	The end of a gene
5’	The start of a gene
PCR	Polymerase Chain Reaction
NaOAc	Sodium acetate
Tris HCL	Tris hydrochloride
BSA	Bovine serum albumin
DMSO	Dimethyl sulfoxide
LME	Linear mixed effects model

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I would also like to thank Dr Ed Smith and Dr Emma Monaghan for the initial data collection and an extra thanks to Emma for all the excellent lab and bioinformatics advice.

I would also like to thank my close friends and family who's unwavering encouragement and support throughout has undoubtedly got me where I am today.

Finally, I would like to thank my mum, although lost along the way, has never stopped being my number 1 cheerleader.

Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. The work presented (including data generated and data analysis) was carried out by the author except in the cases outlined below:

- Collection, transportation and storage of milk samples for Herd 1 and Herd 2 were carried out by Dr Ed Smith and Dr Emma Monaghan.
- DNA extraction, sequencing, processing of sequence data into OTU tables for Herd 1 milk samples was conducted by Dr Ed Smith and Dr Emma Monaghan.

Abstract

Mastitis, the inflammation of the mammary gland, is the most prevalent disease among UK dairy cattle, imposing a significant burden on animal welfare, food security and antibiotic use. It is complex disease, with over 140 bacterial species being identified as etiological agents and general dysbiosis of the mammary gland bacterial community also being associated with mastitis incidence.

A major driver of antibiotic use in dairy cows is in the administration of an intramammary infusion antibiotic and a teat sealant at drying off. This is to treat and prevent mastitis over the dry period and into early lactation when cows are particularly susceptible to mastitis. For responsible and justified use of antibiotics the effect of therapy on the mammary gland microbiota and udder health in sub-clinical mastitis cases need to be understood.

In this study high-throughput sequencing techniques were used to analyse the bacterial community from 1231 milk samples collected from the udder quarters of 40 cows, in 2 independent farm studies, over 11 time points across the dry period into early lactation. Conclusions drawn from the first herd directed the specific selection of samples to analyse in the second herd, allowing the impact of antibiotic therapy on the milk microbiota to be more explicitly addressed. Data analysis and statistical modelling revealed that cows receiving antibiotic therapy had a similar outcome in the udder health and diversity metrics of the mammary gland microbiota in early lactation compared to those cows receiving just teat-sealant therapy. A highly diverse composition and a dynamic nature of the milk microbiota was reported in both farms and treatment groups. Reporting that rapid changes to the bacterial community can occur within udder quarters, over subsequent time points, just 1 to 2 days apart. This study demonstrated the complexity and difficulty in describing a normal, stable microbiota in the mammary gland. It also suggests, in the context of these two farms, more evidence is required to show that omission of antibiotic therapy at drying off would be detrimental to healthy cows or cows with sub-clinical levels of mastitis.

1. Introduction

1.1. The Mammary gland microbiome

1.1.1. Defining the microbiome

Microbiomes are often defined as all of the microorganisms (bacteria, archaea, fungi, protists and viruses), their genomes and the environmental conditions of an area in which they exist (Marchesi & Ravel, 2015). Microbiomes generally have a core community of microorganisms that exist in their host environment. There is consensus, and a growing body of evidence, that there is a mutually positive relationship between the core microbes and the host environment. This has been evidenced in the human body, describing the microbiome as the 'hidden organ' of the human body for the impact the microbes in our body have on helping to regulate health and disease (Cho & Blaser, 2012; Gilbert *et al.*, 2018). Evidence for a core microbiome and its role in health and disease has been characterised in body sites such as the gut, skin, oropharyngeal tract, urinary and genital tract (Faith *et al.*, 2013; Faner *et al.*, 2017; Grice & Segre, 2011; Whiteside *et al.*, 2015).

1.1.2. Bacteria that colonise the mammary gland

Presence of a core protective microbiome in the dairy cow mammary gland is a hotly debated topic (Rainard, 2017; Taponen *et al.*, 2019). It is accepted that the mammary gland is colonised by bacteria (Derakhshani *et al.*, 2018a). However there is no common consensus if this relationship is strictly host-pathogen, with infection of the mammary gland by pathogens associated with mastitis triggering an inflammatory immune response, or if there is a commensal core microbiota associated with healthy udder quarters (Derakhshani *et al.*, 2018a).

Research elucidating if there is a protective microbiome that could be exploited for prophylactic treatment is ongoing. It is known dysbiosis of the mammary gland bacterial community is associated with mastitis incidence (Andrews *et al.*, 2019; Oikonomou *et al.*, 2014; Oikonomou *et al.*, 2012). However, it is unknown if dysbiosis of the mammary gland microbiota in healthy udder quarters is a risk factor for developing mastitis. Understanding

the impact of antibiotics on the mammary gland microbiome is important for both deciphering the implication of antibiotics on the mammary gland community and in justifying antibiotic use.

1.2. Mastitis and the disease burden

1.2.1. Burden of disease

Bovine mastitis is the inflammation of the udder tissue of the mammary gland in response to pathogens or physical trauma. It can be defined by different levels of severity. Clinical mastitis is visibly detectible in the cow, associated with fever, udder swelling, redness and in the appearance of the milk; discolouring, becoming watery and forming clots (Zhao & Lacasse, 2008). Severe cases can cause death or result in death from culling. Sub-clinical mastitis is difficult to visibly detect, with diagnosis usually occurring by presence of an increase in immune response cells in the milk (Cheng & Han, 2020). Subclinical mastitis is often diagnosed by an increase in the somatic cell count (SCC). The SCC is a quantitative measure of immune cells in the mammary gland, it is used as a proxy for measuring mastitis infection (Schukken *et al.*, 2003).

Risk of infection is considerably higher across the dry period and in early lactation than at any other time period during lactation (Bradley & Green, 2004). The dry period is the non-lactating period for a cow prior to parturition, important for cattle recovery, calving and preparation for the next lactation cycle.

1.2.2. Mastitis and Mastitis Associated Pathogens

Mastitis is the most common and most costly disease in dairy cows. Economic losses are due to treatment costs, culling, reduced conception rates, reduced milk production and discarding poor quality milk (Halasa *et al.*, 2007). The average cost of a mastitis case per cow in a herd across the year can be up to $400-500 (Aghamohammadi *et al.*, 2018; Rollin *et al.*, 2015), with an estimated global yearly cost of $2 billion. Mastitis is significant animal welfare issue as severe and chronic mastitis are associated with pain reducing the wellbeing of diseased animals. Reducing mastitis incidence is of primary importance for food security, productivity and to improve cattle welfare.

Mastitis is a complicated disease, with over 140 causal agents identified (Watts, 1988). The multifactorial etiology of mastitis contributes to the continued challenges in preventing and treating disease (Ganda *et al.*, 2016). Cases of clinical mastitis have been associated with specific pathogens such as *Escherichia coli, Klebsiella spp., Staphylococcus aureus, Streptococcus uberis* and *Pseudomonas spp.* (Erskine *et al.*, 1988; Levison *et al.*, 2016; Olde Riekerink *et al.*, 2008)

Streptococcus uberis is largely considered to cause environmental mastitis, present in straw bedding systems in the cow environment (Leigh, 1999). Mastitis caused by *S. uberis* has a rapid, acute onset however a range of antibiotics are effective against this pathogen (Hillerton & Kliem, 2002). Fellow Gram-Positive, *Staphylococcus aureus,* is more difficult to treat and is the cause of the most common type of contagious mastitis in dairy cattle, primarily spread between cows during the milking process (Capurro *et al.*, 2010; Petzl *et al.*, 2018). While *S. aureus* can cause severe clinical mastitis, the pathogen most often causes subclinical, chronic mastitis (Watts, 1988). *S. aureus* produces toxins which damages the lining of teat and gland cisterns in the udder quarter. Toxins can induce cytoskeletal rearrangement to assist invasion across the epithelial barrier. The pathogen then colonises secretory tissue and cells (alveoli) which can result in the formation of abscesses. *Escherichia coli* is an environmental pathogen present in across many farm environments due to its abundance in faeces. It is a toxin producing bacteria can rapidly cause severe mastitis.

The innate immune response in dairy cows comprises of the physical barrier at the tip of the udder teat and the immune cell types that constitute somatic cells, these include neutrophils (which make up 90% of cells when somatic cell count increases during infection), macrophages, cytokines, natural killer cells and compliment (Goulart & Mellata, 2022).

The immune response elicited by *S. aureus* and *E. coli* varies particularly during host recognition. *E. coli* is perceived by Toll-Like Receptors (TLR) inducing a generalised immune response and strong inflammatory response whereas Gram-positive bacteria do not activate TLR-signalling (Petzl *et al.*, 2018). *E. coli* is associated with more severe clinical mastitis, there is a more rapid increase in cytokines and in the level of somatic cells compared with *S. aureus* which often reflects a more moderate and delayed increase in SCC (Bruckmaier & Wellnitz, 2017; Petzl *et al.*, 2018).

It is not fully understood how the different responses of the immune system following infection from different pathogens may effect the long term mammary gland microbiome health. A study by Falentin *et al.* (2016) reported a correlation between the teat microbiome and mastitis history, showing a reduced diversity in udders with a history of mastitis. Raising interesting questions how historic infection may effect the mammary gland microbiome or if prior infection may lead to a continued dysbiosis.

The emergence of high-throughput sequencing techniques, has begun to identify the vast number of bacteria colonising the udder(Figure 1.). It has been suggested in many mastitis cases, and in particular subclinical cases, disease is also caused by dysbiosis of the udder microbiota rather than just a single primary infection (Kuehn *et al.*, 2013; Oikonomou *et al.*, 2014; Oikonomou *et al.*, 2012).

It is widely accepted that colonisation of the teat canal and apex play a significant role in the entry of pathogens and in the development of intramammary infections. It has been suggested that the communities colonising the teat could play a major role in modulating udder health status and therefore in the susceptibility to new infections (Derakhshani *et al.*, 2018a). At drying off, the cow is particularly susceptible to new infections. Effective therapy, including treatment of the teat, outlined fully in the next section, is important in mastitis prevention.

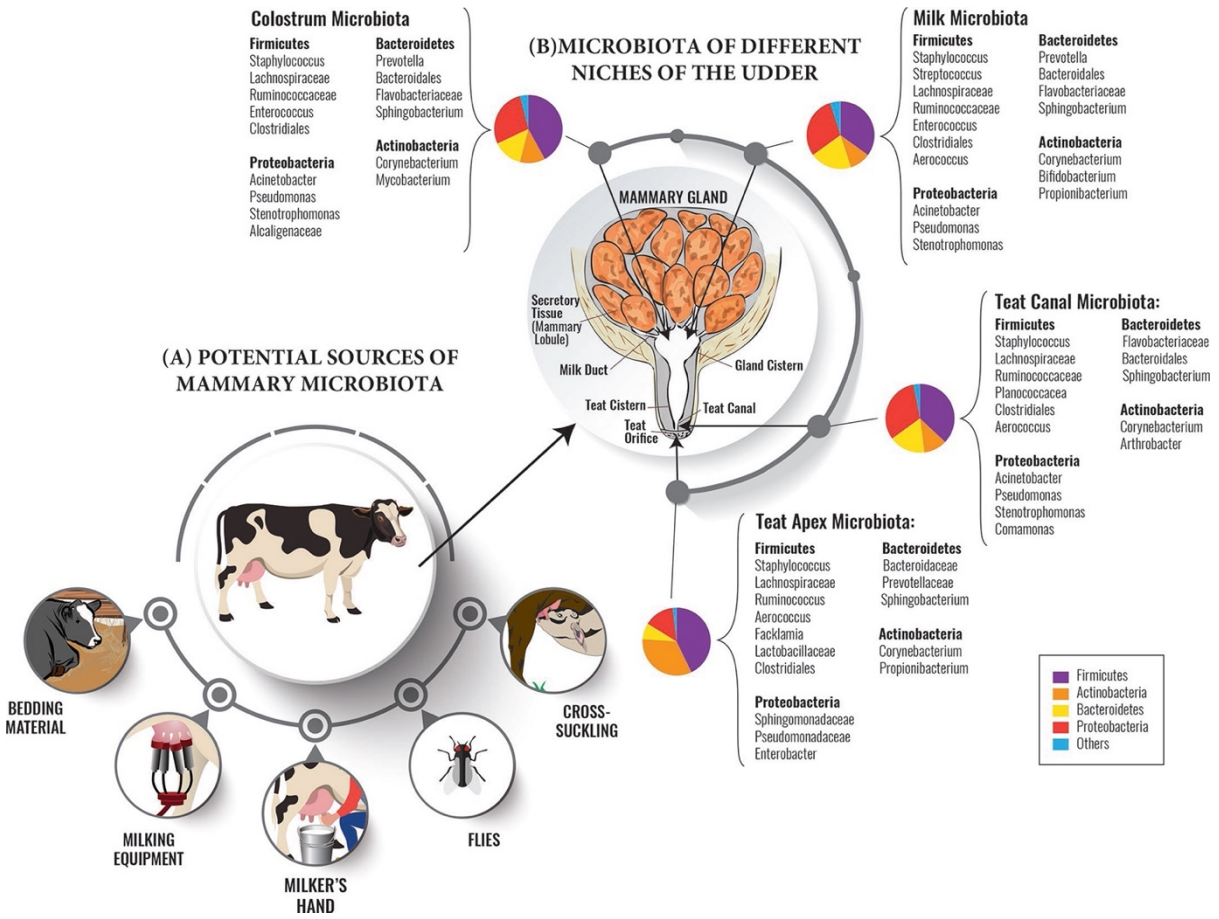


Figure 1. An overview of potential sources of the mammary gland microbiota (A.) and the different niches in which different bacterial Phyla colonise (B.) complied and presented in a review by Derakhshani *et al.* (2018a) highlights the vast diversity of the udder microbiota revealed by 16S rRNA sequencing and the potential sources for colonisation and dysbiosis of the udder community.

1.3. Antibiotic use

Overuse of antibiotics leading to the development of antimicrobial resistance is of global concern for human and animal health. Currently, 73% of all antimicrobials sold globally are used in animal production (Van Boeckel *et al.*, 2019). It is estimated global consumption of antimicrobials could increase by 11.5% by 2030 to meet production demands of a growing population and to facilitate the shift in middle-income countries to larger scale farming (Tiseo *et al.*, 2020; Van Boeckel *et al.*, 2015).

Prophylactic use of antibiotics in farming has been under much scrutiny over the past decade in many countries, there is new pressure to ensure antimicrobial use is targeted and justified (O'Neil, 2015). Policy in dairy farming has begun pivoting away from blanket therapies, which involve treating every quarter of every cow with antibiotics to selective therapy; treating all quarters of cows diagnosed with active mastitis or recurrent mastitis (Hillerton *et al.*, 2017). Lowering the level of unjustified antimicrobial use is a key aim in reducing overall antimicrobial use (O'Neil, 2015), in order to do this understanding the effect of antibiotic treatment on the mammary gland is imperative to defining necessary use.

Dry cow therapy (DCT) is the administration of intramammary antibiotics at the beginning of the dry period (end of lactation). Whether used as selective or blanket therapy, DCT is one of the main drivers for antibiotic use on farms (Bradley & Green, 2004; Hillerton *et al.*, 2017). The risk of infection is considerably higher across the dry period and in early lactation than at any other time period during lactation (Bradley & Green, 2004). Therefore, DCT is an integral part of mastitis management programs, with the aim of treating existing mammary gland infections and preventing the development of new infections across the dry period (Derakhshani *et al.*, 2018b). Entry of pathogens through the teat canal is a risk factor for new infections (Paulrud, 2005). The teat canal can be an effective barrier to environmental pathogens from entering the udder, however this first line of defence can be compromised. Following the prepartum loss of the keratin plug of the teat canal and following repeated milking, sphincter muscles of the teat canal are less effective at contracting quickly, increasing the susceptibility of invasion and colonisation of a range of extramammary microbes. Typically, a teat-sealant is used in combination with antibiotic intramammary treatment in at risk and mastitic cows, and in all cows at the beginning of the dry period to prevent infection of the mammary gland (Berry & Hillerton, 2002; Huxley *et al.*, 2002). The teat-sealant is non-antibiotic. It is administered at drying off and acts as a physical barrier, mimicking the effect of the keratin plug within the teat canal to prevent invasion and colonisation of potential extramammary pathogens.

Despite success in reducing mastitis incidence with a combination of teat-sealant and antibiotic infusion into the mammary gland over recent years, mastitis is still a common and

persistent disease. Understanding the mammary gland microbiome and the changes following antibiotic and teat-sealant only treatment at drying off is important for identifying areas for novel treatment and in ensuring that antibiotic use is responsible and necessary. Defining necessary antibiotic use is important not only for prevention of antibiotic resistance, but in reducing farm costs, and in improving cattle health. Dysbiosis is the imbalance and disruption of the microbial community, often resulting in a reduced bacterial diversity. In the udder microbiota, dysbiosis is associated with mastitis incidence and potential dysbiosis may be a predisposing factor for developing mastitis. Furthermore, in human studies, dysbiosis of the gut following antibiotic treatment has been shown to reduce bacterial diversity and increase the pool of resistance genes present in the gut (Ramirez *et al.*, 2020). Therefore, understanding the impact of antibiotics on the mammary gland is important for sustainable antibiotic use and cattle health.

1.4. Methods for profiling the Microbiome

The emergence and reducing costs of high-throughput sequencing methods has provided exciting platforms to investigate microbial communities rapidly on a large scale in a range of ecosystems; including the characterisation of bacteria found in the dairy cow mammary gland (Addis *et al.*, 2016). 16S rRNA sequencing is the most popular method to characterise the taxonomy of the microbiome. rRNA genes are highly conserved, allowing the design of universal primers to bind the conserved region of the gene and then amplification of the variable regions, capturing taxonomic information (Kuczynski *et al.*, 2011). Raw sequences are passed through quality filtering and chimera checking to reduce the effects of sequencing artifacts. Th reads are then clustered in to operational taxonomic units (OTUs) which group together similar sequences, typically based on 97% sequence similarity, which then represents organisms. The taxonomy is inferred using a database of known OTUs (Kuczynski *et al.*, 2011). A limitation of this method is that it can result in reduced taxa resolution depending on the region chosen for sequencing, furthermore 16S rRNA sequencing only characterises bacteria, this limits the true picture of the microbiome and the impact of the microbiome on health.

Shotgun metagenomics is another method to study non-culturable bacteria. It allows sampling of all genes in all organisms not just bacterial DNA. DNA is fragmented and sequenced, producing numerous DNA sequences that align to multiple genomic locations, allowing analysis that can provide insight on taxonomy but also encoded function (Sharpton, 2014). However this method results in large, complex data requiring complicated bioinformatics analysis. It is also a method that requires a large volume of data to identify meaningful results because of the vast quantity of genomic information being sampled, leading to increased costs (Sharpton, 2014).

While there are other methods to inform different information about the microbial community; gene potential (metatranscriptomics), protein expression (metaproteomics) and metabolite fluctuations (metametabolomics); 16S rRNA sequencing was deemed the most appropriate for the aims of this study, which will be outlined in full in section 1.6. The advantages of 16S rRNA is that is a relatively rapid, accurate way to characterise taxonomy to the Genus level, providing a good representation of the community diversity which can be measured through analysis of OTUs. It allows the identification of low abundance bacteria, important in microbiome studies of the dairy cow microbiome where there is often a high proportion of low abundant taxa. While there are error rates associated with both methods this can be well controlled for during sequence processing and in the inclusion of control samples. It is a cost effective method allowing the combination of samples in to a single sequencing run.

1.5. Current understanding

Analysis of the mammary gland microbiota has focussed on providing insights into the microbiota by profiling the community of bacteria residing in the mammary gland and teat canal, both in healthy cows, mastitic cows and those with a history of mastitis (Braem *et al.*, 2012; Falentin *et al.*, 2016; Kuehn *et al.*, 2013; Oikonomou *et al.*, 2014; Oikonomou *et al.*, 2012). While numerous studies have also investigated the effectiveness of antibiotics on treating and controlling infection from known mastitis pathogens (Barlow, 2011; Royster &

Wagner, 2015; Suojala *et al.*, 2013; Wilson *et al.*, 1999), only a few studies have begun to explore the effect of antibiotic treatment on the mammary gland microbiota.

In two longitudinal studies of the effect of the antimicrobial ceftiofur on naturally occurring mastitis caused by *Escherichia coli* (Ganda *et al.*, 2016) and mastitis experimentally induced by pathogenic *E. coli* (Ganda et al., 2017), researchers showed that following resolution of disease, the milk microbiota returned to a 'healthy' composition within 14 days regardless of intramammary antimicrobial administration. Furthermore, ceftiofur did not appear to provide an advantage in clinical cure, bacterial load or clearance rate of the pathogen compared to no treatment (Ganda *et al.*, 2016). Following induced infection with *E.coli* the diversity of the udder quarter microbiotas dramatically decreased, however the microbiome returned to a similar state to that of unchallenged quarters just 9 days after experimentally induced infection, regardless of intervention with ceftiofur (Ganda *et al.*, 2017).

A study by Bonsaglia *et al.* (2017) also revealed no significant effect on the mammary gland microbiome or bacterial load following drying off intramammary antibiotic treatment with a teat sealant compared to teat sealant treatment alone. They reported no significant difference between bacterial abundance and richness measures between the two time points, dying off and 7 days postpartum, with or without antibiotics (Bonsaglia *et al.*, 2017). However, Derakhshani *et al.* (2018b) had contrasting findings to Bonsaglia *et al.* (2017) where they found that dry cow antibiotic therapy showed changes in the microbiota when sampling the teat canal and milk at drying off and at calving, with a slight reduction in species richness. However, they also suggested the udder microbiota is resilient against exposure to long-acting antimicrobials over the dry period (Derakhshani *et al.*, 2018b).

Finally, Biscarini *et al.* (2020) tested the effect of two antibiotics (cephalonium and cloxacillin) and a teat sealant on the dairy cow milk microbiome from the quarters of 5 cows, finding no significant differences in the major diversity indices and abundancies of specific bacteria between drying off, calving and 5 days post partum.

1.6. Aims of this study

The overall aim of this study was to understand the impact of antibiotic dry cow therapy on the dairy cow mammary gland microbiome in early lactation using high-throughput sequencing of the bacterial community and analysis of the immune response.
In order to address this aim, a longitudinal study was designed to test the following overarching hypothesis on two independent farms:

Antibiotic dry cow treatment has only a transient impact on the dairy cow mammary gland bacterial community that does not reduce immune marker levels associated with sub-clinical mastitis in dairy cows early in lactation.

This study builds upon research outlined in section 1.4. by exploring the change in mammary gland microbiota and udder health, following antibiotic and non-antibiotic treatment in two large, longitudinal studies on independent farms, using a combination of high-throughput sequencing, statistical modelling and Operational Taxonomic Unit (OTU) analysis to analyse changes to udder health and the microbiota.

Changes to udder health were assessed in terms of the somatic cell count (SCC). Cows included in the study were those without a history of clinical mastitis. Those defined to have a low SCC (below 200,000 cells mL^{-1}) at the drying off timepoint received a non-antibiotic treatment and those with an SCC greater than 200,000 cells mL^{-1} (pooled between udder quarters) received antibiotic treatment in addition to a teat-sealant at drying off. Changes to the mammary gland microbiota were reported through analysis of the bacterial abundance measure, the Chao1 index, and the bacterial evenness measure, the Shannon index. The Chao1 index is an abundance-based estimator of species richness, in this context it is a measure of expected OTUs in each sample based on the OTUs identified in all samples (Chao *et al.*, 2006). The Shannon diversity index considers relative abundance while estimating species richness and evenness, this index increases as the both the number of OTUs increases and the distribution amongst the different OTUs becomes more even (Kim *et al.*, 2017; Lemos *et al.*, 2011). In this context an increase in both of these indices would infer a greater bacterial diversity in the sample relative to the measured population. These three parameters are

visualised in each herd, between treatment groups and at a quarter level. Changes in these parameters between treatment groups across the dry period will be compared and statistically modelled. Furthermore, the composition of the microbiota between treatment groups will be compared through the analysing the ranked abundance of OTUs and by comparing the taxonomy of the microbiota.

1.7. Thesis Overview

Chapter 2 will outline the methods used to generate milk microbiota data and to perform analysis of the microbiota and udder health.

In Chapter 3, data from Herd 1 is explored. Over 790 quarter milk samples obtained from 18 cows across 11 time points from drying off into 28 days post calving is analysed. This analysis generated further more-specific hypotheses which were then tested in the second farm. This allowed a more specific understanding of the implications of antibiotic treatment on the udder microbiota.

In Chapter 4, the analysis of Herd 2 data is presented. A total of 440 quarter milk samples obtained from 22 cows across 5 time points were specifically selected based on criteria generated from the analysis of the first farm. This offered a unique perspective, analysing the effect of treatment in 2 independent longitudinal studies across the dry period.

In Chapter 5 the taxonomy of the bacterial community identified in the treatment groups of both Herds will be compared to further explore changes to the diversity of the milk microbiota between treatment groups over time.

Finally, in Chapter 6, the findings, conclusions and implications of this study will be discussed.

2. Methods

2.1. Introduction

In this thesis, the effect of antibiotic treatment on the mammary gland microbiome was investigated. The change in the bacterial community in the mammary gland following a non-antibiotic and antibiotic treatment was compared along with the change in the immune response of the udder. This effect was analysed in 2 herds. Patterns found in Herd 1 informed the selection of milk samples to be analysed from Herd 2. The same methodology for sample collection, storage, DNA extraction, sequence processing and data analysis were used for both herds to allow comparison. The process of this methodology will be outlined in this chapter.

Milk sample collection and storage for both herds was carried out by Ed Smith and Emma Monaghan. For Herd 1, Bacterial DNA extraction, sequencing and OTU alignment was carried out by Ed Smith and Emma Monaghan.

2.2. Sample information

2.2.1. Herd information and milk sample collection

Milk samples were collected from the udder quarters of two summer grazed, winter housed Holstein-Friesian dairy cow herds in a longitudinal study of two separate English farms in 2014. Both herds were milked twice daily in a hygienic rotary parlour. Herd 1 constituted a large dairy herd of 663 cows. In total 109 cows were enrolled into the Herd 1 study. A total of 82 cows were enrolled in the Herd 2 study from the separate second farm. All cows in the study had not been treated for clinical mastitis in the previous month and all had four functional mammary gland quarters.

In both herds, milk samples were collected at 11 time points from individual udder quarters of each cow. The first sample was taken at the end of lactation, drying off, the next was taken following the dry period after calving. Samples were then taken on days 1, 3, 5, 7, 10, 14, 17, 21 and 28 post calving. Sampling was conducted at these time points to assess changes over the dry period. Following calving, samples were taken frequently at intervals beginning at 2

days to 7 days between later time points. This is because after calving the somatic cell count and the microbiome can change rapidly early in lactation. As cows are also being milked twice daily the udder is highly perturbed, taking more frequent samples can provide a more accurate picture of the changes to the SCC and microbiome.

On all sampling days, 5 mL of milk was collected aseptically from each quarter. A calving control was taken on each calving occasion and dairy parlour controls were taken on every sampling occasion. The controls were collected in the respective calving and milking parlour environments by aerating a sampling tube containing 5 mL of phosphate buffered saline for the duration of time it took to collect the corresponding milk sample.

Immediately after collection, all samples were stored at 4°C and transferred on ice to the Warwick School of Life Sciences laboratory. Upon arrival, milk and control samples were aliquoted into a 2 mL barcoded sterile tube and a sterile glycerol solution was added to a final concentration of 10% (v/v). Samples were then stored at -80 °C until DNA extraction for determination of the milk microbiome.

From the large study enrolments a final dataset was selected for each Herd. This was based upon cows with no or very few missing, failed or contaminated samples. Herd 1 was filtered following data processing to contain 18 cows and a total of 791 samples for data analysis.
The Herd 2 dataset was also filtered based upon having cows with no or very few missing, failed or contaminated samples; then, filtered based on patterns established from the analysis of Herd 1. This focussed a selection of a balanced dataset in Herd 2 that could address hypotheses and questions raised from Herd 1. Herd 2 consisted of 22 cows and a total of 440 samples.

2.2.2. Antibiotic treatment

In both herds, immediately following the final milking at drying off, the teats were thoroughly disinfected and all cattle were treated. Half of the herd were allocated to receive an intramammary infusion antibiotic and a teat sealant and the other half received just a teat sealant. Allocation of antibiotic treatment was selected on a commercial basis by the farmer.

Cows with a somatic cell count over 200,000 cells/mL in pooled milk samples (from all quarters of the udder) recorded before drying off were selected for antibiotic treatment. Those below this threshold received just the non-antibiotic teat sealant. Analysis will be carried out at a quarter level to understand the dynamics of the udder within cows. This means that some individual quarters with a low SCC at drying off may have been selected for antibiotic treatment based on the pooled value of the whole udder.

All cows in Herd 1 were treated with a non-antibiotic teat sealant, active ingredient Bismuth Subnitrate (OrbeSeal® Dry Cow, Zoetis UK limited, Surrey, UK), in each of the four quarters at drying off. Half of the herd selected for data analysis (9 cows) received an antibiotic treatment in addition to the teat sealant with an active ingredient of either; Cefquinome (Cephaguard DC® 150 mg, Virbac, Suffolk, UK; 5 cows), Cephalonium (Cepravin Dry Cow® 250 mg, MSD Animal Health, Milton Keynes, UK; 3 cows) or Cloxacillin Benzathine (Orbenin® Extra Dry Cow 600 mg, Zoetis UK Limited, Surrey, UK; 1 cow).

All cows in Herd 2 were treated with the non-antibiotic teat sealant, active ingredient Bismuth Subnitrate (OrbeSeal® Dry Cow, Zoetis UK limited, Surrey, UK), in each of the four quarters at drying off. Half of the herd, 11 cows, received and antibiotic treatment in addition to the teat sealant with the active ingredient Cephalonium (Cepravin Dry Cow® 250 mg, MSD Animal Health, Milton Keynes, UK).

The teat sealant provides a physical barrier to environmental pathogens. The intramammary gland antibiotics used are broad spectrum antibiotics active against Gram negative and positive bacteria.

2.2.3. Somatic Cell Counts

Somatic cell counts (SCC) are a quantitative measure of the dairy cow mammary gland immune response that can be used as a proxy to quantify the level of infection (Schukken *et al.*, 2003). SCC for each milk sample were determined by standard industry protocol (QMMS ltd., Somerset, UK; (Bradley & Green, 2005)). SCC were chosen as the most appropriate measure of host immune response as it is the most widely used measure of mastitis incidence

and well trusted across industry. Counts above 200,000 cells ml^{-1} are considered indicative of clinical mastitis cases.

2.2.4. Experimental workflow and controls

The experimental workflow for profiling the bacterial communities from milk samples is summarised in Figure 1. Samples were randomly assigned a position in 96-well plates to minimise systematic bias. For each plate, a calving and parlour control from the same sampling days were randomly selected and randomly assigned to each of the 96-well plates for DNA extraction. A negative control for the DNA extraction was also included on each plate, with nuclease free water used in place of a milk sample. A positive control constituting a model community (containing DNA from *Escherichia coli, Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae and Streptococcus uberis*) was used as a positive PCR and sequencing control. A blank PCR control containing no DNA in the reaction mix was also added to each reaction batch for each PCR stage, giving 2 PCR negative controls (PCR1 and PCR2). PCR controls ensured contamination was not introduced. All controls were carried through to sequencing. Parlour and Calving controls were used to removed contaminating species from the sequencing library. Stage 1 PCR amplified the V1-V3 variable region of the bacterial 16S rRNA gene. This region was selected as it is highly informative and produces a high-resolution for low ranked taxa on the Illumina MiSeq patfrom (Johnson *et al.*, 2019; Jumpstart Consortium Human Microbiome Project Data Generation Working, 2012). Sequencing of the V1-V3 region using the 27F and 534R primers captures a length of around 300 bp of the bacterial 16S rRNA . Stage 2 PCR attaches index primers with barcodes that can identify samples following pooled sequencing.

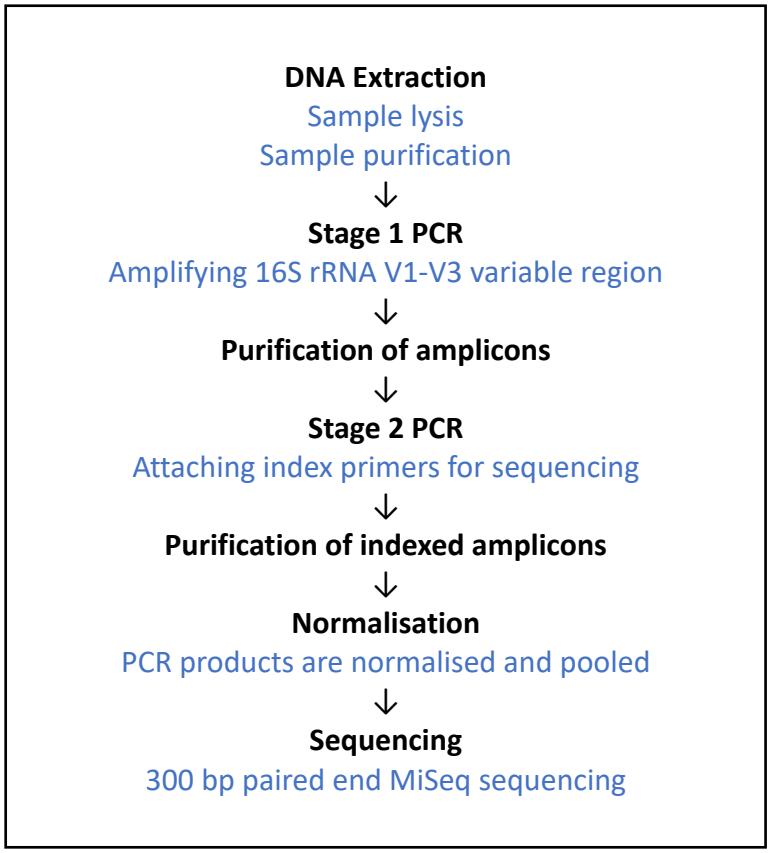


Figure 1. Workflow outline for the extraction, purification and sequencing of bacterial 16S rRNA from milk samples.

2.3. DNA extraction

The milk samples selected for the final datasets were randomised and assigned a position in 96 well plates to reduce systematic bias. The results outlined in this section are from the DNA extraction of herd 2 samples.

2.3.1. Sample lysis

DNA extraction followed published methods adapted from Hunt *et al.* (2011) and Yuan *et al.* (2012) to include an additional enzymatic incubation. Milk samples were defrosted, individually mixed and 550 µL was aliquoted into the assigned position on the 96-well plates.

A 50 µL enzyme mix (25 µL lysozyme [20 mg mL^{-1}]; 19 µL lysostaphin [650 U mL^{-1}]; 6 µL mutanolysin [25 KU / mL^{-1}]; all Sigma-Aldrich, UK) was added to the 550 µL of milk sample, mixed by swirling and incubated at 37°C for 1 hour in a static incubator. Cells were mechanically lysed by adding 0.2 g of 0.1 mm glass beads to the sample lysate and were bead-beaten at 18000 rpm in 3, 30 second rounds (Mini-Beadbeater-96, Biospec Products Inc, USA). Further purification and isolation of bacterial DNA from the milk samples was completed using a Qiagen QIAamp 96 DNA Blood kit (Qiagen, UK).

Plates were centrifuged at 4000 rpm (3200 g) for 2 minutes to pellet the glass beads [Eppendorf 5810R; A-4-62]. The sample lysate was aliquoted into a fresh deep well 96 plate [Axygen P-DW-20-C-S] containing 50 µL Proteinase K (11.1 mg/mL, Sigma P2308) and 500 µL of buffer AL, the sample was mixed by pipetting up and down 5 times and incubated at 56 °C for 30 minutes in a static incubator.

2.3.2. Sample Purification

Sample lysate was combined with 500 µL absolute ethanol and 100 µL 3M NaOAc pH 5.2 (Fisher Scientific: BP334-500, UK) and mixed. The lysate was applied to the 96-well QIAamp plate held in an S-block (QIAmp kit). The plate was sealed with an AirPore tape sheet (QIAmp kit) and centrifuged at 3500 rpm (2,013 × *g*) for 8 min, with the flow though discarded. Two washing steps were carried out, first 500 µL Buffer AW1 (QIAamp kit) was added to each well and centrifuged at 3500 rpm (2,013 × *g*) for 4 min. Then 500 µL Buffer AW2 (QIAamp kit) was added to each well in the plate and centrifuged at 3500 rpm (2,013 × *g*) for 25 min. 30 µL of 70 °C 10 nM Tris HCl pH 7.5 was applied to the elution tube membrane (Tris, Fisher Scientific: BP154-1; HCl, Sigma H1758), incubated at room temperature for 1 minute and centrifuged 3500 rpm (2,013 × *g*) for 8 min to elute the DNA. Eluted DNA was stored at -80 °C until PCR amplification.

2.4. PCR amplification and Sequencing

2.4.1. Stage 1 PCR: Amplifying the bacterial 16S rRNA gene

Stage 1 PCR amplified the V1-V3 variable region of the bacterial 16S rRNA gene (Figure 2.). 27F and 534R primers were modified for the Illumina MiSeq platform (Illumina, San Diego, CA, USA) to include an overhang adapter sequence, Table 1. (Jumpstart Consortium Human Microbiome Project Data Generation Working, 2012; Ravi *et al.*, 2018). This allows for the complimentary addition of a barcode sequence in the second stage PCR. The reactions were performed by adding 1 µL of DNA product to a master mix consisting of, 0.5 µL (0.1 µM) of each primer (forward and reverse), 25 µL Bioline MyFi mix 2X (Mederian Bioscience, USA), 0.5 µL BSA (0.1 mg/ml, Sigma-Aldrich, UK), 2.5 µL DMSO (5%, Sigma-Aldrich, UK) and 20 µL nuclease free water (VWR Chemicals, UK) to reach a final reaction volume of 50 µL. The PCR conditions for amplification were: 1) An initial denaturation of 95 °C for 1 minute; 2) 35 amplification cycles of 95 °C for 15 seconds, 58 °C for 15 seconds and 72 °C for 15 seconds; and 3) Final extension at 72 °C for 2 minutes. Samples were stored at -20°C until purification.

Primer	Sequence 5'-3' (adapter sequence underlined)
Rd1_27F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGAGTTTGATCCTGGCTCAG
Rd2-534R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATTACCGCGGCTGCTGG

Table 1. Stage 1 PCR primers for the amplification of the V1-V3 variable region of the 16S rRNA bacterial gene. 27F and 534R primers are modified for the Illumina MiSeq platform to include adapter sequences (underlined) that are complimentary to barcode sequences added in the second stage of PCR (Chapter 2. Section 2.4.3.).

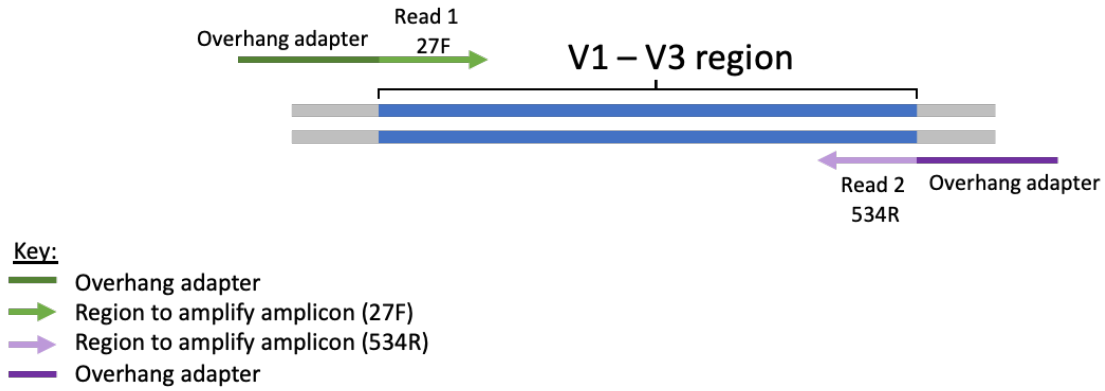


Figure 2. Amplification of the V1-V3 region of the bacterial 16S rRNA. Forward (27F) and Reverse (534R) primers were modified to include an overhanging adapter sequence complimentary to the stage 2 PCR primers which are used to attach a barcode sequence and a region specific for flow cell attachment on the illumina MiSeq system (Jumpstart Consortium Human Microbiome Project Data Generation Working, 2012).

To check the success of the PCR, 2 randomly selected samples, the positive control (model community) and PCR negative control were visualised by gel electrophoresis 1% (wt/vol) agarose gels stained with GelRed 1X concentration (Biotium, USA); using HyperLadder 1 kb as a scale (Meridianlifescience, Memphis, TN, USA). In Herd 2 the PCR was successful showing a band at the expected range for the model community and no DNA was present in the PCR blank (Figure 3.).

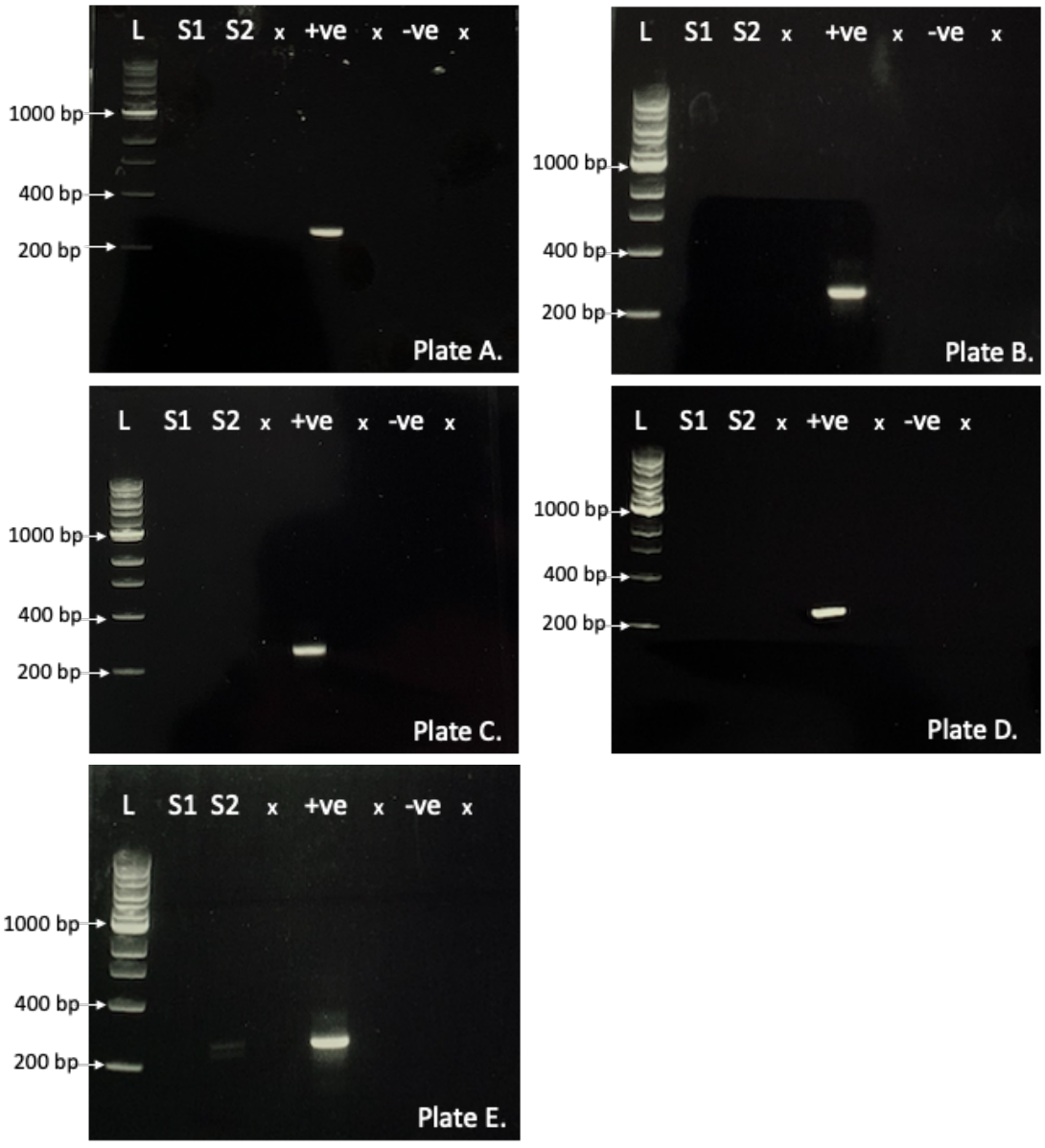


Figure 3. Gel electrophoresis of amplicons for stage 1 PCR, the amplification of the V1-V3 region of the 16S rRNA variable region of bacterial DNA, for the Herd 2 data set. The 440 milk samples were divided over 5 96-well plates. L, HyperLadder 1 kb; S1 and S2, random samples taken from the milk samples on that plate; x, blank lane; +ve, positive control (model community of known bacteria); -ve, PCR blank. The positive control was successful in all plates and there is no contamination of the negative control. Lack of DNA in the random milk samples is not of concern as it is unknown if or how many bacteria there will be in each sample.

2.4.2. Purification of stage 1 PCR products

Before the second stage PCR, all PCR products, including controls, were purified using the AMPure XP magnetic beads kit as recommended by the manufacturer (Beckman Coutler, High Wycomb, UK).

2.4.3. Stage 2 PCR: Adding index primers for the Illumina MiSeq platform

Unique dual index primer pairs were added to each sample in preparation for sample pooling and sequencing on the Illumina MiSeq platform (Illumina, San Diego, CA, USA). The primers comprised of; a sequence complimentary to the overhang adapter sequence added in stage 1 PCR, a unique 8 base index to identify the amplicons sample origin and a sequencing adaptor for the MiSeq platform (Table 2, Figure 4.).

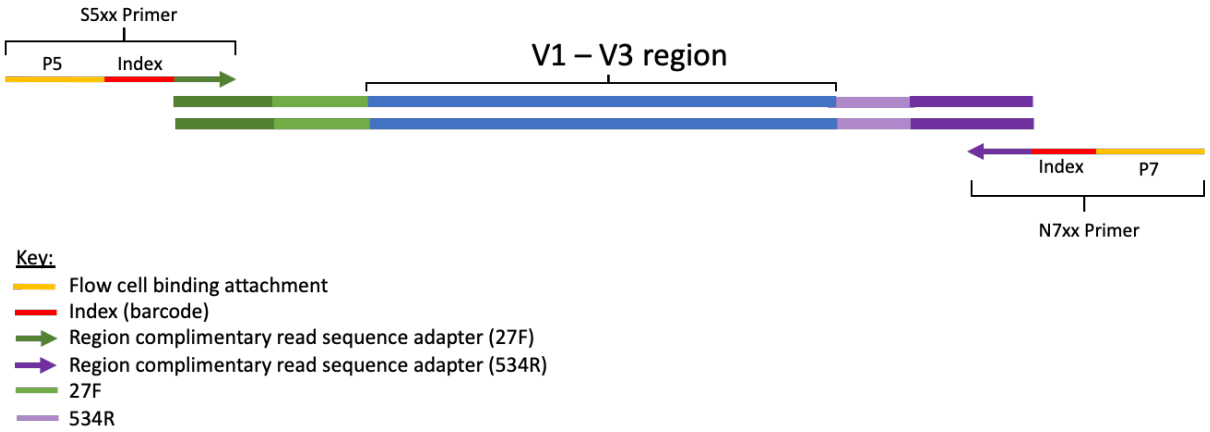


Figure 4. Amplification of the read sequence amplicon with addition of barcodes and flow cell adapter sequences complimentary to the Illumina MiSeq platform. A Unique dual index combination of Nextera S5 and N7 primers (S5xx and N7xx, Table 2.) were added to the Stage 1 PCR amplicons. Each primer consists of a region complimentary to the read sequence adapter sequence, an 8 base index and a region which binds to the flow cell of the Illumina platform. The 8 base index sequence is unique to each primer variation (Table 2.) allowing for pooling of samples for the sequencing platform and later sequence identification to the sample origin.

Primer	Sequence (5' - 3')
S502	AAT GAT ACG GCG ACC ACC GAG ATC TAC AC**C TCT CTA T**TC GTC GGC AGC GTC
S503	AAT GAT ACG GCG ACC ACC GAG ATC TAC AC**T ATC CTC T**TC GTC GGC AGC GTC
S505	AAT GAT ACG GCG ACC ACC GAG ATC TAC AC**G TAA GGA G**TC GTC GGC AGC GTC
S506	AAT GAT ACG GCG ACC ACC GAG ATC TAC AC**A CTG CAT A**TC GTC GGC AGC GTC
S507	AAT GAT ACG GCG ACC ACC GAG ATC TAC AC**A AGG AGT A**TC GTC GGC AGC GTC
S508	AAT GAT ACG GCG ACC ACC GAG ATC TAC AC**C TAA GCC T**TC GTC GGC AGC GTC
S510	AAT GAT ACG GCG ACC ACC GAG ATC TAC AC**C GTC TAA T**TC GTC GGC AGC GTC
S511	AAT GAT ACG GCG ACC ACC GAG ATC TAC AC**T CTC TCC G**TC GTC GGC AGC GTC
S513	AAT GAT ACG GCG ACC ACC GAG ATC TAC AC**T CGA CTA G**TC GTC GGC AGC GTC
S515	AAT GAT ACG GCG ACC ACC GAG ATC TAC AC**T TCT AGC T**TC GTC GGC AGC GTC
S516	AAT GAT ACG GCG ACC ACC GAG ATC TAC AC**C CTA GAG T**TC GTC GGC AGC GTC
S517	AAT GAT ACG GCG ACC ACC GAG ATC TAC AC**G CGT AAG A**TC GTC GGC AGC GTC
S518	AAT GAT ACG GCG ACC ACC GAG ATC TAC AC**C TAT TAA G**TC GTC GGC AGC GTC
N701	CAA GCA GAA GAC GGC ATA CGA GAT **TCG CCT TA**G TCT CGT GGG CTC GG
N702	CAA GCA GAA GAC GGC ATA CGA GAT **CTA GTA CG**G TCT CGT GGG CTC GG
N703	CAA GCA GAA GAC GGC ATA CGA GAT **TTC TGC CT**G TCT CGT GGG CTC GG
N704	CAA GCA GAA GAC GGC ATA CGA GAT **GCT CAG GA**G TCT CGT GGG CTC GG
N705	CAA GCA GAA GAC GGC ATA CGA GAT **AGG AGT CC**G TCT CGT GGG CTC GG
N706	CAA GCA GAA GAC GGC ATA CGA GAT **CAT GCC TA**G TCT CGT GGG CTC GG
N707	CAA GCA GAA GAC GGC ATA CGA GAT **GTA GAG AG**G TCT CGT GGG CTC GG
N710	CAA GCA GAA GAC GGC ATA CGA GAT **CAG CCT CG**G TCT CGT GGG CTC GG
N711	CAA GCA GAA GAC GGC ATA CGA GAT **TGC CTC TT**G TCT CGT GGG CTC GG
N712	CAA GCA GAA GAC GGC ATA CGA GAT **TCC TCT AC**G TCT CGT GGG CTC GG
N714	CAA GCA GAA GAC GGC ATA CGA GAT **TCA TGA GC**G TCT CGT GGG CTC GG
N715	CAA GCA GAA GAC GGC ATA CGA GAT **CCT GAG AT**G TCT CGT GGG CTC GG
N716	CAA GCA GAA GAC GGC ATA CGA GAT **TAG CGA GT**G TCT CGT GGG CTC GG
N718	CAA GCA GAA GAC GGC ATA CGA GAT **GTA GCT CC**G TCT CGT GGG CTC GG
N719	CAA GCA GAA GAC GGC ATA CGA GAT **TAC TAC GC**G TCT CGT GGG CTC GG
N720	CAA GCA GAA GAC GGC ATA CGA GAT **AGG CTC CG**G TCT CGT GGG CTC GG
N721	CAA GCA GAA GAC GGC ATA CGA GAT **GCA GCG TA**G TCT CGT GGG CTC GG
N722	CAA GCA GAA GAC GGC ATA CGA GAT **CTG CGC AT**G TCT CGT GGG CTC GG
N723	CAA GCA GAA GAC GGC ATA CGA GAT **GAG CGC TA**G TCT CGT GGG CTC GG
N724	CAA GCA GAA GAC GGC ATA CGA GAT **CGC TCA GT**G TCT CGT GGG CTC GG
N726	CAA GCA GAA GAC GGC ATA CGA GAT **GTC TTA GG**G TCT CGT GGG CTC GG
N727	CAA GCA GAA GAC GGC ATA CGA GAT **ACT GAT CG**G TCT CGT GGG CTC GG
N728	CAA GCA GAA GAC GGC ATA CGA GAT **TAG CTG CA**G TCT CGT GGG CTC GG
N729	CAA GCA GAA GAC GGC ATA CGA GAT **GAC GTC GA**G TCT CGT GGG CTC GG

Table 2. Stage 2 PCR primers, a unique dual index combination of Nextera S5 and N7 primers were added to the purified stage 1 PCR products. The sequence region at the 5' end identified the sequencing adaptor complimentary to the MiSeq platform, in bold a unique 8 base index

for identification and underlined a sequence complimentary to the overhang adaptor added in stage 1 PCR.

The 5 sample batches were split over 2 sequencing runs (Figure 5.). Batch E was split over run 1 and 2 each containing 44 samples and the 2 sampling controls (Calving and Parlour), the DNA extraction negative control, the positive model community control, the stage 1 PCR control and a second PCR stage 2 negative control. The index primers were added in pairings (Figure 5., Table 2.).

All controls from the previous stage 1 PCR were carried through into this reaction, (Sampling controls – calving and parlour controls, DNA extraction negative control, positive model community control, PCR negative control. A second PCR negative control was included for each sample batch in stage 2 PCR, containing no DNA in the reaction mix.

The reactions were performed by adding 5 µL of purified PCR product to a mastermix containing 25 µL 2X Bioline MyFi mix (Mederian Bioscience, USA), 16 µL nuclease free water (VWR Chemicals, UK), 2 µL i5 adapter (0.4 µM) and 2 µL i7 adapter (0.4 µM) to a final reaction volume of 50 µL. The PCR conditions for amplification were: 1) An initial denaturation of 95 °C for 3 minutes; 2) 8 amplification cycles of 95 °C for 15 seconds, 55 °C for 15 seconds and 72 °C for 15 seconds; and 3) Final extension at 72 °C for 5 minutes. Samples were stored at -20°C until a second purification.

Run 1

A		1	2	3	4	5	6	7	8	9	10	11	12
		N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
A	S502	1	2	3	4	5	6	7	8	9	10	11	12
B	S503	13	14	15	16	17	18	19	20	21	22	23	24
C	S505	25	26	27	28	29	30	31	32	33	34	35	36
D	S506	37	38	39	40	41	42	43	44	45	46	47	48
E	S507	49	50	51	52	53	54	55	56	57	58	59	60
F	S508	61	62	63	64	65	66	67	68	69	70	71	72
G	S510	73	74	75	76	77	78	79	80	81	82	83	84
H	S511	85	86	87	88	PC_A	CC_A	Pos_A	Neg_A	PCR_1_A	PCR_2_A		

B		1	2	3	4	5	6	7	8	9	10	11	12
		N716	N718	N719	N720	N721	N722	N723	N724	N726	N727	N728	N729
A	S502	1	2	3	4	5	6	7	8	9	10	11	12
B	S503	13	14	15	16	17	18	19	20	21	22	23	24
C	S505	25	26	27	28	29	30	31	32	33	34	35	36
D	S506	37	38	39	40	41	42	43	44	45	46	47	48
E	S507	49	50	51	52	53	54	55	56	57	58	59	60
F	S508	61	62	63	64	65	66	67	68	69	70	71	72
G	S510	73	74	75	76	77	78	79	80	81	82	83	84
H	S511	85	86	87	88	PC_B	CC_B	Pos_B	Neg_B	PCR_1_B	PCR_2_B		

E.i		1	2	3	4	5	6	7	8	9	10	11	12
		N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
A	S513	1	2	3	4	5	6	7	8	9	10	11	12
B	S515	13	14	15	16	17	18	19	20	21	22	23	24
C	S516	25	26	27	28	29	30	31	32	33	34	35	36
D	S517	37	38	39	40	41	42	43	44	PC_E	CC_E	Pos_E	Neg_E
E	S518	PCR_1_E	PCR_2_E										
F	S520												
G	S521												
H	S522												

		1	2	3	4	5	6	7	8	9	10	11	12
		N716	N718	N719	N720	N721	N722	N723	N724	N726	N727	N728	N729
A	S513												
B	S515												
C	S516												
D	S517												
E	S518												
F	S520												
G	S521												
H	S522												

Run 2

C		1	2	3	4	5	6	7	8	9	10	11	12
		N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
A	S502	1	2	3	4	5	6	7	8	9	10	11	12
B	S503	13	14	15	16	17	18	19	20	21	22	23	24
C	S505	25	26	27	28	29	30	31	32	33	34	35	36
D	S506	37	38	39	40	41	42	43	44	45	46	47	48
E	S507	49	50	51	52	53	54	55	56	57	58	59	60
F	S508	61	62	63	64	65	66	67	68	69	70	71	72
G	S510	73	74	75	76	77	78	79	80	81	82	83	84
H	S511	85	86	87	88	PC_C	CC_C	Pos_C	Neg_C	PCR_1_C	PCR_2_C		

D		1	2	3	4	5	6	7	8	9	10	11	12
		N716	N718	N719	N720	N721	N722	N723	N724	N726	N727	N728	N729
A	S502	1	2	3	4	5	6	7	8	9	10	11	12
B	S503	13	14	15	16	17	18	19	20	21	22	23	24
C	S505	25	26	27	28	29	30	31	32	33	34	35	36
D	S506	37	38	39	40	41	42	43	44	45	46	47	48
E	S507	49	50	51	52	53	54	55	56	57	58	59	60
F	S508	61	62	63	64	65	66	67	68	69	70	71	72
G	S510	73	74	75	76	77	78	79	80	81	82	83	84
H	S511	85	86	87	88	PC_D	CC_D	Pos_D	Neg_D	PCR_1_D	PCR_2_D		

E.ii		1	2	3	4	5	6	7	8	9	10	11	12
		N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
A	S513	45	46	47	48	49	50	51	52	53	54	55	56
B	S515	57	58	59	60	61	62	63	64	65	66	67	68
C	S516	69	70	71	72	73	74	75	76	77	78	79	80
D	S517	81	82	83	84	85	86	87	88	PC_E	CC_E	Pos_E	Neg_E
E	S518	PCR_1_E	PCR_2_E										
F	S520												
G	S521												
H	S522												

		1	2	3	4	5	6	7	8	9	10	11	12
		N716	N718	N719	N720	N721	N722	N723	N724	N726	N727	N728	N729
A	S513												
B	S515												
C	S516												
D	S517												
E	S518												
F	S520												
G	S521												
H	S522												

Figure 5. Layout of Herd 2 samples for sequencing runs on the Illumina MiSeq platform, with the dual index primers orientated for the Nextera index N7 primers 1-12 and S5 A-H. 440 samples (numbered in grey) are spilt across 2 sequencing runs. In blue: PC, parlour control; CC, calve control; Pos, positive control (model community); Neg, negative control (experimental); PCR_1, PCR stage 1 blank; PCR_2, PCR stage 2 blank.

Following stage 2 PCR, 2 randomly selected samples, the PCR positive and the second stage PCR negative controls were visualised by gel electrophoresis 1% (wt/vol) agarose gels stained with GelRed 1X concentration (Biotium, USA), to check for contamination and successful amplification; using HyperLadder 1 kb as a scale (Meridianlifescience, Memphis, TN, USA). In Herd 2 the PCR was successful showing a band at the expected range for the model community and no DNA was present in the PCR blank (Figure 6.).

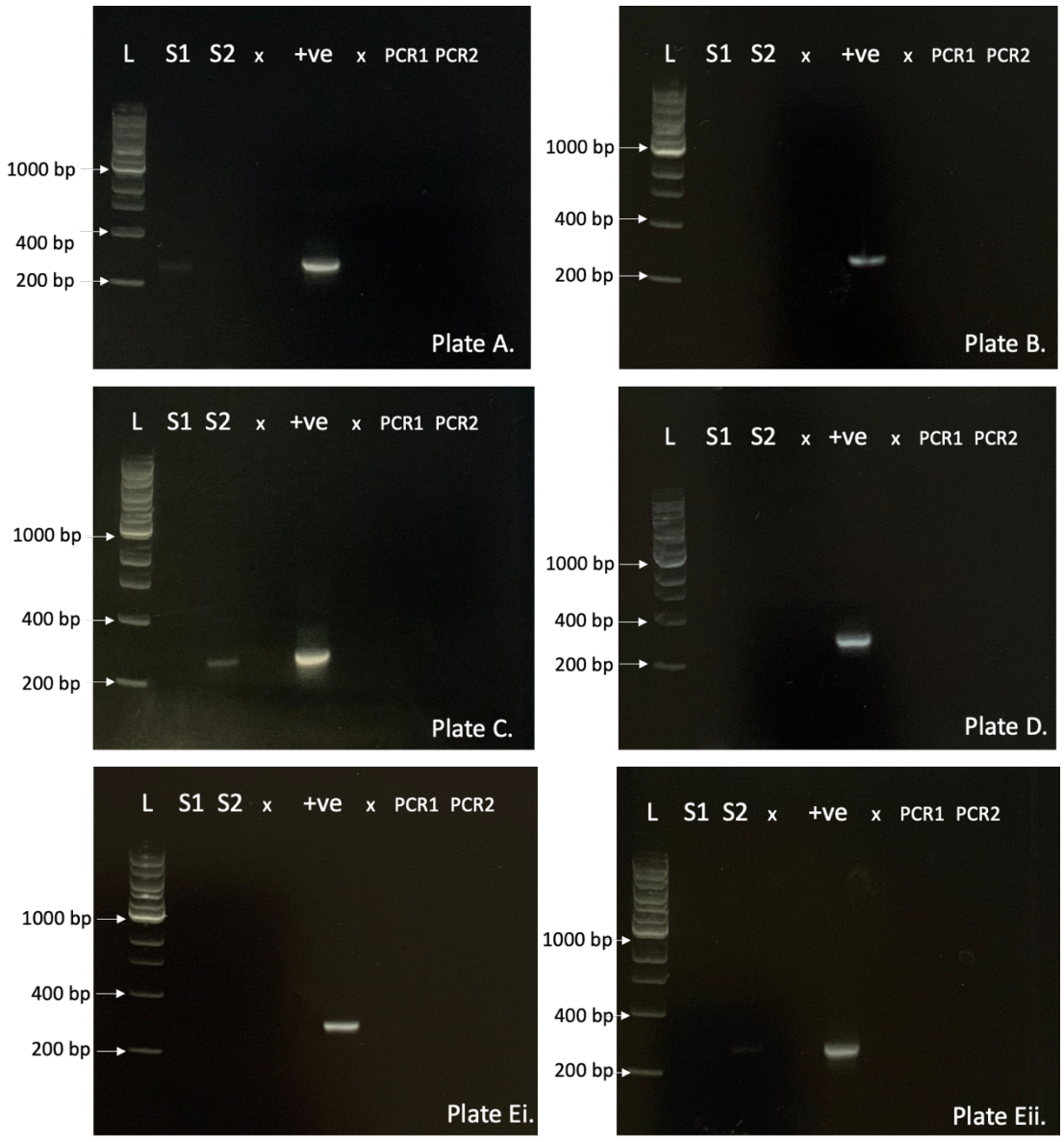


Figure 6. Gel electrophoresis of amplicons for stage 2 PCR, the addition of barcode sequences for sequencing for the Herd 2 data set. The 440 milk samples were divided over 5 96-well plates. L, HyperLadder 1 kb; S1 and S2, random samples taken from the milk samples on that plate; x, blank lane; +ve, positive control (model community of known bacteria); PCR1, PCR

stage 1 blank; PCR2, PCR stage 2 blank. The positive control was successful in all plates and there is no contamination of the negative control. Lack of DNA in the random milk samples is not of concern as it is unknown if or how many bacteria there will be in each sample. A faint band can be seen in S2 on plate C at the expected base pair length.

2.4.4. Purification of stage 2 PCR products

Before sequencing preparation, all PCR products, including controls, were purified using the AMPure XP magnetic beads kit as recommended by the manufacturer (Beckman Coutler, High Wycomb, UK).

2.4.5. Normalisation of PCR products and Sequencing

All sample and control amplicons were purified, pooled and normalised using the SequalPrep Normalisation Plate Kit and the sequential elution method recommended by the manufacturer (Invitrogen, Walton, MA, USA). The pooled samples for both library sample runs were normalised to a 4nM concentration using the Qubit dsDNA HS Assay kit (ThermoFisher Scientific, Waltham, MA, USA) and submitted to the University of Warwick Genomics Facility for 300bp end sequencing on the Illumina MiSeq Platform.

2.5. Processing sequencing data

This section details the methodology for Illumina MiSeq analysis of the 16S rRNA V1-V3 variable region for Herd 2 mammary gland milk samples. The sequencing library preparation protocol, data analysis pipeline and results for Herd 2 data processing.

2.5.1. Sequencing Metrics summary and processing work flow

2 libraries (238 samples in each) were prepared for Illumina MiSeq 300 bp paired-end sequencing as outlined in the methods above (Chapter 2 Section 2.4.). Library run data was downloaded and viewed in Illumina BaseSpace. Yields reported in reads 1 and 4 for both libraries are 7.47 Gbp and 6.71 Gbp which is within the expected range. Totalling 15.29 Gbp and 13.73 Gbp respectively, within the expected range for this type of Illumina Sequencing

(Illumina, 2022). The total number of reads generated in library 1 was 54,675,428 and library 2 generated 48,945,332 (Supplementary Materials Table 1.).

Raw sequence data was processed using custom Perl scripts and software platforms USEARCH8.1 (Edgar, 2010) and UPARSE (Edgar, 2013), the processing workflow is summarised in Figure 7.

Figure 7. Data processing of raw sequencing data was conducted using a combination of custom Perl scripts and software platforms USEARCH8.1 (Edgar, 2010) and UPARSE (Edgar, 2013).

2.5.2. Processing metrics

Read quality was checked on the fastQC and MultiQC platforms (Andrews, 2010; Ewels *et al.*, 2016). Read quality on the reverse reads tended to diminish across most samples for both libraries, this is a common quality issue with the sequencing kits used. To resolve this issue truncation between 10-30 bp was tested in data processing.

2.5.2.a. Merging forward and reverse reads

Forward and Reverse reads for each sample were merged using USEARCH8.1 testing the truncation of 0-30 base pairs on the Reverse reads allowing for 2 mismatches per sequence. Truncation of 30 bps was decided based on the % reads merged (Supplementary Materials Table 2). The mean percentage of reads merged in library 1 was 42.35% and 50.61% in library 2 (Figure 8.A. and Figure 8.B. respectively). The mean number of reads per file was 40,043 in library 1 and 42,965 in library 2.

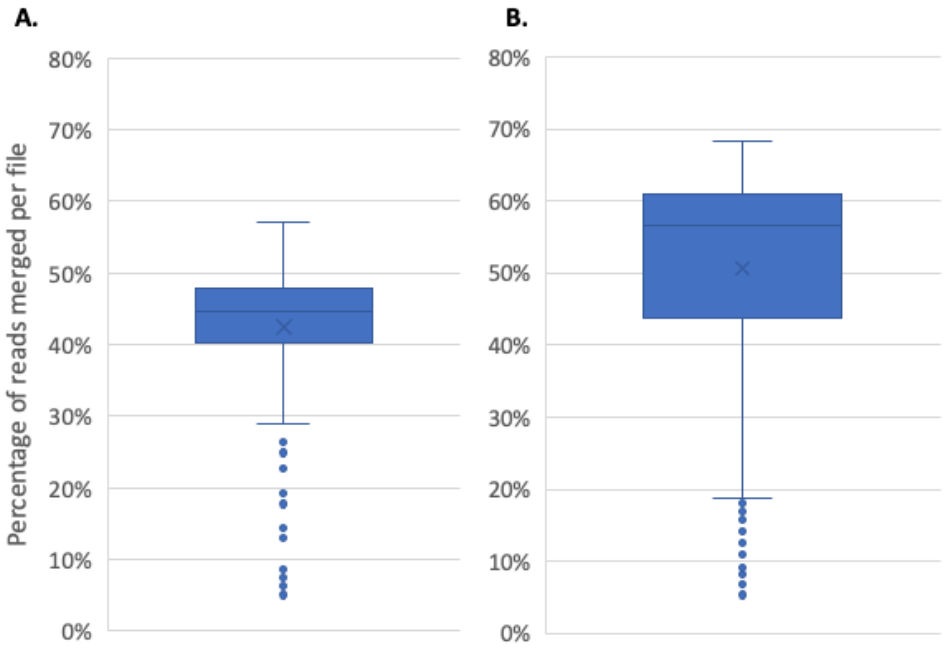


Figure 8. The distribution of the percentage of reads merged per file in library 1 (A.) and library 2 (B.) for the Herd 2 sequencing data, number of merged samples in both libraries = 476.

2.5.2.b. Quality filtering and dereplication

Sequence headers for the merged files were relabelled using a custom Perl script providing the correct formatting for quality filtering on the USEARCH8.1 platform. A maximum error rate of 0.005 (2 errors per 400 bp) was used to determine if the sequences were of sufficient quality. A minimum sequence length of 425 bp was assigned as the minimum acceptable sequence read length. Files were then dereplicated using USEARCH8.1, this removes identical sequences, leaving representative sequences for each sample (Table 3.A.). Control files for

both libraries were concatenated into a control libraries containing negative and positive controls, the sample libraries were also combined (Table 3.B.). All libraries were sorted by size using USEARCH8.1 and assigned a minimum size of 2 to remove all singleton files from the dataset (Table 3.B.).

A.

		Number of files	Dereplication 1
Library 1	Samples	220	6633947
	Negative Controls	15	213080
	Positive Controls	3	109678
Library 2	Samples	220	6649219
	Negative Controls	15	165327
	Positive Controls	3	51373

B.

Library	Number of files	Concatenated file	Sorted	Dereplication 2
Samples	440	13283166	2446696	2164173
Negative Controls	30	378407	61266	58145
Positive Controls	6	161051	20488	17644

Table 3. Files in both Herd 2 libraries were separated into sample, negative controls and positive control files and dereplicated removing identical sequences, the number of sequences in each file in each library is shown in A. Files from both libraries were concatenated, sorted by size using the minimum size of 2 to remove singletons and dereplicated again (B.).

2.5.2.c. Negative control filtering

To remove contaminants from the sample files, sequences recorded in the negative control files were filtered out of the sample files. 97, 98 and 99% matches to the sequences in the negative control files were tested using USEARCH8.1. (Table 4.).

Filtering score	% Reads matched	Sequences remaining in sample library	Sequences removed from sample library	Sort by size
0.97	56.8	935010	1229163	935010
0.98	51.2	1056890	1056890	1056890
0.99	40.9	1278958	885215	1278953

Table 4. Sequences found in the negative control samples were removed from the samples library to remove potential contamination using filtering scores of 97, 98 and 99% sequence matching using USEARCH8.1.

2.5.2.d. OTU clustering and chimera checking

Files for each of the negative control filtering scores were carried through the processing. Reads were clustered into Operational Taxonomic Unit (OTUs) based on 97% identity using UCLUST (Edgar, 2010) and checked for chimeras in UPARSE (Edgar, 2013) using the recommended 16S reference database from USEARCH8.1, RDP Gold (Table 5.). Chimeric sequences are artefacts that can be formed by the incorrect joining of 2 or more sequences, often occurring during PCR (Smyth *et al.*, 2010). The library is checked for these artifacts formed between parent sequences as they can be incorrectly interpreted as novel sequences, inflating diversity.

Filtering score	Number of OTUs	Chimeras
0.97	3495	155 (4.4%)
0.98	3685	146 (4.0%)
0.99	3817	135 (3.5%)

Table 5. The sequencing reads in the library were clustered into OTUs and checked for chimeras for each of the filtering scores tested.

2.5.2.d. Mapping Reads and creating an OTU table

The OTUs that have been defined for this library are used to map all of the quality filtered reads from the dataset with a similarity score of 97%. The number of unique OTUs in the dataset for each of the filtering scores tested is presented in Table 6.

Filtering score	% Reads matched	Hits	Non-hits	Number of unique OTUs
0.97	65.1	11835718	6355472	3340
0.98	83.7	15221841	2969349	3539
0.99	94.0	17099439	1091751	3682

Table 6. Sequences from the quality filtered dataset are matched to sequences in the representative OTU files generated based on a 97% similarity score.

The OTU table generated using a filtering score of 97% was chosen to proceed forward. Details of sequences and OTUs present in the negative control samples are detailed in Supplementary Materials Figure 1.

2.5.3. Assigning Taxonomy and control filtering

To further control for contamination, OTUs identified in negative controls were filtered out of the sample library. Unexpected OTUs identified in the positive control (the model community) were also filtered out of the library. Taxonomy was assigned to each OTU using the SILVA ACT SINA alignment service (Pruesse *et al.*, 2012).

162 OTUs out of 3340 were identified to be removed, 119 in negative controls, 14 in the positive controls, 29 were found in both controls (Figure 9.). All OTUs from the DNA extraction negative, Parlour controls and both PCR controls were removed. Positive controls which were removed were ones that did not belong to the genera from the model community (these were *Streptococcus, Staphylococcus* and *Escherichia*).

After removal of contaminant OTUs the total number of unique OTUs reduced from 3340 to 3178. 57.78% of the total reads were removed, equating to 4.85% of unique OTUs being removed. A conservative approach was taken to avoid misrepresenting the community. However this results in the removal of a large portion of reads, this can be expected in communities with lower levels of biomass present (Salter *et al.*, 2014). This was the case in Herd 2 compared to Herd 1, which had a low level of DNA in samples and thus more susceptible to higher levels of kitome contamination.

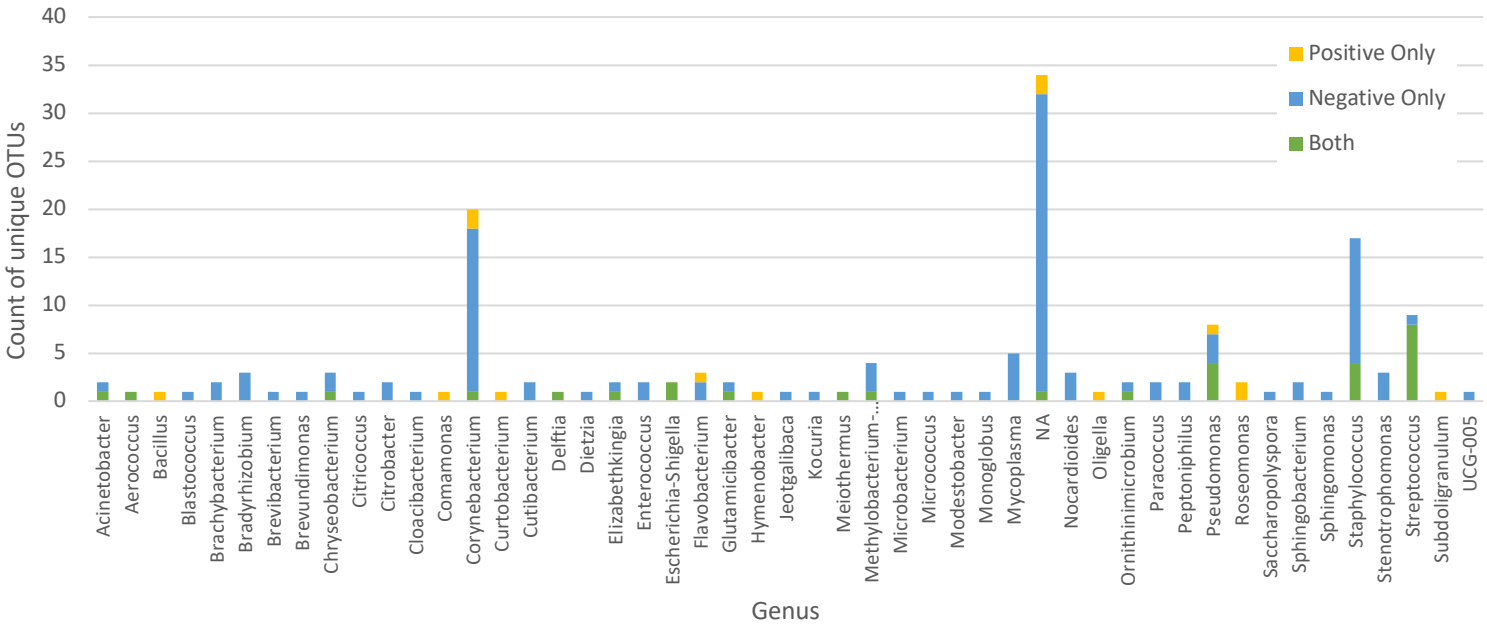


Figure 9. Summary of the unique OTUs filtered from the sample dataset identified in either the negative controls, positive control or both controls. The genus assigned to these OTUs is summarised. NA pools samples not identified to Genus level.

The quality filtered sample library was then reclustered against this filtered OTU library. At 97% clustering of the new filtered file, 3170 unique OTUs were detected 41% of reads were matched, with a total of 7,484,819 reads.

From the 3170 OTUs, 119 were unclassified and 3 were classified as Eukaryotes, these were removed resulting in a final OTU dataset for Herd 2 of 3048 unique OTUs and a total of 7,300,426 total reads.

2.5.3. Model community analysis

The model community contained DNA from *Escherichia coli, Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae and Streptococcus uberis.* The largest proportion of reads were assigned to the Genera *Streptococcus* and *Staphylococcus*, representing 51.1% and 48.7% of total reads (Table 7.). *Escherichia-Shigella* represents the next largest proportion however at only 0.2%, indicating it may not have been successful in the starting model community stocks.

Analysing OTU read counts, 3 of the 5 model community species were successful and represented the largest proportion of all reads (*Streptococcus* and 2 of the *Staphylococcus* species), with 1 of the *Streptococcus* species and the *Escherichia* genus appearing in low quantities (Table 7.). This indicates an issue with the level of a *Streptococcus* species and the *Escherichia coli* in the starting model community stocks which were from the same stocks as Herd 1.

Model community samples from Plate A-Ei are successful with the almost all reads originating from *Streptococcus* and *Staphylococcus* genera. The model community sample from plate Eii appears to have been unsuccessful with only 92 reads recorded.

Genus	Plate A	Plate B	Plate C	Plate D	Plate Ei	Plate Eii	Proportion represented (%)	Unique OTU count
Acinetobacter	0	0	2	0	0	0	0.0013	1
Aerococcus	4	0	0	0	0	0	0.0027	1
Bacillus	1	0	0	0	0	0	0.0007	1
Chryseobacterium	0	0	0	0	1	0	0.0007	1
Comamonas	0	0	0	1	0	0	0.0007	1
Corynebacterium	0	2	0	0	0	48	0.0335	3
Curtobacterium	0	0	0	2	0	0	0.0013	1
Delftia	0	1	0	0	0	0	0.0007	1
Elizabethkingia	0	2	0	0	0	1	0.0020	1
Escherichia-Shigella	53	34	57	40	92	0	0.1850	2
Flavobacterium	1	0	0	0	0	0	0.0007	1
Glutamicibacter	0	0	0	0	0	1	0.0007	1
Hymenobacter	1	0	0	0	0	0	0.0007	1
Meiothermus	0	0	1	0	0	0	0.0007	1
Methylobacterium-Methylorubrum	0	0	0	0	2	0	0.0013	1

Oligella	0	0	0	0	0	1	0.0007	2
Ornithinimicrobium	2	1	0	0	0	0	0.0020	1
Pseudomonas	1	0	0	0	1	40	0.0282	1
Roseomonas	0	1	0	0	0	1	0.0013	5
Staphylococcus	22190	14215	12076	10738	16946	0	51.0626	2
Streptococcus	8961	13812	9615	13780	26406	0	48.6551	4
Subdoligranulum	1	0	0	0	0	0	0.0007	8
Unclassified	0	1	0	0	0	0	0.0007	1
NA	5	4	3	3	9	0	0.0161	1
Grand Total	**31220**	**28073**	**21754**	**24564**	**43457**	**92**	**100**	**43**

Table 7. Summary of the number of reads for each Genus identified in the 6 model community samples (1 for each sampling plate) and the unique OTUs assigned to each genus. The largest proportion of OTU reads were seen in the Genus for the expected model community, *Staphylococcus* and *Streptococcus*, representing 51.06% and 48.66% respectively. These two genera contained 3 of the 5 model community species, the final model community species from the genus *Escherichia* was less prolific than expected, indicating an issue with the original stocks.

2.6. Analysing microbial communities

The mammary gland microbiome for each sample was defined as the OTUs identified from bacterial 16S rRNA sequencing. The bacterial community in milk samples will be analysed using raw OTU data, described in section 2.6.3. and through computation and analysis of alpha diversity metrics.

2.6.1. Calculating diversity and richness indices

The phyloseq package in R (McMurdie & Holmes, 2013; R Core Team, 2017) was used to calculate diversity and abundance indices for each milk sample from the OTU reads. The Chao1 index is a non-parametric alpha diversity abundance-based estimator of species richness. In this context, the Chao1 index is a measure of expected OTUs in each sample based on OTUs identified in all samples (Chao *et al.*, 2006). The Shannon diversity index is a metric which estimates species richness and evenness while considering relative abundance; the

Shannon index score will increase as the number of OTUs increase and the distribution amongst the different OTUs become more even (Kim *et al.*, 2017; Lemos *et al.*, 2011). Together, an increase in both the Chao1 index and the Shannon diversity index would indicate a greater bacterial diversity in the sample relative to the measured population.

2.6.2. Data visualisation

The somatic cell counts (2.2.3.) and the diversity and abundance measures (2.6.1) were visualised using R. The distribution and variation of these variables for each treatment group was assessed and the variation seen within the cows udder over time for each treatment group was also assessed. The distribution of cattle parity between treatment groups was also visualised in Herd 1 and the distribution of the dry period length for both herds was compared between treatment groups.

2.6.3. Statistical analysis of OTUs

Methods of analysis were tested, developed and determined from the analysis of the Herd 1 dataset. The most effective analysis methods are described below. Then, the same methods of analysis were applied to the Herd 2 dataset, which was specifically selected to be more balanced and have more power in addressing hypotheses generated from the data exploration of Herd 1.

A non-parametric Spearman's rank correlation of the abundant OTUs were calculated and determined the association between the ranked order of abundant OTUs in the milk sample communities in the udder quarter of each cow across time. This gives insight in to how the microbial community changes overtime for each treatment group. A Kruskal-Wallis test was then performed to statistically compare the correlation scores between each treatment group.

2.6.4. Statistical modelling

Linear mixed effects (LME) models allow the prediction or inference of a continuous outcome variable using multiple measured independent variables. A quantitative relationship between

the outcome variable and independent variables (termed effects) is defined by the statistical model. In LME models both fixed and random effects are in-cooperated into the model. Fixed effects assume values/observations are independent, whereas random effects assume that there is some type of relationship between some of the values/observations of that variable, that there is not complete independence.

LME models were built using the lmer package in R (Bates *et al.*, 2015; R Core Team, 2017). Linear mixed effects analysis was conducted to compare the effect of antibiotic treatment and non-antibiotic treatment on the outcome variables; somatic cell count, Chao1 index and the Shannon index.

The model took the general form:

$$Y_{si} = \beta_0 + S_{0s} + \beta_i X_i + e_{si}$$

Where Y_{si} is the dependent variable, the outcome, predicted by the model. For the lmer models built these outcomes were the somatic cell count (SCC, mastitis infection level proxy) and the bacterial abundance and diversity measures, the Chao1 index and Shannon index (Figure 10.). β_0 denotes the intercept value. β_i describes the regression coefficient for the explanatory variable (X_i). These variables are termed fixed effects, effects which are assumed to be constant from one experiment to another (Barr *et al.*, 2013). The explanatory variables, described as fixed effects in the model, tested include treatment group, parity, the SCC and Chao1 index at the drying off time point (before treatment was administered), the length of the dry period and the udder quarter (Figure 10.).
S_{0s} is an offset term, this accounts for the deviation from the intercept β_0 caused by clustering in the data from a certain variable. Inclusion of this term allows for predictions for each grouped variable, in this model, Cow Id. The variation in the individual cows is accounted for by inclusion of the variable as a random effect, the model produces random intercepts for each individual cow. The individual effect of each cow (S_{0s}) is not estimated, but rather the model estimates the population distribution from which the effect of individual cows were drawn (Barr *et al.*, 2013). e_{si} is the error term accounting for the unexplained, unobserved, variation in the model.

Each fixed effect were tested for significance individually then a multivariable model was built with treatment group, the main explanatory variable of interest. Variables were added to the treatment models in a forward stepwise approach testing for confounding significance. Cow Id was used in all models as a grouping factor, telling the model Cow Id is a random effect and to assume the intercept is different for each Cow.

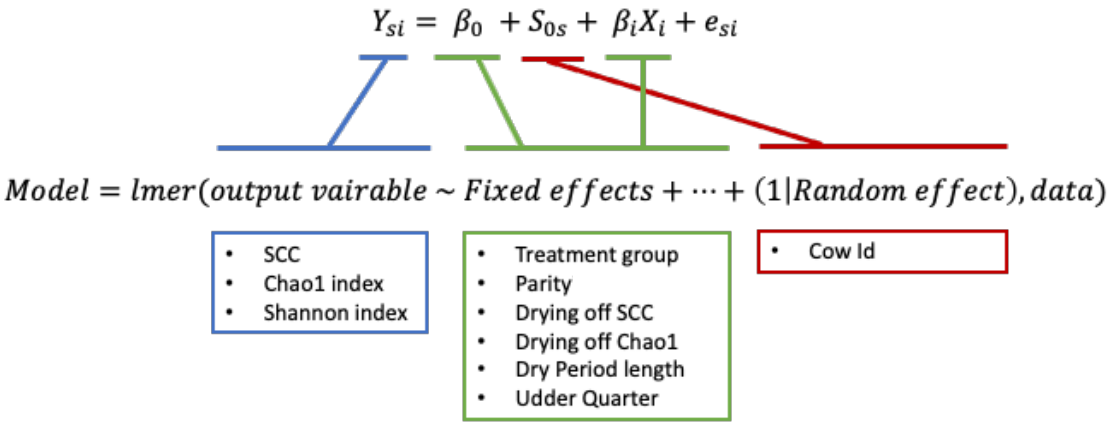


Figure 10. The general mathematical equation for the linear mixed effects models and the model format used in the lme4 package in R. Models were constructed to assess the effect of treatment on the somatic cell count (SCC), Chao1 index and Shannon index. Y_{si} is the term for the outcome variable predicted by the model described in the blue left-hand side box. β_0 denotes the intercept value. $\beta_i X_i$ is the term for the fixed effects, described in the green middle box. S_{0s} describes the inclusion of the random effect cow which allows estimation of random intercepts for this grouped variable in the model. e_{si} is the error term accounting for the unexplained, unobserved variation in the model.

Model performance was assessed by checking the model assumptions. Normality of the model residuals was visualised through a histogram and normal Q-Q plot. Outliers were checked for by assessing the deviance of the sample points against the theoretical values, from the theoretical normal line.

The model for each output variable was then statistically tested to further determine the effect of the fixed effect of interest, treatment group, on the model result. A likelihood ratio test of the model and a null model (with the treatment group fixed effect omitted), was conducted. The likelihood ratio test assesses the probability of the collected data being represented in the model (Bolker *et al.*, 2009). An Anova test was conducted to compare the likelihood ratios of the two models. If there is a significant difference, it can be inferred that the treatment group is influential to the model and therefore in influencing the result of the model outputs.

3. Analysis of Herd 1

3.1. Introduction

In this Chapter, the results for analysis of the Herd 1 dataset will be presented and discussed. Changes to udder health in terms of the somatic cell count and changes to the udder microbiota in terms of diversity indices and OTU correlations will be explored through data visualisations and statistical analysis. A comparison between the antibiotic and non-antibiotic treatment groups will be discussed in order to address the hypothesis outlined in Chapter 1, to investigate the impact of antibiotics on the mammary gland across the dry period into early lactation.

3.2. Methods

Methods outlining the collection, sequencing and analysis of the 16S rRNA microbiota of milk samples is outlined in Chapter 2. Briefly, to address the hypotheses outlined in the introduction, the impact of antibiotic treatment on the mammary gland microbiome was tested by the following analysis methods (outlined in detail in Chapter 2.6.):

- Visualising the change in the immune response (SCC) and in the diversity and abundance metrics (Chao1 and Shannon indices).
- Using statistical models to test if there is a clear difference in the above metrics between treatment groups.
- Statistical analysis of the ranked abundance of OTUs identified in individual milk samples with statistical comparison between groups.

3.3. Results

3.3.1. Overview of the Herd 1 dataset

The variables included in the filtered Herd 1 dataset were; Cow ID, udder quarter sample parity, treatment group, dates sampled, sample time point, somatic cell count (SCC) and the length of dry period (days).

From OTU counts, diversity and abundancy indices for each sample were calculated using the phyloseq package (McMurdie & Holmes, 2013) in R (R Core Team, 2017). These indices were the Chao1 index and Shannon index. The Chao1 index is a non-parametric alpha diversity abundance-based estimator of species richness. In this context, the Chao1 index is a measure of expected OTUs in each sample based on OTUs identified in all samples (Chao *et al.*, 2006). The Shannon diversity index is a metric which estimates species richness and evenness while considering relative abundance; the Shannon index score will increase as the number of OTUs increase and the distribution amongst the different OTUs become more even (Kim *et al.*, 2017; Lemos *et al.*, 2011). Together, an increase in both the Chao1 index and the Shannon diversity index would indicate a greater bacterial diversity in the sample relative to the measured population. The SCC and Chao1 index scores were $\log_{10}$ transformed to normalise the data for analysis.

Herd 1 constituted a large Holstein-Frisian dairy herd of 663 cows. 109 cows were enrolled into a wider study and 22 were selected for sequencing in this study. Of the 22 cows, 18 were selected for further data analysis, this was based on having full datasets with all 4 udder quarters of each cow sampled and having a balance between the treatment groups.

All four udder quarters (LF, left fore; LH, left hind; RF, right fore; RH, right hind) of each cow were independently sampled at 11 time points across the dry period, giving a total of 72 samples at each time point. The time points are D, C0, PC1, PC3, PC5, PC7, PC10, PC14, PC17, PC21, PC28 (D, Drying off; C0, Calving day; PC, Post-Calving, 1-28 days post calving). One sample was missing from one quarter of cow 584, at time point PC28. Since the effect of treatment across the dry period into early lactation is of main interest for deeper analysis in this thesis, the inclusion of this cow in the data was permitted; this gave a total of 791 samples

in the whole dataset. Nine cows received an intramammary antibiotic treatment and a teat sealant at drying off (AB) and nine cows received just a teat sealant at drying off (Orb Only), those with a pooled milk sample from all udder quarters of the udder greater than 200,000 cells mL^{-1} were selected for the AB group, those with less than 200,000 cells mL^{-1} were selected for the Orb Only group.

Balancing of the dataset between the treatment groups was based on the SCC at the drying off time point. The SCC distribution was visualised. To improve the evenness of the distribution between treatment groups, removal of outlier samples was tested. Filtering of samples with SCC values between certain thresholds were also tested. This often resulted in removing individual quarter samples from different cows. Ultimately, it was decided that retaining cows with all four quarters of the udder sampled was the priority. This allowed the effect of possible relationships between udder quarters within and between cows, and the changes to the microbiome to be explored. Resulting in the herd of 22 filtering to the final data set size of 18 cows, by retaining only cows with 4 udder quarters sampled at each time point.

The SCC was log_{10} transformed to normalise the data. There is a greater mean log_{10}SCC in the AB group compared to the Orb Only group (2.13 and 1.96 respectively, Supplementary Materials Table 4.), this is equal to roughly 135,000 cells mL^{-1} in the AB group and 91,000 cells mL^{-1} (Figure 1.). The variation in SCC of samples taken at drying off is comparable between treatment groups ($F = 1.24$, $p > 0.05$; Supplementary Materials Table 5.A.). A two sample t-test comparing the means of the treatment groups reported there is not sufficient evidence to show the two treatment group means are not equal ($t(70) = 0.91$, $p = 0.36$, Supplementary Materials Table 5.B.).

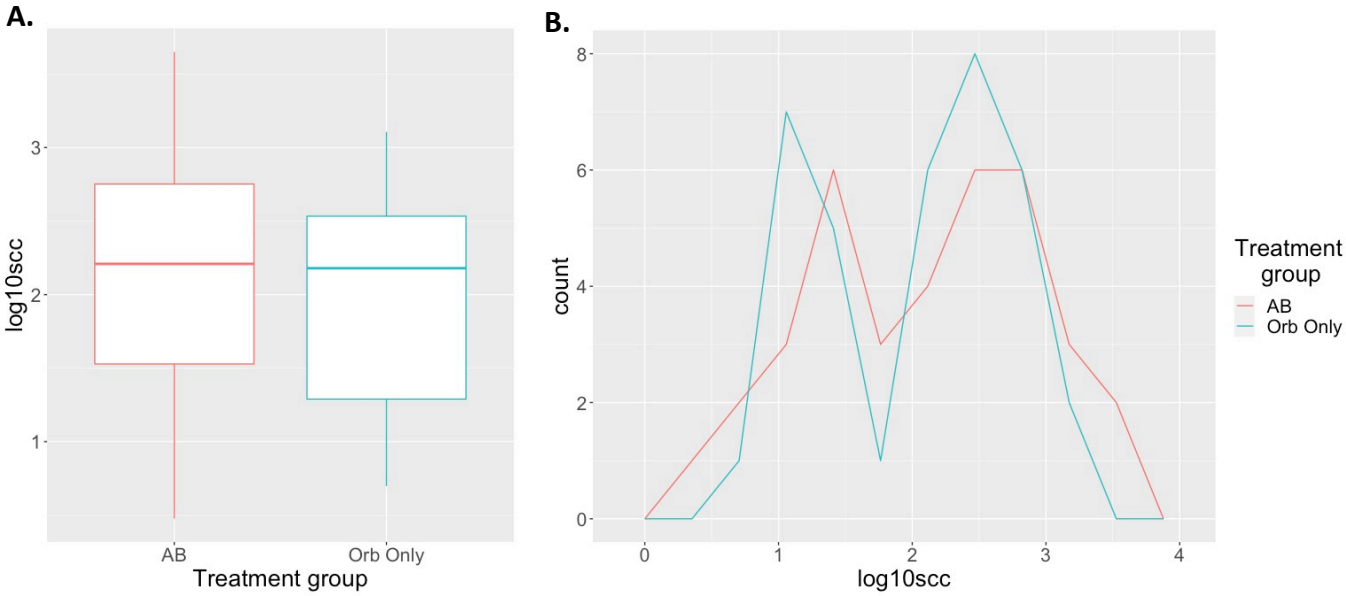


Figure 1. Variation in the somatic cell count (log_{10}SCC) between the two treatment groups (Antibiotic, AB and Non-antibiotic, Orb Only) at the drying off time point (n=72). Treatment was administered following sampling at this time point. The data is fairly well balanced between treatment groups with both showing wide variation within group (A). There is a greater mean log_{10}SCC in the AB group compared to the Orb Only group (2.13 and 1.96 respectively; B). A two sample t-test comparing the means of the treatment groups reported there is not sufficient evidence to show the two treatment group means are not equal ($t(70) = 0.91$, $p = 0.36$, Supplementary Materials Table 5.B.). The impact of any difference in SCC at drying off between treatment groups will be considered in later model analysis.

Variation in the other measured variables was considered. The majority of cows in the the herd are parity 2 (61%, Figure 2.A.). Parity describes the number of times the cow has calved, it is accepted that an increasing parity is associated with an increased susceptibility to clinical mastitis early in lactation (Green *et al.*, 2002). There are limited studies on the effect of increasing parity on changes to the microbiome, however it has been reported that primiparous cows have a significantly richer colostrum microbiome compared to multiparous cows, but showed no difference in the Shannon indices (Lima *et al.*, 2017). Parity will be considered, along with treatment group, in later analysis to assess potential confounding effects on the SCC, Chao1 index and Shannon index following the dry period.

The dry period is the the period of time prior to calving in which the cows are not milked. Having a dry period is important to cattle health, welfare, fertility and to the quality of milk (Kok *et al.*, 2019). The length of the dry period is generally shorter in the Orb Only group compared to the AB group, median values of 44 days and 50 days respectively (Figure 2.B.). The length of the dry period is not expected to have a confounding effect on treatment outcome but the effect will be tested in later analysis.

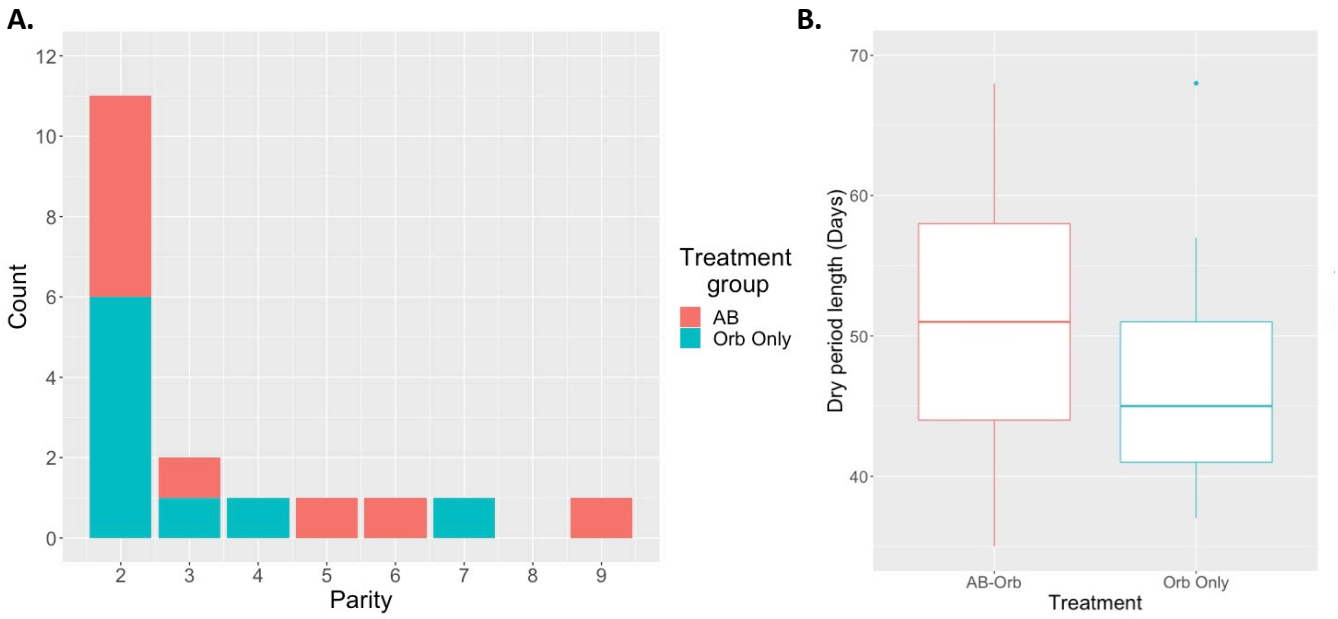


Figure 2. 61% of Herd 1 are Parity 2 cows, with mean parity in the antibiotic treatment group (AB) of 3.7 and an mean parity in the non-antibiotic group (Orb Only) of 2.9. There is not enough evidence to suggest the mean parity is significantly different between the two treatment groups (t=0.77, p>0.05;Figure 2.A). The median length of the dry period is 50 days in the AB group and 44 days in the Orb Only group and there is not enough evidence to suggest the mean dry period length of the two treatment groups is different (t= 0.64, p >0.05; Figure 2.B.). There are limitations in the statistics due to the small sample sizes (n=9) for each treatment group.

3.3.2. Somatic Cell Count

3.3.2.a Visualising somatic cell count variation following antibiotic treatment

The Somatic Cell Count (SCC) is a quantitative measure of immune cells in the mammary gland and is used as a proxy for measuring mastitis infection (Schukken *et al.*, 2003). The variation in the SCC between treatment groups across the dry period into early lactation will be presented. Linear mixed effects model analysis to determine the effect of antibiotic treatment on the early lactation SCC will be conducted.

The SCC increases following drying off and is highest at the calving time point (C0) in both treatment groups with a mean $\log_{10}$ SCC of 2.71 in the AB group and 2.98 in the Orb only group (Supplementary Materials Table 4.). This decreases most rapidly until 3 days post calving (PC3) and slowly decreases towards 28 days post calving (PC28). The median SCC remains higher in the Orb Only group across most time points compared to the AB treatment group (Figure 3.). The lowest mean $\log_{10}$SCC of 1.18 is reached at PC28 in the AB group and the lowest mean $\log_{10}$SCC for the Orb Only group is recorded as 1.26, 21 days post calving (Supplementary Materials Table 4.).

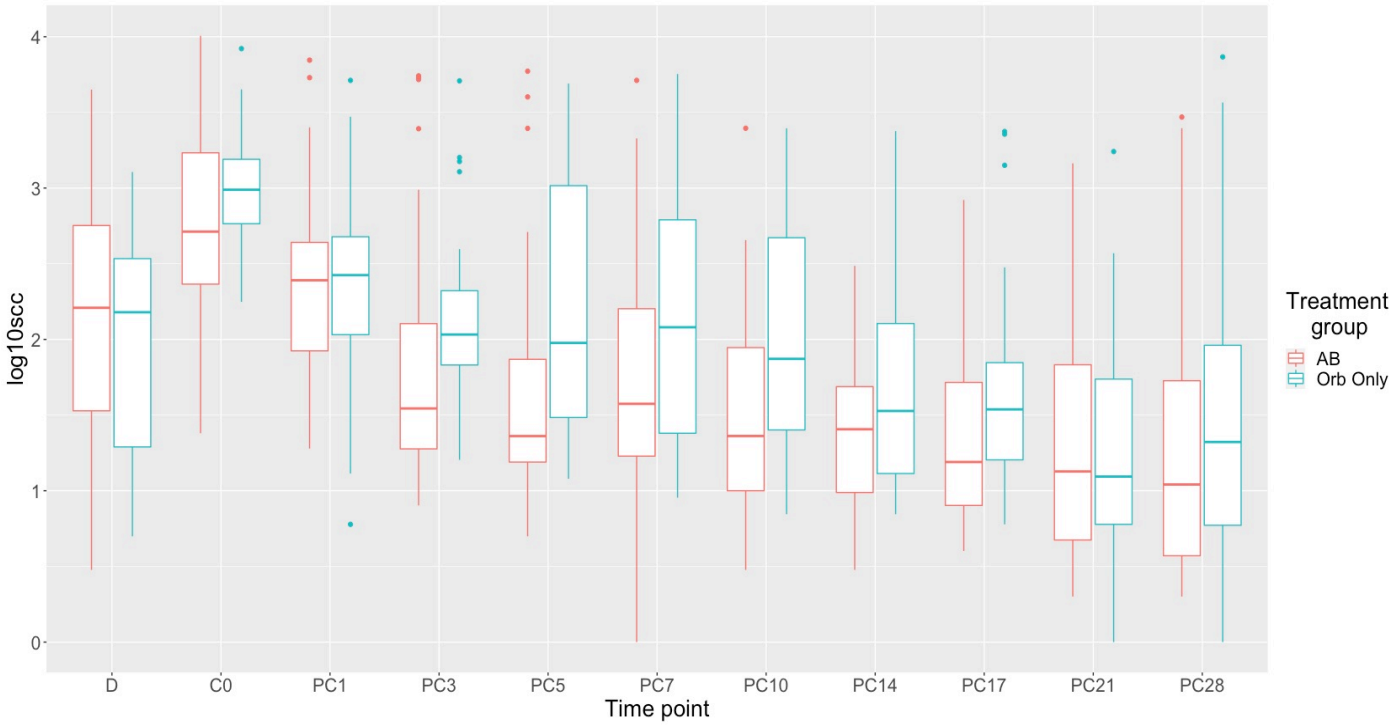


Figure 3. The mean log_{10}SCC peaks following the dry period at the calving (C0) time point for both the antibiotic (AB) and non-antibiotic (Orb Only) treatment groups, with a log_{10}SCC of 2.71 and 2.98 respectively (Supplementary Materials Table 4.). The median log_{10}SCC remains highest in the Orb Only group compared to the AB group across all time points following the dry period (C0 onwards) and decreases steeply until 3 days post calving (PC3) where the median SCC for both groups reduces below the drying off (D) level. (D, Drying off; C0, Calving day; PC, Post-Calving, 1-28 days post calving; n = 72 per time point, total n = 791).

There is great variation in the SCC at each time point and within each treatment group (Figure 3.). Visualisation of the changes in SCC per udder quarter per cow displays the volatility in SCC in each udder quarter (Figure 4., Figure 5.). While some udder quarters within the same cow appear to follow a similar pattern in SCC variation (e.g. Cow 599, Figure 4.B., Figure 5.B.), other udder quarters within the same cow experience high SCC spikes in only one quarter (e.g. Cow 371, Figure 4.B., Figure 5.B.). While the level of interaction between udder quarters within a cow on the subsequent incidence of infection is not fully understood, and is out of the scope of this thesis, its potential impact as a confounding effect for the impact of treatment group will be considered in later analysis.

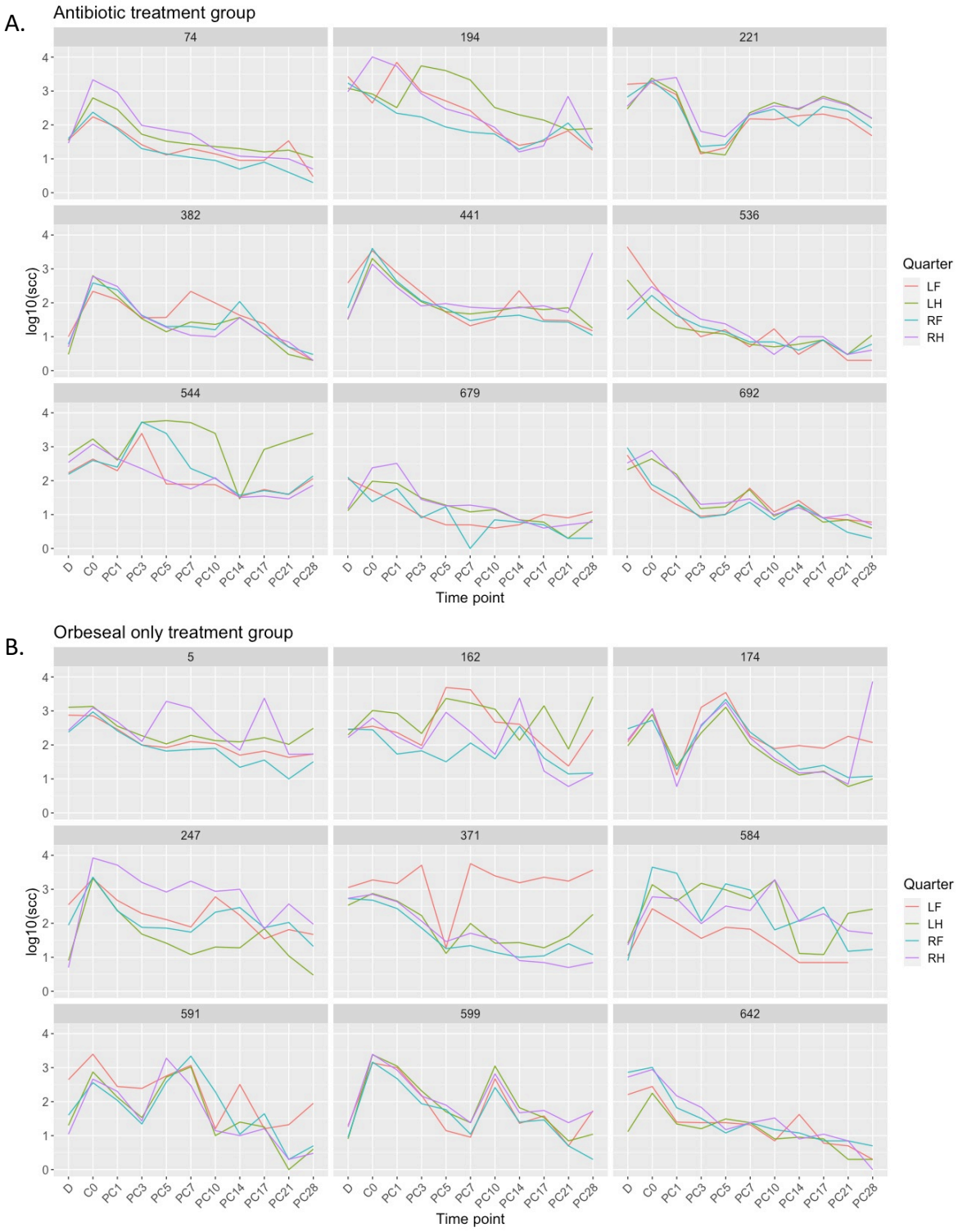


Figure 4. The change in somatic cell count (log_{10}SCC) per cow (numbered) per udder quarter over time for each treatment group, antibiotic (Figure 4.A.) and non-antibiotic (Figure 4.B.). Udder quarters: LF, Left Fore; LH, Left Hind; RF, Right Fore; RH, Right Hind. Time points: D, Drying off; C0, Calving day; PC, Post-Calving, 1-28 days Post Calving. Total n = 791. SCC tends to decrease over time for both treatment groups, but SCC levels can be turbulent within and between cows, increasing and decreasing drastically in a couple of days, this illustrates the high level of variation seen in Figure 3.

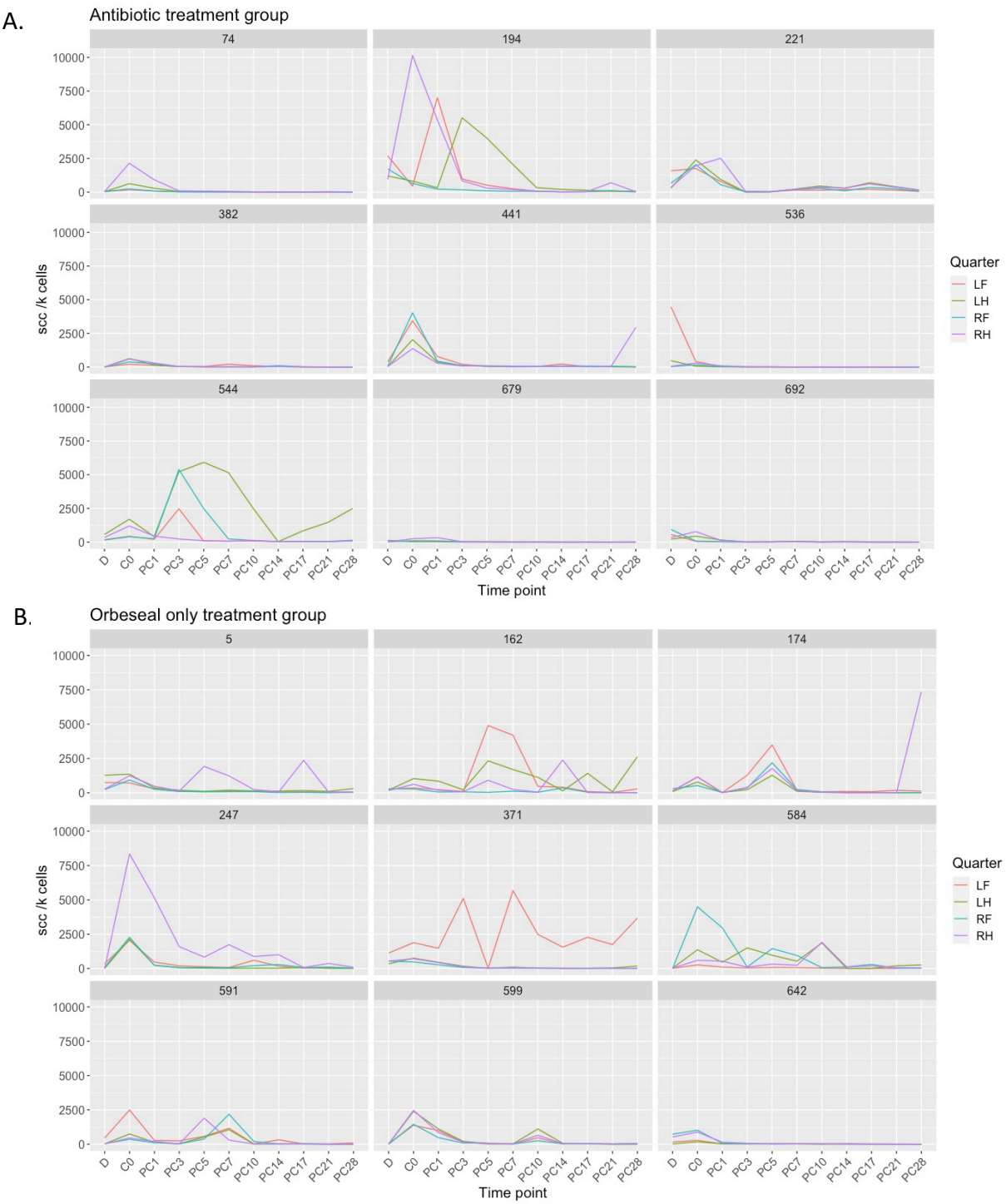


Figure 5. The change in somatic cell count (scc/ 1000 cells) per cow (numbered) per udder quarter over time for each treatment group, Antibiotic (Figure 5.A.) and non-antibiotic (Figure 5.B.). Udder quarters: LF, Left Fore; LH, Left Hind; RF, Right Fore; RH, Right Hind. Time points: D, Drying off; C0, Calving day; PC, Post-Calving, 1-28 days Post Calving. Total n = 791. Log transformation of Figure 4. to display the scale of the SCC spikes in the udder quarters as a measure per 1000 cells (k cells).

The first few days post partum are associated with an increased incidence of mastitis (Green *et al.*, 2002). To explore the effect of antibiotic treatment further following the dry period, a mean log_{10}SCC value for each of the samples at the time points calving (C0), 1 day post calving (PC1) and 3 days post calving (PC3) was calculated. Following the dry period there is an increase in the median SCC for both treatment groups (Figure 6.). In the antibiotic treatment group (AB) there was an increase in the mean SCC from 2.13 to 2.30 following the dry period representing an increase of 63,000 cells mL^{-1} (Supplementary Materials Table 4.). In the non-antibiotic group (Orb Only) there is an increase in the mean SCC from 1.96 to 2.48 representing an average increase in 210,000 cells mL^{-1} over the dry period.

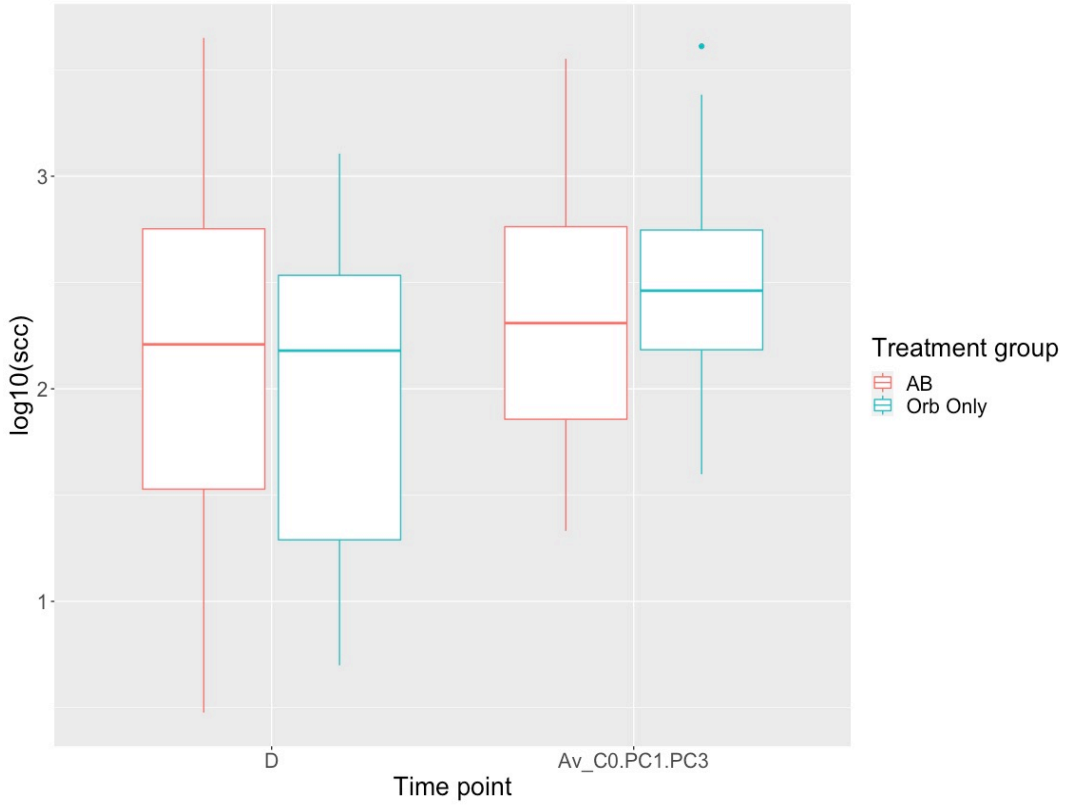


Figure 6. Distribution of the somatic cell count (log_{10}SCC) at drying off (D) and the average log_{10}SCC for samples at time points calving (C0), 1 day post calving (PC1) and 3 days post calving (PC3) termed Av_C0.PC1.PC3. Comparing the antibiotic (AB) and non-antibiotic (Orb Only) treatment groups, the mean log_{10}SCC for the AB group increases from 2.131 to 2.297 following the dry period, whereas, in the Orb Only group there is a larger increase in the mean log_{10}SCC from 1.963 at D to 2.477 at Av_C0.PC1.PC3.

3.3.2.b. Modelling somatic cell count following antibiotic treatment

The lme4 function from the lmer package in R (Bates *et al.*, 2015; R Core Team, 2017) was used to conduct linear mixed effects (LME) analysis of somatic cell counts (SCC) in early lactation following antibiotic treatment. The fixed effect of interest, treatment group (AB_Orb) was defined in all models tested. The mean value for SCC at the first three time points following the dry period (Calving, C0; 1 day post calving, PC1 and 3 days post calving, PC3) was calculated and used to reflect prolonged changes across the dry period following treatment. This was the outcome variable, denoted Av_log10scc.

The following fixed effects were tested in the model in a stepwise approach;

- Treatment group (AB_Orb, AB and Orb Only)
- Parity, binned into Parity 2 and > Parity 2 (Parity 2, Parity 3+)
- Somatic cell count at drying off (D.log10scc_centred)
- Chao1 index score at drying off (D.log10Chao1_centred)
- Length of the dry period in days (DPL_centred)
- Udder quarter (quarter; LF, left fore; LH, left hind; RF, right fore; RH, right hind)

Each were tested independently with the model outcome and included in all combinations with the fixed effect of interest, treatment group. Treatment group (AB_Orb) was included in all models as the aim was to understand if there is a difference between treatment groups on the outcome variable. The other fixed effects were tested and chosen if it were determined they were important to the model based on the T-value in the model and level of standard error, then, assessing if they had a confounding effect on the effect of treatment on the outcome variable testing if there was an increase in the T-value of the AB_Orb group if the fixed effect tested was included. All cows with a parity greater than 2 were grouped together giving a dichotomous variable (Parity 2/ Parity 3+), this was due to the low numbers of cows at the higher parity values (Figure 2.A.). The individual effect of Cow variation within the herd was assessed in the model by including it as a random effect. The log_{10}SCC and log_{10}Chao1 values at drying off (D), and dry period length effect were centred before addition into the

model. This allows for a more accurate representation of the intercept value and therefore a more meaningful interpretation of the variation between treatment groups in real terms.

The model chosen to best infer the impact of treatment group on the SCC following the dry period is presented. Model diagnostics, to assess if model assumptions are fulfilled, and further statistical analysis, testing the significance of the fixed effect treatment in the model, using the likelihood ratio test will also be described (Bolker *et al.*, 2009).

LME analysis of the relationship between SCC and antibiotic treatment was performed. As fixed effects, AB_Orb, Parity, D.log10scc_centred, D.log10Chao1_centred and quarter were entered into the model. As a random effect, intercepts for Cow Id were entered into the model (Table 1.).

Model equation is as follows:

Av_log10scc_C0.PC1.PC3 ~ AB_Orb + Parity + D.log10scc_centred + D.log10Chao1_centred + quarter + (1|cow)

Fixed effects	**Estimate**	**Std. Error**	**df**	**t value**	**Pr(>|t|)**	
(Intercept)	2.093	0.171	19.946	12.216	0.000	***
AB_Orb: Orb Only	**0.227**	**0.189**	**14.517**	**1.203**	**0.248**	
Parity 3+	0.353	0.207	17.620	1.705	0.106	
D.log10scc_centred	0.020	0.097	63.878	0.211	0.834	
D.log10Chao1_centred	0.062	0.128	58.950	0.485	0.630	
Quarter LH	0.086	0.115	53.009	0.752	0.455	
Quarter RF	-0.096	0.110	50.536	-0.869	0.389	
Quarter RH	0.207	0.116	52.445	1.774	0.082	.

Random effects			
Groups	Name	Variance	Std.Dev.
Cow	(Intercept)	0.132	0.364
Residual		0.100	0.316

Significance codes: 0 '***', 0.001'**', 0.01'*', 0.05'.', 0.1 ' , 1 '

Table 1. Linear mixed effects model predicting the influence of antibiotic treatment on the outcome of the predicted variable, the mean somatic cell count (SCC) following the dry period (Av_log10scc_C0.PC1.PC3), specifically the mean $\log_{10}$ somatic cell count for the time points

calving (C0), 1 day post calving (PC1) and 3 days post calving (PC3). Antibiotic treatment (AB_Orb), Parity and udder quarter were built into the model as fixed effects (LF, left fore; LH, left hind; RF, right fore; RH, right hind). The log_{10} of SCC and Chao1 index values at the drying off time point were centred and included as fixed effects in the final model (D.log10scc_centred and D.log10Chao1_centred, respectively). The effect of individual cow variation was accounted for in the model, included as a random effect. 72 observations, 18 groups.

Addition of the estimate values for the fixed affects allows comparisons of the predicted effect of treatment between cows. For example, the model reflects an estimate of the SCC following the dry period of an AB treated, Parity 2, LF quarter of a cow to be 123,880 cells $mL^{-1} \pm$ 1,483 (standard errors). The model estimates that in an Orb Only, Parity 3+, LH quarter of a cow the SCC would be 693,425 cells $mL^{-1} \pm$ 8,072 (standard errors). The estimate values generated by the model for each fixed effect are summed and the inverse log is calculated to determine the overall estimate value for the model outcome. The result is multiplied by 1000 to adjust the number of cells (SCC are recorded per 1000 cells).

The model estimates for the differences between the treatment groups vary compared to the SCC values recorded (AB 198,000 cells mL^{-1}, Orb Only 279,900 cells mL^{-1}; Figure 6., Supplementary Material Table 4.). Inspection of whether the effect of Parity could account for the disparity shows Parity 3+ cows have a much greater SCC compared to Parity 2 in both treatment groups (Figure 7.). However the model estimates are still not accurate. Comparing the estimate log_{10}SCC for the measured value Orb Only Parity 3+ cow (mean 338,844 cell mL^{-1}; Figure 1.) is far from the model estimate of over 690,000 cells mL^{-1}. However, when grouped by treatment and Parity there is an outlier value in the Orb Only, 3+ Parity group which may account for the discrepancy (Figure 7.). The log_{10}SCC value measured for this outlier is 3.38 equating to 2,398,832 cells mL^{-1} . Removal of the outlier value was tested but did not improve model performance, it is also not warranted as the value was within reasonable bounds of the whole dataset.

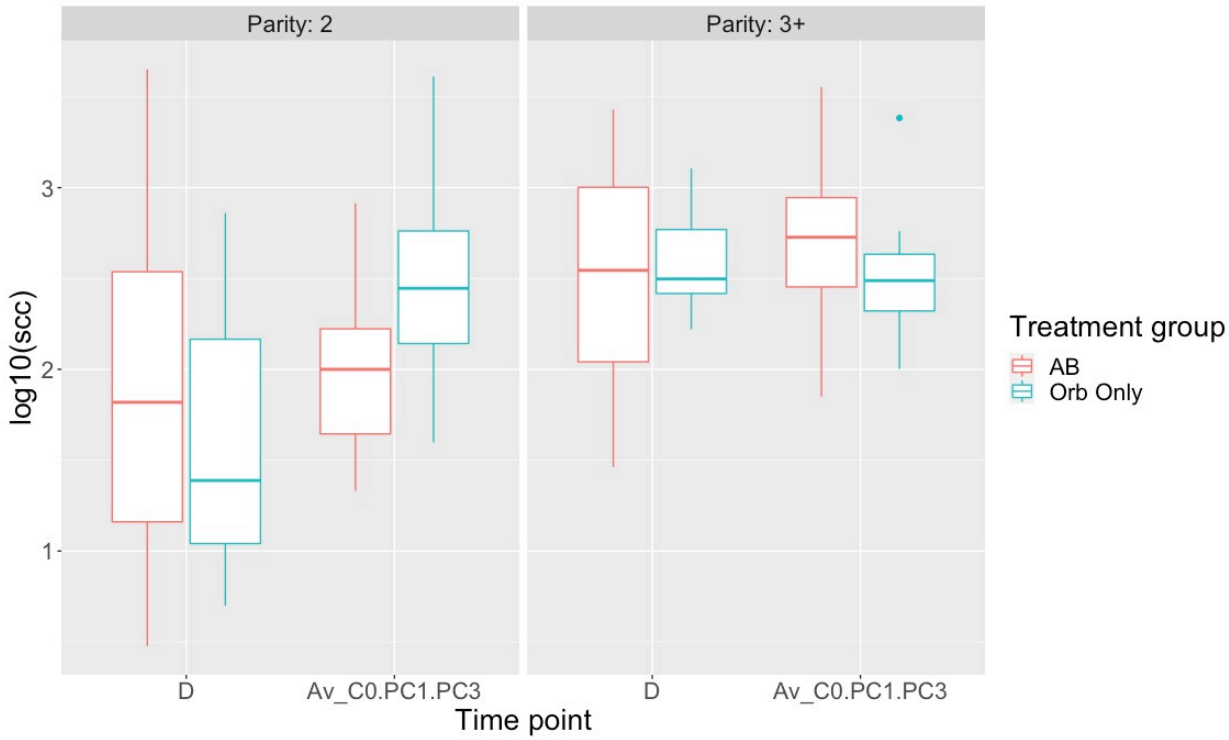


Figure 7. The somatic cell count (SCC) at the drying off time point (D) and the mean SCC for time points calving (C0), 1 day post calving (PC1) and 3 days post calving (PC3). Data is grouped into parity 2 and parity 3+. Boxplots compare the variation within and between treatment groups (AB, antibiotic; Orb Only, non-antibiotic). Mean SCC is higher in parity 3+ cows for both treatment groups compared to parity 2 cows. There is wide variation in SCC for both parities overall. There is a difference in means based on parity and treatment group following drying off (Av_C0.PC1.PC3). $\log_{10}$SCC: AB parity 2, 1.99; AB parity 3+, 2.68; Orb Only parity 2, 2.45; Orb Only parity 3+, 2.53.

Visual inspection of the model assumption parameters indicated no major deviation from homoscedasticity or normality of the residuals (Figure 8.).

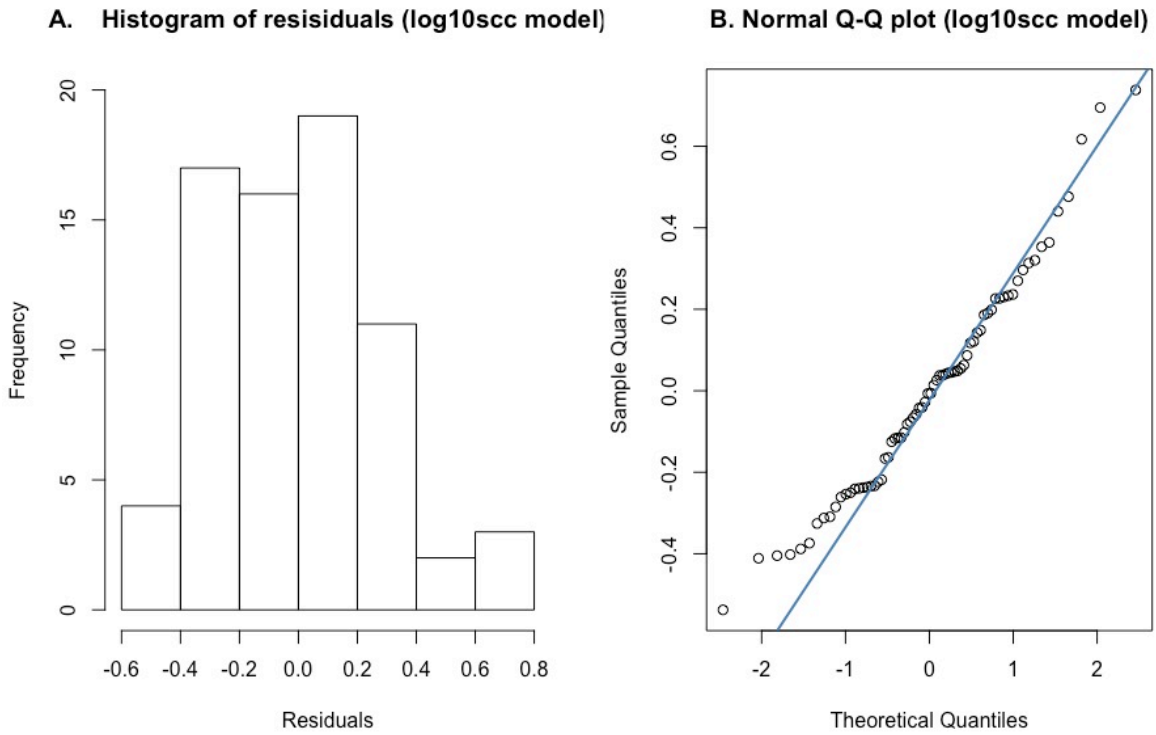


Figure 8. Histogram of residuals (A) and normal Q-Q plot (B) for the somatic cell count model. Residuals appear normally distributed with slight right-hand skewing. The Q-Q plot also shows some deviation from the theoretical normal line but not at a great enough threshold to violate the model assumption parameters.

To assess model significance a likelihood ratio test of the somatic cell count (SCC) model and a null model was conducted. The null model uses the same parameters as the SCC model but omits the fixed effect of interest, treatment group (AB_Orb). The likelihood ratio test assesses the probability of the collected data being represented in the model (Bolker *et al.*, 2009). An Anova test was conducted to compare the likelihood ratios of the two models. If there is a significant difference, it can be inferred that the treatment group is influential to the model and therefore in influencing the result of the model outputs.

Model equations are as follows:

SCC model: Av_log10scc_C0.PC1.PC3 ~ AB_Orb + Parity + D.log10scc_centred + D.log10Chao1_centred + quarter + (1|cow)

SCC null model: Av_log10scc_C0.PC1.PC3 ~ Parity + D.log10scc_centred + D.log10Chao1_centred + quarter + (1|cow)

Herd	Model	Df	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
1	SCC null	9	82.63	103.12	-32.31	64.63			
	SCC	10	82.93	105.69	-31.46	62.93	1.70	1	0.192

Significance codes: 0 '***', 0.001'**', 0.01'*', 0.05'.', 0.1 ' , 1 '

Table 2. Anova analysis comparing the likelihood ratio tests for the SCC model model and SCC null model. The fixed effect treatment group was removed in the null model allowing the effect of treatment group to be statistically assessed. A significant difference between the two models could not be determined ($\chi^2(1)= 1.70$, $p = 0.192$). It cannot be concluded that with the inclusion of treatment group in the SCC model that it is more likely to see data collected in the model to when the effect of treatment group is omitted.

While explicit significance scores were not seen in the model (Table 1.) or in Anova analysis (Table 2.), it is important to consider this analysis together with the context of measured SCC values (Figure 6.) to fully address the impact of antibiotic treatment on the early lactation SCC in Herd 1. This will be discussed later in the Chapter.

3.3.3. Chao1 index

3.3.3.a Chao1 index variation following antibiotic treatment

The Chao1 index is a non-parametric abundance-based estimator of species richness (Chao *et al.*, 2006). In this context, the Chao1 index is a measure of expected OTUs in each sample based on OTUs identified in all samples. The Chao1 index was calculated using the phyloseq package in R (McMurdie & Holmes, 2013; R Core Team, 2017). The impact on species richness of the mammary gland microbiome following antibiotic treatment will be explored using the same analysis applied to somatic cell counts (Chapter 3. Section 3.3.2).

The Chao1 index decreases following drying off (D), reaching the lowest levels across the week following calving (C0 to PC7). There is slight rise in both treatment groups in the median Chao1 index towards 28 days post calving (PC28), however there is large variation (Figure 9).

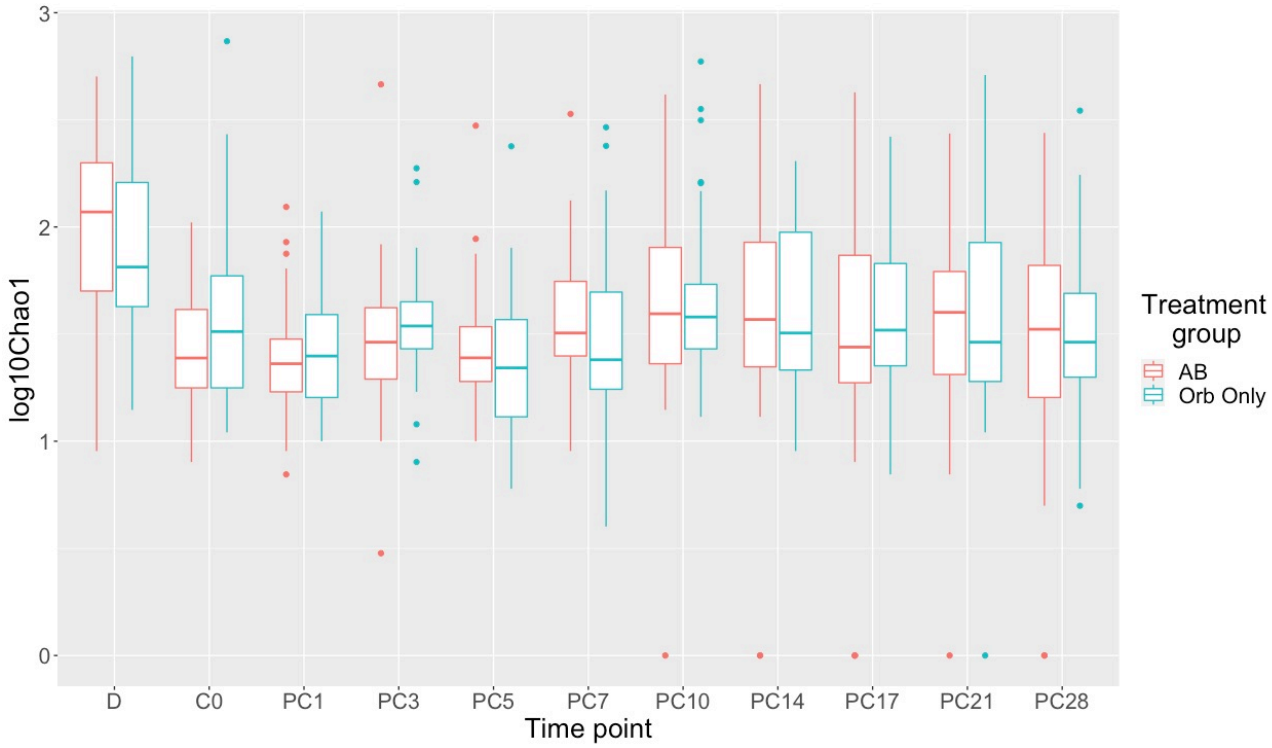


Figure 9. The change in log_{10}Chao1 index from drying off (D) to 28 days post calving (PC28) for antibiotic (AB) and non-antibiotic (Orb Only) treatment groups. The mean Chao1 index score is largest at D for both treatment groups, with a log_{10}Chao1 index score of 1.98 in the AB group and 1.86 in the Orb Only group. The Chao1 index score lowers following calving (C0 to PC1) and begins to level out in both groups across the remaining time points. (D, Drying off; C0, Calving day; PC, Post-Calving, 1-28 days post calving; n = 72 per time point, total n = 791).

There is great variation in the Chao1 index scores at each time points for both treatment groups (Figure 9.). To explore this variation the changes in Chao1 index scores at each time point, for each cow and each quarter was visualised (Figure 10., Figure 11.).

While some udder quarters within the same cow appear to show a fairly consistent Chao1 index scores (e.g. Cow 591, Figure 10.B., Figure 11.B.), often the Chao1 index within the udder of each cow can vary greatly even between consecutive time points (e.g. Cow 194, Figure 10.A., Figure 11.A.).

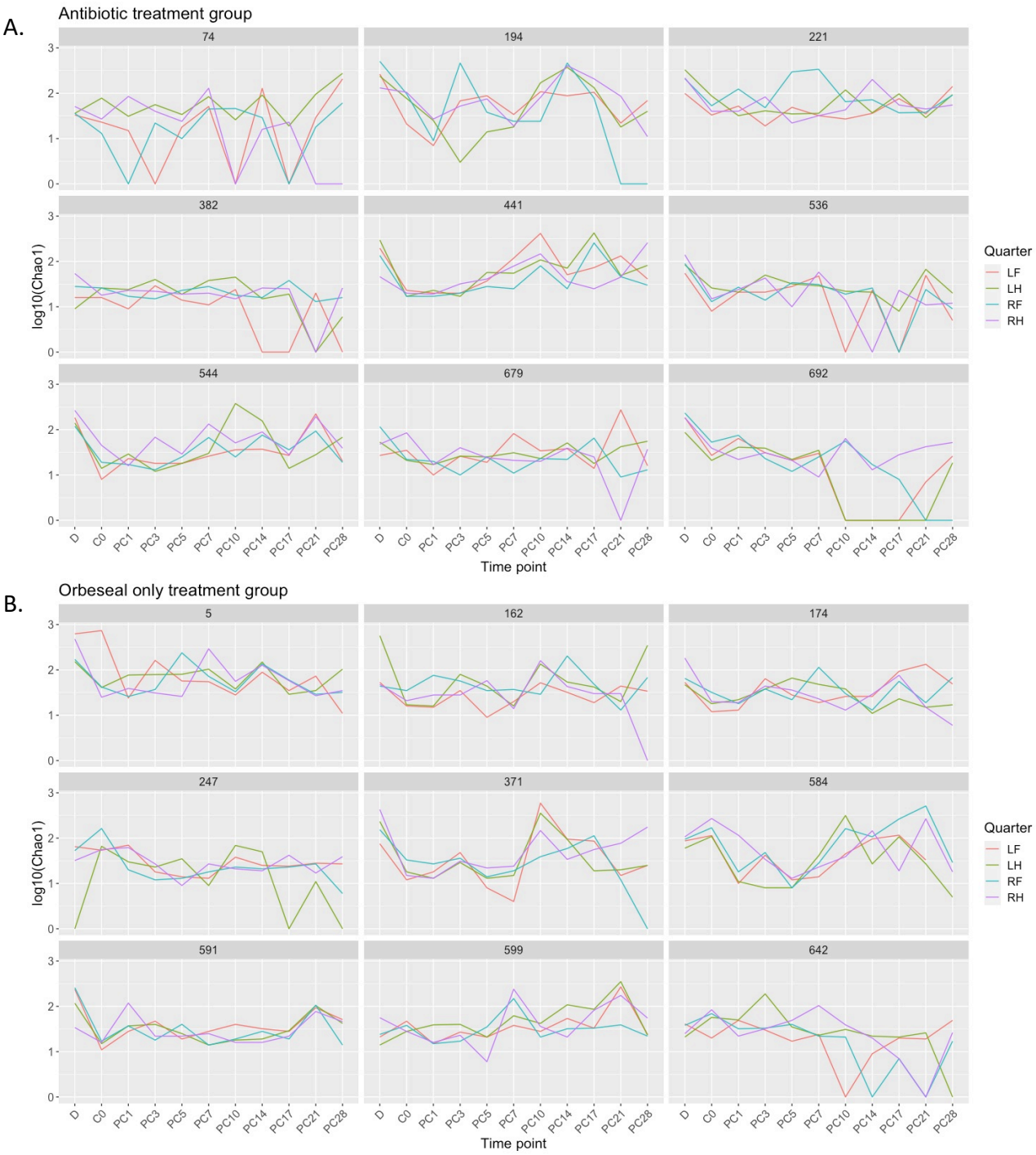


Figure 10. The change in the Chao1 index (log_{10}Chao1) per cow (numbered) per udder quarter over time for each treatment group, antibiotic (Figure 10.A.) and non-antibiotic (Figure 10.B.). Udder quarters: LF, Left Fore; LH, Left Hind; RF, Right Fore; RH, Right Hind. Time points: D, Drying off; C0, Calving day; PC, Post-Calving, 1-28 days post calving. Total n = 791. Chao1 index scores appear high in most samples at (D) with an unclear pattern in the following 28 days post calving.

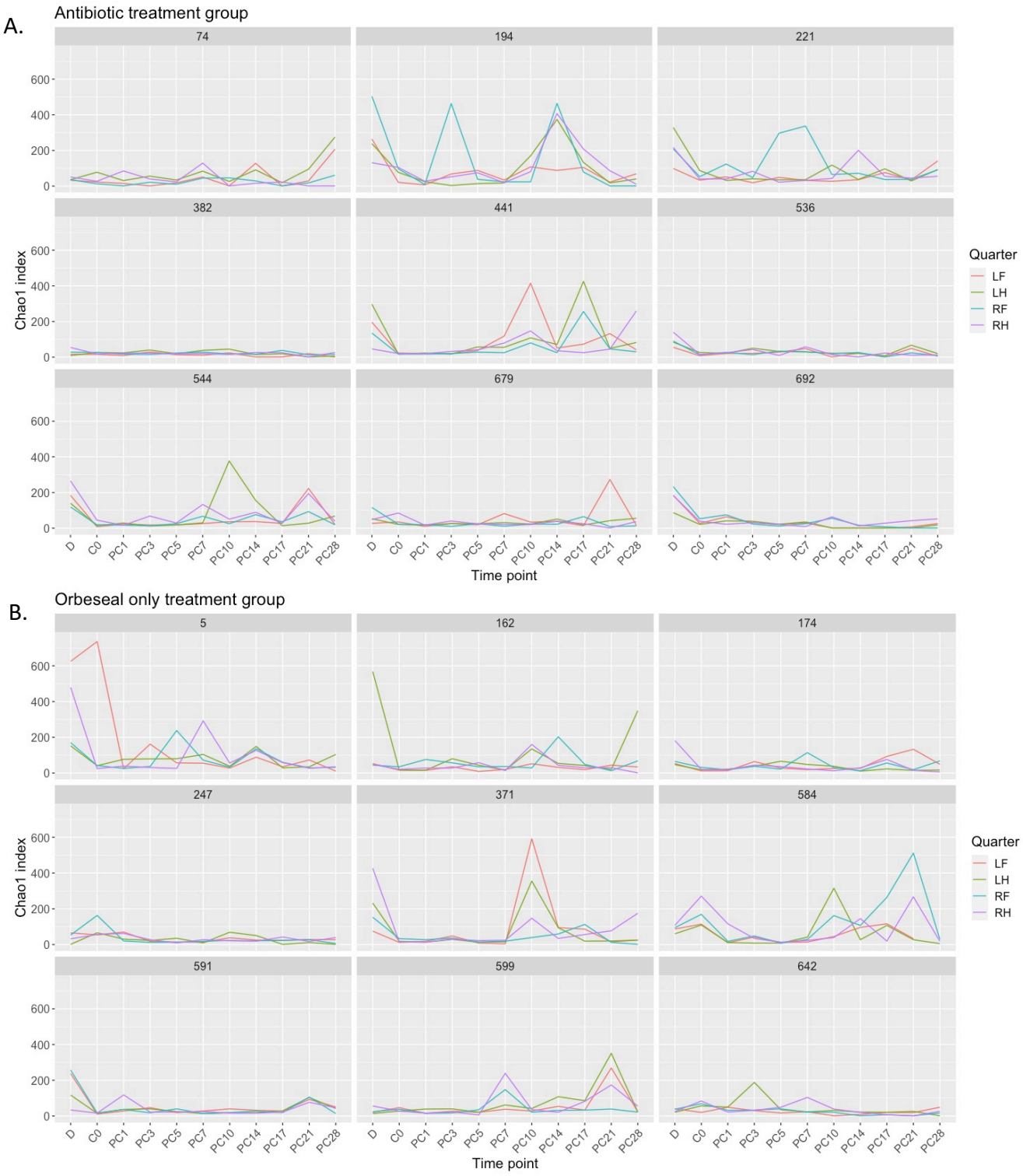


Figure 11. Variation in the Chao1 index per cow (numbered) per udder quarter over time for each treatment group, antibiotic (Figure 11.A.) and non-antibiotic (Figure 11.B.). Udder quarters: LF, Left Fore; LH, Left Hind; RF, Right Fore; RH, Right Hind. Time points: D, Drying off; C0, Calving day; PC, Post-Calving, 1-28 days post calving. Total n = 791. Plotting on a non-log scale highlights the spikes in the Chao1 index experienced in some cows (e.g. Cow 441 and Cow 371, Figure 11. A. and B. respectively).

To explore the effect of antibiotic treatment in the first few days post-partum following the dry period, a mean log_{10}Chao1 index score for each of the samples at the time points calving (C0), 1 day post calving (PC1) and 3 days post calving (PC3) was calculated. Following the dry period there is a decrease in the median Chao1 index for both treatment groups (Figure 12.). In the antibiotic treatment group (AB) there was a decrease in the mean log_{10}Chao1 from 1.98 to 1.40 following the dry period. In the non-antibiotic group (Orb Only) there was a decrease in the mean log_{10}Chao1 from 1.86 to 1.52 over the dry period (Supplementary Materials Table 6.).

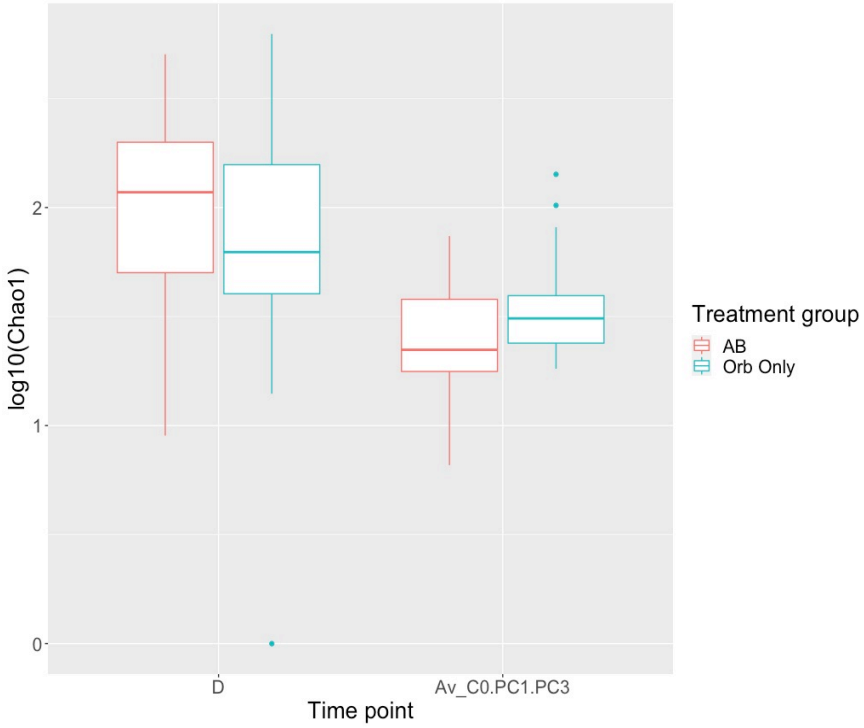


Figure 12. Distribution of the Chao1 index (log_{10}Chao1) at drying off (D) and the average log_{10}Chao1 for samples at time points calving (C0), 1 day post calving (PC1) and 3 days post calving (PC3) termed Av_C0.PC1.PC3. Comparing the antibiotic (AB) and non-antibiotic (Orb Only) treatment groups, the mean log_{10}Chao1 for the AB group decreases from 1.98 to 1.40 following the dry period, whereas, in the Orb Only group there is a lower decrease in the mean log_{10}Chao1 from 1.86 at D to 1.52 at Av_C0.PC1.PC3.

3.3.3.b. Modelling Chao1 richness index following antibiotic treatment

Linear mixed effects analysis exploring the relationship between the Chao1 index and antibiotic treatment was performed using the lmer package in R (Bates *et al.*, 2015; R Core Team, 2017). As fixed effects, AB_Orb, Parity, D.log10scc_centred, D.log10Chao1_centred and quarter were entered into the model. As a random effect, intercepts for Cow were entered into the model (Table 3).

Model equation:

Av_log10Chao1_C0.PC1.PC3 ~ AB_Orb + Parity + D.log10scc_centred + D.log10Chao1_centred + quarter + (1 | cow)

Fixed Effects	**Estimate**	**Std. Error**	**df**	**t value**	**Pr(>|t|)**	
(Intercept)	1.341	0.070	31.010	19.267	<2e-16	***
AB_Orb: Orb Only	**0.125**	**0.067**	**14.329**	**1.864**	**0.083**	**.**
Parity3+	-0.010	0.078	17.741	-0.123	0.903	
D.log10scc_centred	0.044	0.051	52.989	0.871	0.388	
D.log10Chao1_centred	0.032	0.072	63.620	0.447	0.656	
Quarter LH	0.109	0.069	54.975	1.571	0.122	
Quarter RF	0.066	0.068	51.919	0.977	0.333	
Quarter RH	0.148	0.071	54.769	2.098	0.041	*

Random effects

Groups	Name	Variance	Std.Dev.
Cow	(Intercept)	0.010	0.101
Residual		0.038	0.196

Significance codes: 0 '***', 0.001'**', 0.01'*', 0.05'.', 0.1 ' , 1 '

Table 3. Linear mixed effects model describing the influence of antibiotic treatment on the outcome variable, the mean species richness index (log_{10}Chao1) following the dry period, specifically the mean values of the time points calving (C0), 1 day post calving (PC1) and 3 days post calving (PC3). The fixed effects treatment group (AB_Orb), Parity and udder quarter were included in the model (LF, left fore; LH, left hind; RF, right fore; RH, right hind). The log_{10} of SCC and Chao1 index values at the drying off time point were centred and included as fixed effects in the final model (D.log10scc_centred and D.log10Chao1_centred, respectively). The effect of individual cow variation was accounted for in the model by inclusion of Cow as a random effect. 72 observations, 18 groups.

Accumulation of the estimate values for the fixed effects allows for comparison of the predicted effect of antibiotic treatment between cows (Table 3.) For example, following an inverse log transformation of the outcome variable (log_{10}Chao1), the model reflects an estimate of the Chao1 index following the dry period of an AB treated, Parity 2, LF quarter of a cow to be 21.93 $\pm$ 1.74 (standard errors). The model estimates that in an Orb Only, Parity 3+, LH quarter of a cow the Chao1 index would increase to 43.75 $\pm$ 2.55 (standard errors). Compared to the measured Chao1 index scores following the dry period the model predictions are fairly accurate with a measured Chao1 index score in the AB treated group of 25.12 and in the Orb Only group a value of 33.11 (Figure 12., Supplementary Materials Table 6.).

Visual inspection of the model assumption parameters indicated no major deviation from homoscedasticity or normality of the residuals (Figure 13.).

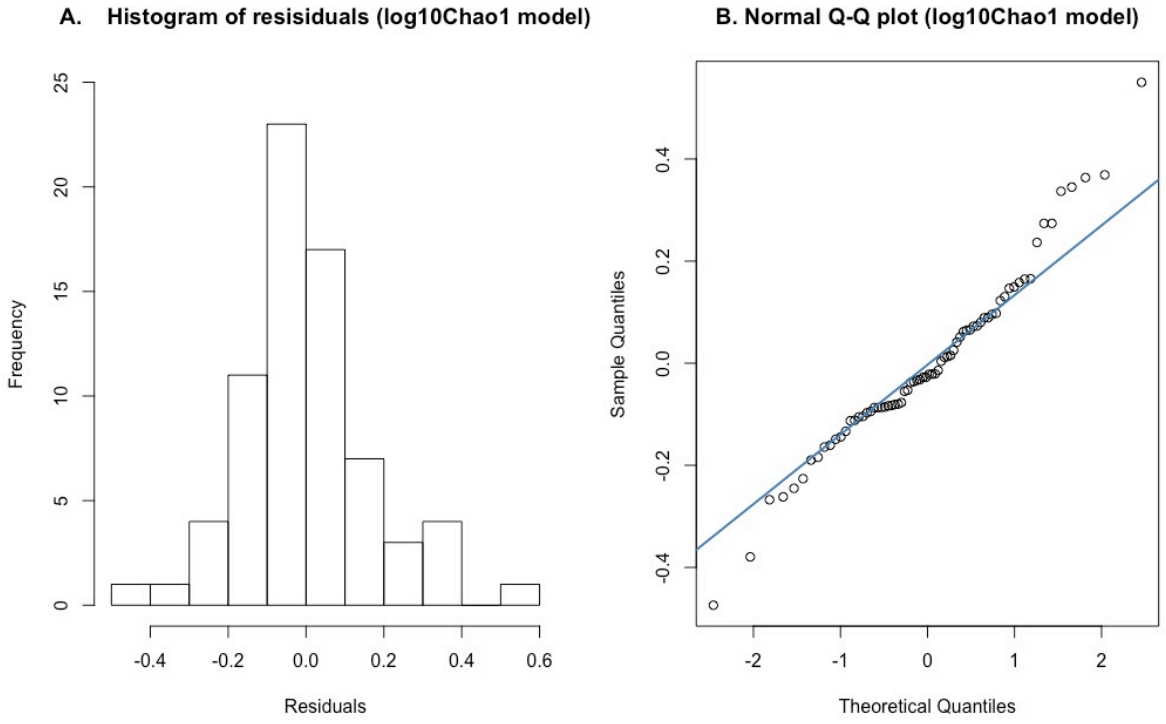


Figure 13. Residual distribution (A) and Normal Q-Q plot (B) for the outcome of the log_{10}Chao1 linear mixed effects model. Residuals are normally distributed, there is some deviation of the of values from the theoretical normal line in the Q-Q plot at the extremities, but does not cause a great enough shift to violate the model assumption parameters.

Treatment group (AB_Orb) has a significant influence on the outcome of the Chao1 index following the dry period ($p < 0.1$, Table 3.), that is difference between the effect of non-antibiotic and antibiotic treatment on the Chao1 index is likely not zero. However, lmer models are not designed to produce a straightforward p value to determine the significance of the model. To test the significance of the model further, a likelihood ratio test was conducted (Table. 4.).

A likelihood ratio test of the Chao1 index model was compared to the likelihood ratio test of a null Chao1 model using Anova analysis. The null model uses the same model parameters as the Chao1 model, but excludes the fixed effect of interest, treatment group. A significant difference was seen between the two models ($\chi^2(1)= 3.93$, $p < 0.05$; Table 4.). This indicates that the inclusion of the fixed effect treatment group makes it more likely to see the data collected in the model than when the effect of treatment is omitted.

<u>Model equations are as follows:</u>

Chao1 model:	Av_log10Chao1_C0.PC1.PC3 ~ AB_Orb + Parity + D.log10scc_centred + D.log10Chao1_centred + quarter + (1 | cow)
Chao1 null model:	Av_log10Chao1_C0.PC1.PC3 ~ Parity + D.log10scc_centred + D.log10Chao1_centred + quarter + (1|cow)

Herd	Model	Df	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)	
1	Chao1_null	9	-3.97	16.52	10.99	-21.97				
	Chao1	10	-5.90	16.87	12.95	-25.90	3.93	1	0.047	*

Significance codes: 0 '***', 0.001'**', 0.01'*', 0.05'.', 0.1 ' , 1 '

Table 4. Anova analysis comparing the likelihood ratio tests for the Chao1 model and the Chao1 null model. The fixed effect treatment group was removed in the null model allowing the effect of treatment group to be statistically assessed. A significant difference between the two models was determined ($\chi^2(1)= 3.93$, $p = 0.047$).

3.3.4. Shannon index

3.3.4.a. Shannon index variation following antibiotic treatment

The Shannon index is a metric for quantifying community diversity by estimating both species richness and evenness while considering relative abundance; in this context, the Shannon index score will increase as the number of OTUs increase and the distribution amongst the different OTUs becomes more even (Kim *et al.*, 2017; Lemos *et al.*, 2011).

The Shannon index was calculated using the phyloseq package in R (McMurdie & Holmes, 2013; R Core Team, 2017). The impact on species diversity of the mammary gland microbiome following antibiotic treatment will be analysed using the same process as applied to the somatic cell count and Chao1 index (Chapter 3. Section 3.3.2 and Section 3.3.3).

The Shannon index is greatest for both treatment groups at the drying off time point (D, Figure 14.), with mean index scores of 3.08 in the antibiotic group (AB) and 3.29 in the non-antibiotic group (Orb Only; Supplementary Materials, Table 7.). A low diversity is reached 1 day post calving (PC1) for both treatment groups; mean Shannon Index of 1.76 and 2.00 in the AB and Orb Only groups respectively (Supplementary Materials, Table 7.). The Shannon index score begins to level out 3 days post calving (PC3, Figure 14.).

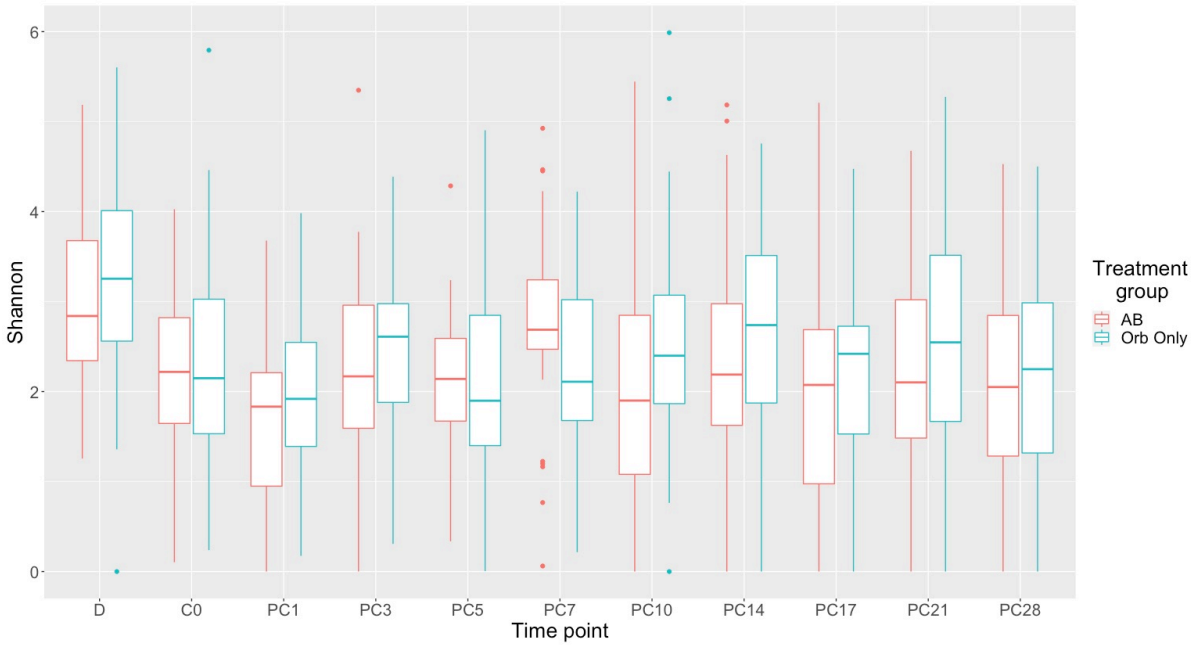


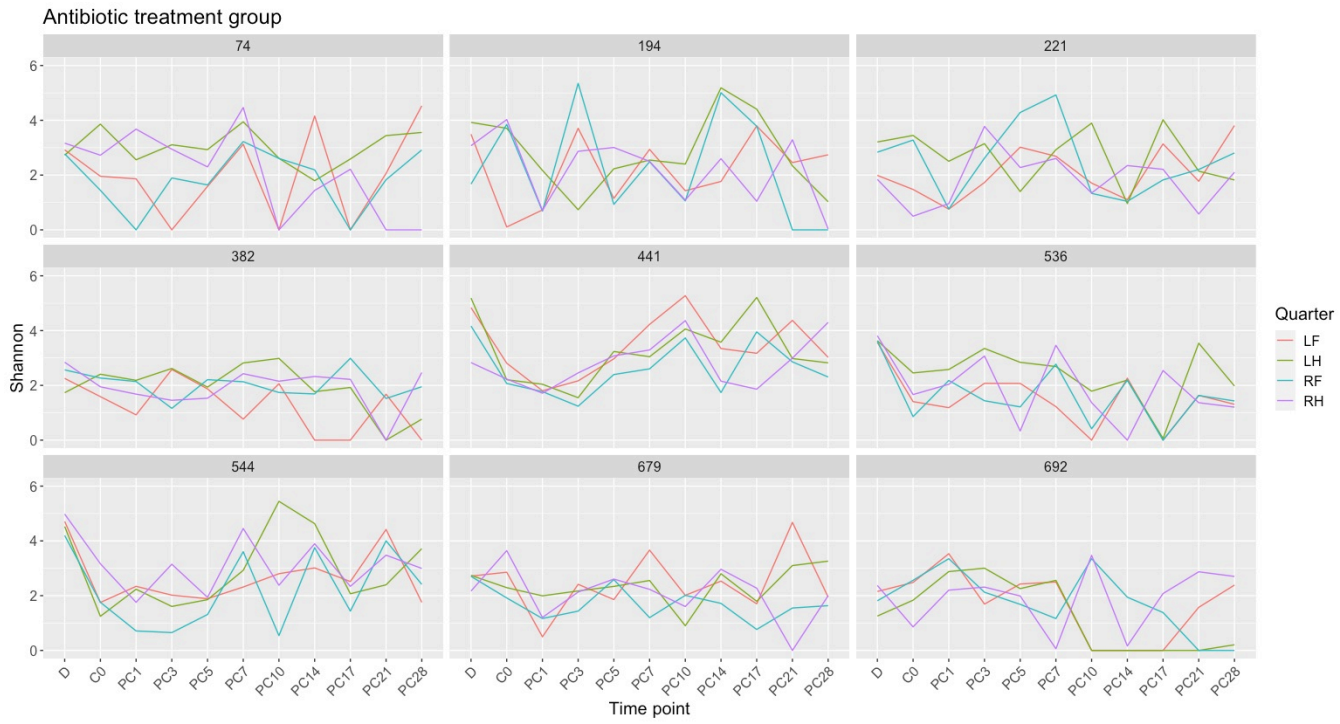
Figure 14. The change in Shannon index from drying off (D) to 28 days post calving (PC28) for antibiotic (AB) and non-antibiotic (Orb Only) treatment groups. The median Shannon index score is greatest at D for both treatment groups, with scores of 2.84 in the AB group and 3.25 in the Orb Only group (Supplementary Materials Table 7.). The Shannon index score decreases following the dry period with the lowest diversity at C0 for the AB group (median 1.83) and at PC5 for the Orb Only group (median 1.90). The Shannon index scores level out for both treatment groups following PC1 and there is wide variation in scores at all timepoints. (D, Drying off; C0, Calving day; PC, Post-Calving, 1-28 days post calving; n = 72 per time point, total n = 791).

Plotting Shannon index data per cow per udder quarter for each treatment group and each time point displays the level of variation within cows between time points, this changeable pattern is seen in both treatment groups (Figure 15.)

To explore the effect of antibiotic treatment on the time points of interest, from the dry period to the first few days post-partum, the Shannon index scores across this period were directly compared. The mean Shannon index score for the first 3 time points following the dry period (calving, C0; 1 day post calving, PC1 and 3 days post calving, PC3) was calculated and compared to the drying off samples. Following the dry period there is a decrease in the

median Shannon index scores for both treatment groups (Figure 16.). In the antibiotic treatment group (AB) there was a decrease in the mean Shannon index score from 3.08 to 2.09 and in the non-antibiotic group (Orb Only) there was a decrease from 3.29 to 2.25 following the dry period (Supplementary Materials Table 7.).

A.



B.

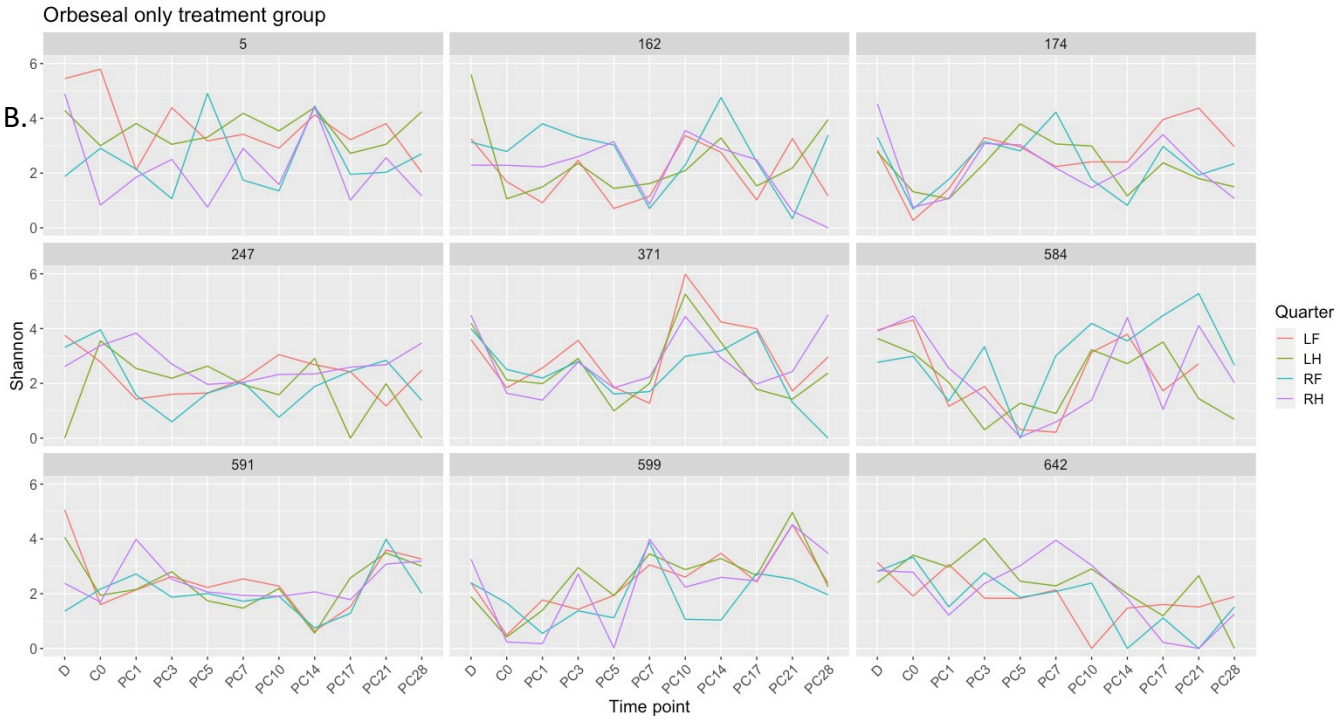


Figure 15. Variation in the Shannon index per cow (numbered) per udder quarter over time for each treatment group, antibiotic (Figure 15.A.) and non-antibiotic (Figure 15.B.). Udder quarters: LF, Left Fore; LH, Left Hind; RF, Right Fore; RH, Right Hind. Time points: D, Drying off; C0, Calving day; PC, Post-Calving, 1-28 days post calving. Total n = 791. Shannon index scores appear high in most samples at (D) with a very variable scores in the following 28 days post calving for cows in both treatment groups.

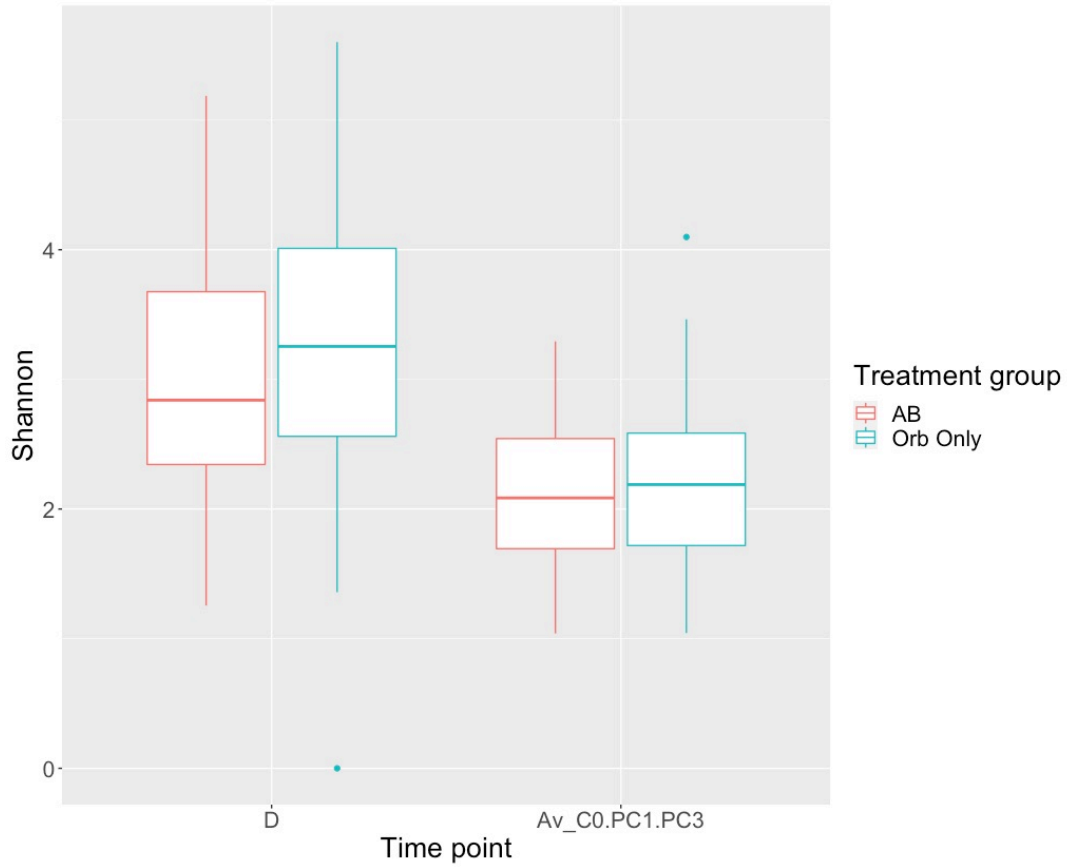


Figure 16. Distribution of the Shannon index at the drying off (D) time point and the mean Shannon index scores for samples at time points calving (C0), 1 day post calving (PC1) and 3 days post calving (PC3) termed Av_C0.PC1.PC3. The non-antibiotic treatment group (Orb Only) has a higher median Shannon index score at D, 3.25, compared to the antibiotic treatment group (AB) with a median score of 2.84 (Supplementary Materials Table 7.) The median Shannon index scores decreases in both treatment groups over the dry period. The median for the Orb Only group decreases by 1.06 to 2.19 at Av_C0.PC1.PC3 and the median for the AB group decreases by 0.75 to 2.09 following the dry period (Supplementary Materials Table 7.).

3.3.4.b. Modelling the Shannon diversity index following antibiotic treatment

Linear mixed effects model analysis was conducted to explore the relationship between the Shannon index and antibiotic treatment using the lmer package in R (Bates *et al.*, 2015; R Core Team, 2017). Models were constructed testing all fixed effects outlined in Chapter 3. Section 3.3.2.b., here the 'best' model is presented. The fixed effects treatment group (AB_Orb), Parity, Somatic Cell Count at drying off (D.$\log_{10}$scc_centred), Chao1 index at drying off

(D.log_{10}Chao1_centred), dry period length (DPL_centred) and udder quarter were entered into the model. As a random effect, intercepts for each Cow were entered into the model (Table 5).

Model equation:

av_Shannon_C0.PC1.PC3 ~ AB_Orb + Parity + D.log10scc_centred + D.log10Chao1_centred + DPL_centred + quarter + (1 | cow)

Fixed Effects	**Estimate**	**Std. Error**	**df**	**t value**	**Pr(>|t|)**	
(Intercept)	1.820	0.205	31.101	8.883	4.87E-10	***
AB_Orb: Orb only	**0.205**	**0.183**	**12.801**	**1.122**	**0.282**	
Parity 3+	0.212	0.251	17.545	0.847	0.409	
D.log_{10}scc_centred	0.031	0.158	48.793	0.197	0.845	
D.log_{10}Chao1_centred	0.016	0.215	58.832	0.073	0.942	
DPL_centred	0.006	0.011	14.517	0.568	0.579	
Quarter LH	0.376	0.217	54.673	1.731	0.089	.
Quarter RF	0.059	0.212	51.677	0.280	0.781	
Quarter RH	0.220	0.221	54.629	0.999	0.322	

Random effects

Groups	Name	Variance	Std.Dev.
Cow	(Intercept)	0.046	0.214
Residual		0.377	0.614

Significance codes: 0 '***', 0.001'**', 0.01'*', 0.05'.', 0.1 ' , 1 '

Table 5. Linear mixed effects model predicting the Shannon index scores as a mean value of the time points C0 (calving), PC1 (1 day post calving) and PC3 (3 days post calving). The fixed effects treatment group (AB_Orb), Parity and udder quarter were included in the model (LF, left fore; LH, left hind; RF, right fore; RH, right hind). The log_{10} of SCC and Chao1 index values at the drying off time point were centred and included as fixed effects in the final model (D.log_{10}scc_centred and D.log_{10}Chao1_centred, respectively), the length of the dry period was also centred and included in the model as a fixed effect (DPL_centred). The effect of individual cow variation was accounted for in the model by inclusion of Cow as a random effect. 72 observations, 18 groups.

The model intercept value reflects the output Shannon index score following the dry period of 1.82 $\pm$ 0.205 (standard errors) for the left fore udder quarter of an AB treated, Parity 2 cow (Table 5.). Addition of the fixed effect estimate scores in the model allows for comparisons of cows in each treatment group. For example, for the left hind udder quarter of an Orb Only treated parity 3+ cow the Shannon index score would increase to 2.67 $\pm$ 1.398 (standard errors, Table 5.). However the model has not reported a significant difference between the treatment groups, there is not enough evidence to confirm that the effect between the different treatment groups on the Shannon index following the dry period is not zero.

Visual inspection of the model assumption parameters indicated no major deviation from homoscedasticity or normality of the residuals (Figure 17.).

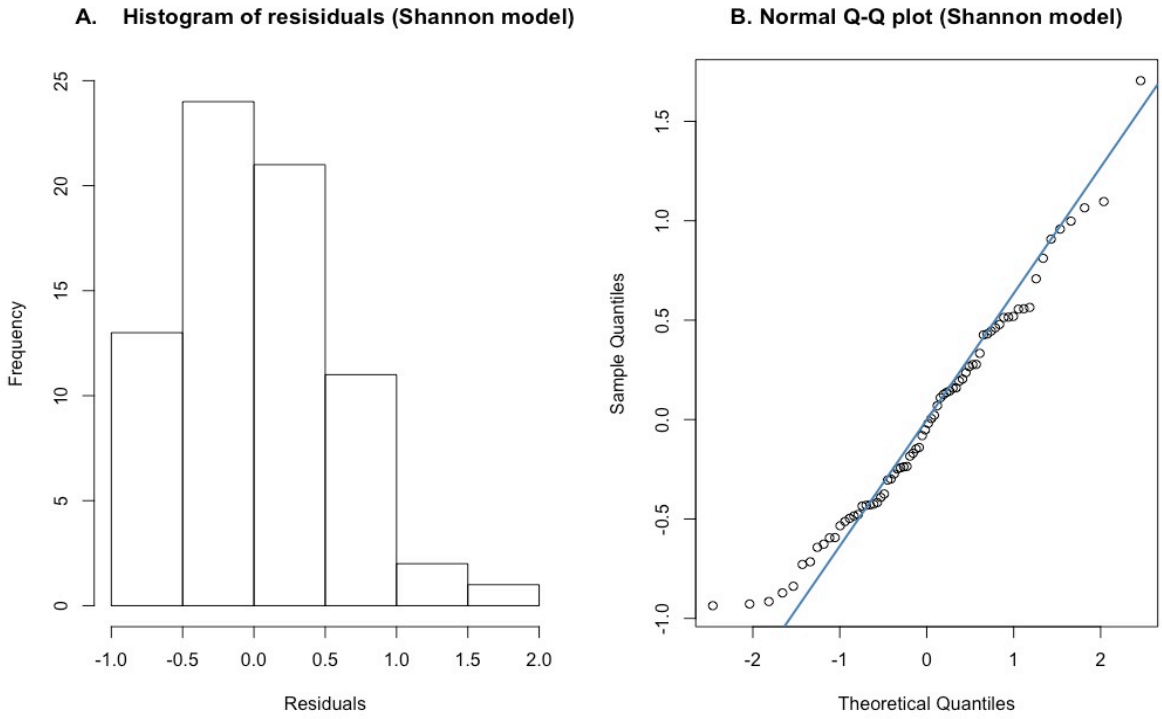


Figure 17. Histogram of model residuals for the Shannon model (A) show a relatively normal distribution with a slight rightward skew. Normal Q-Q data plotted against the expected normality line (B) is not perfectly aligned, however it does not deviate enough to violate the model assumptions.

To further explore the effect of treatment on the outcome of the Shannon index model, a likelihood ratio test was conducted for both the Shannon model and a null model using Anova analysis. The null model includes the same model parameters as the Shannon model, but omits the fixed effect of interest, treatment group. No significant difference was determined between the two models ($\chi^2(1)= 1.67$, $p > 0.05$, Table 6.). This indicates that the omission of the fixed effect treatment group does not effect the likelihood of seeing the data collected in the model.

Model equations are as follows:

Shannon model:	av_Shannon_C0.PC1.PC3 ~ AB_Orb + Parity + D.log10scc_centred + D.log10Chao1_centred + DPL_centred + quarter + (1 | cow)
Shannon null model:	av_Shannon_C0.PC1.PC3 ~ AB_Orb + Parity + D.log10scc_centred + D.log10Chao1_centred + DPL_centred + quarter + (1 | cow)

Herd	Model	Df	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
1	Shannon_null	10	152.91	175.68	-66.46	132.91			
	Shannon	11	153.25	178.29	-65.62	131.25	1.67	1	0.197

Significance codes: 0 '***', 0.001'**', 0.01'*', 0.05'.', 0.1 ' , 1 '

Table 6. Anova analysis comparing the likelihood ratio tests for the Shannon model and the Shannon null model. The fixed effect treatment group was omitted from the null model allowing the absence of treatment group to be statistically assessed. No significant difference between the two models could be determined ($\chi^2(1)= 1.67$, $p = 0.197$).

3.3.5. OTU Analysis

3.3.5.a. OTUs Overview

Operational Taxonomic Units (OTUs) are in context defined as a cluster of 16S rRNA reads with at least 97% similarity, which can approximately correspond to a bacterial genus. Here OTUs are used as a tool to estimate the richness and diversity of the bacteria in milk samples from the mammary gland. In previous sections OTU values have been used to calculate richness and diversity indices, in this section OTUs will be used directly to assess how the OTU community changes across the dry period and if there is a difference between treatment groups. Summary information of OTU and herd information for context is provided in Table 7. Further community analysis will be explored in Chapter 5.

OTU information		**Herd 1**
Total unique OTUs in dataset		8680
Number OTUs identified per sample		10.97
Mean $\log_{10}$(total OTU count) per sample		5.28
Mean % of non-hits per sample		99.3%
Mean unique OTUs per sample		58.7
Mean unique OTUs per treatment group:		
	AB-Orb	57.0
	Orb Only	60.4
Mean $\log_{10}$(total OTU count) per treatment group:		
	AB-Orb	5.02
	Orb Only	5.55

Table 7. Summary of OTU information for Herd 1. The mean unique number of OTUs per sample in the antibiotic treatment group (AB) is lower than in the non-antibiotic treatment group (Orb Only), 57 and 60 respectively. The $\log_{10}$ total number of OTUs is also greater in the Orb Only group (5.55) compared to the AB group (5.02). Number of samples 791.

3.3.5.b. Statistical analysis of OTU correlations

A Spearman's rank correlation of the abundance of unique OTUs in each udder quarter of each cow with the following time points for each treatment group was performed. The ranked composition of OTUs can be used to understand the retention of similar abundancies of OTUs between sampling time points. The time points drying off (D), calving (C0), 1 day post calving (PC1) and 3 days post calving (PC3) were used to correlate ranked OTUs between drying off and the the 3 time points following the dry period (Table 8.A., Figure 18.A.) and to correlate ranked OTUs between consecutive time points (Table 8.B., Figure 18.B.). The Spearman's rank correlation scores were averaged within each treatment group and summarised in Table 8. and Figure 18.

A higher correlation between the two time points (-1 to 1) in the samples indicates that the proportion of OTUs found are more similar and in a more similar abundance. Indicating the mammary gland microbiome has been less perturbed over the dry period; or less new bacteria have entered the udder system. The strength of correlation is directive of the similarity in the microbiota between sampling time points.

Overall there is a low correlation of ranked OTU abundancies between time points tested in both treatment groups Figure 18. The mean correlation scores vary between 0.128 and 0.235 indicating the composition of OTUs changes greatly between the sampling time points (Table 8.).

	Time points correlated	Treatment	count	mean	sd	median	IQR
A.		AB	36	0.142	0.078	0.120	0.088
	D_C0	Orb_only	36	0.148	0.076	0.132	0.094
		AB	36	0.134	0.078	0.124	0.086
	D_PC1	Orb_only	36	0.128	0.067	0.121	0.079
		AB	36	0.146	0.081	0.148	0.093
	D_PC3	Orb_only	36	0.157	0.081	0.153	0.134
B.		AB	36	0.142	0.078	0.120	0.088
	D_C0	Orb_only	36	0.148	0.076	0.132	0.094
		AB	36	0.191	0.081	0.178	0.098
	C0_PC1	Orb_only	36	0.182	0.081	0.180	0.131
		AB	36	0.235	0.128	0.230	0.142
	PC1_PC3	Orb_only	36	0.197	0.094	0.182	0.143

Table 8. Summary of Spearman's rank correlation scores of the ranked abundancies of OTUs for samples taken at drying off (D) to the 3 sampling time points following the dry period (C0, calving; PC1, 1 day post calving; PC3, 3 days post calving) for each treatment group (antibiotic, AB and non-antibiotic, Orb Only). Correlations between drying off to the 3 time points following the dry period are low for both treatment groups, with little difference between the mean correlation scores of each treatment group (A). Comparing subsequent time points, there is also a low mean correlation between timepoints (B). This indicates that between each sampling time point the ranked abundance of OTUs in each mammary gland quarter changes greatly.

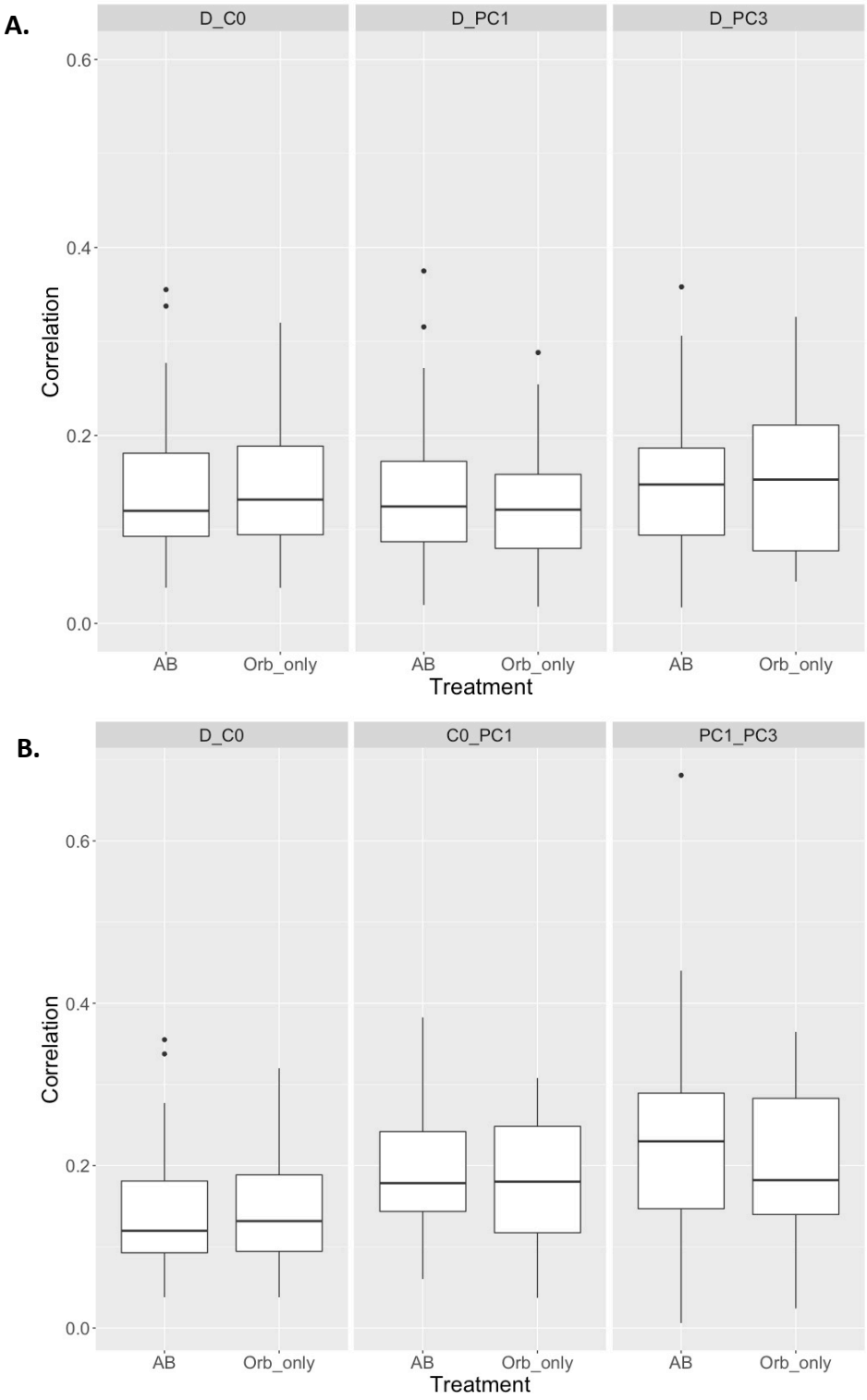


Figure 18. Visualisation of the summary of Spearman's rank correlation scores for the the ranked OTU abundancies in milk samples taken from the mammary gland across the dry period (Table 8.).Correlations between the drying off timepoint (D) and the 3 time points following the dry period show little retention of common abundancies between samples with a mean correlation ranging between 0.128 and 0.157 for the antibiotic treated group (AB) and non-antibiotic (Orb Only) groups (Table 9.). The scores are slightly higher in when

correlating subsequent time points with the mean ranging between 0.142 and 0.235 but there is little visible difference between the two treatment groups. D, drying off; C0, calving; PC1, 1 day post calving; PC3, 3 days post calving.

A Kruskal-Wallis test was performed to compare the OTU correlations between treatment groups. There is not enough evidence to support that the differences in OTU correlations is significantly different between treatment groups ($p > 0.05$; Table 9). Indicating that overall the composition of the OTUs in the mammary gland varies greatly between sampling time points, with not enough evidence to suggest a difference between treatment groups.

Time points correlated	p-value	Test statistic	parameter
D_C0	0.652	0.203	1
D_PC1	0.866	0.029	1
D_PC3	0.581	0.305	1

D_C0	0.652	0.203	1
C0_PC1	0.822	0.051	1
PC1_PC3	0.300	1.074	1

Significance codes: 0 '***', 0.001'**', 0.01'*', 0.05'.', 0.1 ' , 1 '

Table 9. The Kruskal-Wallis test determined no significant difference ($p>0.05$) between the two treatment groups in the Spearman's rank correlation scores for the ranked abundance of OTUs between time point pairs tested. D0, drying off; C0, calving; PC1, 1 day post calving; PC3, 3 days post calving.

3.4. Discussion and Conclusions

Udder quarter milk samples from a herd of 18 cows, 9 receiving antibiotic treatment and 9 receiving non-antibiotic teat sealant, were collected across 11 time points from drying off until 28 days post calving. The effect of antibiotic treatment on the somatic cell count, Chao1 and Shannon indices was analysed and will be discussed along with limitations of the findings.

Somatic cell count (SCC) is an immune response proxy quantifying the level of infection in the mammary gland. The SCC increased in both treatment groups following the dry period before

decreasing steadily across the next 10 time points (Figure 3.). The median SCC following the dry period was generally lower in all time points in the antibiotic group apart from 21 days post calving. To further assess the effect of antibiotics on the early lactation SCC, the average of the first three time points following the drying off period was analysed and statistically modelled. Linear mixed effects modelling (LME) revealed no significant difference in the SCC in early lactation between the two treatment groups (Table 1. And Table 2.). This was slightly unexpected based upon visualisation, however there is a great deal of variation (Figure 3.). In infected cows the effect of cattle parity on SCC levels can be significant compared to lower parity cows (Laevens *et al.*, 1997). Laevens *et al.* (1997) also showed there was no significant between different parity cows in terms of the SCC of bacteriologically negative cows. Variation due to parity was seen in the early lactation SCC (Figure 7.). Although the potential confounding effect of parity was accounted for in the model. In Herd 2 analysis being able to control for parity and better balance the drying off SCC levels may improve interpretation.

Visualisation of the bacterial abundance measure, the Chao1 index, showed the highest level of diversity for both groups at drying off which decreased for both groups into early lactation and began to slightly increase, then level, across the remaining time points, however variation was wide within each treatment groups (Figure 9.). LME modelling revealed a significant difference between the Chao1 index in early lactation, that it was lower in the antibiotic group compared to the non-antibiotic group (Table 3. and Table 4.). This contrasts results seen by Bonsaglia *et al.* (2017) who found a lower Chao1 and Shannon index in their non-antibiotic teat sealant group 7 days into milking, and in results from Biscarini *et al.* (2020) who found in their teat-sealant only treated quarters, a reduction in the Chao1 and Shannon indices measures; however in both of these studies, they found the reductions were not significant between treatment groups. This discrepancy could also be due to the averaging of the sampling time points calving, 1 day post calving and 3 days post calving which were modelled in this study, whereas 5 days (Biscarini *et al.*, 2020) and 7 days (Bonsaglia *et al.*, 2017) post calving were sampled in previous studies. Furthermore, in this study, only a small significance could be reported ($p < 0.05$, Table 4.). It should also be considered that no significant difference between the two treatment groups in early lactation was found for the Shannon diversity indices (Table 5. and Table 6.). Considered together with the Chao1 index it is more

difficult to conclude with certainty that the diversity of the mammary gland microbiota is significantly different following antibiotic treatment in early lactation.

Finally a Spearman's rank correlation of the ranked abundancies of OTUs from individual quarter samples to corresponding quarter samples in across the dry period into early lactation were compared. Correlation scores were very low between corresponding time points and no significant difference was seen in the correlation scores between the antibiotic and non-antibiotic treatment groups. This suggests the mammary gland milk microbiota is highly changeable, regardless of treatment group, not only between milkings when the udder is highly perturbed but also across the dry period when the udder is 'undisturbed'. It has been reported that the microbiota of udder quarters is highly dynamic in previous studies (Andrews *et al.*, 2019; Porcellato *et al.*, 2020). The dynamic nature of the microbiota will be further explored in Chapter 5 on a taxonomic level and will be compared between treatment groups.

While these findings suggest there is little lasting impact of antibiotics on mammary gland health and diversity following antibiotic dry therapy, confounding factors such as varying parity and varying drying off SCC will be considered in the selection of samples in Herd 2, to more specifically address the impact of antibiotics on the mammary gland microbiome in Chapter 4.

4. Analysis of Herd 2

4.1. Introduction

In this Chapter, following analysis of the Herd 1 data set, a more specific set of samples were selected for DNA extraction and sequencing of the mammary gland microbiome for Herd 2 and is outlined fully in Chapter 4.3. Again, changes to udder health in terms of the somatic cell count and changes to the udder microbiota in terms of diversity indices and OTU correlations will be explored through data visualisations and statistical analysis. A comparison between the antibiotic and non-antibiotic treatment groups will be discussed to more specifically address the hypothesis outlined in Chapter 1. To again investigate the impact of antibiotics on the mammary gland across the dry period into early lactation with a purposely chosen dataset.

4.2. Methods

Methods outlining the collection, sequencing and analysis of the 16S rRNA microbiota of milk samples is outlined fully in Chapter 2. Analysis of the Herd 1 dataset generated specific hypotheses, outlined in the introduction, to be tested on the Herd 2 dataset. To address these, milk samples were specifically selected for sequencing to address these hypotheses from a more balanced dataset, described in Chapter 4.3. For comparison between herds the same methods of analysis were applied (outlined in chapter 2.6. and summarised below) to address the main aim of the thesis, to understand the impact of antibiotic treatment on the mammary gland microbiome.

- Visualise the changes in the immune response and in the diversity and abundance metrics following antibiotic and non-antibiotic treatment over 5 time points.

- Use statistical models to test if there is a clear difference in the immune response and in the diversity and abundance metrics between treatment groups following drying off.

- Conduct statistical analysis of the ranked abundance of OTUs identified in individual milk samples with statistical comparison between treatment groups, to further understand the changes in the microbiota over early lactation time points.

4.3. Results

4.3.1. Overview of the Herd 2 dataset

The variables included in the Herd 2 data set were; Cow ID, udder quarter sampled, cow, parity, treatment group, sampling time point, somatic cell count (SCC) and the length of the dry period (days). The SCC is a quantitative measure of immune cells in the mammary gland and is used as a proxy for measuring mastitis infection (Schukken *et al.*, 2003).

From OTU data collected from milk samples, diversity and abundancy indices for the bacterial community in each sample were calculated using the phyloseq package in R and included as variables in the dataset (McMurdie & Holmes, 2013; R Core Team, 2017). The indices calculated were the Chao1 index and Shannon index. The Chao1 index is an abundance-based estimator of species richness, in this context it is a measure of expected OTUs in each sample based on the OTUs identified in all samples (Chao *et al.*, 2006). The Shannon diversity index considers relative abundance while estimating species richness and evenness, the index increases as the both the number of OTUs increases and the distribution amongst the different OTUs becomes more even (Kim *et al.*, 2017; Lemos *et al.*, 2011). In this context an increase in both of these indices would infer a greater bacterial diversity in the sample relative to the measured population. The SCC and Chao1 were $\log_{10}$ transformed to normalise the data for analysis.

Herd 2 comprised of 82 Holstein-Friesan dairy cows. From this study enrolment, cows were selected to specifically test the hypotheses from Herd 1 and to address the hypotheses generated from the analysis of Herd 1 cows. It was important to select a balanced dataset to address specifically if there is any difference between the microbiome of antibiotic and non-antibiotic treated cows.

The data set comprised of a herd of 22 cows. All 4 udder quarters (LF, left fore; LH, left hind; RF, right fore; RH, right hind) of each cow were independently sampled at the 5 chosen time points (D0, Drying-off; C0, Calving day; PC1, 1 day post calving; PC3, 3 days post calving; PC17, 17 days post calving). Giving 88 samples at each time point, totalling 440 samples in the whole dataset. 11 cows received an intramammary antibiotic treatment and a teat sealant at drying off (antibiotic treatment group, AB) and 11 cows received just a teat sealant at drying off (non-antibiotic treatment group, Orb Only). All cows in the dataset were parity 2 cows, meaning the cow has calved twice.

The aim in choosing cows for the Herd 2 dataset was to improve the balance between treatment groups compared to Herd 1, in order to specifically test whether there is any difference between antibiotic and non-antibiotic treated cows. This was achieved in part by removing variation caused by cattle parity, as all cows selected in Herd 2 were Parity 2. Then, selection was based on the SCC at drying off. The cows selected for this dataset are well balanced, the mean $\log_{10}$SCC at drying off was 1.45 in the AB group and 1.44 in the Orb Only group (Supplementary Materials Table 8.). The variation in SCC between treatment groups is similar and the distribution is also well balanced (Figure 1.A., Figure 1.B.).

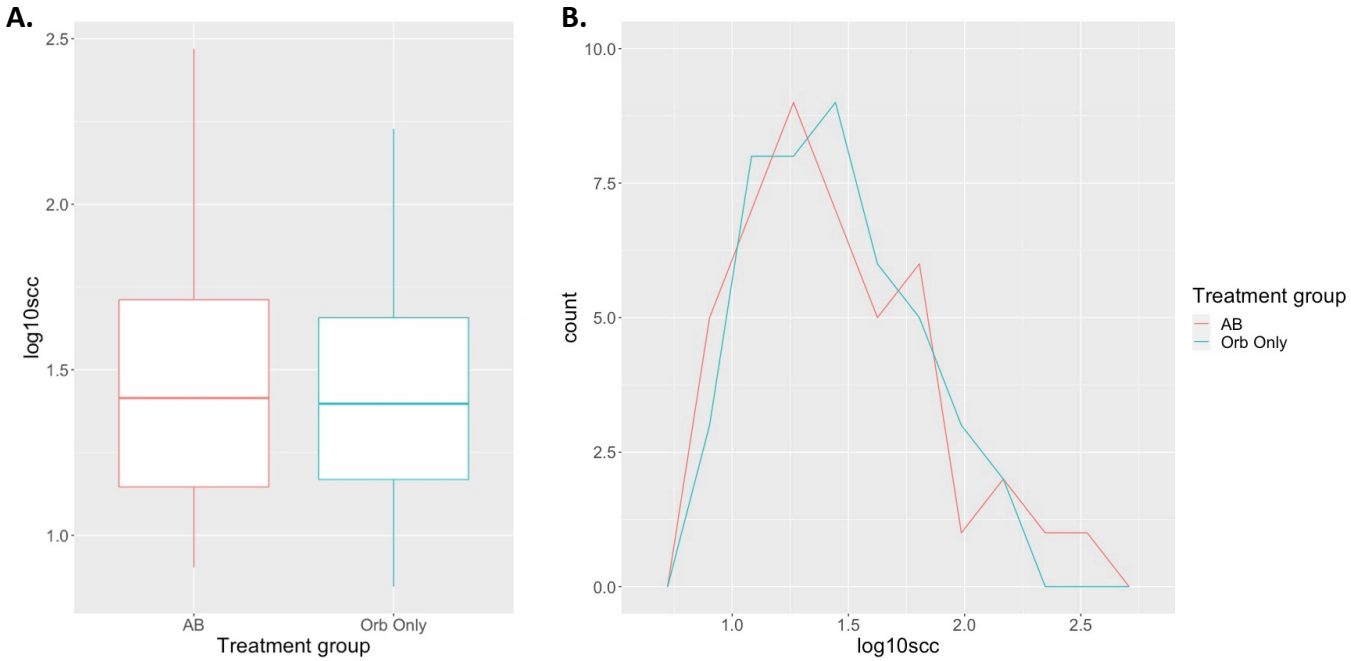


Figure 1. Variation in the somatic cell count (log_{10}SCC) of the two treatment groups, antibiotic (AB) and non-antibiotic (Orb Only), at the drying off time point (n = 88). The data is well balanced between the two treatment groups, with the median SCC for the AB group 1.45 and a median SCC for the Orb Only group of 1.44 (Figure 1.A.). The distribution of the SCC for both treatment groups is also well matched (Figure 1.B.).

Since all cows in the Herd are Parity 2, the potential confounding effect of parity does not need to be accounted for in this dataset. The length of the dry period is roughly equal between the two treatment groups, with median values of 50 days in the AB group and 51 days in the Orb Only group (Figure 2.). The length of the dry period is not expected to have a confounding effect on treatment outcome but the effect will be tested in later analysis.

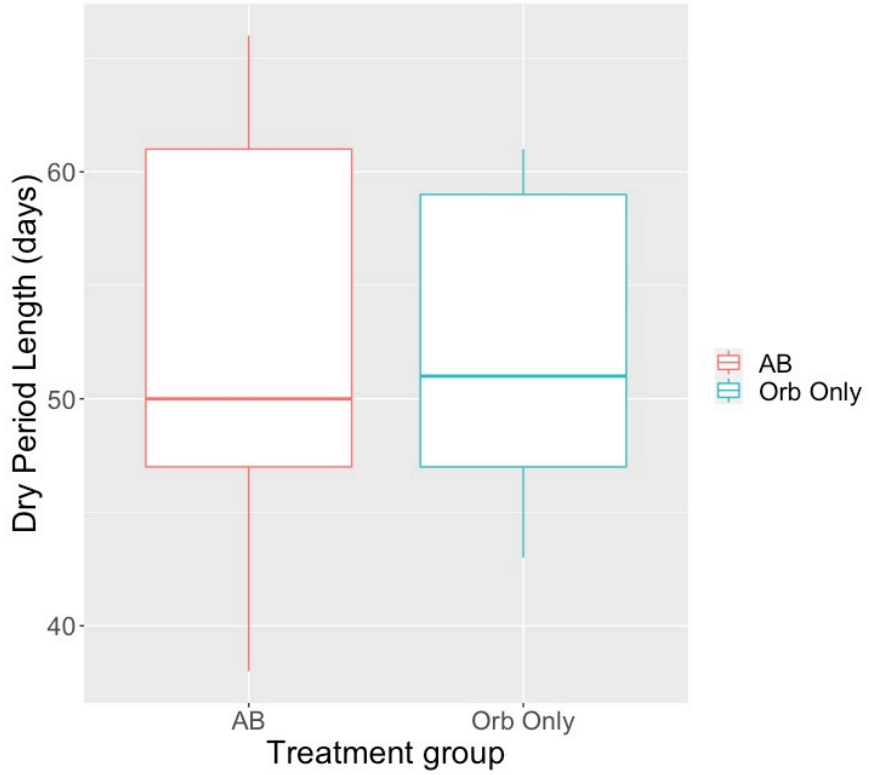


Figure 2. The variation in the length of the dry period in days for both treatment groups is well balanced. The median length in the antibiotic (AB) treatment group is 50 days and is 51 days in the non-antibiotic treatment group (Orb Only).

4.3.2. Somatic Cell Count

The somatic cell count (SCC), the proxy for assessing mastitis, was measured across the dry period. The log_{10}SCC increases greatly following the dry period and is highest at calving (C0) in both treatment groups before consistently decreasing at each subsequent time point (Figure 3.). In the AB group the mean log_{10}SCC increases from 1.45 at D to 2.68 at C0 and in the Orb Only group there is a higher mean log_{10}SCC across the dry period from 1.44 at D to 2.80 at C0 (Supplementary Materials Table 8.). The AB group median log_{10}SCC remains lower than the Orb Only group throughout all time points, in both treatment groups the lowest mean log_{10}SCC is recorded 17 days post calving (AB, 1.04 and Orb Only, 1.24; Figure 3., Supplementary materials Table 8.).

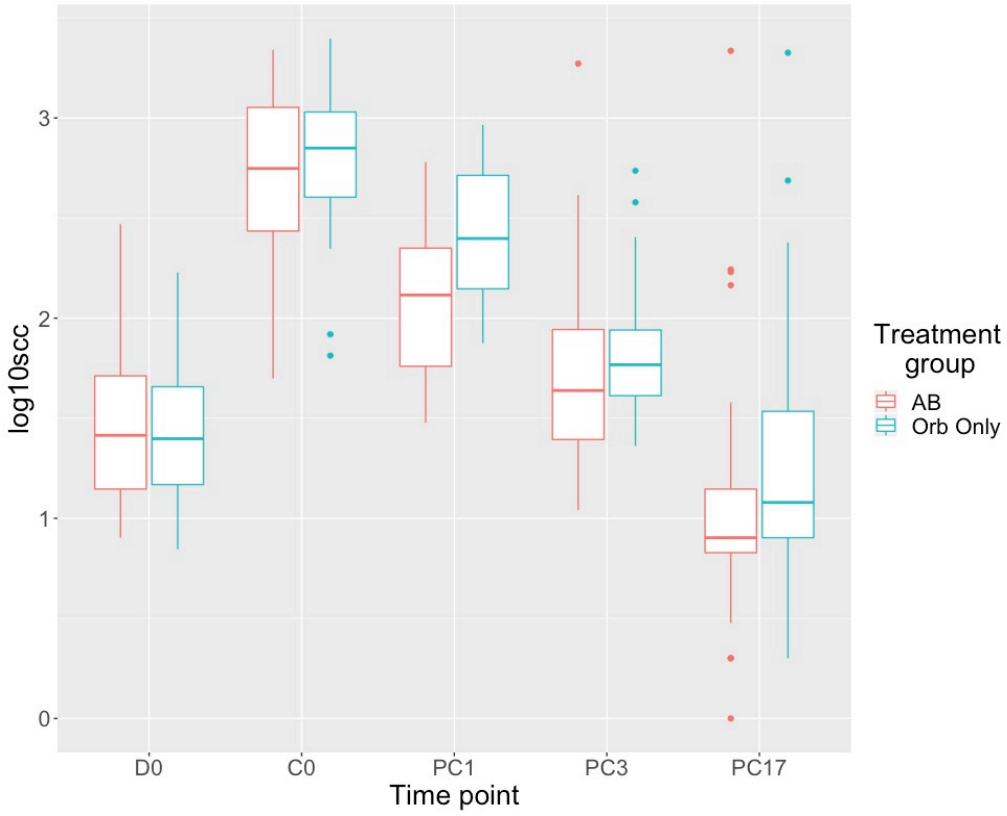


Figure 3. Variation in the log_{10}SCC over the dry period for each treatment group, antibiotic (AB) and non-antibiotic (Orb Only). The Mean log_{10}SCC peaks at calving (C0) following the drying off time point (D0), increasing from 1.45 to 2.68 int the AB group and increasing from 1.44 to 2.80 in the Orb Only group. There is a rapid decline in log_{10}SCC in early lactation, with the mean log_{10}SCC decreasing below values at D0 at 17 days post calving (PC17, AB = 1.04, Orb Only 1.23). n= 44 per treatment group per time point; total n= 440. (D0, Drying-off; C0, Calving day; PC1, 1 day post calving; PC3, 3 days post calving; PC17, 17 days post calving).

The variation in the SCC at each time point within the udder quarter per cow for each treatment group was visualised. There is a similar pattern in SCC changes over time for each udder quarter of each cow, generally peaking at calving (C0) and following a decline in subsequent time points (Figure 4., Figure 5.). While some udder quarters within the same cow appear to follow a similar pattern in SCC variation (e.g. Cow 802 and 857, Figure 4., Figure 5.), other udder quarters within the same cow experience higher SCC spikes in a quarter (e.g. Cow 850, Figure 4., Figure 5.).

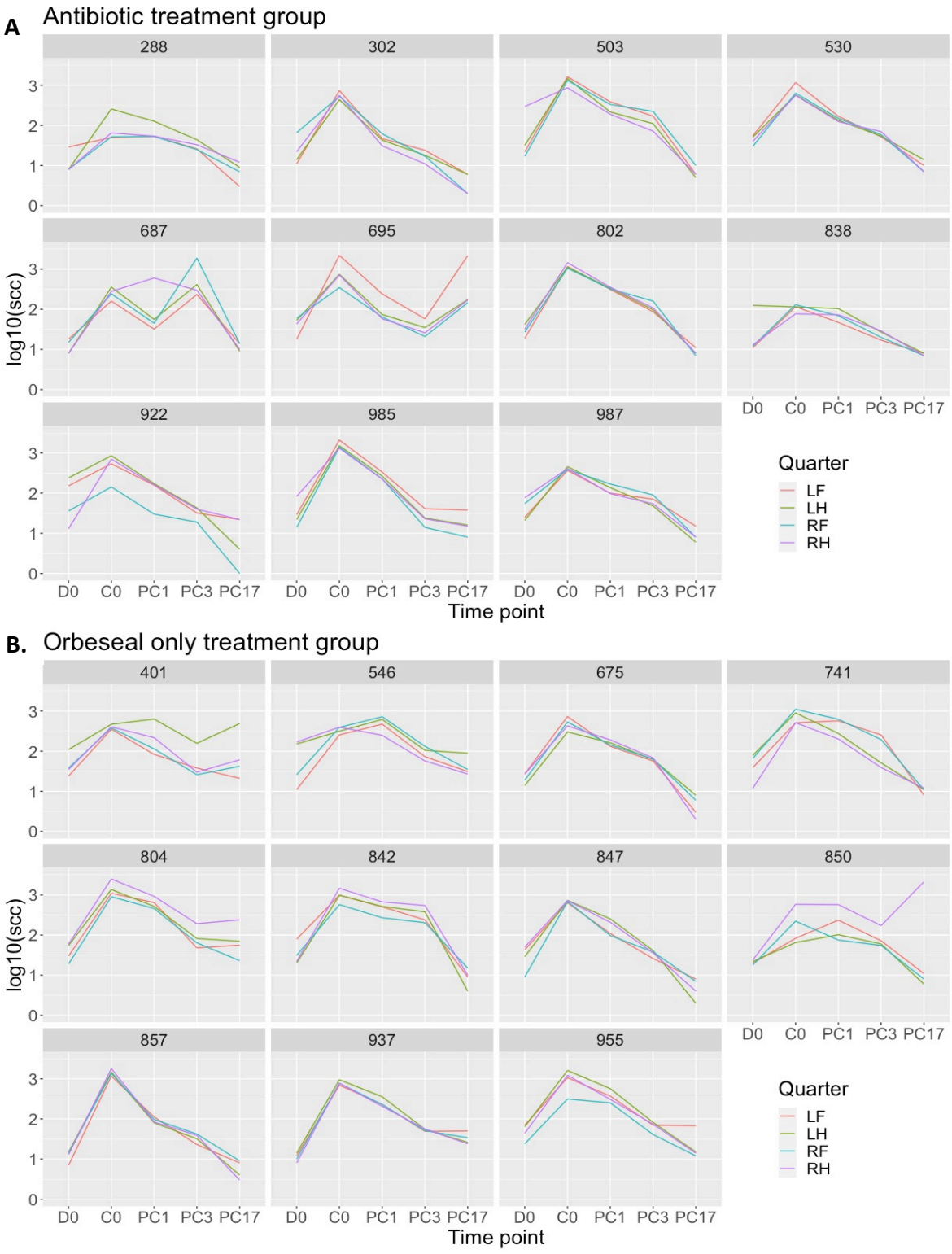


Figure 4. The change in somatic cell count (log_{10}SCC) per cow (numbered) per udder quarter over time for each treatment group, Antibiotic (Figure 4.A.) and non-antibiotic (Figure 4.B.). Udder quarters: LF, Left Fore; LH, Left Hind; RF, Right Fore; RH, Right Hind. Time points: D, Drying off; C0, Calving day; PC1, 1 day post calving; PC3, 3 days post calving; PC17, 17 days post calving. Total n = 440. SCC decreases over time for both treatment groups after peaking at the C0 time point.

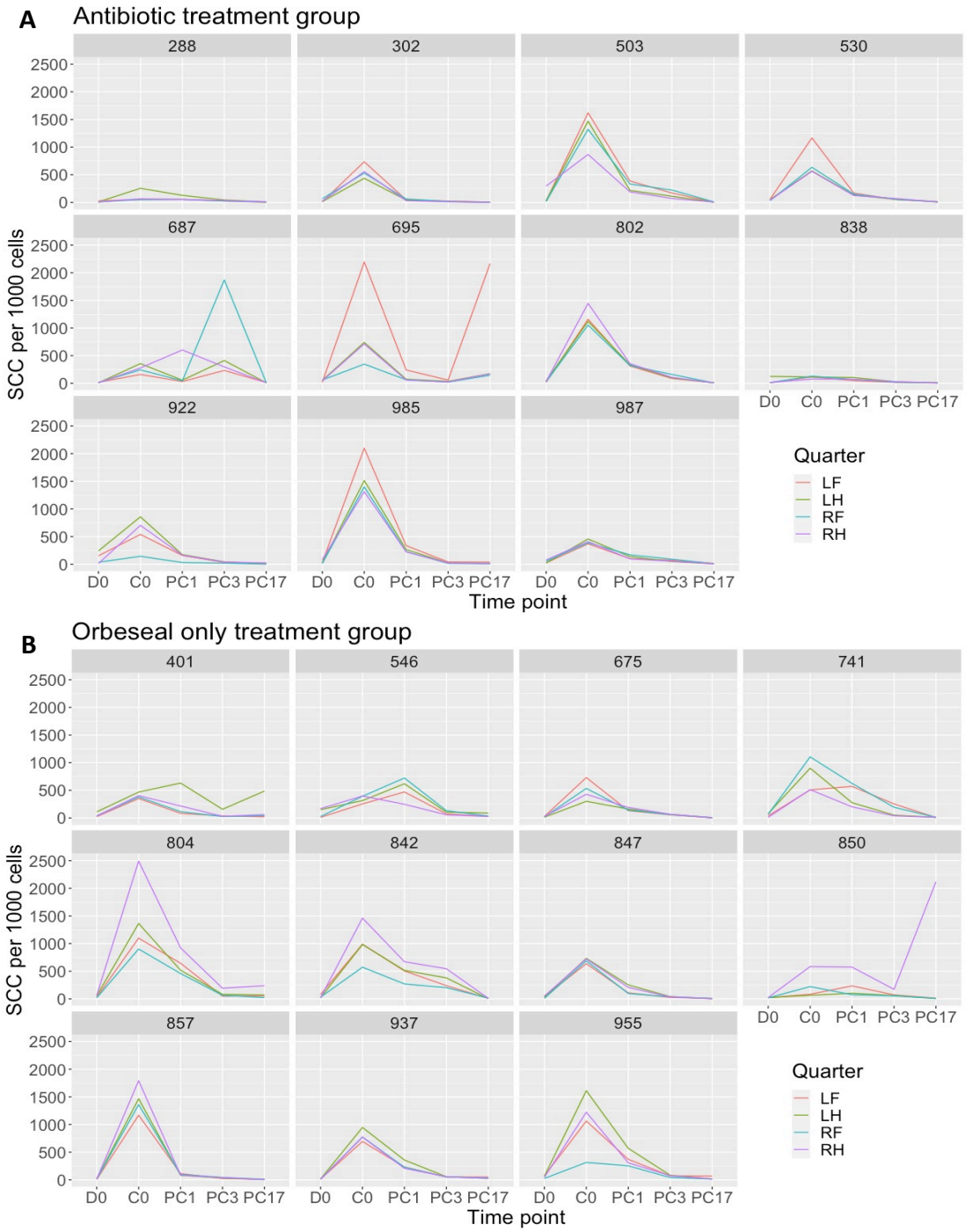


Figure 5. The change in somatic cell count per 1000 cells, per cow (numbered), per udder quarter over time for each treatment group, antibiotic (Figure 5.A.) and non-antibiotic (Figure 5.B.). Udder quarters: LF, Left Fore; LH, Left Hind; RF, Right Fore; RH, Right Hind. Time points: D, Drying off; C0, Calving day; PC1, 1 day post calving; PC3, 3 days post calving; PC17, 17 days post calving. Total n = 440. Log transformation of Figure 4. to display the scale of the SCC spikes in the udder quarters as a measure per 1000 cells. The scale of some peaks in SCC can be appreciated in udder quarters of each cow on this scale, for example in cows 695 and 687.

The first few days post partum are associated with an increased incidence of mastitis (Green *et al.*, 2002). The mean value of the log_{10}SCC for each of the sample time points calving (C0), 1 day post calving (PC1) and 3 days post calving (PC3) were calculated to assess the sustained effect of antibiotic treatment following the dry period and compared to the drying off time period (D0). Following the dry period there is an increase in the median SCC for both treatment groups (Figure 6.). In the antibiotic treatment group (AB) there was an increase in the mean SCC from 1.45 to 2.16 following the dry period representing an increase of 116,000 cells mL^{-1}. In the non-antibiotic group (Orb Only) there is an increase in the mean SCC from 1.44 to 2.35 representing an average increase in 196,000 cells mL^{-1} over the dry period (Supplementary Materials Table 8.).

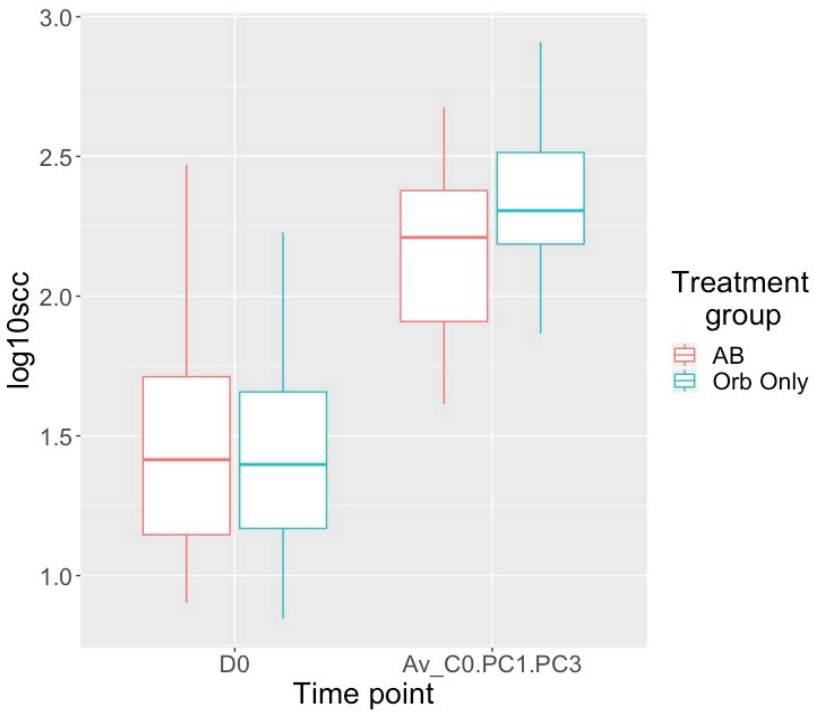


Figure 6. Distribution of the somatic cell count (log_{10}SCC) at drying off (D0) and the average log_{10}SCC for samples at time points calving (C0), 1 day post calving (PC1) and 3 days post calving (PC3) termed Av_C0.PC1.PC3. Comparing the antibiotic (AB) and non-antibiotic (Orb Only) treatment groups, the mean log_{10}SCC for the AB group increases from 1.45 to 2.16 following the dry period, whereas, in the Orb Only group there is a larger increase in the mean log_{10}SCC from 1.44 at D0 to 2.35 at Av_C0.PC1.PC3 (Supplementary Materials Table 8.).

4.3.2.b. Modelling somatic cell count following antibiotic treatment

Linear mixed effects model analysis was performed to explore the relationship between the somatic cell count (SCC) and antibiotic treatment following the dry period using the lmer package in R (Bates *et al.*, 2015; R Core Team, 2017). As fixed effects, treatment group (AB_Orb), somatic cell count at drying off (D0_log10scc_centred), dry period length (DPL_centred) and udder quarter (Qrt) were included in the model. Random intercepts were introduced in the model to account for within cow variability by including cow as a random effect (Table 1.).

Model equation:

Av_C0.PC1.PC3_log10scc ~ AB_Orb + D0_log10scc_centred + DPL_centred + Qrt + (1 | Cow)

Fixed Effects	**Estimate**	**Std. Error**	**df**	**t value**	**Pr(>|t|)**	
(Intercept)	2.156	0.078	26.901	27.540	<2e-16	***
AB_Orb: Orb Only	**0.194**	**0.101**	**18.699**	**1.922**	**0.070**	**.**
D0_log10scc_centred	0.043	0.065	71.974	0.661	0.511	
DPL_centred	-0.009	0.007	18.744	-1.250	0.227	
Quarter LH	0.034	0.053	62.144	0.635	0.528	
Quarter RF	-0.044	0.053	61.903	-0.827	0.412	
Quarter RH	0.021	0.053	61.947	0.398	0.692	

Random effects

Groups	Name	Variance	Std.Dev.
Cow	(Intercept)	0.048	0.220
Residual		0.031	0.175

Significance codes: 0 '***', 0.001'**', 0.01'*', 0.05'.', 0.1 ' , 1 '

Table 1. Linear mixed effects model predicting the somatic cell count following the dry period. The model output is the mean value of the three time points following the dry period; calving (C0), 1 day post calving (PC1) and 3 days post calving (PC3), calculated. The fixed effects included in the model are treatment group (AB_Orb), log_{10}SCC at drying off (D0_log10scc_centred), dry period length (DPL) and udder quarter (Qrt). Cow was included as a random effect. Total of 88 observations in the predicted variable, in 22 groups (number of cows).

The model predicts the outcome of the mean log_{10}SCC following the dry period for the time points calving (C0), 1 day post calving (PC1) and 3 days post calving (PC3). The estimate for the intercept is the mean value for the left fore (LF) udder quarter of an antibiotic treated (AB) cow. Taking the inverse log and multiplying by 1000 to adjust the number of cells (SCC are recorded per 100 cells), the estimate is 143,000 cells $mL^{-1} \pm$ 1,197 (standard errors). Whereas in the left hind (LH) quarter of a non-antibiotic treated cow (Orb Only), there is a larger estimate of 262,000 cells $mL^{-1} \pm$ 2,014 (standard errors). The recorded mean log_{10}SCC following the dry period measured values of 145,000 cells mL^{-1} for the AB group and 224,000 cells mL^{-1}.

Visualisation of the model assumption parameters indicated no major violation in the distribution of residuals and in the expected normal Q-Q plot (Figure 7.)

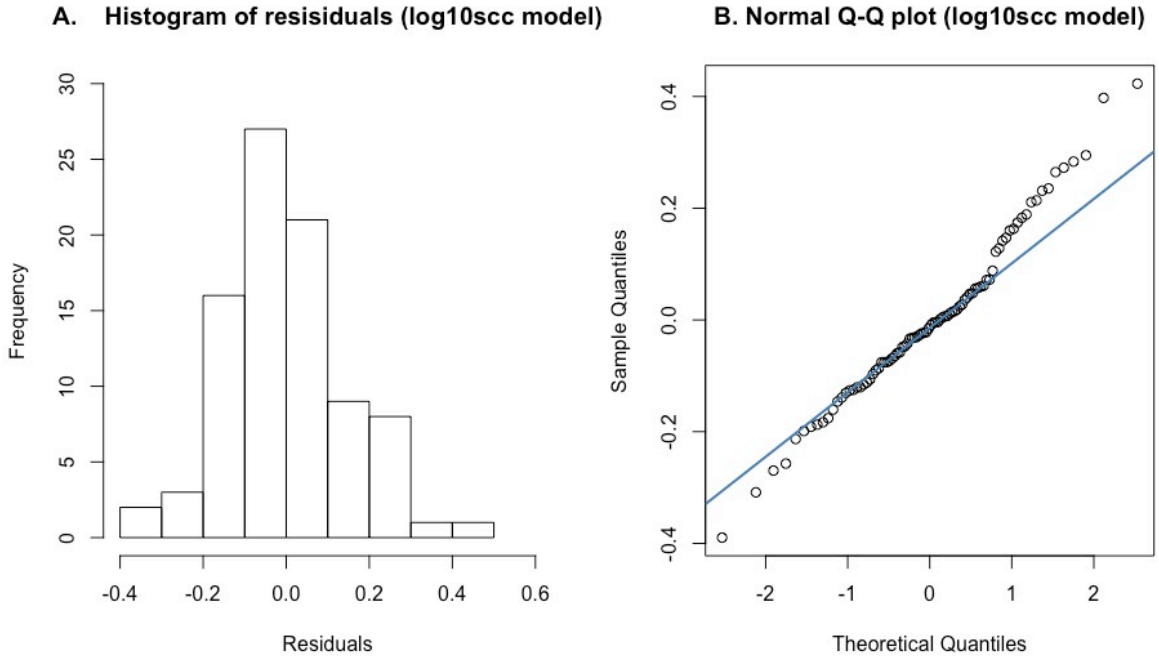


Figure 7. Histogram of model residual values (A.) and the normal Q-Q plot for the somatic cell count model. The residuals are normally distributed, there is some deviation in the upper quantiles from the theoretical normal line in the Q-Q plot, but not at a great enough threshold to violate the model assumption parameters.

The fixed effect treatment group (AB_Orb) had a significant effect on the outcome variable, mean log_{10}SCC following the dry period ($p < 0.1$, Table 1.), that is the difference between the effect of treatment group on the SCC is likely not zero. To further test the significance of the model a likelihood ratio test of the SCC model was compared to the likelihood ratio test of a null SCC model using Anova analysis. The null model uses the same model parameters as the SCC model but omits the fixed effect of interest, AB_Orb. There was a significant difference between the two models (χ^2 (1)= 3.92, $p < 0.05$; Table 2.). This indicates that the inclusion of the fixed effect treatment group makes it more likely to see the data collected in the model than when the effect of treatment is removed.

Model equations are as follows:

SCC model: Av_C0.PC1.PC3_log10scc ~ AB_Orb + D0_log10scc_centred + DPL_centred + Qrt + (1 | Cow)

SCC null model: Av_C0.PC1.PC3_log10scc ~ D0_log10scc_centred + DPL_centred + Qrt + (1 | Cow)

Herd	Model	Df	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)	
2	SCC _null	8	-0.80	19.02	8.40	-16.80				
	SCC	9	-2.73	19.57	10.36	-20.73	3.92	1	0.048	*

Significance codes: 0 '***', 0.001'**', 0.01'*', 0.05'.', 0.1 ' , 1 '

Table 2. Anova analysis comparing the likelihood ratio tests for the SCC model and the SCC null model. The fixed effect treatment group was removed in the null model allowing the effect of treatment group to be statistically assessed. A significant difference between the two models was determined (χ^2 (1)= 3.92, $p = 0.048$).

4.3.3. Chao1 index

4.3.3.a Chao1 index variation following antibiotic treatment

The Chao1 index is a species richness measure, estimating the number of OTUs present in each sample based on the OTUs identified in all samples (Kim *et al.*, 2017). The Chao1 index was calculated using the phyloseq package in R (McMurdie & Holmes, 2013; R Core Team, 2017). The impact of antibiotic treatment on species richness will be explored using the same analysis as applied to somatic cell counts (Chapter 4. Section 4.3.2.)

The median log_{10}Chao1 index is highest in both treatment groups at the drying off time point (D0), a index score of 1.56 in the antibiotic (AB) group and 1.66 in the non-antibiotic (Orb Only) group (Supplementary Materials Table 9.), but across the 5 time points remains fairly constant with the mean value in the AB group ranging between 1.36 and 1.53, and in the Orb Only group ranging between 1.42 and 1.64 (Figure 8., Supplementary Materials Table 9.).

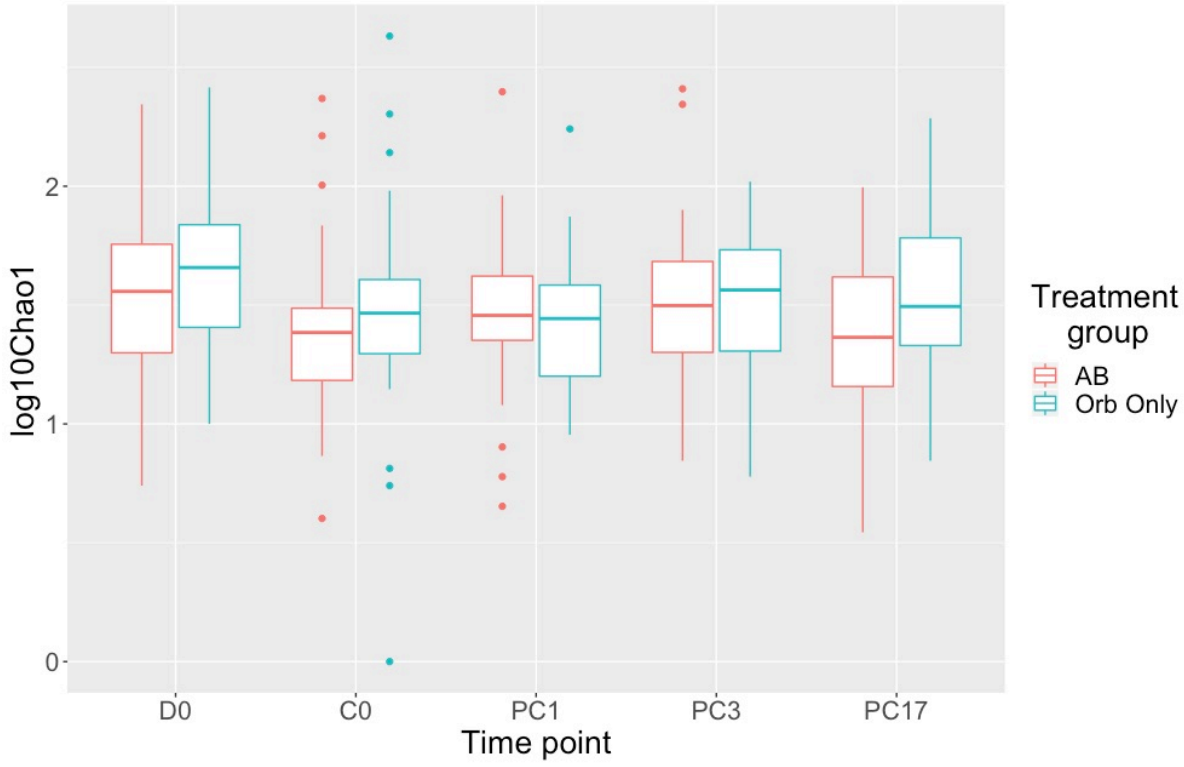


Figure 8. The change in log_{10}Chao1 index across the dry period for antibiotic (AB) and non-antibiotic (Orb Only) treatment groups. The mean Chao1 index score is marginally largest at D0 for both treatment groups, with a log_{10}Chao1 index score of 1.53 in the AB group and 1.64 in the Orb Only group. The Chao1 index score lowers following the dry period to its lowest

mean level in the AB group at C0 (1.36) and at PC1 in the Orb Only group (1.42), however there is little variation in the mean Chao1 index score across the 5 time points. (D, Drying off; C0, Calving day; PC1, 1 day post calving; PC3, 3 days post calving; PC17, 17 days post calving; n = 88 per time point, total n = 440).

To assess the variation within each udder quarter the change in the Chao1 index for each cow per time point for each treatment was plotted (Figure 9., Figure 10.). There was a fairly consistent mean $\log_{10}$Chao1 index across the time points (Figure 8.), on a cow level there is no obvious patterns emerging (Figure 9.), however it does show the variation in the index score between quarters of the same udder. With an inverse log transformation, the spikes in Chao1 index scores can be seen and displays the outlier values seen at post calving time points in Figure 8. Often it is just one udder quarter at a singular time point that has a spike in the Chao1 index which then recovers by the subsequent time point (e.g. Cow 695 and Cow 937, Figure 10.A. and Figure 10.B. respectively). There is no visible difference between the two treatment groups in the change and variation in Chao1 index scores within the udders over time.

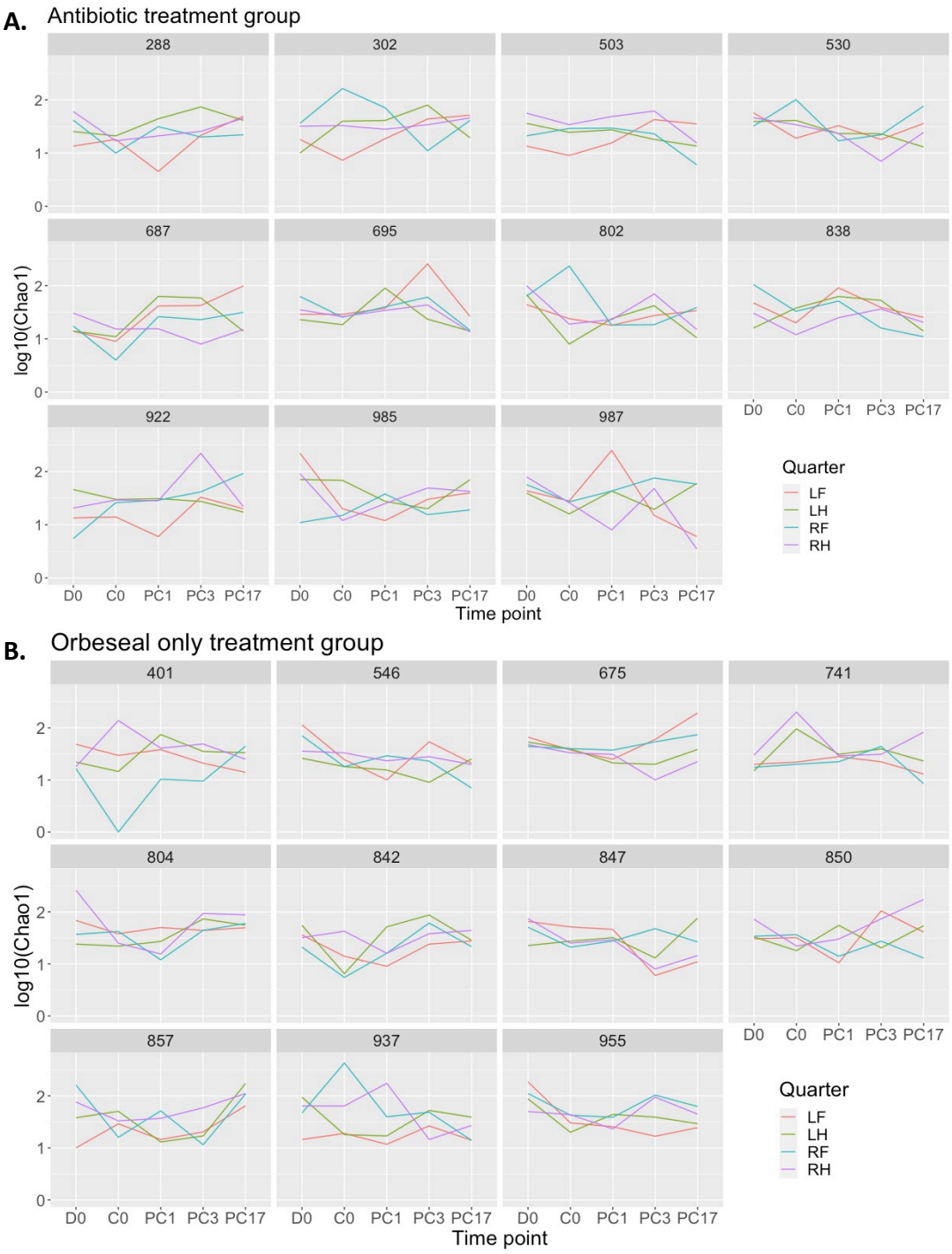


Figure 9. The change in the Chao1 index (log_{10}Chao1) per cow (numbered) per udder quarter over time for each treatment group, antibiotic (Figure 9.A.) and non-antibiotic (Figure 9.B.). Udder quarters: LF, Left Fore; LH, Left Hind; RF, Right Fore; RH, Right Hind. Time points: D, Drying off; C0, Calving day; PC1, 1 day post calving; PC3, 3 days post calving and PC17, 17 days post calving. Total n = 440.

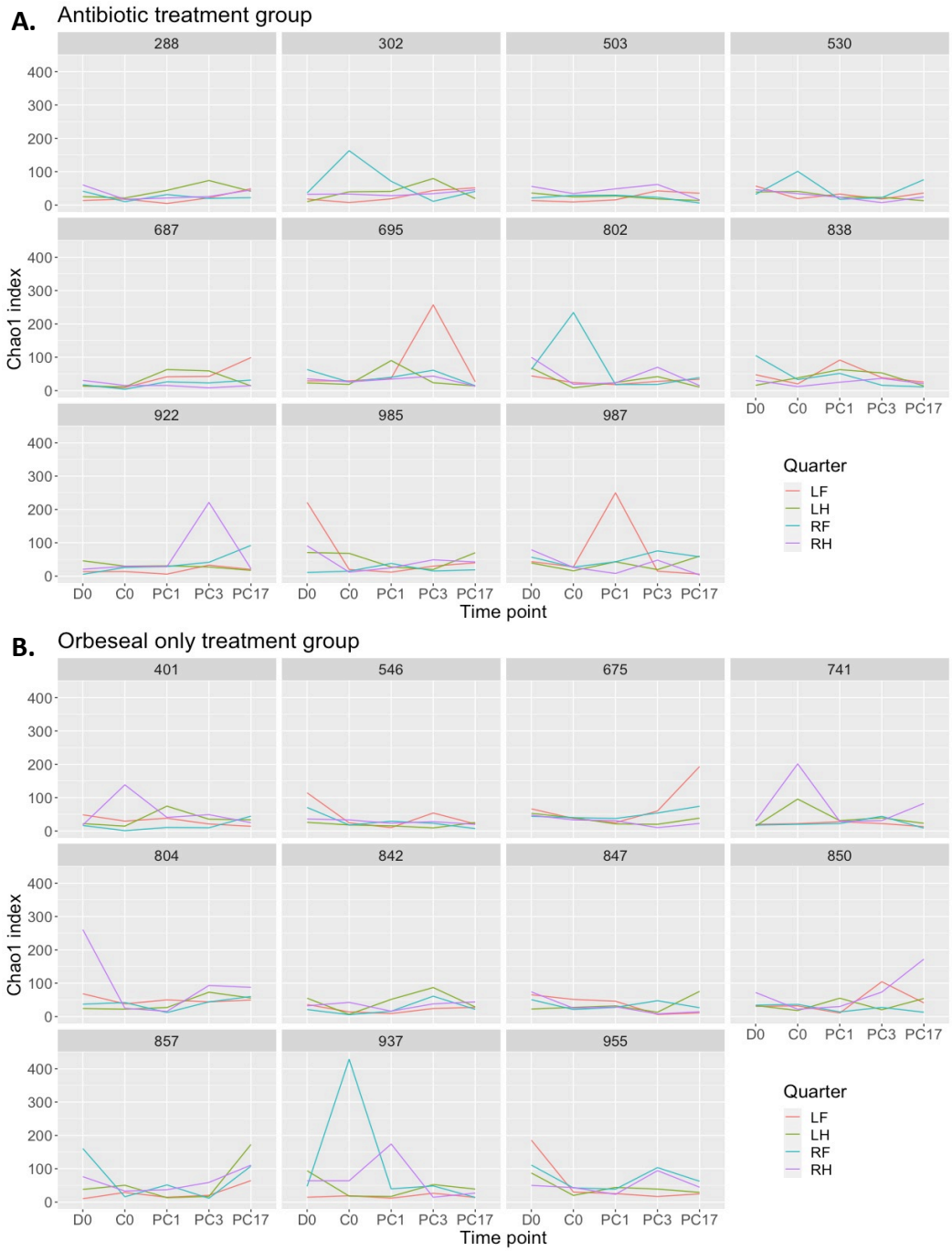


Figure 10. Variation in the Chao1 index per cow (numbered) per udder quarter over time for each treatment group, antibiotic (Figure 10.A.) and non-antibiotic (Figure 10.B.). Udder quarters: LF, Left Fore; LH, Left Hind; RF, Right Fore; RH, Right Hind. Time points: D, Drying off; C0, Calving day; PC1, 1 day post calving; PC3, 3 days post calving and PC17, 17 days post calving. Total n = 440. Plotting on a non-log scale highlights the spikes in the Chao1 index experienced in some cows (e.g. Cow 937 Figure 10. B.).

To further explore the effect of antibiotic treatment in the first few days post partum, the mean of the Chao1 index scores for each of the samples at the time points calving (C0), 1 day post calving (PC1) and 3 days post calving (PC3) were calculated (termed Av_C0.PC1.PC3). Following the dry period there is a decrease in the median log_{10}Chao1 in both treatment groups and a narrowing in the variation (Figure 11.). In the AB treatment group there was a decrease in the mean log_{10}Chao1 at drying off (D0) from 1.53 to 1.45 following the dry period. In the Orb Only group there was a reduction from 1.64 to 1.46 in the mean log_{10}Chao1 following the dry period (Supplementary Materials Table 9.).

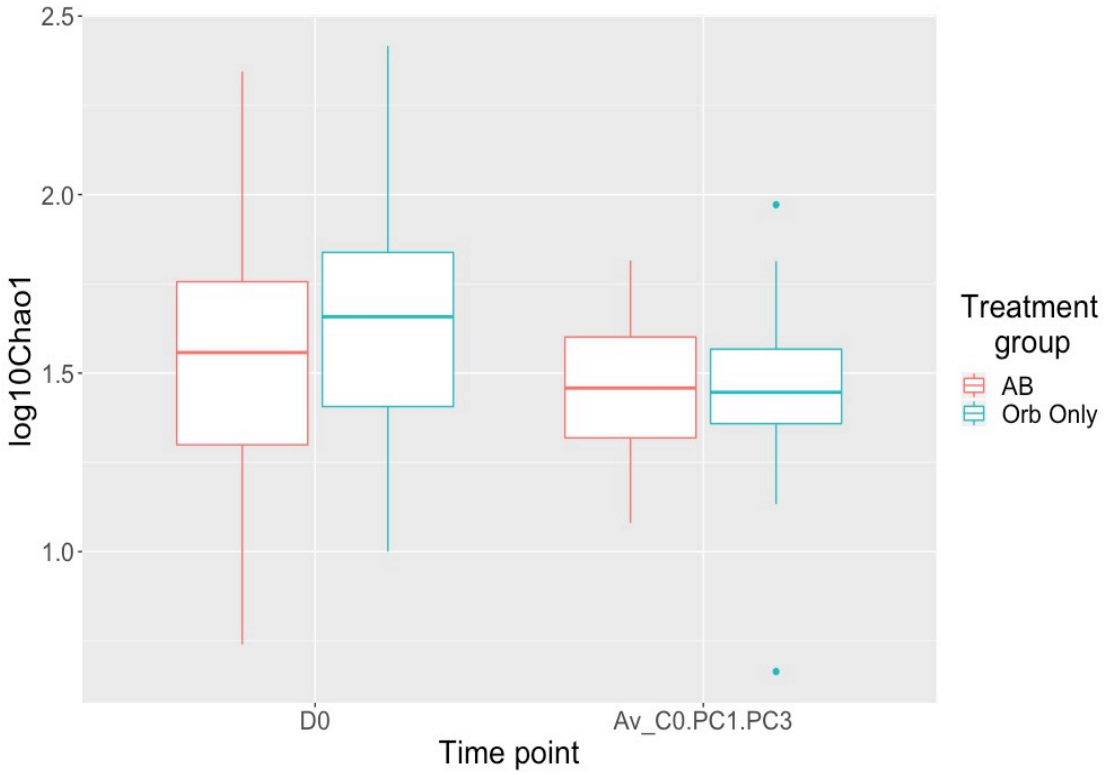


Figure 11. Change in log_{10}Chao1 over the dry period for the antibiotic (AB) and non-antibiotic (Orb Only) treatment groups. The time points Drying off (D0) and the mean log_{10}Chao1 for samples taken from each quarter in each cow at calving, 1 day post calving and 3 days post calving (Av_C0.PC1.PC3), were compared. There is a reduction in the mean log_{10}Chao1 index of 0.08 across the dry period in the AB group and a reduction in the mean log_{10}Chao1 index of 0.18 in the Orb Only group (Supplementary Materials Table 9.).

4.3.3.b. Modelling Chao1 richness index following antibiotic treatment

Linear mixed effects analysis exploring the relationship between the Chao1 index and antibiotic treatment was performed using the lmer package in R (Bates *et al.*, 2015; R Core Team, 2017). As fixed effects, treatment group (AB_Orb), somatic cell count at drying off (D0.log_{10}scc_centred) and udder quarter were entered into the model. As a random effect, intercepts for Cow were entered into the model (Table 3).

Model equation:
Av_C0.PC1.PC3_log10Chao1 ~ AB_Orb + D0_log10scc_centred + Qrt + (1 | Cow)

Fixed Effect	**Estimate**	**Std. Error**	**df**	**t value**	**Pr(>|t|)**	
(Intercept)	1.388	0.048	69.776	28.960	<2e-16	***
AB_Orb: Orb Only	**0.009**	**0.044**	**19.077**	**0.214**	**0.833**	
D0_log10scc_centred	-0.032	0.060	72.258	-0.532	0.596	
Quarter LH	0.083	0.060	62.727	1.370	0.176	
Quarter RF	0.054	0.060	61.965	0.909	0.367	
Quarter RH	0.101	0.060	62.103	1.682	0.098	.

Random effects

Groups	Name	Variance	Std.Dev.
Cow	(Intercept)	0.001	0.025
Residual		0.039	0.199

Significance codes: 0 '***', 0.001'**', 0.01'*', 0.05'.', 0.1 ' , 1 '

Table 3. Linear mixed effects model describing the effect of antibiotic treatment on the outcome variable, the mean species richness index (log_{10}Chao1) following the dry period, specifically the mean values of the time points calving (C0), 1 day post calving (PC1) and 3 days post calving (PC3). The fixed effects treatment group (AB_Orb) and udder quarter were included in the model (LF, left fore; LH, left hind; RF, right fore; RH, right hind). The somatic cell count (log_{10}SCC) at the drying off time point was centred and included as fixed effect in the final model (D.log10scc_centred). The effect of individual cow variation was accounted for in the model by inclusion of Cow as a random effect. 88 observations, 22 groups.

The mean log_{10}Chao1 index for the time points following the dry period (calving, 1 day post calving and 3 days post calving) were estimated by the model. Following an inverse log

transformation, the model estimates that the mean Chao1 index following the dry period for the left fore udder quarter of an antibiotic (AB) treated cow would be 24.43 ± 1.12 (standard errors). For the left hind udder quarter of a non-antibiotic (Orb Only) treated cow there is an estimate Chao1 index score of 28.05 ± 1.63 (standard errors). For the measured Chao1 index scores the estimate would be between 28-29 for both treatment groups following the dry period (Figure 11., Supplementary Materials Table 9.).

Visual inspection of the model model parameters shows a relatively normal distribution with some outlier values at the negative end of the residual scale (Figure 12.A.). There is a clear outlier value at the lower quartiles of the normal Q-Q plot that deviates from the expected normality line (Figure 12. B.). As outliers violate the model assumption, the value was removed and the model re-tested.

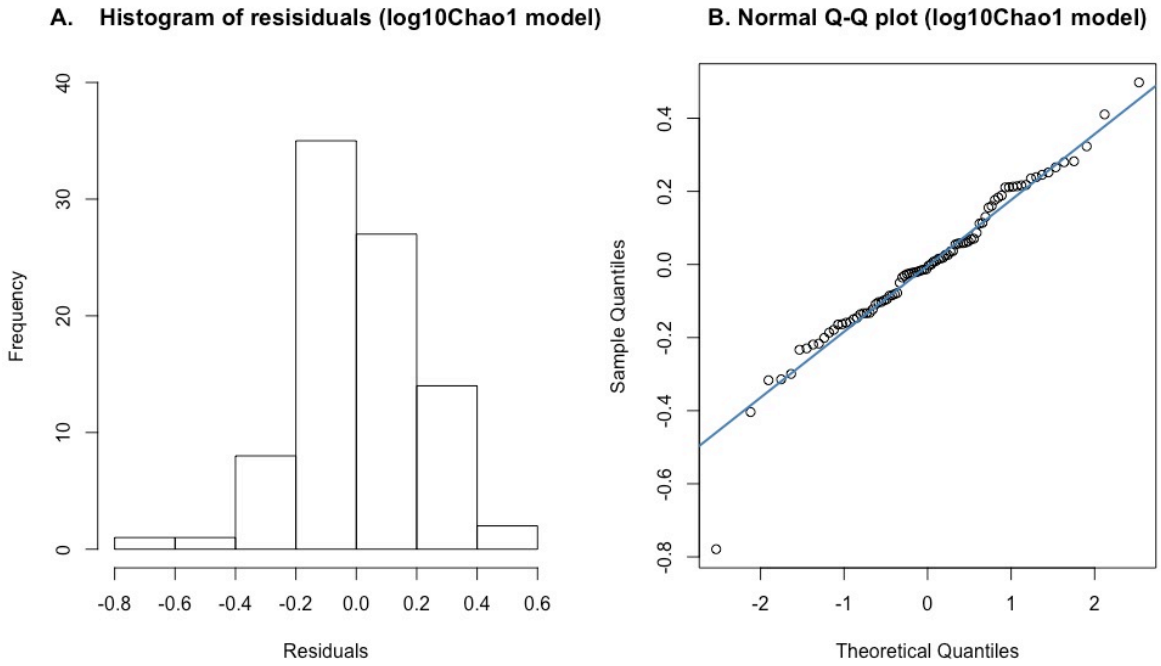


Figure 12. Histogram of residuals for the log_{10}Chao1 index linear mixed effects model shows a relatively normal distribution with some clear outlier values at the lower residual end of the plot (A.). The normal Q-Q values fit well along the expected normality line with a clear outlier value at around (-2.6,-0.8, B.). This outlier violates the model assumptions, the model was identified and re-tested (Table.4).

The outlier value was identified as a sample from the right fore of Cow 401, with a mean log_{10}Chao1 index following the dry period of 0.66. For this sample the log_{10}Chao1 index scores for the other time points as follows: Drying off (D0), 1.22; Calving (C0), 0; 1 day post calving, 1.01 (PC1) and 3 days post calving (PC3), 0.98. This is likely to be an experimental failure, as all other samples in the dataset recorded an OTU count greater than 0 (Figure 9.). The mean of the two successful samples, PC1 and PC3, was calculated (1.00) and replaced the post drying off Chao1 score for the RF of Cow 401 in the model.

The model with the outlier removed (Table 4.) no longer violates the model assumptions (Figure 13.). The model estimates a mean Chao1 index following the dry period for the LF of a AB treated cow to be 24.27 ± 1.11 (standard errors) and a mean Chao1 index of 28.44 ± 1.59 (standard errors). The t value and p value are improved in this model (t = 0.396, p = 0.697; Table 4.) compared to the model including the outlier (t = 0.214, p =0.83, Table 3.), however not at a threshold that confidently identifies that the difference between the Chao1 index score following the dry period is not equal to 0 when comparing the two treatment groups. In addition as p value reporting is disputed in lmer models, to more robustly test for significance a likelihood ratio test was conducted (Table 5.).

Model formula:
Av_C0.PC1.PC3_log10Chao1 ~ AB_Orb + D0_log10scc_centred + Qrt + (1 | Cow)

Fixed effect	**Estimate**	**Std. Error**	**df**	**t value**	**Pr(>|t|)**	
(Intercept)	1.385	0.045	65.893	30.590	<2e-16	***
AB_Orb: Orb only	**0.017**	**0.043**	**18.909**	**0.396**	**0.697**	
D0_log10scc_centred	-0.031	0.057	75.344	-0.539	0.591	
Quarter LH	0.083	0.056	62.468	1.487	0.142	
Quarter RF	0.070	0.055	61.726	1.264	0.211	
Quarter RH	0.101	0.055	61.860	1.828	0.072	.

Random effects

Groups	Name	Variance	Std.Dev.
Cow	(Intercept)	0.002	0.041
Residual		0.033	0.182

Significance codes: 0 '***', 0.001'**', 0.01'*', 0.05'.', 0.1 ' , 1 '

Table 4. Linear mixed effects model predicting the log10Chao1 index following the dry period as the mean of time points calving (C0), 1 day post calving (PC1) and 3 days post calving (PC3). 1 outlier value was removed from Cow 401,RH at C0. The average value for the log10Chao1 index was calculated as a mean of values at PC1 and PC3. Fixed effects treatment group (AB_Orb), log10scc at drying off and udder quarter were included in the model. Variation between cows was accounted for by adding Cow as a random effect. Number of observations 88, 22 groups (number of cows).

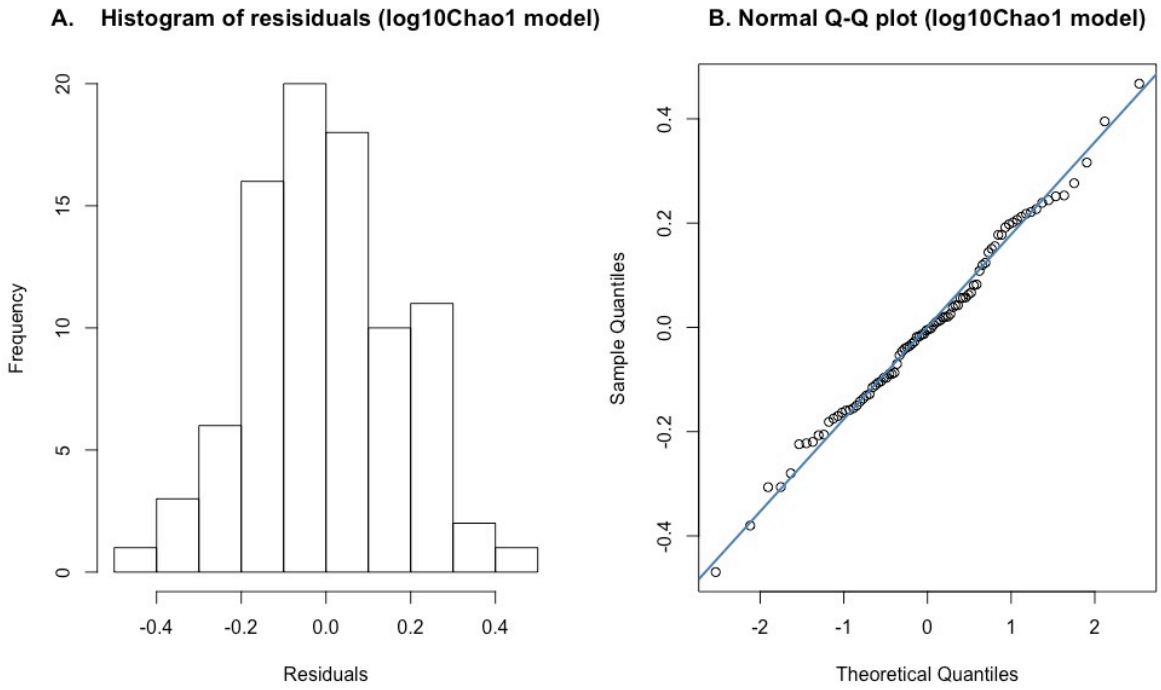


Figure 13. Histogram of residuals for the log10Chao1 model with outlier value removed shows a normal distribution (A). The normal Q-Q plot values also follow the expected normal line (B). The removal of the outlier value improves the quality of the residual and Q-Q plot compared to the inclusion of the outlier value (Figure 5.), the model parameter assumptions have been met.

A likelihood ratio test of the Chao1 index model was compared to the likelihood ratio test of a null Chao1 model using Anova analysis. The null model uses the same model parameters as the Chao1 model, but excludes the fixed effect of interest, treatment group. A significant difference was not determined between the two models ($\chi^2(1)= 0.21$, $p > 0.1$; Table 5.). This indicates that the inclusion of the fixed effect treatment group does not have an effect on making it more likely to see the data collected in the model than when the effect of treatment group is removed.

Model equations are as follows:

Chao1 model:	Av_log10Chao1_C0.PC1.PC3 ~ AB_Orb + D.log10scc_centred + quarter + (1 | cow)
Chao1 null model:	Av_log10Chao1_C0.PC1.PC3 ~ D.log10scc_centred + quarter + (1|cow)

Herd	Model	Df	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
2	Chao1_ null	7	-37.71	-20.37	25.86	-51.71			
	Chao1 (outlier removed)	8	-35.89	-16.07	25.94	-51.89	0.18	1	0.675

Significance codes: 0 '***', 0.001'**', 0.01'*', 0.05'.', 0.1 ' , 1 '

Table 5. Anova analysis comparing the likelihood ratio tests for the Chao1 model and the Chao1 null model. The fixed effect treatment group was removed in the null model allowing the effect of treatment group to be statistically assessed. A significant difference between the two models was not determined ($\chi^2(1)= 0.18$, $p = 0.675$).

4.3.4. Shannon index

4.3.4.a. Shannon index variation following antibiotic treatment

The Shannon index is a metric for community diversity by estimating both species richness and species evenness while considering the relative abundance, the Shannon index score in this context will increase when both the number of OTUs increase and the distribution amongst the different OTUs becomes more even (Kim *et al.*, 2017; Lemos *et al.*, 2011).
The Shannon index was calculated using the phyloseq package in R (McMurdie & Holmes, 2013; R Core Team, 2017). The effect of antibiotic treatment on species diversity of the mammary gland microbiome will be explored using the same analysis as previous sections.

The median Shannon index score is highest at the drying off time point (D0) and reaches the lowest value at calving (C0), before levelling off between 1 and the days post calving for both treatment groups (Figure 14.). The mean Shannon index score for the antibiotic (AB) treatment group reduces from 2.08 at D0 to 1.29 at C0. The mean Shannon index for the non-

antibiotic group (Orb Only) also reduces across the dry period from 2.10 at D0 to 1.48 at C0 (Supplementary Materials Table 10.). The largest variation between the mean Shannon index score between the two treatment groups is at the final time point, 17 days post calving (PC17; Figure 14.), where there is a mean Shannon index score of 1.53 in the AB treatment group and a score of 1.91 in the Orb Only treatment group (Supplementary Materials Table 10.).

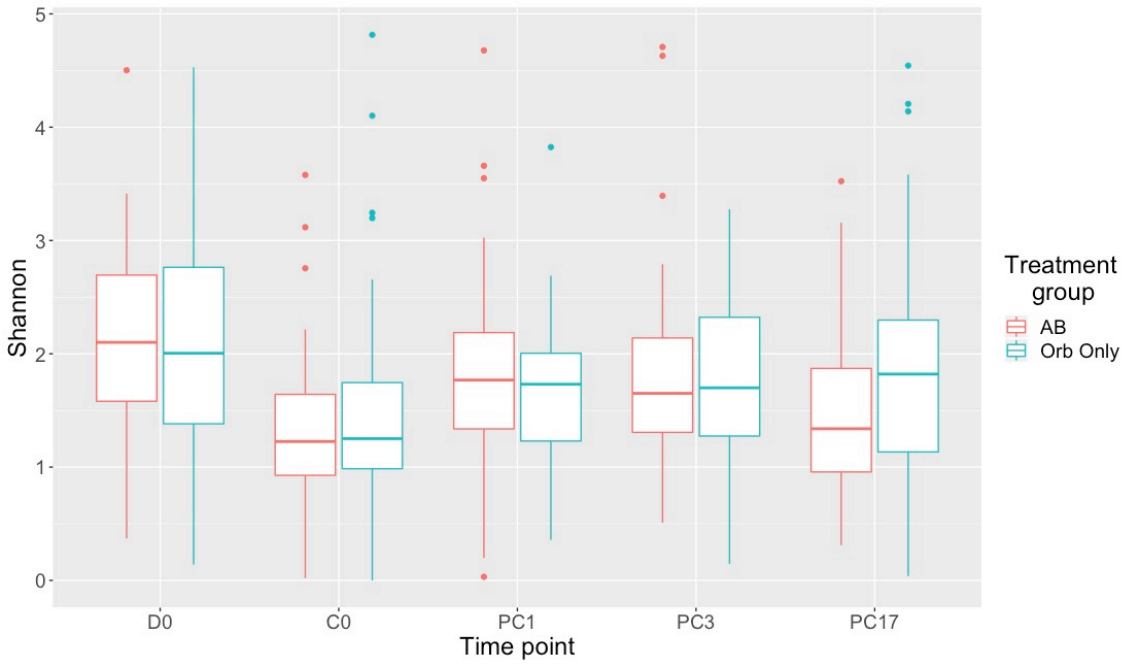


Figure 14. The variation in the Shannon index across the dry period to 17 days post calving for the antibiotic (AB) and non-antibiotic (Orb Only) treatment groups. The median Shannon index score is greatest at D0 for both treatment groups, with scores of 2.10 in the AB group and 2.01 in the Orb Only group. The Shannon index score decreases following the dry period with the lowest diversity at C0 (AB group median 1.23, Orb Only group median 1.25; (Supplementary Materials Table 10.). D, Drying off; C0, Calving day; PC1, 1 day post calving; PC3, 3 days post calving; PC17, 17 days post calving; n = 88 per time point, total n = 440).

Plotting Shannon index data per udder quarter per cow for each treatment group and each time point displays the level of variation within cows between time points, this changeable pattern is seen in both treatment groups (Figure 15.)

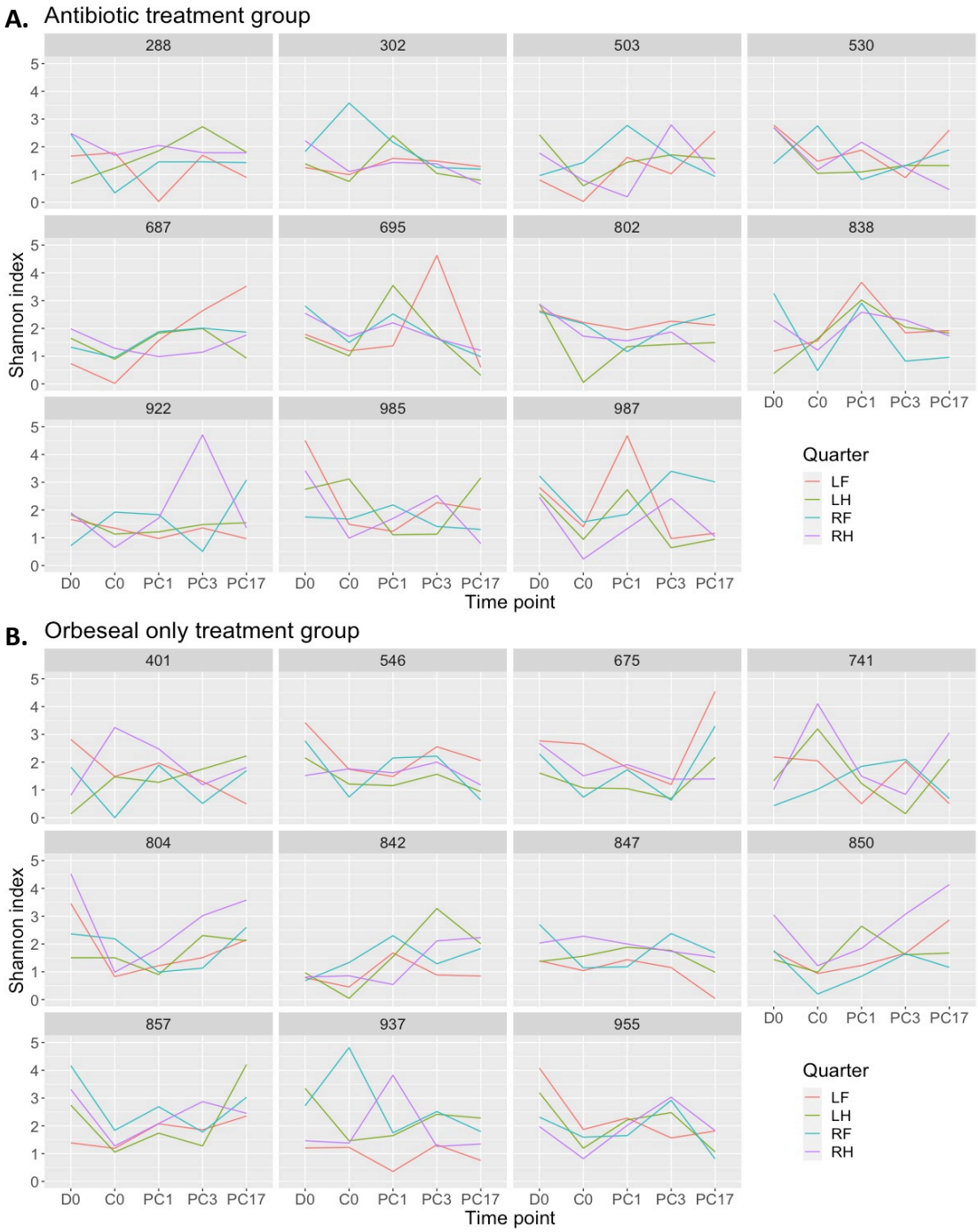


Figure 15. Variation in the Shannon index per cow (numbered) per udder quarter over time for the antibiotic treatment group (A.) and non-antibiotic treatment group (B.). Udder quarters: LF, Left Fore; LH, Left Hind; RF, Right Fore; RH, Right Hind. Time points: D, Drying off; C0, Calving day; PC1, 1 day post calving; PC3, 3 days post calving; PC17, 17 days post calving. Total n = 440. Shannon index scores appear very changeable across the udder quarters within cows over the time points.

To further explore the effect of antibiotic treatment on the time points of interest, the mean of the Shannon index scores following the dry period (calving, C0; 1 day post calving, PC1 and 3 days post calving, PC3) was calculated and compared with the drying off (D0) Shannon index score. The within treatment group variation in the Shannon index score narrows across the dry period for both the antibiotic (AB) and non-antibiotic (Orb Only) treatment groups (Figure 16.). The mean Shannon index score reduces in both treatment groups over the dry period, reducing by 0.43 in the AB group and reducing by 0.45 in the Orb Only group (Supplementary Materials Table 10.)

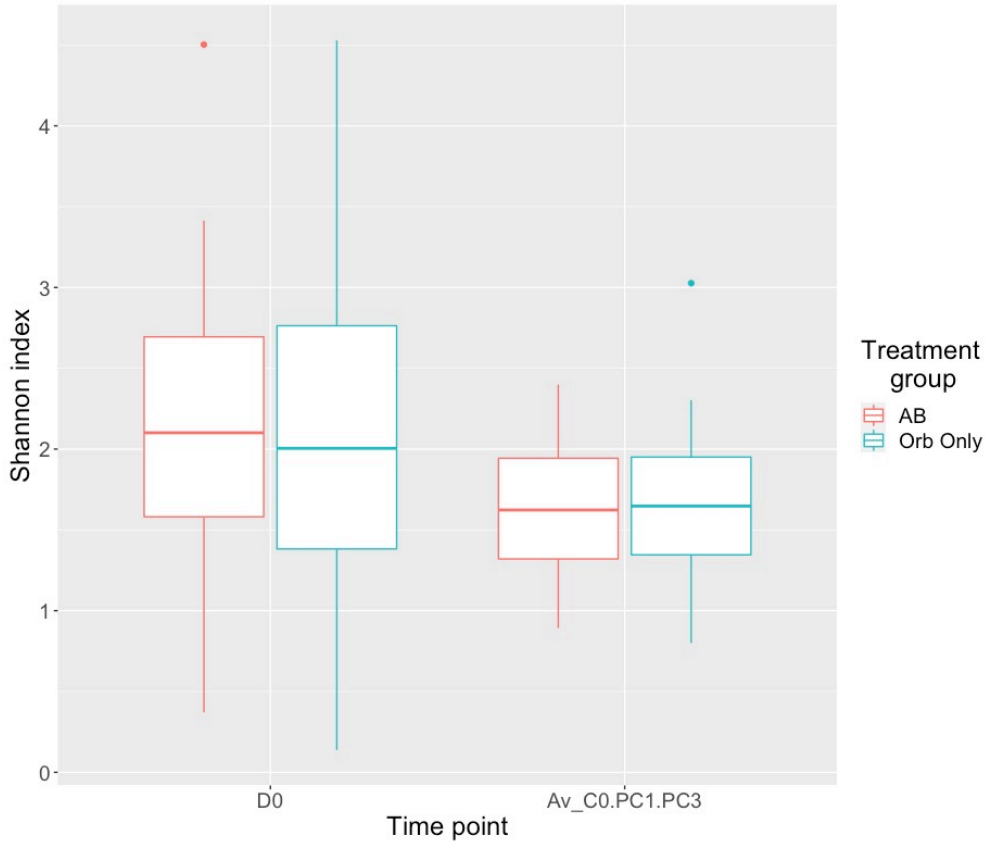


Figure 16. Variation in the Shannon index at the drying off time point (D0) and the mean of the samples at the time points following the dry period, calving (C0), 1 day post calving (PC1) and 3 days post calving (PC3), termed Av_C0.PC1.PC3. There is a higher median Shannon index score in the antibiotic (AB) treatment group compared the non-antibiotic (Orb Only) treatment group at D0, 2.10 and 2.01 respectively. The Shannon index score reduces over the dry period for both treatment groups to a median value of 1.62 in the AB group and 1.65 in the Orb Only group (Supplementary Materials Table 10.). The within treatment group variation narrows over the dry period for both treatment groups.

4.3.4.b. Modelling the Shannon diversity index following antibiotic treatment

Linear mixed effects model analysis was conducted to explore the relationship between the Shannon index and antibiotic treatment using the lmer package in R (Bates *et al.*, 2015; R Core Team, 2017). The model output was the prediction of the mean Shannon index score for the time points following the dry period (calving, C0; 1 day post calving, PC1 and 3 days post calving, PC3; termed Av_C0.PC1.PC3_Shannon). The fixed effects treatment group (AB_Orb), Somatic Cell Count at drying off (D.log_{10}SCC_centred), Chao1 index at drying off (D.log_{10}Chao1_centred) and dry period length (DPL_centred) were included in the model. As a random effect, intercepts for each cow were entered into the model (Table 6.).

Model equation:

Av_C0.PC1.PC3_Shannon ~ AB_Orb + D0_log10scc_centred + D0_log10Chao1_centred + DPL_centred + (1 | Cow)

Fixed Effect	**Estimate**	**Std. Error**	**df**	**t value**	**Pr(>|t|)**	
(Intercept)	1.669	0.066	18.696	25.211	6.80E-16	***
AB_Orb: Orb only	**-0.041**	**0.094**	**19.044**	**-0.430**	**0.672**	
D0_log10scc_centred	-0.154	0.126	73.539	-1.224	0.225	
D0_log10Chao1_centred	0.316	0.145	75.586	2.181	0.032	*
DPL_centred	0.006	0.007	18.499	0.896	0.382	

Random effects

Groups	Name	Variance	Std.Dev.
Cow	(Intercept)	0.004	0.063
Residual		0.174	0.417

Significance codes: 0 '***', 0.001'**', 0.01'*', 0.05'.', 0.1 ' , 1 '

Table 6. Linear mixed effects model predicting the Shannon index scores as a mean value of the time points C0 (calving), PC1 (1 day post calving) and PC3 (3 days post calving). The main fixed effect of interest, treatment group (AB_Orb), was included in the model. The log_{10} of SCC and Chao1 index values at the drying off time point were centred and included as fixed effects in the final model (D.log_{10}scc_centred and D.log_{10}Chao1_centred, respectively), the length of the dry period was also centred and included in the model as a fixed effect

(DPL_centred). The effect of individual cow variation was accounted for in the model by inclusion of Cow as a random effect. 88 observations, 22 groups.

The model intercept value reflects the output Shannon index score following the dry period of 1.669 $\pm$ 0.066 (standard errors) for an AB treated cow (Table 6.). Addition of the fixed effect estimate scores in the model allows for comparisons of cows in each treatment group. For example, for an Orb Only treated cow the Shannon index score would increase to 1.796 $\pm$ 0.438 (standard errors, Table 6.). The model has not reported a significant difference between the treatment groups; there is not enough evidence to confirm that the effect of treatment on the Shannon index following the dry period is not zero.

Visualisation of the model assumption parameters indicated no major violation in the distribution of residuals and in the expected normal Q-Q plot (Figure 17.). There is one potential outlier value at the upper quantile range, but upon inspection the value is not out of the normal range of the data in context.

To further explore the effect of treatment on the outcome of the Shannon index model, a likelihood ratio test was conducted for both the Shannon model and a null model using Anova analysis. The null model includes the same model parameters as the Shannon model, but omits the fixed effect of interest, treatment group. No significant difference was determined between the two models ($\chi^2(1)= 0.21$, $p > 0.1$, Table 7.). This indicates that the omission of the fixed effect treatment group does not effect the likelihood of seeing the data collected in the model.

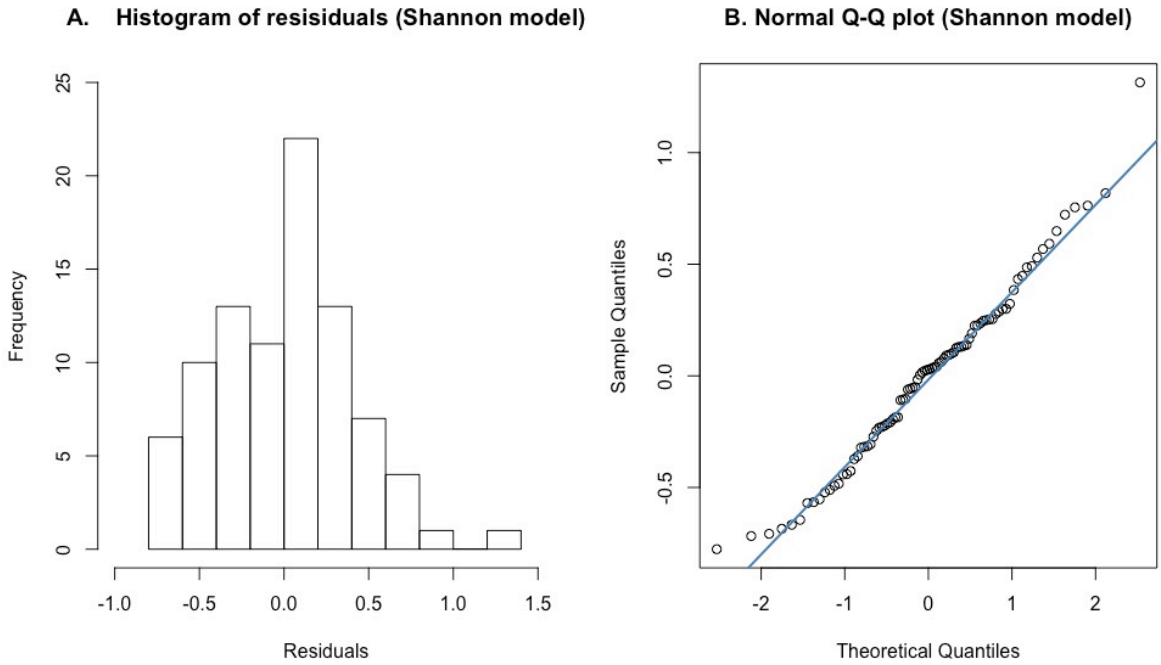


Figure 17. Histogram of model residuals for the Shannon model (A.) show a relatively normal distribution. Normal Q-Q data plotted against the expected normality line (B) has one value in the upper quantiles deviating from the expected normality line, however overall there is not enough deviation to violate the model assumptions.

Model equations are as follows:

Shannon model:	av_Shannon_C0.PC1.PC3 ~ AB_Orb + D.log10scc_centred + D.log10Chao1_centred + DPL_centred + (1 | cow)
Shannon null model:	av_Shannon_C0.PC1.PC3 ~ AB_Orb + D.log10scc_centred + D.log10Chao1_centred + DPL_centred + (1 | cow)

Herd	Model	Df	AIC	BIC	logLik	deviance	Chisq	Df	Pr(>Chisq)
2	Shannon _null	6	104.76	119.62	-46.38	92.76			
	Shannon	7	106.55	123.89	-46.27	92.55	0.21	1	0.645

Significance codes: 0 '***', 0.001'**', 0.01'*', 0.05'.', 0.1 ' , 1 '

Table 7. Anova analysis comparing the likelihood ratio tests for the Shannon model and the Shannon null model. The fixed effect treatment group was omitted from the null model allowing the absence of treatment group to be statistically assessed. No significant difference between the two models could be determined ($\chi^2(1)= 0.21$, $p = 0.645$).

4.3.5. OTU Analysis

4.3.5.a. OTUs Overview

Operational Taxonomic Units (OTUs) are in context defined as a cluster of 16S rRNA reads with at least 97% similarity, which can approximately correspond to a bacterial genus. Here OTUs are used as a tool to estimate the richness and diversity of the bacteria in milk samples of the mammary gland. In previous sections OTU values have been used to calculate richness and diversity indices, in this section OTUs will be used directly to assess how the OTU community changes across the dry period and if there is a difference between treatment groups.

Summary information of OTU and herd information for context is provided in Table 8. Deeper community analysis will be explored in Chapter 5.

OTU information	**Herd 2**
Total unique OTUs in dataset	3048
Number OTUs identified per sample	6.93
Mean $\log_{10}$(total OTU count) per sample	3.72
Mean % of non-hits per sample	98.6%
Mean unique OTUs per sample	31.3
Mean unique OTUs per treatment group:	
AB-Orb	29.2
Orb Only	33.3
Mean $\log_{10}$(total OTU count) per treatment group:	
AB-Orb	3.78
Orb Only	3.67

Table 8. Summary of OTU information for Herd 2. The mean unique number of OTUs per sample in the antibiotic treatment group (AB) is lower than in the non-antibiotic treatment group (Orb Only), 29 and 33 respectively. Whereas the $\log_{10}$ total number of OTUs is greater in the AB group (3.78) compared to the Orb Only group (3.67). Number of samples 440.

4.3.5.b. Statistical analysis of OTU correlations

A Spearman's rank correlation of the abundance of unique OTUs in each udder quarter of each cow with the following time points for each treatment group was performed. The

ranked composition of OTUs can be used to understand the change in abundancies of OTUs between sampling time points. The time points drying off (D), calving (C0), 1 day post calving (PC1), 3 days post calving (PC3) and 17 days post calving (PC17) were used to correlate ranked OTUs between drying off and the the 4 time points following the dry period (Table 9.A., Figure 18.A.) and to correlate ranked OTUs between consecutive time points (Table 9.B., Figure 18.B.). The Spearman's rank correlation scores were averaged within each treatment group and summarised in Table 9. and Figure 18.

A higher correlation between the two time points (-1 to 1) in the samples indicates that the proportion of OTUs found are more similar and in a more similar abundance. Indicating the mammary gland microbiome has been less perturbed over the dry period; or less new bacteria have entered the udder system. The strength of correlation is directive of the similarity in the microbiota between sampling time points.

Overall there is a low correlation of ranked OTU abundancies between time points tested in both treatment groups (Figure 18). The mean correlation scores vary between 0.097 and 0.149 indicating the composition of OTUs changes greatly between the sampling time points (Table 8.). There is a slightly greater level of correlation when comparing the median values later time point correlations with those across the dry period, for example there are median correlation scores of 0.091 (AB) and 0.099 (Orb Only) across D0 to C0 and median scores of 0.138 (AB) and 0.143 (Orb Only) across D0 to PC17 (Table 9.A.). However, there is little difference between treatment groups.

	Time points correlated	Treatment	n	mean	sd	median	IQR
A.	**D0_C0**	AB	44	0.120	0.092	0.091	0.140
		Orb_only	44	0.123	0.100	0.099	0.163
	D0_PC1	AB	44	0.132	0.081	0.130	0.134
		Orb_only	44	0.132	0.093	0.118	0.118
	D0_PC3	AB	44	0.140	0.078	0.138	0.121
		Orb_only	44	0.149	0.108	0.135	0.138
	D0_PC17	AB	44	0.132	0.085	0.138	0.140
		Orb_only	44	0.148	0.097	0.143	0.130
B.	**D0_C0**	AB	44	0.120	0.092	0.091	0.140
		Orb_only	44	0.123	0.100	0.099	0.163
	C0_PC1	AB	44	0.097	0.080	0.075	0.092
		Orb_only	44	0.106	0.076	0.089	0.125
	PC1_PC3	AB	44	0.117	0.071	0.119	0.114
		Orb_only	44	0.133	0.091	0.118	0.118
	PC3_PC17	AB	44	0.118	0.076	0.117	0.177
		Orb_only	44	0.149	0.095	0.140	0.120

Table 9. Summary of Spearman's rank correlation scores of the ranked abundancies of OTUs for samples taken at drying off (D0) to 4 sampling time points following the dry period (C0, calving; PC1, 1 day post calving; PC3, 3 days post calving; PC17, 17 days post calving) for each treatment group (antibiotic, AB and non-antibiotic, Orb Only). Correlations between drying off to the time points following the dry period are low for both treatment groups, with little difference between the mean correlation scores of each treatment group (A). Comparing subsequent time points, there is also a low mean correlation between timepoints (B). This indicates that between each sampling time point the ranked abundance of OTUs in each mammary gland quarter changes greatly.

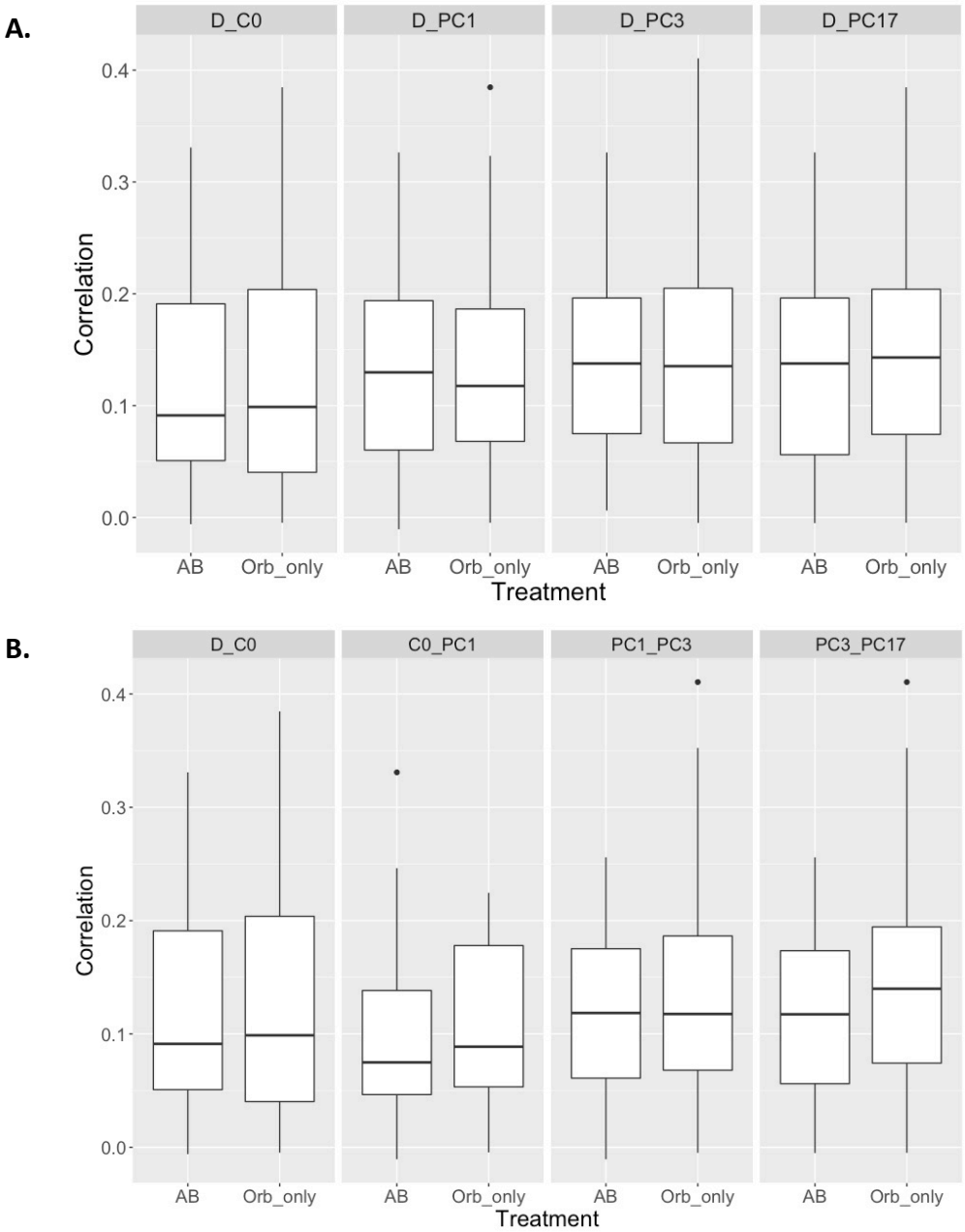


Figure 18. Visualisation of the summary of Spearman's rank correlation scores for the the ranked OTU abundancies in milk samples taken from the mammary gland across the dry period (Table 9.).Correlations between the drying off timepoint (D0) and the time points following the dry period show little retention of common abundancies between samples with a mean correlation ranging between 0.120 and 0.149 for the antibiotic treated group (AB) and non-antibiotic (Orb Only) groups (Table 9.). There is a similarly low range in correlations when correlating subsequent time points with the mean, ranging between 0.097 and 0.149. There is little visible difference between the two treatment groups. D, drying off; C0, calving; PC1, 1 day post calving; PC3, 3 days post calving; PC17, 17 days post calving.

To test if there is a significant difference between the mean OTU correlation scores between treatment groups a Kruskal-Wallis test was performed. There is not enough evidence to support that the differences in OTU correlations is significantly different between treatment groups ($p > 0.05$; Table 10.). Indicating that overall the composition of the OTUs in the mammary gland varies greatly between sampling time points, with not enough evidence to suggest a difference between treatment groups.

Time points correlated	p-value	statistic	parameter
D0_C0	0.933	0.00696	1
D0_PC1	0.940	0.00564	1
D0_PC3	0.973	0.00111	1
D0_PC17	0.570	0.32204	1
D0_C0	0.933	0.00696	1
C0_PC1	0.418	0.65500	1
PC1_PC3	0.599	0.27600	1
PC3_PC17	0.217	1.53000	1

Significance codes: 0 '***', 0.001'**', 0.01'*', 0.05'.', 0.1 ' , 1 '

Table 10. The Kruskal-Wallis test determined no significant difference ($p>0.05$) between the two treatment groups in the Spearman's rank correlation scores for the ranked abundance of OTUs between time point pairs tested. D0, drying off; C0, calving; PC1, 1 day post calving; PC3, 3 days post calving; PC17, 17 days post calving.

4.4. Discussion and conclusions

For analysis of Herd 2, samples were specifically selected based upon conclusions of Herd 1 analysis, to specifically address the effect of antibiotic treatment on the early lactation mammary gland microbiota. This resulted in the selection of udder quarter milk samples from a herd of 22 cows, 11 receiving antibiotic treatment and 11 receiving a non-antibiotic teat sealant at drying off, as in Herd 1. Samples were chosen across 5 time points, drying off (D0), calving (C0), 1 day post calving (PC1), 3 days post calving (PC3) and 17 days post calving (PC17),

resulting in a total of 440 samples. All cows selected for data analysis in Herd 2 were of the same parity, parity 2. The somatic cell count (SCC) at D0 was generally lower than in Herd 1, and was better balanced between the samples at D0; mean $\log_{10}$ SCC of 1.45 in the antibiotic treatment group and a mean $\log_{10}$ SCC of 1.44 in the non-antibiotic treatment group (Figure 1.). This selection was chosen to reduce possible confounding caused by parity and variation in starting infection levels.

The SCC increased in both treatment groups at calving following the dry period, and steadily decreased in both treatment groups across the next 3 time points (Figure 3.). Linear mixed effects (LME) modelling of the SCC reported a that the SCC was significantly higher in the non-antibiotic treatment group in the average of the first 3 time points following the dry period, however, fairly low significance ($p<0.05$, Table 1. and Table 2.).

Similar to Herd 1, the Chao1 index was highest at drying off, in Herd 2 the reduction in the Chao1 index was less dramatic than in Herd 1 following the dry period (Figure 8. and Chapter 3. Figure 9.). LME modelling of the Chao1 index and the Shannon index in early lactation reported no significant difference between the non-antibiotic and antibiotic treatment groups (Table 4., Table 5., and Table 6., Table 7., respectively). These results are similar to Herd 1; there is not enough evidence to conclude that the mammary gland health status or diversity of the milk microbiota is significantly different in early lactation following antibiotic treatment. This finding is in line with previous studies on herds with healthy udders and sub-clinical levels of mastitis, which found no major difference in the diversity indices between antibiotic and non-antibiotic treatment groups 5 days and 7 days post calving (Biscarini *et al.*, 2020; Bonsaglia *et al.*, 2017).

To explore the change in the the microbiota sampling time points, a Spearman's rank correlation of OTUs present in each milk sample was carried out, correlating the ranked abundancies of the OTUs in present in subsequent corresponding quarter samples across the dry period into early lactation. Although there were overall less OTUs present in the Herd 2 samples compared to Herd 1 (Table 8. and Chapter 3. Table 7.), a similar level of OTUs were correlated between subsequent time points (Table 9. and Chapter 3. Table 8.). A mean correlation score averaged between 0.097 and 0.149 in Herd 2, displaying how much the milk

microbiota changes between time points. The perturbation is of similar levels regardless of treatment group (Table 10.).

The dynamic nature of the microbiota will be further explored in Chapter 5 on a taxonomic level and will be compared between treatment groups. In Chapter 6, together with analysis of Herd 1, the taxonomic analysis in Chapter 5 and in the context of previous studies, the effect of antibiotics on the early lactation microbiome will be further discussed.

5. Mammary gland taxonomy

5.1. Introduction

As described in Chapter 3 and Chapter 4, the composition of bacteria in the mammary gland is dynamic for both treatment groups. This is evidenced in the large changes to the ranked OTU abundancies for corresponding milk samples over the dry period into early lactation (Chapter 3. Figure 18., and Chapter 4. Figure 18.).

Several studies have aimed to identify specific microbiota associated with healthy and mastitic mammary glands. However, profiling the mammary gland microbiome at a lower taxonomic level is challenging and can produce conflicting results (Ganda *et al.*, 2016; Oikonomou *et al.*, 2014; Taponen *et al.*, 2019). There are many reasons contributing to the difficulty in defining a 'stable' microbiota. Farming practices (Doyle *et al.*, 2017; Metzger *et al.*, 2018), sampling site (colostrum, milk, teat canal, teat apex), infection status, time (Andrews *et al.*, 2019), genetics (Cremonesi *et al.*, 2018), cattle parity (Lima *et al.*, 2017), mastitis history (Falentin *et al.*, 2016), the environment (Oikonomou *et al.*, 2014), sequencing techniques and contamination (Metzger *et al.*, 2018; Salter *et al.*, 2014) can all influence the diversity of the microbiota.

Aiming to define specific bacteria or a specific microbiome associated with antibiotic and non-antibiotic treated cows is outside of the aims of this thesis. Instead, in this chapter, the taxonomic profile of the mammary gland milk microbiota will be explored on a higher taxonomic level. With the aim of expanding upon analysis from Chapter 3. and Chapter 4. to further describe the changes to the mammary gland microbial community over time following antibiotic treatment. Furthermore to ask whether the dynamic changes to the microbiota occur regardless of treatment group over the dry period into early lactation.

5.2. Methods

Methods for identifying the microbiota in the milk samples are outlined in detail in Chapter 2. Taxonomy was assigned separately to the OTUs identified in Herd 1 and Herd 2 using the SILVA ACT SINA alignment service (Pruesse *et al.*, 2012). Analysis of the taxonomy identified in the milk microbiotas was conducted using the phyloseq package (McMurdie & Holmes, 2013) in R (R Core Team, 2017).

Herd information and milk sample collection is outlined in detail in Chapter 2. Briefly, Herd 1 constituted 18 cows; 9 in the antibiotic treatment group, 9 in the non-antibiotic treatment group. For taxonomic analysis, milk samples collected from the time points drying off, calving, 1 day post calving, 3 days post calving and 5 days post calving were selected as the drying off and early lactation time points are of most interest to understand the impact of antibiotic treatment in early lactation. This gave a sample size of 360 for Herd 1. Herd 2 constitutes 22 cows, 11 in the antibiotic treatment group and 11 in the non-antibiotic treatment group. Milk microbiota are analysed from the time points drying off, calving, 1 day post calving, 3 days post calving and 17 days post calving; providing a total sample size of 440.

5.3. Results

OTUs identified in the mammary gland milk microbiotas were assigned to a diverse range of taxa, with bacteria from 30 different identified Phyla in Herd 1 and 24 different Phyla identified in Herd 2 (Table. 1.). The abundance of different taxa was comparable between treatment groups in both Herds. In Herd 1, bacteria from 406 different Genera in the antibiotic group and 394 different Genera in the non-antibiotic group were identified. In the Herd 2 dataset, bacteria from 351 different Genera in the antibiotic group and from 345 different Genera were identified (Table 1.).

Farm	Treatment group	Number of samples	Phylum	Class	Order	Family	Genus
Herd 1	Antibiotic	180	29	70	154	250	406
	Non-antibiotic	180	27	65	151	247	394
	Total dataset	360	30	73	173	276	464
Herd 2	Antibiotic	220	24	54	129	216	351
	Non-antibiotic	220	23	52	128	215	345
	Total dataset	440	24	58	140	239	409

Table 1. Count of the different taxonomic hierarchal ranks present in the microbiota of the antibiotic and non-antibiotic treatment groups of Herd 1 and Herd 2.

5.3.1. Herd 1

The community of bacteria identified in the individual milk sample microbiotas are incredibly diverse. This will be evidenced for each treatment group and within the individual cows of each group. The microbiota is highly dynamic across sampling time points, even between individual udder quarters at subsequent time points, this will also be demonstrated in an example cow from each treatment group.

To describe the overall structure of the microbiota within Herd 1, the prevalence of different Phyla and the proportion of samples they were identified in was plotted. The overall community is diverse, with many low prevalence Phyla, indicated by the grey dotted line representing presence in 5% of samples (Figure 1.). Generally, bacteria from 4 Phyla dominate the milk microbiota, these are the *Actinobacteria, Bacteroidota, Firmicutes* and *Proteobacteria* (Figure 1.).

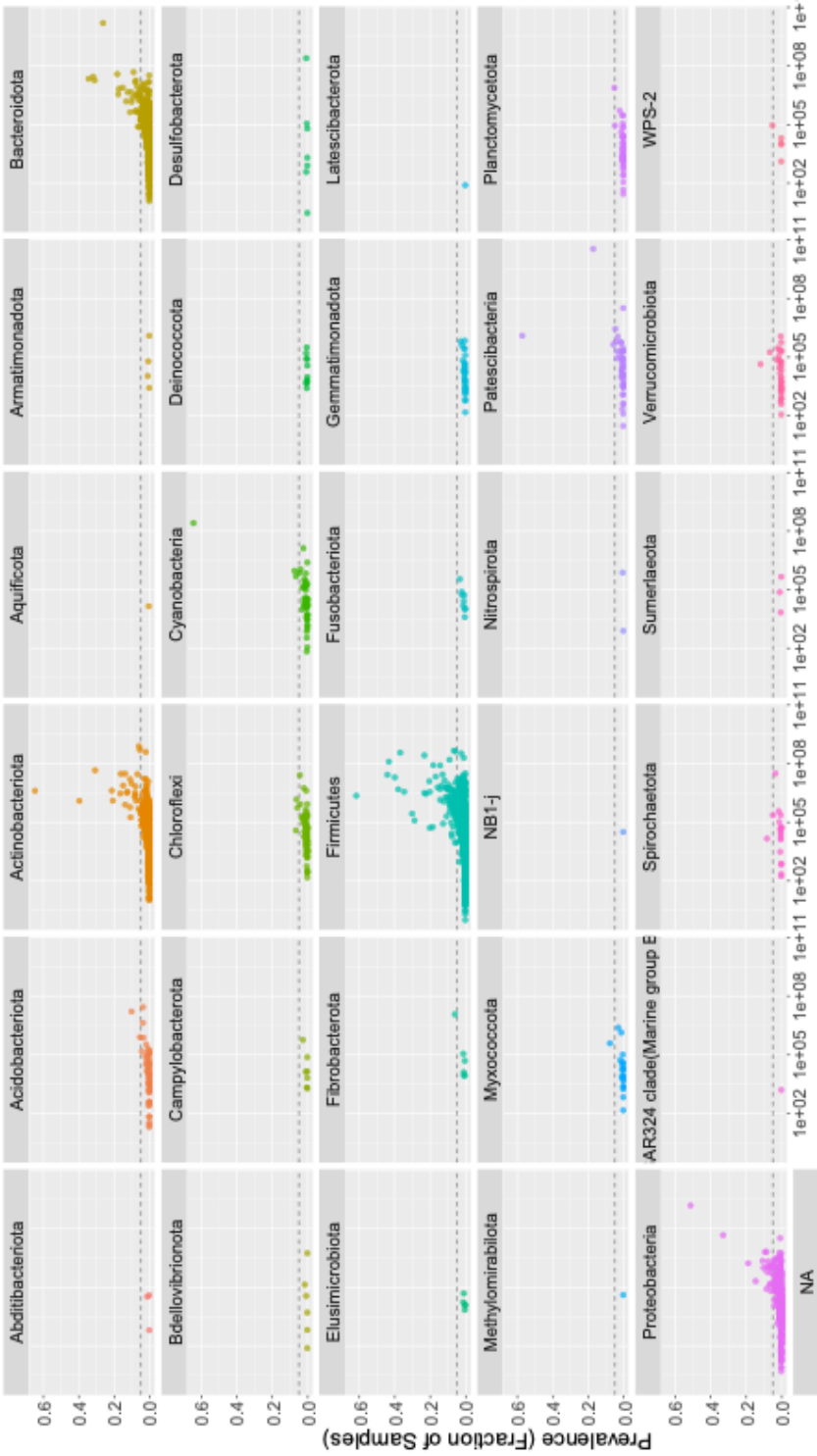


Figure 1. Prevalence of Phyla identified in the mammary gland milk microbiota of the Herd 1 samples. The majority of Phyla are not prevalent in 5% of samples, indicated by the grey dotted line. The community is overall dominated by bacteria from 4 Phyla, the *Actinobacteria, Bacteroidota, Firmicutes* and *Proteobacteria*.

The relative abundance of Phyla identified in the microbiota of milk samples for each the non-antibiotic (Orb Only) and antibiotic (AB) treatment groups shows a high diversity with bacteria

from 30 different phyla identified (Figure 2.). The most abundant Phyla in both treatment groups and across the 5 time points taken at drying off into early lactation are from the *Firmicutes, Bacteroidota, Actinobacteriota* and *Proteobacteria.* The largest change in proportion of Phyla is seen between the drying off (D) and Calving (C0) time points in the non-antibiotic treatment group, with the proportion of *Firmicutes* decreasing and the proportion of *Bacteroidota* increasing (Figure 2.). However the *Firmicutes* tend to recover their proportion in the non-antibiotic microbiota by 5 days post calving (PC5). The mammary gland microbiota varies within treatment groups between individual cows and between subsequent time points for the same cow (Figure 3.).

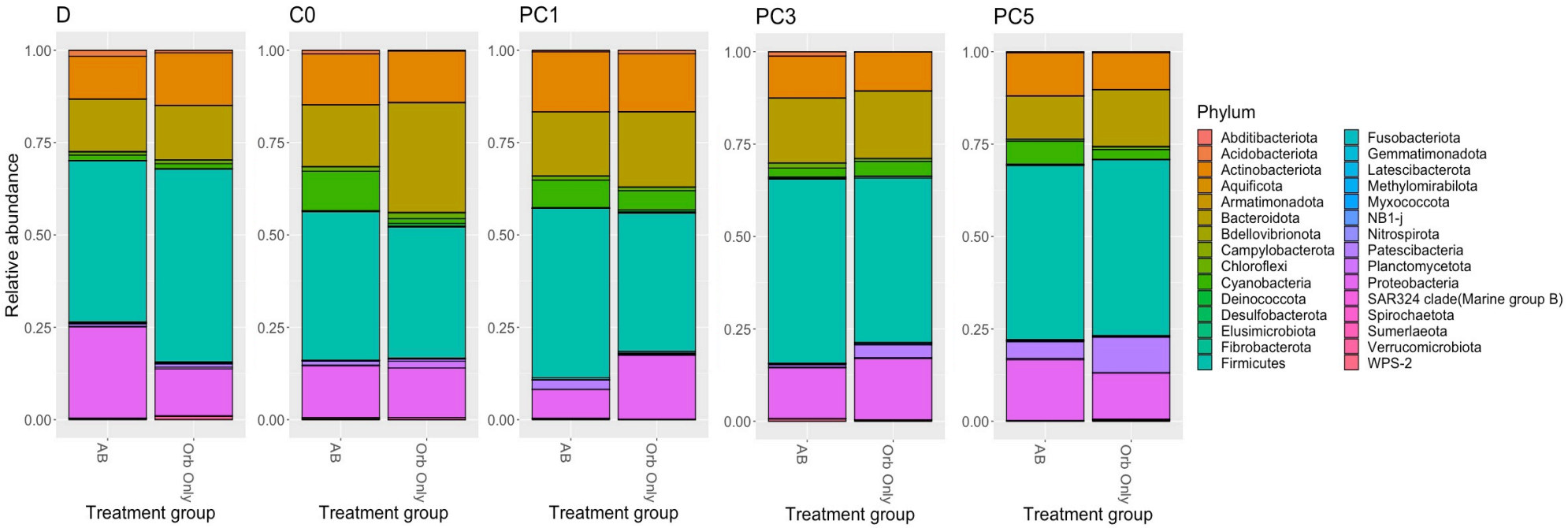


Figure 2. The relative abundancies of Phyla assigned to OTUs identified from Herd 1 mammary gland milk samples for the non-antibiotic (Orb Only) and antibiotic (AB) treatment groups across the drying off period into early lactation. Time points: D, drying off; C0, Calving; PC1, 1 day post-calving; PC3, 3 days post calving; PC5, 5 days post calving. 36 samples per time point, per treatment group. 360 total sample size.

The largest proportion of Phyla across all time points in both treatment groups is from the Firmicutes Phylum, which narrows following drying off in the non-antibiotic group, but recovers in its portion size up to 5 days post calving.

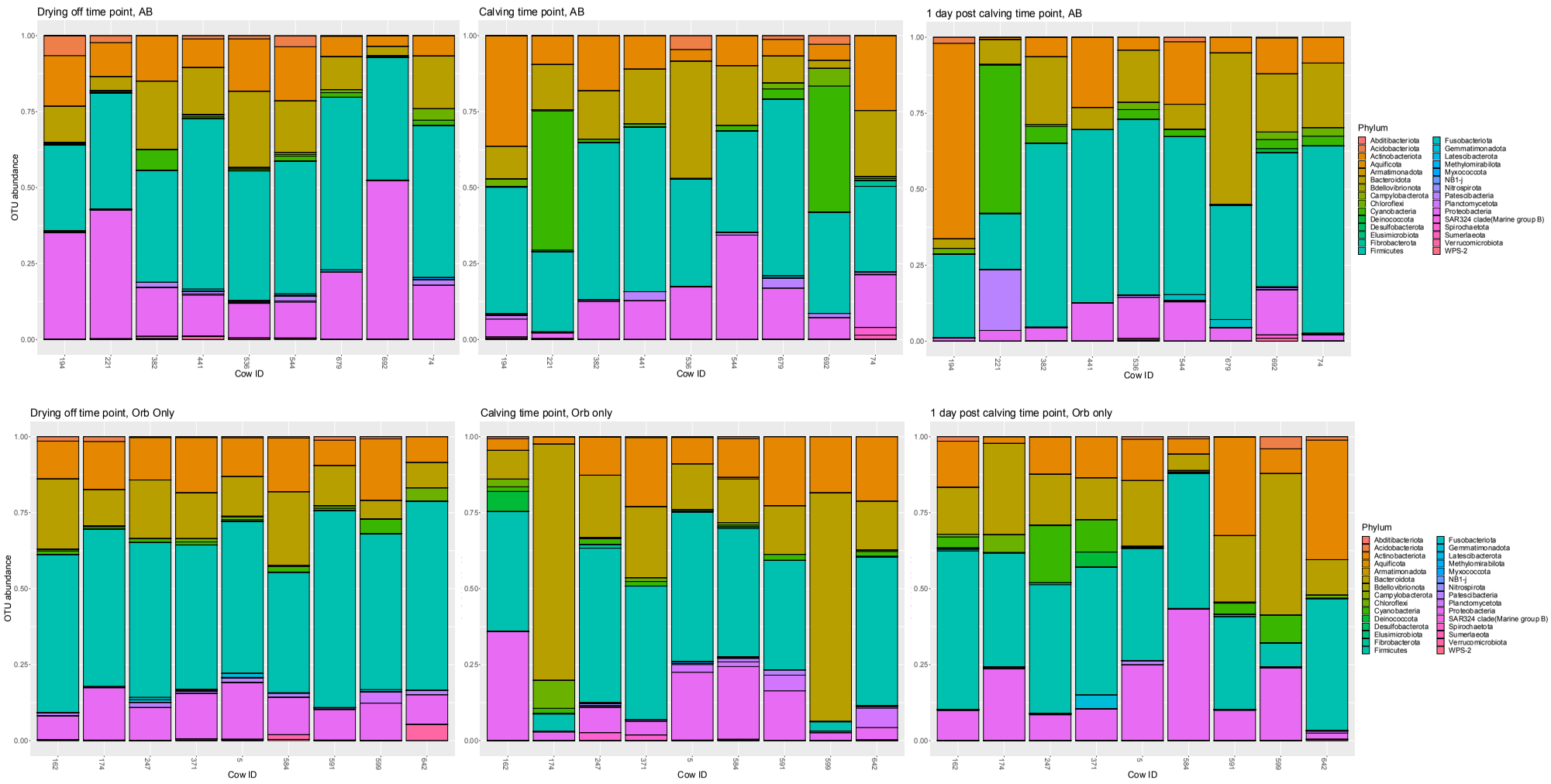


Figure 3. The relative abundance of Phyla of the milk microbiota for each cow (numbered on x-axis) for the antibiotic (AB, top row) and non-antibiotic (Orb Only, bottom row) treatment groups for the drying off, calving and 1 day post calving time points. There is variation in both treatment groups in the Phyla that constitute the milk microbiota between cows within treatment groups and for the same cow on subsequent sampling time points.

As the microbiota of the milk samples is incredibly diverse, to explore the taxonomy at a lower hierarchy, the taxa were filtered on the parameters of at least 10 reads identified in at least 1% of samples. This filtered the number of taxa in the 360 sample dataset from 4314 to 1229. For presenting Order the taxa were also filtered to include those with over 10000 reads to aide interpretation.

An exemplar cow from the non-antibiotic treatment group (Figure 4.) and the antibiotic treatment group (Figure 5.) were randomly selected to display the diversity in the bacteria in the udder and the dynamic nature of the udder quarters within the cow and over subsequent time points.

The abundance of bacteria can spike in a single udder quarter and clear significantly by the subsequent time point, for example in the Left Hind (LH) and Right Hind (RH) quarters of cow 5 (Figure 4.A.). In the RH the microbiota appears to be dominated by bacteria from the Order *Enterobacterales* from the *Proteobacteria* Phylum and *Oscillospirales* from the *Firmicutes* Phylum.

An exemplar cow (Cow 544) from the antibiotic treatment group also displays great differences in the structure of the mammary gland microbiota between the different udder quarters and between subsequent time points (Figure 5.). For example, in the LH udder quarter the relative abundance changes dramatically between each time point. At Calving (C0), the most abundant Order is *Bacillales;* then 1 day post calving (PC1), the most abundant orders become *Erysipelotrichales* and *Lachnospirales*; 3 days post calving (PC3), the 2 most abundant orders become *Burkholderiales* and *Oscillospirales* and finally 5 days post calving (PC5), the microbiota of the LH is dominated by OTUs from the orders *Oscillospirales* and *Peptostreptococcales-Tissierellales* (Figure 5.B.).

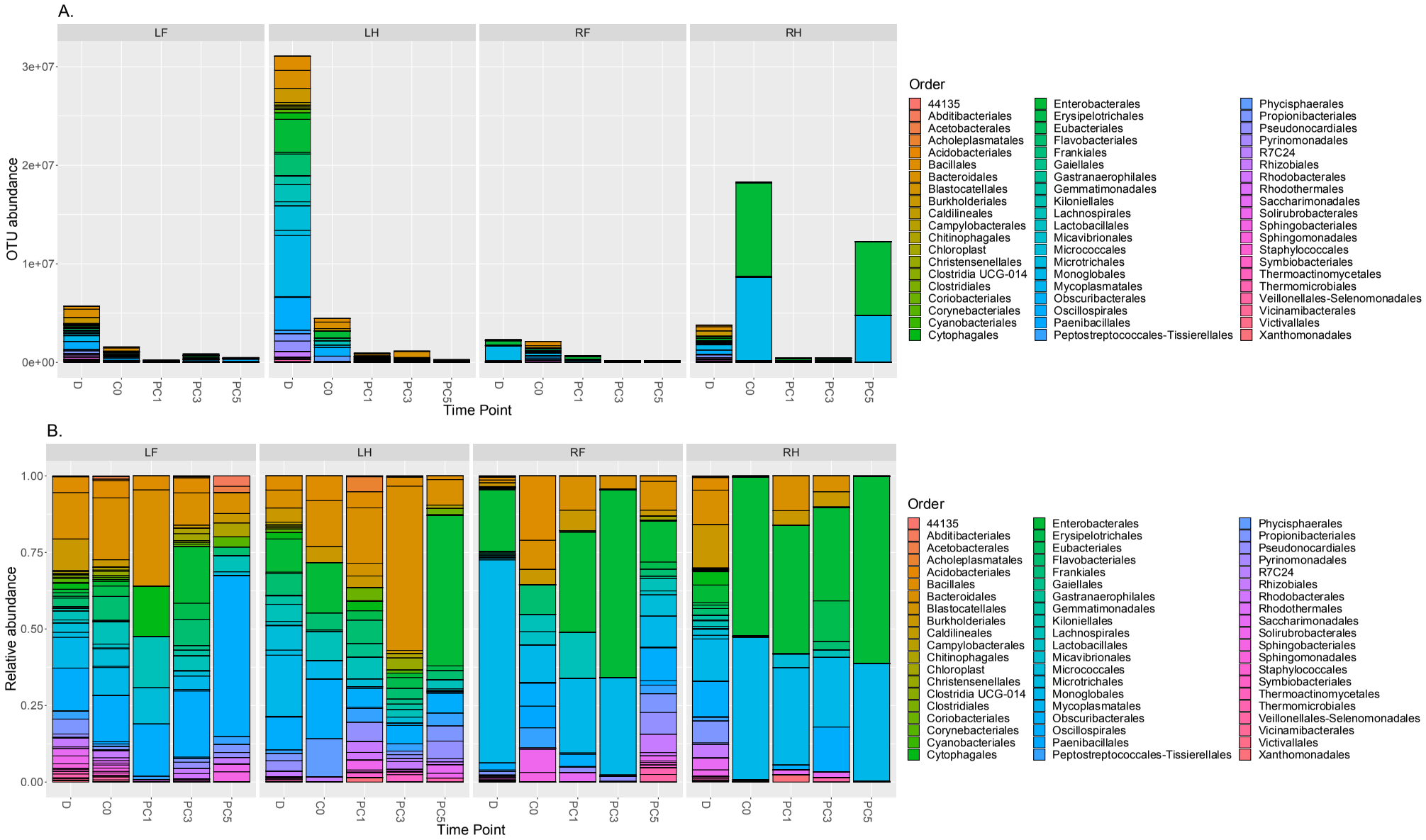


Figure 4. An example of the dynamic nature of the mammary gland microbiota for a cow in the non-antibiotic treatment group (Cow 5) across the dry period into early lactation, in terms of bacterial abundance (A.) and relative abundance (B.). Bacteria from 30 Orders are present across the udder quarters of Cow 5. Time point, D, Drying off; C0, Calving; PC1, 1 day post calving; PC3, 3 days post calving; PC5, 5 days post calving. Udder quarter, Left Fore, LF; Left Hind, LH; Right Fore, RF; Right Hind, RH.

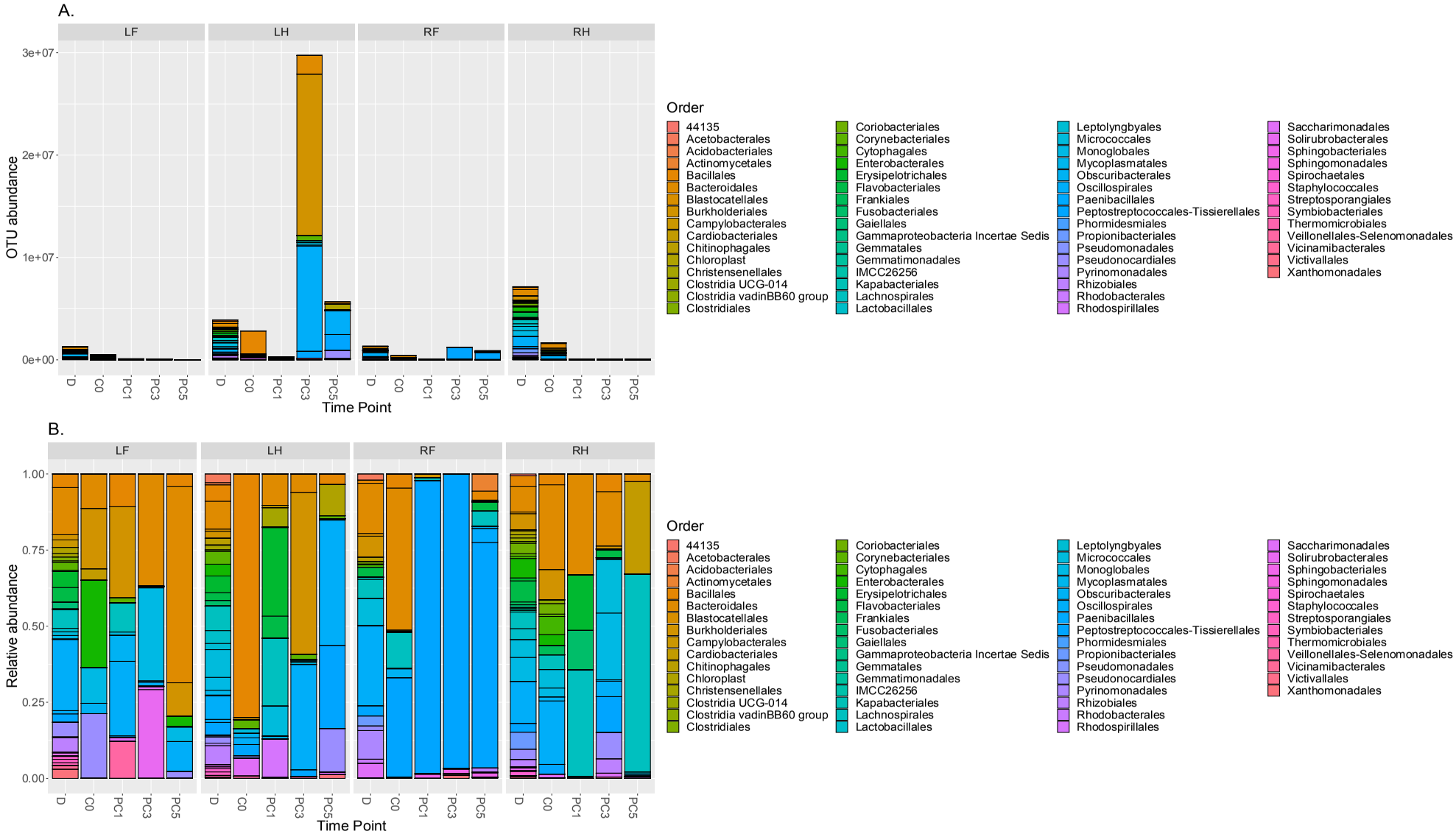


Figure 5. An example of the dynamic nature of the mammary gland microbiota for a cow in the antibiotic treatment group (Cow 544) across the dry period into early lactation, in terms of bacterial abundance (A.) and relative abundance (B.). Bacteria from 60 Orders are present across the udder quarters of Cow 544. Time point, D, Drying off; C0, Calving; PC1, 1 day post calving; PC3, 3 days post calving; PC5, 5 days post calving. Udder quarter, Left Fore, LF; Left Hind, LH; Right Fore, RF; Right Hind, RH.

5.3.2. Herd 2

The community of bacteria identified in the individual milk sample microbiotas of Herd 2 were assigned to 3048 taxa across the total 440 samples. Overall, there is a lower abundance of bacteria in Herd 2 compared to Herd 1.

To describe the overall structure of the microbiota within Herd 2, the prevalence of different Phyla and the proportion of samples they were identified in was plotted. The overall community is diverse, like Herd 1, with many low prevalence Phyla, indicated by the grey dotted line representing presence in 5% of samples (Figure 6.). Generally, in a similar manner to Herd 1, bacteria from 4 Phyla dominate the milk microbiota, these are the *Actinobacteria, Bacteroidota, Firmicutes* and *Proteobacteria* (Figure 6.).

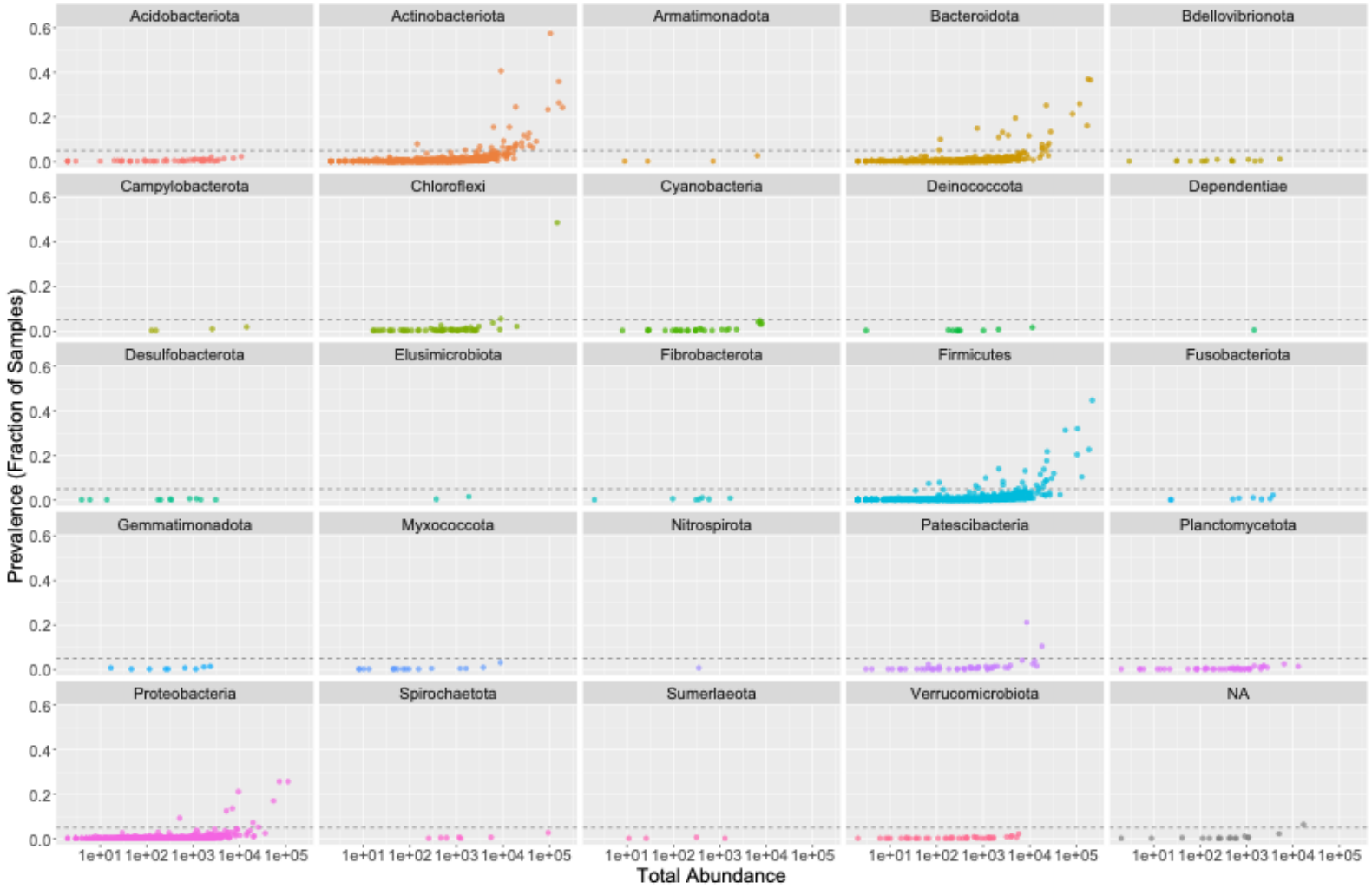


Figure 6. Prevalence of Phyla identified in the mammary gland milk microbiota of the Herd 2 samples. The majority of Phyla are not prevalent in 5% of samples, indicated by the grey dotted line. The community is overall dominated by bacteria from 4 Phyla, the *Actinobacteria, Bacteroidota, Firmicutes* and *Proteobacteria.*

The relative abundance of Phyla identified in the microbiota of milk samples for each the non-antibiotic (Orb Only) and antibiotic (AB) treatment groups shows a high diversity with bacteria from 24 different Phyla identified (Figure 7.). The most abundant Phyla across in both treatment groups and across the 5 time points taken at drying off into early lactation are from the *Firmicutes, Bacteroidota, Actinobacteriota* and *Proteobacteria.* The proportions of the different Phyla are relatively similar between time points and between the antibiotic and non-antibiotic treatment groups.

The mammary gland microbiota is dynamic and diverse for cows within the antibiotic (AB) and non-antibiotic (Orb Only) treatment groups (Figure 8.). The relative abundance of different Phyla within the same cow between time points is also dynamic and can change greatly between subsequent time points. For example in cow 741 (Orb Only), the Phylum *Actinobacteriota* dominates the microbiota at drying off (D0); at calving (C0), the microbiota has increased proportions of *Firmicutes* and *Bacteroidota,* and by 1 day post calving (PC1), the microbiota is largely populated by *Firmicutes* and *Bacteroidota* (Figure 8.).

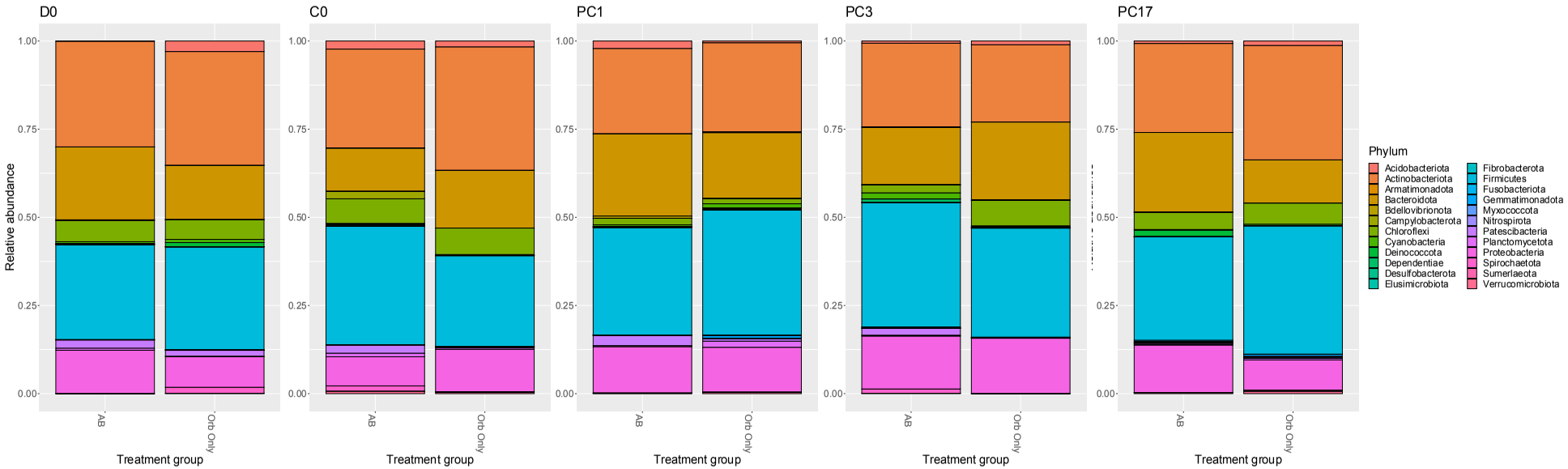


Figure 7. The relative abundancies of Phyla assigned to OTUs identified from Herd 2 mammary gland milk samples for the non-antibiotic (Orb Only) and antibiotic (AB) treatment groups across the drying off period into early lactation. Time points: D0, drying off; C0, Calving; PC1, 1 day post-calving; PC3, 3 days post calving; PC17, 17 days post calving. 44 samples per time point, per treatment group. 440 total sample size.

The largest proportion of Phyla across all time points in both treatment groups are from the *Firmicutes* and *Actinobacteriota* Phyla. The proportions of different Phyla for each treatment group is fairly even across the time points.

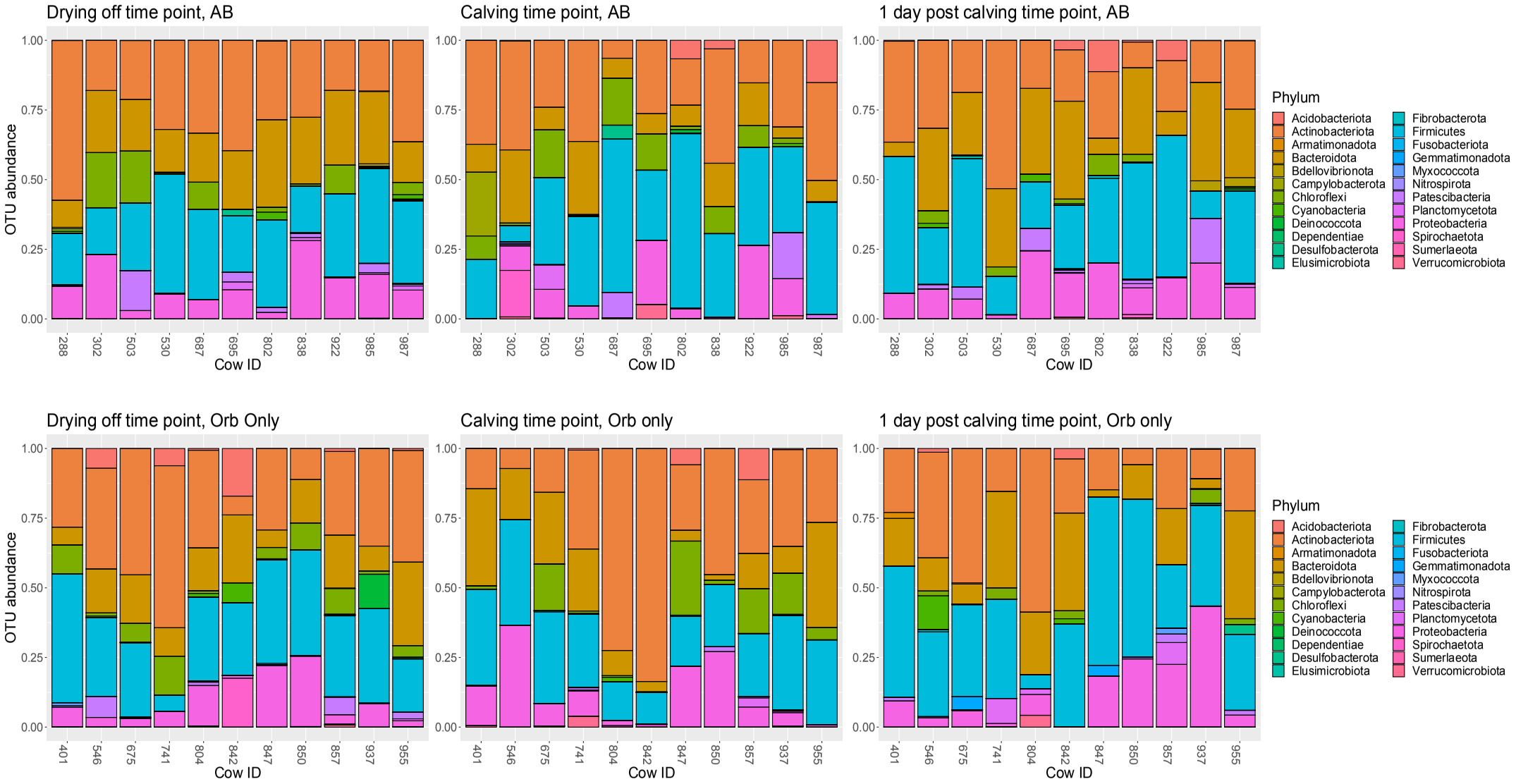


Figure 8. The relative abundance of the OTUs assigned to the Phyla identified in the the milk microbiota for each cow (numbered on x-axis) for the antibiotic (AB, top row) and non-antibiotic (Orb Only, bottom row) treatment groups at the drying off, calving and 1 day post calving time points. There is variation in both treatment groups in the Phyla that constitute the milk microbiota between cows within treatment groups and for the same cow on subsequent sampling time points.

As the microbiota of the milk samples are incredibly diverse, to explore the taxonomy at a lower hierarchy, the taxa were filtered on the parameters of at least 10 reads identified in at least 1% of samples. This filtered the number of taxa in the 440 sample dataset from 3048 to 273, highlighting that the Herd 2 bacterial community contains many low prevalence taxa. For presenting Order, the taxa were also filtered to include those with over 1000 reads to aide interpretation.

An exemplar cow from the non-antibiotic treatment group (Figure 9.) and the antibiotic treatment group (Figure 10.) were randomly selected to display the diversity in the bacteria in the udder and the dynamic nature of the udder quarters within the cow and over subsequent time points.

Much like in Herd 1, abundance of bacteria can spike in a single udder quarter and clear significantly by the subsequent time point, for example in the Left Fore (LF) of cow 955 from the non-antibiotic treatment group (Figure 9.A.). The microbiota composition can change dramatically between time points in the udder, for example in the Right Fore (RF) microbiota of cow 955, at drying off (D0), the dominant bacteria are from the Orders *Flavobacteriales* and *Lactobacillales.* By the next sampling time point, calving (C0), the biggest proportion of bacteria in the RF udder quarter sample is from the Order *Bacteroidales.* Then at the next time point, 1 day post calving (PC1), the bacterial Order *Lachnospirales* is most relatively abundant in the community (Figure 9.B.).

The dynamism and diversity of the mammary gland milk microbiota within the udder quarter is also seen in the antibiotic treated group. For example in the Left Hind (LH) of cow 288 from the antibiotic treatment group, there is a spike in abundance of bacteria (C0 to PC1) before reducing again by 3 days post calving (Figure 10.A.). The relative abundance of the bacteria also changes drastically between time points, for example in the LH the largest proportion of bacteria is from the Order *Cyanobacteriales* at D0*,* then at C0 it becomes *Micrococcales* and at PC1 the largest proportion is from the *Peptostreptococcales-Tissierellales* (Figure 10.B).

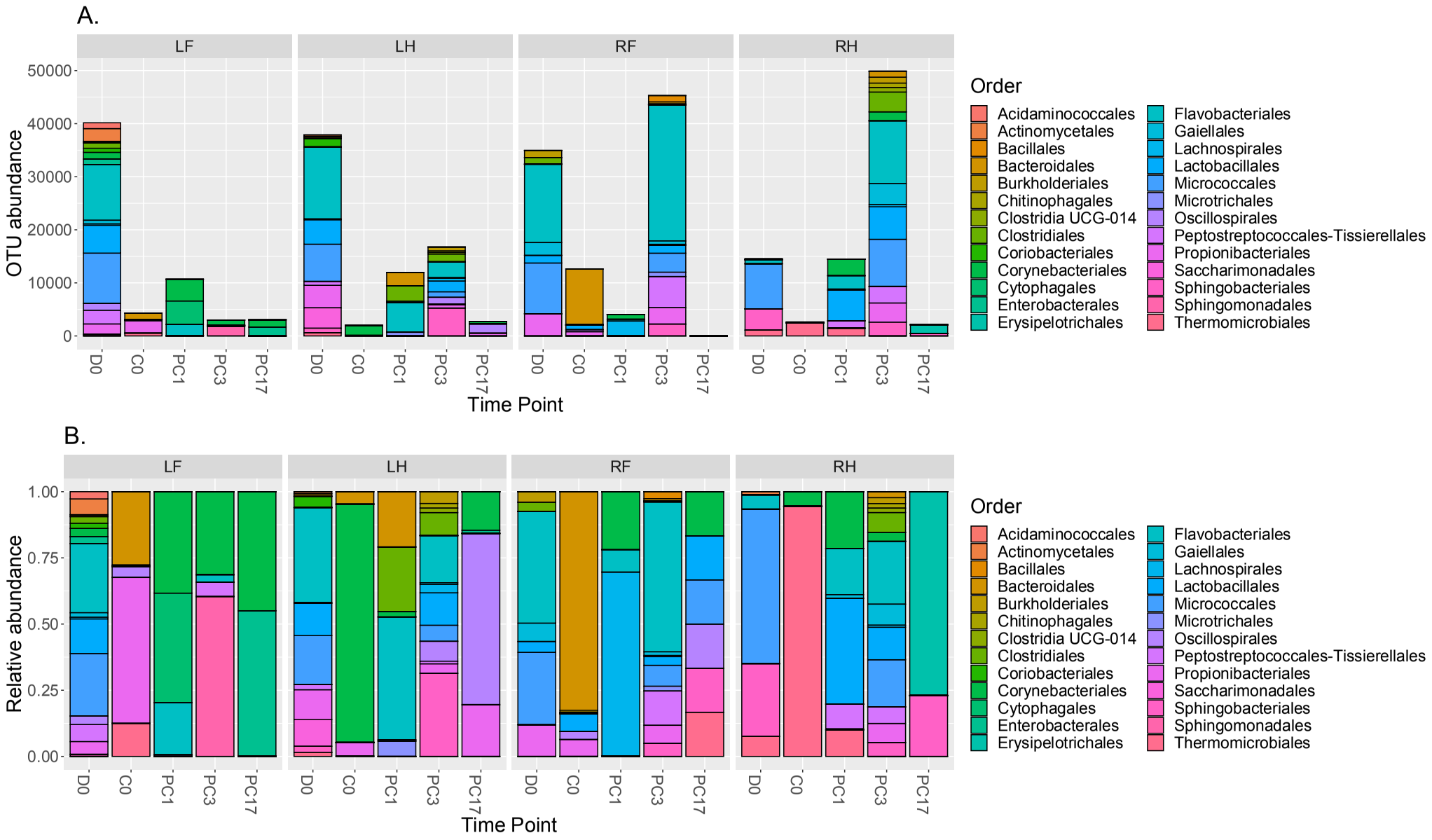


Figure 9. An example of the dynamic nature of the mammary gland microbiota for a cow in the non-antibiotic treatment group (Cow 955) across the dry period into early lactation, in terms of bacterial abundance (A.) and relative abundance (B.). Bacteria from 26 Orders are present across the udder quarters of Cow 955. Time point, D0, Drying off; C0, Calving; PC1, 1 day post calving; PC3, 3 days post calving; PC17, 17 days post calving. Udder quarter, Left Fore, LF; Left Hind, LH; Right Fore, RF; Right Hind, RH.

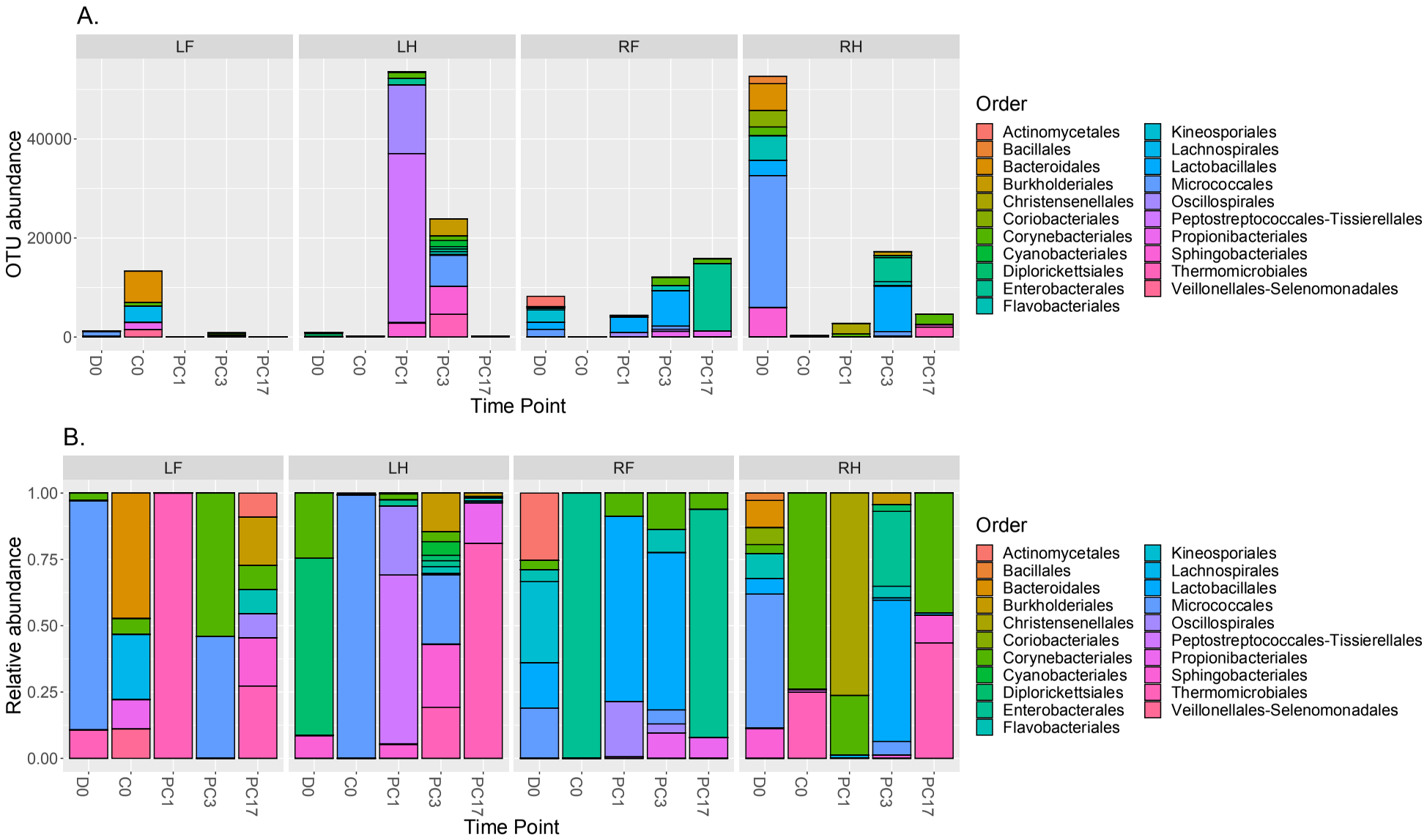


Figure 10. An example of the dynamic nature of the mammary gland microbiota for a cow in the antibiotic treatment group (Cow 288) across the dry period into early lactation, in terms of bacterial abundance (A.) and relative abundance (B.). Bacteria from 21 Orders are present across the udder quarters of Cow 288. Time point, D0, Drying off; C0, Calving; PC1, 1 day post calving; PC3, 3 days post calving; PC17, 17 days post calving. Udder quarter, Left Fore, LF; Left Hind, LH; Right Fore, RF; Right Hind, RH.

5.4. Discussion

The microbiota of mammary gland milk samples in both Herd 1 and Herd 2 are diverse, regardless of treatment group. 406 different Genera in the antibiotic group and 394 different Genera in the non-antibiotic group were identified in Herd 1, from a total of 360 samples. In the Herd 2 dataset, bacteria from 351 different Genera in the antibiotic group and from 345 different Genera were identified from a total of 440 samples.

The overall microbiota of each herd was diverse and contained many low prevalence Phyla (Figure 1. and Figure 6.). The Phyla *Firmicutes, Bacteroidota, Actinobacteriota* and *Proteobacteria* comprised the largest proportion of the microbiota in each herd, each treatment group and across all sampling time points from the dry period into early lactation (Figure 2. And Figure 7.). These findings consistent with the most common Phyla reported in previous studies (Bonsaglia *et al.*, 2017; Lima *et al.*, 2017; Taponen *et al.*, 2019).

The mammary gland milk microbiota over the dry period into early lactation is diverse and dynamic. As described in Chapter 3. and Chapter 4. with the changes in ranked OTU abundancies (Chapter 3. Figure 18., and Chapter 4. Figure 18.), composition of the bacteria in the udder quarter of the same cow can change dramatically between time points, for cows in both treatment groups (Figure 4., Figure 5., Figure 9., and Figure 10.). For Example, in the exemplar cow in Herd 2 from the antibiotic group in almost every subsequent time point for each of the 4 udder quarters, different Orders of bacteria comprise the microbiota (Figure 10.B.). In a study on the composition of the mammary gland milk microbiota, Porcellato *et al.* (2020) also also reported there was high microbial diversity between cows and between quarters of the same cow. They reported the milk microbiota diversity at two time points, once during early lactation and once in later lactation and revealed great diversity between udder quarters of the same cows on the two sampling occasions (Porcellato *et al.*, 2020). In this study, it was shown that the mammary gland milk microbiota changes significantly even over short periods of time, from drying off to calving (roughly 48 days), and between 1-2 days during early lactation.

In a similar study comparing the mammary gland milk microbiota at drying off and 7 days postpartum following antibiotic treatment (ceftiofur hydrochloride) and teat sealant with just teat sealant treatment, it was shown that there was no shift in the mammary gland microbiota regardless of treatment group (Bonsaglia *et al.*, 2017). Here, along with evidence from Chapter 3 and Chapter 4, no clear difference between the microbiota of the mammary gland at Phylum and Order taxonomic levels was shown, and both treatment groups showed similar levels of disruption between time points.

While it was out of the scope for the aims of this thesis to discover specific taxa associated with reduced diversity or with the effect of antibiotic treatment, the level of diversity and the rapid nature in which the milk microbiota changes its composition over the dry period into early lactation was shown, regardless of treatment group.

During milking almost all of the udder is emptied. The continued perturbation of the mammary gland microbiota could result in the large differences in the mammary gland microbiota between sampling time points as the bacteria community are emptied then compete to recolonise the mammary gland. At drying off, the mammary gland is treated with either the antibiotic and teat sealant or just the teat sealant. Over the dry period, the mammary gland milk microbiome is not perturbed by milking. Bonsaglia *et al.* (2017) showed that from drying off to 7 days postpartum, there was no shift in the microbiota following antibiotic treatment compared to a teat sealant alone. This study built upon their findings showing that even at calving, the first sampling time following the dry period, there was no difference in the microbiota diversity between treatment groups. The community of bacteria is dynamic and any lasting effects of the antibiotic on the microbiota may have been reversed by the end of the dry period. Ganda *et al.* (2016) compared the microbiota of cows with *Escherichia coli* mastitis in a controlled longitudinal trial treating with and without the antibiotic ceftiofur. They showed that active mastitis quarter microbiotas are less diverse than healthy quarters. But, in mild and moderate cured cases, by day 14, the mammary gland microbiota returned to the pervious composition for both the ceftiofur treated and control group; indicating no long term dysbiosis of the microbiota following treatment and no difference when using antibiotic treatment. Showing in a short space of time how dynamic the microbial community of milk is, and in this study showing how changeable the microbiota

is over a much shorter space of time between 1-2 days. This raises questions into the usefulness of antibiotic treatment in mild and moderate mastitis cases and is an interesting area for further studies.

6. General Discussion and Conclusions

6.1. Introduction

Understanding the impact of antibiotics on the mammary gland microbiome and understanding the effectiveness of non-antibiotic alternatives is imperative to ensure justified and responsible antibiotic use, while improving mastitis treatment.

The overall aim of this study was to understand the impact of antibiotic dry cow therapy on the dairy cow mammary gland microbiome in early lactation using high-throughput sequencing of the bacterial community and analysis of the immune response.

In order to address this aim, a longitudinal study was designed to test the following overarching hypothesis on two independent farms:

Antibiotic dry cow treatment has only a transient impact on the dairy cow mammary gland bacterial community that does not reduce immune marker levels associated with sub-clinical mastitis in dairy cows early in lactation.

Using data from a large study investigating whether the dairy cow mammary gland microbiome has a functional role in bovine mammary gland health and well-being the above hypothesis was first addressed in analysis of Herd 1 data, containing 791 milk samples collected from 18 multiparous cows across 11 time points. In Herd 1 half of the herd (9 cows) received an antibiotic treatment plus a teat sealant (antibiotic group) and the other half (9 cows) received just a teat sealant at drying off. Analysis of the somatic cell count (SCC), abundance and diversity metrics through data visualisation and statistical modelling showed minimal differences between the two treatment groups. This generated more specific hypotheses to be addressed by the second herd. Cows were specifically selected to provide a more balanced dataset to reduce outside variation when addressing the effect of antibiotic therapy on the mammary gland. This resulted in the selection of 440 samples from the udder quarters of 22 cows, 11 received antibiotic treatment and a teat sealant (antibiotic group) at drying off and 11 received just the teat sealant (non-antibiotic group). Unlike herd 1, all cows

were in their second lactation (Parity 2) to reduce variation in the microbiota associated with parity (Lima *et al.*, 2017). 5 time points were selected ranging from drying off to early lactation and cows were selected for both treatment groups to have as similar as possible somatic cell counts (SCC) at drying off. This was based on the mean values and distribution of the SCC to provide a more focussed comparison (Described in Chapter 4.). The same methods for processing, identifying and analysing the milk microbiota was conducted in Herd 1 and Herd 2 to assess if conclusions drawn from the first herd were also true in the second herd (Outlined in Chapter 2.). Herd 2 had overall a much lower level of infection compared to Herd 1, and was much more balanced between the treatment groups at drying off (Chapter 3. Figure 6. and Chapter 4. Figure 1.).

The research findings of this study in respect to determining the effect of antibiotic treatment on the SCC, diversity and abundance measures, and on the general diversity of the microbiotas for both herds will be discussed in the next section. Implications and limitations of this study will also be discussed.

6.2. Research findings and implications

Sequencing of the 16S rRNA V1-V3 variable region of the bacterial microbiota in milk was an effective high-throughput method to analyse the mammary gland community from 1231 milk samples across two farms and 11 time points. Analysing a comparatively large dataset allowed a wider comparison of diversity and abundance metrics and community analysis between the non-antibiotic and antibiotic treatment groups than seen in current literature analysing the impact of antibiotics on the dairy cow microbiome (Biscarini *et al.*, 2020; Derakhshani *et al.*, 2018b; Ganda *et al.*, 2016; Ganda *et al.*, 2017). Furthermore, targeting selection of samples for herd 2 based on findings from herd 1 allowed a conclusions drawn to be re-evaluated in a more balanced dataset, providing arguably more powerful conclusions than can be drawn from smaller studies in current literature. Through data visualisation, statistical analysis and taxonomic analysis, patterns in the SCC diversity and abundance metrics following antibiotic treatment were analysed. Robust statistical analysis through conducting OTU correlations and statistical modelling is also fairly unique in the current literature when comparing the effect of antibiotics on the dairy cow microbiome. OTU correlation analysis and statistical modelling

offers an insight into the complex bacterial community that is and is often difficult to elucidate, and that fluctuates rapidly in the dairy cow mammary gland during the drying off period

Antibiotics have little impact on the SCC, Chao1 and Shannon indices

Data visualisation and linear mixed effects modelling of Herd 1 data showed that antibiotic treatment at drying off did not have a significant impact on the somatic cell count (SCC) or Shannon diversity index following the dry period (Chapter 3. Table 2. and Table 6. respectively). Modelling of the Chao1 index revealed a slightly greater Chao1 index score in the non-antibiotic treatment group following the dry period at a threshold of $p<0.05$ ($\chi^2(1)= 3.93$, $p = 0.047$; Chapter 3. Table 4.).

In Herd 2 antibiotic treatment at drying off did not have a significant impact on the Chao1 and Shannon indices following the dry period (Chapter 4. Table 5. and Table 7.). However there was a lower SCC in the antibiotic treated group at a threshold of $p<0.05$ (χ^2 (1)= 3.92, $p = 0.048$; Chapter 4. Table 2.). Taken together, it is not strong evidence to indicate that antibiotic treatment had a significant impact on the diversity and abundance metrics and the SCC following the dry period. Previous studies support these conclusions, by also finding little effects of antibiotic treatment on the Chao1 and Shannon indices following the dry period. Bonsaglia *et al.* (2017) showed no significant reduction in the Shannon and Chao1 indices at 7 days in milk in an antibiotic and teat sealant treatment group compared to a teat sealant treatment alone.

Furthermore, Biscarini *et al.* (2020) showed a similar finding to this study in the analysis of Herd 2, that there was a reduced SCC in antibiotic treated groups compared to teat sealant alone by 5 days in milk, however they did not find a strongly significant difference. It was also shown there was no major difference between treatment groups in terms of the Chao1 and Shannon indices (Biscarini *et al.*, 2020). However, it should be noted these are cases of sub-clinical mastitis and conclusions cannot be applied to more severe mastitis cases. The effectiveness of antibiotics on the long-term mammary gland diversity in clinical mastitis cases should be continually explored. Reduced richness and evenness has been associated

with udder quarters with clinical mastitis, but it has yet to be elucidated if dysbiosis of the mammary gland microbiota is the cause or effect of mastitis (Oikonomou *et al.*, 2014; Oikonomou *et al.*, 2012).

It should also be noted the effectiveness of teat sealant can be partially attributable to its action as a physical barrier to the environment but also in the active ingredient used in the teat sealant in this study and in those by Bonsaglia *et al.* (2017) and Biscarini *et al.* (2020), which is bismuth-subnitrate. Bismuth-based teat sealants have been shown to have inhibitory effects on bacterial growth and have been shown to be more effective in the prevention of intramammary infections than other teat sealant products over the dry period (Notcovich *et al.*, 2020).

<u>The mammary gland microbiota is diverse and dynamic</u>

8680 unique OTUs across 791 samples were identified in Herd 1, with the $\log_{10}$ mean count of OTU reads for each sample of 5.28 (Chapter 3. Table 7.). 3048 unique OTUs across 440 sample in Herd 2 were identified, with the $\log_{10}$ mean count of OTU reads for each sample of 3.72 (Chapter 4. Table 7.).

Spearman's Rank correlation of the ranked abundance of OTUs between sampling time points across the dry period into early lactation showed a large shift in the composition of bacteria in the same udder quarters between time points. This shift was seen in both herds with no significant difference in the level of perturbation between treatment groups reported (Chapter 3. Figure 18., Table 9. and Chapter 4. Figure 18., Table 10.).

The great diversity of the mammary gland microbiota and the dynamic nature in which it changes in this study has been previously reported (Andrews *et al.*, 2019; Winther *et al.*, 2022). To further explore the rapid flux seen in OTUs in quarter samples over time, the changes in the taxonomy of the milk microbiota was analysed. The Phyla *Firmicutes, Bacteroidota, Actinobacteriota* and *Proteobacteria* comprised the largest proportion of the microbiota in each herd, each treatment group and across all sampling time points from the dry period into early lactation (Chapter 5. Figure 2.and Figure 7.). These findings are in

agreement with the most common Phyla reported in previous studies (Bonsaglia *et al.*, 2017; Derakhshani *et al.*, 2020; Lima *et al.*, 2017; Taponen *et al.*, 2019).

Analysing the microbiota at lower taxonomic levels is complex (Taponen *et al.*, 2019), in this study bacteria from over 464 genera were identified in Herd 1 and bacteria from 409 different Genera were identified in Herd 2. Identifying a 'core' microbiome, a similar community of bacteria that reside in the milk mammary gland over time, was out of the scope of this study. However, taxonomic analysis, even at the Order taxonomic level, revealed the great diversity and dynamic nature of the mammary gland microbiota at quarter level, regardless of treatment group. Porcellato *et al.* (2020) reported the significant changes in the milk microbiota between early and late lactation. In this study it was shown the milk microbiota can change significantly over shorter periods of time, over the dry period (around 50 days) and even between 1-2 days during early lactation.

This rapid flux in the microbiota between early lactation sampling times could be due to the emptying of the udder during milking, meaning 'new' bacteria compete to recolonise between milkings. Furthermore, antibiotics were administered at drying off, any lasting effect of the antibiotic may have worn off by the next sampling point, calving (C0), taken at end of the dry period (around 50 days later). This is supported by Ganda *et al.* (2016), who showed that in mild and moderate cured mastitis cases, the mammary gland microbiota had returned to previous diversity levels 14 days after treatment. Moreover, in preparation for calving, the biology of the udder changes which could alter any existing bacterial community across the dry period. (Green *et al.*, 2008).

In depth analysis of the mammary gland microbiome is complex due to the great diversity within datasets but also the additional diversity seen between outside variants such as breed, sampling site, sampling time or health status (Andrews *et al.*, 2019; Falentin *et al.*, 2016; Oikonomou *et al.*, 2014). Further variation is also caused because of different sampling and experimental techniques (Metzger *et al.*, 2018).

The mammary gland milk microbiota often constitutes many low abundance species, the prevalence of many OTUs identified in this study was low, most apparent in the samples from

Herd 2, in part due to the lower infection rates and thus lower bacterial loads. Contamination from sampling methods can be an issue, as bacteria that colonise the teat cistern, teat canal and udder skin can enter the milk (Rainard, 2017; Vangroenweghe *et al.*, 2001). While this is in part mitigated by the disinfection of the udder teat before sampling in this study, separating bacteria originating from inside the teat canal and cistern from the milk is difficult without more invasive sampling techniques (Vangroenweghe *et al.*, 2001).

Furthermore low bacterial load samples are more prone over representation of low abundance species and to kitome contamination (Salter *et al.*, 2014). To control for these possible sources of contamination, the udder teat was sterilised prior to sample collection, and parlour controls, a DNA extraction negative control and PCR blanks were included. During sequence processing, any OTUs identified in these control samples were removed from the dataset. Although this approach can result in the removal of some genuine sample OTUs, it was important to have a conservative approach that was uniform across all samples.

A unique feature of this study was the longitudinal analysis of the effect of antibiotic treatment on the mammary gland microbiome of one herd; before selecting specific samples from a second herd, to specifically address questions generated form the first herd. Very similar findings were generated from both herds. Evidence suggests there is little or only a transient impact on the mammary gland milk microbiota following drying off antibiotic treatment, and that there is little or only brief reduction in SCC in the early lactation of dairy cows. However as described above, and highlighted in this thesis, the mammary gland microbiota is very diverse and very dynamic and can depend on many variables. Therefore further studies are needed to investigate dry cow therapy (with or without antibiotics) in various herd types, environments and infection statuses.

It should also be considered that the selection of cows selected for antibiotic treatment in this study, and others, is not always randomised. This is often a difficult scenario, unavoidable in commercial settings where the welfare of the herd and productivity of the farm is of up most importance. For example, in this study, cattle were selected to receive antibiotic treatment based on their somatic cell count (SCC) at drying off. Milk samples taken from each udder quarter were pooled giving one count for every cow. If the pooled SCC was greater than

200,000 cells mL^{-1} at drying off the cow was selected to receive an antibiotic treatment as well as a non-antibiotic teat sealant treatment, those cows with SCC less than this threshold received just the non-antibiotic treatment. While this may limit the conclusions that can be drawn in this study, powerful conclusions can still be drawn within this context based on the large study sample sizes considered in two separate herds, with the second selected to answer specific questions.

Implications of this study on wider antimicrobial policies

As the population and demand for antibiotics grows , understanding the impact of antibiotics on the mammary gland microbiome and understanding the effectiveness of non-antibiotic alternatives is imperative to ensure justified and responsible antibiotic use, while improving mastitis treatment. This study aimed to provide more evidence to help support justified and responsible antibiotic use. It was shown in the context of these two herds that there was not enough evidence to conclude that antibiotic use significantly reduces the early lactation SCC compared to using a non-antibiotic treatment alone at the beginning of the dry period. There is also not enough evidence to conclude that antibiotic treatment has a lasting impact on the diversity and abundance measures (Chao1 and Shannon indices) compared to non-antibiotic treatment alone.

While conclusions drawn from the analysis of the data from the two herds in this thesis should be considered within the context of the limitations of the study; evidence from this study has shown there is a need for more understanding on the dynamics of the microbiome to ensure appropriate antibiotic use. In particular the dynamic nature of the microbiome between a matter of 1-2 days, even at a quarter level, offers interesting questions in to the robustness of the mammary gland and questions the ability to be able to characterise a stable core microbiome in the highly perturbed dairy cow mammary gland environment.

Assessing whether there may be an opportunity to reduce antibiotic therapy use whilst not impacting mastitis incidence through selecting cows for treatment at an individual udder quarter level or by re-analysing the SCC threshold at which cows are treated could be considered in further research. For the SCC threshold other factors which can contribute to mastitis incident should be modelled and risk assessed to determine whether certain farms

could increase this threshold. It is also important in both instances to balance the welfare of the animals and the cost/benefits involved in implementing new polices at a farm and community level in order for policy change to be sustainable and beneficial.

6.3. Conclusion

Analysis of Herd 1 revealed the vast diversity and dynamic nature of the mammary gland microbiome and drew suggestions that antibiotic treatment may not have a significant effect on the somatic cell count, bacterial richness (Chao1 index) and evenness (Shannon index) of the milk microbiota in cows with subclinical mastitis and in healthy cows. To specifically and directly test this, samples were selected for analysis from Herd 2 to address confounding variation that could be controlled for such as selecting cattle of the same parity, balancing the immune response status at drying off and focusing on cows at the time points drying off into early lactation. While it should be noted variation in infection levels, bacterial community, diversity and abundance measures can occur for many reasons such as breed, parity, farming practices, environment; in this context it was shown in a second, more selective, herd that antibiotics had little effect on the mammary gland milk microbiota and SCC. Overall, this large longitudinal study on two independent farms adds to a growing body of evidence aiming to ensure antibiotic use is justified and necessary.

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Supplementary Materials

Table 1. Summary metrics for the Herd 1 sequencing libraries generated by the Illumina MiSeq 300 bp paired-end sequencing of the bacterial 16S rRNA V1-V3 variable region. The two libraries contain a forward (R1) and reverse (R2) read for a total of 476 samples split evenly into the two libraries, giving 238 R1 and 238 R2 reads per run totalling 952 sampling reads.

Library 1

Total reads	PF reads	% Reads identified (PF)	CV	Min	Max
54,675,428	49,804,472	82.7373	0.8483	0.0002	1.4545

	Cycles	Yield	Projected yield	Aligned (%)	Error rate (%)	Intensity cycle 1	%>Q30
Read 1	301	7.47 Gpb	7.47 Gpb	5.28	2.25	236.29	74.98
Read 2 (I)	8	174.32 Mbp	174.32 Mbp	0.00	0.00	446.95	81.87
Read 3 (I)	8	174.32 Mbp	174.32 Mbp	0.00	0.00	210.47	81.49
Read 4	301	7.47 Gbp	7.47 Gbp	5.20	2.37	205.87	61.70
Non-Index reads	602	14.94 Gbp	14.94 Gbp	5.24	2.31	221.08	68.34
Totals	**618**	**15.29 Gbp**	**15.29 Gbp**	**5.24**	**2.31**	**274.89**	**68.34**

Library 2

Total reads	PF reads	% Reads identified (PF)	CV	Min	Max
48,945,332	44,734,428	82.8151	1.0267	0.0006	1.6401

	Cycles	Yield	Projected yield	Aligned (%)	Error rate (%)	Intensity cycle 1	%>Q30
Read 1	301	6.71 Gpb	6.71 Gpb	6.69	2.29	261.21	81.61
Read 2 (I)	8	156.57 Mbp	156.57 Mbp	0.00	0.00	438.18	82.26
Read 3 (I)	8	156.57 Mbp	156.57 Mbp	0.00	0.00	236.95	85.66
Read 4	301	6.71 Gbp	6.71 Gbp	6.56	2.53	234.00	64.81
Non-Index reads	602	13.42 Gbp	13.42 Gbp	6.63	2.41	247.60	73.21
Totals	**618**	**13.73 Gbp**	**13.73 Gbp**	**6.63**	**2.41**	**292.59**	**73.46**

Table 2. Forward and Reverse reads for each sample were merged using USEARCH8.1. Due to low quality in the Reverse reads in both libraries, the reads were truncated by up to 30 base pairs, with a mismatch score of between 2 to 5 bases tested per file. 2 samples were randomly selected (S69 and S159) along with one positive and one PCR control and the percentage of reads merged presented below. The truncation of 30 bp was chosen and a mistmatch score of 2 to remain conservative.

		% reads merged (mismatch score 2-5)			
Sample ID	Truncation (bp)	**2**	**3**	**4**	**5**
A-F9-69_S69	0	28.7	35.3	40.4	43.0
	10	34.4	40.6	45.4	47.5
	20	39.2	45.1	48.0	50.0
	30	**44.3**	**49.8**	**52.2**	**54.0**
B-F5-153_S159	0	27.3	33.8	38.4	40.3
	10	32.2	38.6	42.7	44.1
	20	38.2	43.3	45.3	46.6
	30	**41.9**	**46.5**	**48.0**	**48.9**
Ei-D12-Pos	0	31.5	39.5	39.5	39.5
	10	41.4	41.6	41.6	41.6
	20	**48.9**	**48.9**	**48.9**	**48.9**
	30	44.7	44.7	44.7	44.7
Ei-E1-PCR-1	0	27.8	33.6	34.7	35.2
	10	34.7	36.0	36.7	37.1
	20	**39.5**	**40.8**	**41.4**	**41.9**
	30	37.7	38.5	39.0	39.4

Figure 1. Number of OTU sequences identified in the negative control files after reads were mapped back to the reference OTUs generated at 97% (A.), 98% (B.) and 99% (C.) filtering. CC, Calving control; DNA_ex_Neg, DNA extraction negative control; PC, Parlour control; PCR1, PCR stage 1 blank; PCR2, PCR stage 2 blank. As the negative filtereing score is increased more OTUs are present in all controls. There is contamination across the negative controls. This is not suprising in Parlour and Calve controls as these were exposed to the environment in the sampling areas. There is contamination in the DNA extraction negative control, this is also not too suprising, as although best sterile practice was used, many samples were being processed at the same time and stage 1 PCR was highly sensitive.

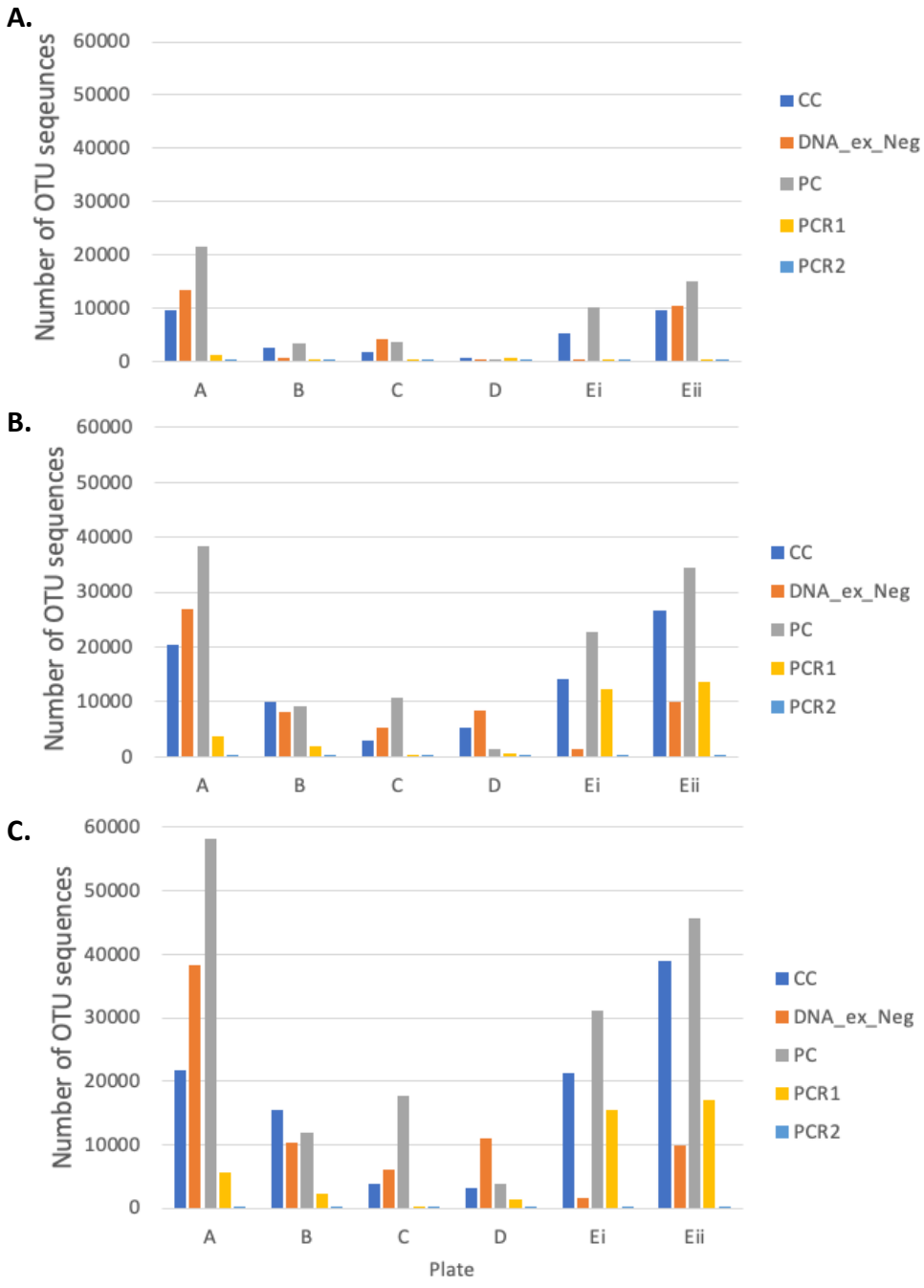

Table 4. Summary of variation in the somatic cell count (log_{10}SCC) over 11 time points for the antibiotic (AB) and non-antibiotic (Orb Only) treatment groups in Herd 1. D, Drying off; C0, Calving day; PC, Post-Calving, 1-28 days post calving; mean value of log_{10}SCC index scores for time points C0, PC1 and PC3, Av_C0.PC1.PC3.

Treatment group	Time point	count	mean	sd	median	IQR
AB	D	36	2.131	0.822	2.209	1.224
	C0	36	2.714	0.601	2.712	0.868
	PC1	36	2.350	0.616	2.391	0.717
	PC3	36	1.827	0.831	1.544	0.828
	PC5	36	1.649	0.727	1.361	0.679
	PC7	36	1.648	0.736	1.575	0.974
	PC10	36	1.531	0.668	1.362	0.946
	PC14	36	1.421	0.549	1.406	0.699
	PC17	36	1.410	0.639	1.190	0.813
	PC21	36	1.302	0.794	1.128	1.158
	PC28	36	1.175	0.827	1.041	1.156
	Av_C0.PC1.PC3	36	2.297	0.579	2.309	0.906
Orb Ony	D	36	1.963	0.737	2.180	1.244
	C0	36	2.984	0.363	2.989	0.426
	PC1	36	2.336	0.658	2.425	0.647
	PC3	36	2.110	0.551	2.032	0.491
	PC5	36	2.242	0.837	1.977	1.532
	PC7	36	2.174	0.775	2.080	1.410
	PC10	36	1.997	0.750	1.872	1.270
	PC14	36	1.710	0.691	1.527	0.991
	PC17	36	1.615	0.666	1.538	0.642
	PC21	36	1.256	0.706	1.094	0.960
	PC28	35	1.486	0.942	1.322	1.189
	Av_C0.PC1.PC3	36	2.477	0.420	2.462	0.563

Table 5. In Herd 1, somatic cell counts (SCC) were log transformed to normalise the data and presented in Chapter 3. Figure 1.A.). The F test was conducted to compare the log_{10}SCC variances between the antibiotic (AB) and non-antibiotic (Orb Only) treatment groups at the drying off time point (Table5.A). There is not sufficient evidence to say that the variances between the two treatment groups are unequal (F = 1.2441, p > 0.05). The two-sample t-test was conducted to compare the means between the AB and Orb Only treatment groups, the means of the two groups are not significantly different (*t* = 0.9093, p >0.05; Table 5.B.).

	Statistical test	**Statistic**	**df**	**95% CI**	**p-value**	**Sample estimates**
A.	F test	F = 1.2441	35	0.6344, 2.4397	0.5217	1.2441*
B.	t-test	*t* = 0.9093	70	-0.1997, 0.5345	0.3663	2.1307, 1.9633**

** Ratio of variances ** mean of x, mean of y*

Table 6. Summary of variation in the Chao1 index (log_{10}Chao1) over 11 time points for the antibiotic (AB) and non-antibiotic (Orb Only) treatment groups in Herd 1. D, Drying off; C0, Calving day; PC, Post-Calving, 1-28 days post calving; mean value of log_{10}Chao1 index scores for time points C0, PC1 and PC3, Av_C0.PC1.PC3.

Treatment group	Time point	count	mean	sd	median	IQR
AB	D	36	1.984	0.404	2.070	0.598
	C0	36	1.445	0.294	1.388	0.366
	PC1	36	1.343	0.353	1.352	0.239
	PC3	36	1.423	0.423	1.439	0.345
	PC5	36	1.433	0.278	1.389	0.256
	PC7	36	1.578	0.326	1.505	0.348
	PC10	36	1.441	0.689	1.481	0.540
	PC14	36	1.510	0.675	1.562	0.597
	PC17	36	1.293	0.750	1.398	0.740
	PC21	36	1.291	0.737	1.462	0.671
	PC28	36	1.381	0.650	1.446	0.692
	Av_C0.PC1.PC3	36	1.404	0.243	1.347	0.331
Orb Only	D	36	1.858	0.532	1.796	0.592
	C0	36	1.579	0.419	1.512	0.523
	PC1	36	1.436	0.285	1.398	0.387
	PC3	36	1.549	0.266	1.538	0.219
	PC5	36	1.373	0.339	1.342	0.454
	PC7	36	1.489	0.400	1.380	0.454
	PC10	36	1.617	0.481	1.580	0.322
	PC14	36	1.576	0.448	1.505	0.652
	PC17	36	1.541	0.428	1.519	0.466
	PC21	36	1.546	0.585	1.455	0.640
	PC28	35	1.317	0.606	1.421	0.488
	Av_C0.PC1.PC3	36	1.521	0.204	1.491	0.218

Table 7. Summary of variation in the Shannon index scores over 11 time points for the antibiotic (AB) and non-antibiotic (Orb Only) treatment groups in Herd 1. D, Drying off; C0, Calving day; PC, Post-Calving, 1-28 days post calving; mean value Shannon index scores for time points C0, PC1 and PC3, Av_C0.PC1.PC3.

Treatment group	Time point	count	mean	sd	median	IQR
AB	D	36	3.084	1.014	2.840	1.333
	C0	36	2.241	0.972	2.219	1.176
	PC1	36	1.764	0.888	1.833	1.261
	PC3	36	2.271	1.014	2.169	1.368
	PC5	36	2.144	0.756	2.140	0.919
	PC7	36	2.752	1.026	2.688	0.771
	PC10	36	2.054	1.465	1.900	1.767
	PC14	36	2.229	1.377	2.189	1.351
	PC17	36	1.981	1.417	2.074	1.712
	PC21	36	2.077	1.388	2.102	1.537
	PC28	36	2.053	1.252	2.050	1.563
	Av_C0.PC1.PC3	36	2.092	0.592	2.085	0.849
Orb Only	D	36	3.289	1.162	3.254	1.450
	C0	36	2.269	1.297	2.149	1.495
	PC1	36	1.998	0.922	1.919	1.156
	PC3	36	2.473	0.879	2.610	1.097
	PC5	36	1.975	1.101	1.898	1.448
	PC7	36	2.247	1.052	2.109	1.343
	PC10	36	2.585	1.194	2.399	1.205
	PC14	36	2.651	1.281	2.739	1.639
	PC17	36	2.237	1.049	2.420	1.197
	PC21	36	2.556	1.360	2.547	1.846
	PC28	35	2.150	1.207	2.249	1.669
	Av_C0.PC1.PC3	36	2.247	0.683	2.189	0.868

Table 8. Summary of variation in the somatic cell count (log_{10}SCC) over 5 time points for the antibiotic (AB) and non-antibiotic (Orb Only) treatment groups in Herd 2. D, Drying off; C0, Calving day; 1 day post calving, PC1; 3 days post calving, PC3; 17 days post calving, PC17; mean value of log_{10}SCC index scores for time points C0, PC1 and PC3, Av_C0.PC1.PC3.

Treatment group	Time point	count	mean	sd	median	IQR
AB	D0	44	1.451	0.392	1.415	0.566
	C0	44	2.679	0.443	2.748	0.618
	PC1	44	2.076	0.346	2.115	0.590
	PC3	44	1.717	0.444	1.638	0.550
	PC17	44	1.044	0.560	0.903	0.318
	Av_C0.PC1.PC3	44	2.157	0.311	2.210	0.469
Orb Only	D0	44	1.443	0.337	1.398	0.489
	C0	44	2.802	0.321	2.850	0.425
	PC1	44	2.412	0.316	2.398	0.566
	PC3	44	1.847	0.321	1.767	0.328
	PC17	44	1.235	0.609	1.079	0.632
	Av_C0.PC1.PC3	44	2.354	0.243	2.306	0.328

Table 9. Summary of variation in the Chao1 index (log_{10}Chao1) over 5 time points for the antibiotic (AB) and non-antibiotic (Orb Only) treatment groups in Herd 2. D, Drying off; C0, Calving day; D, Drying off; C0, Calving day; 1 day post calving, PC1; 3 days post calving, PC3; 17 days post calving, PC17; mean value of log_{10}Chao1 index scores for time points C0, PC1 and PC3, Av_C0.PC1.PC3.

Treatment group	Time point	count	mean	sd	median	IQR
AB	D0	44	1.530	0.321	1.558	0.457
	C0	44	1.361	0.330	1.385	0.303
	PC1	44	1.469	0.306	1.457	0.270
	PC3	44	1.513	0.316	1.498	0.382
	PC17	44	1.389	0.322	1.364	0.461
	Av_C0.PC1.PC3	44	1.448	0.184	1.458	0.282
Orb Only	D0	44	1.639	0.312	1.658	0.432
	C0	44	1.457	0.401	1.466	0.312
	PC1	44	1.415	0.263	1.443	0.383
	PC3	44	1.500	0.325	1.563	0.426
	PC17	44	1.545	0.351	1.494	0.453
	Av_C0.PC1.PC3	44	1.457	0.213	1.446	0.209

Table 10. Summary of variation in the Shannon index over 5 time points for the antibiotic (AB) and non-antibiotic (Orb Only) treatment groups in Herd 2. D, Drying off; C0, Calving day; D, Drying off; C0, Calving day; 1 day post calving, PC1; 3 days post calving, PC3; 17 days post calving, PC17; mean value of Shannon index scores for time points C0, PC1 and PC3, Av_C0.PC1.PC3.

Treatment group	Time point	count	mean	sd	median	IQR
AB	D0	44	2.084	0.858	2.101	1.113
	C0	44	1.292	0.743	1.226	0.715
	PC1	44	1.854	0.871	1.768	0.850
	PC3	44	1.811	0.876	1.651	0.834
	PC17	44	1.525	0.763	1.339	0.914
	Av_C0.PC1.PC3	44	1.652	0.421	1.623	0.623
Orb Only	D0	44	2.096	1.046	2.005	1.381
	C0	44	1.484	0.949	1.251	0.760
	PC1	44	1.681	0.636	1.731	0.775
	PC3	44	1.771	0.750	1.700	1.048
	PC17	44	1.908	1.031	1.821	1.164
	Av_C0.PC1.PC3	44	1.645	0.441	1.647	0.605