INFLUENCE OF ENVIRONMENTAL FACTORS ON BOVINE MAMMARY BACTERIAL COMMUNITIES AND MAMMARY INFLAMMATION

BY

Kelsey B. Fehr

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ABSTRACT

Bovine milk and teat-apex bacterial communities are thought to contribute to mammary health. However, milk and teat-apex bacterial communities likely also differ in bacterial diversity and composition among farm-systems and between seasons, along with differing environmental bacterial communities. To find evidence supporting these notions, the bacterial-profile of milk, teat-apices, used-bedding and feces were assessed on three dairy farms using different bedding [Straw (ST), Sand (SA), and Recycled bedding material (RBM)], and also during summer and winter time-points for farm ST. Differences in between-sample bacterial diversity were identified among farms, and between both time-points for farm ST, for all sample-types ($P_{(PERMANOVA)} \leq 0.0002$). Consistently among farms, a major source of milk microbiota was the teat-apex. Further, four OTUs on the teat-apex were associated with reduced mammary inflammation, and two within milk ($P_{(FDR)} < 0.05$). Overall, this research demonstrates farm and seasonal differences as substantial drivers in milk and teat-apex bacterial diversity and composition.

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DEDICATION

I dedicate this thesis to my family.

Thank you for your love and support!

FOREWORD

This thesis was written in a manuscript format, consisting of the following:

A literature review section, which, with some modifications, will be submitted as a review paper and the main manuscript that will be submitted as “Fehr KB, Derakhshani H, Sepehri S, Plaizier JC, and Khafipour E. Influence of environmental bacterial communities on bovine mammary bacterial communities and mammary inflammation”.

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a given site on a given farm. Phyla shown are those present in at least 25% of samples within a site (e.g. WPS.2 is a phylum shown for bedding because it was in at least 25% of bedding samples but is not shown for milk because it was present in fewer than 25% of milk samples).

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LIST OF ABBREVIATIONS

16S rRNA	16S ribosomal RNA
BTSCC	Bulk tank milk somatic cell count
CBMRN	National Cohort of Dairy farms of the Canadian Bovine Mastitis Research Network
CDIC	Canadian Dairy Information Centre
CNS	Coagulase negative *Staphylococci*
DHI	Dairy herd improvement program
FDR	False discovery rate
Hp	Hepatoglobin
IMI	Intramammary infection
IRCM	Incidence rate of clinical mastitis
LogSCC	Log_{10} of milk somatic cell count
NMC	National Mastitis Council
NCR OTU	New CleanUp Reference OTU
N.R.OTU	New reference OTU
nMDS	Nonmetric multidimensional scaling
OTU	Operational taxonomic unit
RBM	Recycled bedding material, only used to denote bedding use of a specific farm included in the study
RMS	Recycled manure solids
SAA	Serum amyloid A
SA	Sand bedding, only used to denote bedding use of a specific farm included in the study
SCC	Somatic cell count of milk

ρ	Spearman rank-order correlation coefficient
ST	Straw bedding, only used to denote bedding use of a specific farm included in the study

GENERAL INTRODUCTION

Mammary gland inflammation, known as mastitis, is commonly triggered as a defense mechanism against intramammary infections (IMIs) (Pyörälä et al., 1992). Bovine mastitis is considered among the most common diseases on dairy farms (Halasa et al., 2007), and results in major limitations to milk production, milk quality and cow welfare. In Canada, mastitis is the second most common reason for early removal of a cow from the herd (CDIC, 2017). Due at least in part to these limitations, mastitis is considered one of the most costly diseases to the dairy industry (Halasa et al., 2007). It has long been known that mastitis is a multifactorial disease, and the primary factors that influence the risk of mastitis and severity of the disease can be grouped into three general categories: a) factors of the microorganism causing infection such as virulence factors, b) host-factors such as host immunity, and c) environmental factors that can influence both the host and the pathogen causing the infection, such as climate and environmental hygiene (Schroeder, 2009). This research will primarily focus on environmental sources of mammary microbiota within different dairy farm-systems, and also, a less-understood factor that may influence a cow's risk of mastitis, the diversity and composition of microbial communities within the mammary gland, as well as on the teat-apex.

Until recently, the mammary gland of healthy cows has been thought of as a sterile environment (Tolle, 1980), and this notion was likely due to the reliance on standard culturing techniques that detected only bacterial groups that grow under standard laboratory conditions (Hugenholtz et al., 1998). For instance, it has been estimated that 85-99% of microorganisms cannot be grown under standard laboratory conditions (Lok, 2015). However, progress was made in the area of culture-independent research that shed

light on the bacterial diversity of milk even from healthy quarters. Specifically, high-throughput DNA sequencing techniques used to identify a large diversity of bacteria within the bovine mammary gland have allowed for the identification of commensal mammary microbiota, novel associations between bacterial groups in milk and mammary gland inflammatory or health status, and the realization of potentially beneficial bacterial groups that could protect against invasion by pathogens (Kuehn et al., 2013, Oikonomou et al., 2014). Microbial groups that come into contact with a cows teat-apex, the area over and around the route of entry for microbiota into the mammary gland, may be able to influence mammary health in the following ways: Through bypassing barriers of entry into the mammary gland and causing an IMI, or through altering susceptibility to IMIs from either within the mammary gland or on the teat-apex. As examples of how microbiota may alter susceptibility to IMIs, commensal mammary microbiota may inhibit the colonization of mastitis pathogens within the mammary gland, or commensal teat-apex microbiota may inhibit the colonization of mastitis pathogens on the teat-apex. In terms of the origin of commensal mammary microbiota, the possibility exists that some of these microbial groups enter the mammary gland directly from the gut through an entero-mammary pathway within the host (Rodríguez, 2014). The prevailing view however, is that commensal mammary microbiota originate from the external environment, being either natural skin microbiota found on the teat-apex, or microbiota that contact the teat-apex from other environmental sources (Addis et al., 2016), such as bedding and feces. Lactating dairy cows spend approximately 10 to 12 hours resting in stalls per day (Haley et al., 2001), and during this time teats are in contact with bedding material. Increased microbial load of bedding material positively correlates with teat-

apex microbial load (Hogan et al., 1989b, Hogan and Smith, 1997), and along with this, fecal material is likely a main contaminant of used bedding in stalls. Increased teat-apex microbial load has been linked with an increased risks of environmental mastitis (Bey et al., 2002). Though providing an environment that limits contact of the teat-apex to microorganisms, for instance, through the implementation of hygienic practices such as those recommended by the National Mastitis Council (NMC, 2011), the incidence of mastitis on farms can be reduced. However, mastitis is still a major concern for dairy farms, and regardless of total bacterial load, bacterial communities will continue to exist in the dairy environment. More focus is needed, on how bacterial communities within the dairy environment (e.g. those from feces and bedding) influence mammary and teat-apex bacterial communities, and on how differences among farm-systems and seasons, influence environmental, mammary and teat-apex bacterial communities in ways that are important to mammary inflammation.

Using high-throughput sequencing, this research aims to compare the bacterial composition and diversity of potential environmental sources, teat-apices, used bedding and feces, as well as milk of cows between three different farm-systems. Along with this, we aim to estimate and compare the proportional contribution of environmental sources (used bedding, feces and/or teat-apices) to milk microbial communities among farms. A further aim is to identify used bedding, feces and/or teat-apices as possible sources of bacterial groups whose abundance on the teat-apex and in milk were found to be associated with reduced mammary inflammation here, and whether they differ in abundance between farm-systems. Different farm-systems, refers to farms that use different management practices, specifically different bedding management practices

such as the use of different bedding types. To achieve the aforementioned aims, this thesis research focused specifically on three farms-systems, within the same region (Manitoba) and season (Winter), most distinguishable based on differences in bedding material used in lactating herd stalls [One using straw (ST), one using sand (SA) and one using recycled bedding material (RBM)].

In addition to this, the straw bedded farm was sampled during a second summer time-point in order to identify whether for this farm, we could identify an increased mammary inflammation during the summer compared to winter, similar to what has been observed previously (Smith et al., 1985), and whether this may be related to changes in the bacterial diversity and composition of commensal mammary and teat-apex microbiota between summer and winter time-points, which has not been researched previously.

By determining which farm-system has an ideal proportion of bacterial groups potentially beneficial to mammary health, or optimal mammary bacterial diversity, we can help recommend management strategies to improve a herds overall mammary inflammatory status. Additionally, microbiota associated with reduced mammary inflammation can be defined as candidates for use in the development of synthetic microbial communities that may be used as an alternative to antibiotic therapy for the treatment and prevention of mastitis. Further, this thesis research will provide insights regarding the level of consideration that should be placed on variation among microbial communities of healthy mammary glands due to farm and seasonal differences, and in relation to variation in environmental bacterial communities. Understanding the natural variation that may occur in teat-apex and mammary microbial communities depending of environmental differences, is an important first step in identifying compositional differences between

commensal mammary and teat-apex microbial communities that may protect the host against pathogens, and states of mammary and teat-apex microbial communities that predispose a cow to high levels mammary gland inflammation.

1 LITERATURE REVIEW

1.1 Bovine mastitis: Definition and consequences to the dairy industry

Bovine mastitis is defined as mammary gland inflammation, and is most commonly the result of an intramammary infection (Pyörälä et al., 1992). Another cause of mammary gland inflammation that will not be addressed here is injury to the udder (Bryan, 1947). For example, kicking the udder or prolonged milking can cause injury that results in mammary gland inflammation referred to as non-infectious mastitis. Mastitis has been classified into two broad categories based on whether there are visible signs of mammary inflammation. Clinical mastitis presents with visible signs of mammary inflammation that include: Abnormal milk (e.g. clots or blood in milk), possibly a red, hard, and swollen udder, and only in severe cases there are signs of systemic infection, such as a fever, anorexia and abnormal respiration (Hogan et al., 1989a). These signs can be used to both diagnose and identify the severity of clinical mastitis (Hogan et al., 1989a). Meanwhile, subclinical mastitis is mammary inflammation in the absence of visible signs of inflammation, and the indirect measure commonly used for diagnosis of subclinical mastitis, milk somatic cell count (SCC), is discussed in detail within section 1.2.1.

In Canada, mastitis is currently the second most common reason for removal of a cow from the herd (CDIC, 2017). The overall incidence rate of clinical mastitis (IRCM) in Canada has previously been estimated at 23 cases per 100 cow-years, ranging from 0.7 to 97.4 cases per 100 cow-years, with a median of 16.7 based off of 106 farms in 10 Canadian provinces. Note that “100 cow-years”, as a measure for the incidence rate of clinical mastitis, is defined as the number of new clinical mastitis cases for every 36,500 days in the lactating cow herd (Olde Riekerink et al., 2008). The authors further identified the IRCM for each province, which was lowest for Manitoba at 7.6 cases per

100 cow-years in Manitoba, and the highest for Ontario and Québec, being 31.6 and 29.7 cases per 100 cow-years respectively. On the other hand, while there are no visual signs of subclinical mastitis and limited to no recent publications on its prevalence, overall, it is thought to be a more common condition compared to clinical mastitis. Similarly, there is said to be considerably more published data on the economic impact of clinical mastitis compared to subclinical mastitis (Rollin et al., 2015). This is by in large due to difficulties in estimating the economic impact due to variation in the definition of subclinical mastitis (e.g. SCC threshold used to define it) and in the level of screening for the disease from herd to herd (Rollin et al., 2015).

Kirkpatrick and Olson (2015) evaluated production losses of a high first test somatic cell count, or in other words, subclinical mastitis within 5 to 45 days of lactation or early lactation, using the most common definition of subclinical mastitis in the dairy industry, a somatic cell count above 200,000 cells/ml (Dohoo and Leslie, 1991, Harmon, 1994). Alongside this, production losses of a clinical mastitis case in the first 60 days of lactation were evaluated. Mastitis in early lactation can be particularly costly, since losses in milk production can extend out to late lactation due to long-term effects on milk production. While clinical mastitis in the first 60 days of lactation were found to reduce milk production by 457 kg across lactation ($181 USD lost milk yield) compared to cows without clinical mastitis during the first 60 days of lactation, cows with a high SCC (≥ 200,000 cells/ml) in early lactation had reduced milk production by 718 kg across lactation ($285 USD lost milk yield), compared to cows with a low SCC (< 200,000 cells/ml) in early lactation. Based on these results from this large-scale study of 164,423 cow records from 22 herds in the USA (Washington, Oregon and Idaho), it was suggested

that at least in terms of lost milk production, subclinical mastitis during the first test day (5-45 days of lactation) is costlier compared to clinical mastitis within the first 60 days of lactation. Additional cost considerations not included in the research were treatment costs, reduced reproductive ability, mastitis recurrence and early removal from the herd. However, in comparison to cows with a low first test SCC, this research found cows with a high SCC during the first test to be 2.48 times more likely to also develop clinical mastitis within the first 60 days of lactation (Kirkpatrick and Olson, 2015).

Recent in-depth cost estimates of clinical mastitis occurring within the first 30 days of lactation, that encompassed costs of diagnostics, therapies, discarded milk, veterinary services, labor, death, premature removal from the herd and replacement costs, along with losses to future milk production and future reproduction found the average case to cost $444 USD (Rollin et al., 2015). In this study, the greatest costs of clinical mastitis were due to future milk production losses that amounted to 28% of the total cost, and when added to discarded milk costs, totaled at $150 USD for an average case of clinical mastitis, based on an average net price of $0.461 USD per kilogram of milk received by farmers (Net price: all payments received from and costs from marketing milk). These estimates were also based on both economic data from scientific literature and actual herd data across the USA, including 30,000 lactating cows.

1.2 Known bacterial causative agents and diagnosis of mastitis

1.2.1 Measures of mammary inflammation

While clinical mastitis is typically diagnosed by visibly looking at milk, and signs of udder inflammation, as previously discussed, other measures of mammary inflammation are necessary to identify subclinical mastitis. Most commonly, milk somatic cell count

(SCC) is used as a relatively accurate indirect measurement of subclinical mastitis (Holdaway et al., 1996). While somatic cells are generally defined as any host-cell other than reproductive cells, those in milk are primarily from the immune system, with an estimated 80% of total somatic cells being immune cells in uninfected quarters and up to approximately 99% in mastitic quarters (Sordillo et al., 1997), with large numbers of leukocytes, macrophages and other immune cells moving into the mammary gland as part of the defense mechanism against intramammary infections (Pillai et al., 2001). Milk SCC thresholds are frequently used to classify cows as having subclinical mastitis; a threshold that has previously been identified as ideal being a quarter or composite milk SCC of 200,000 cells/ml, above which cows are diagnosed as having subclinical mastitis (Dohoo and Leslie, 1991). Composite milk SCC samples are less accurate compared to quarter milk samples since mammary inflammatory status is frequently different between quarters of a cow (Schukken et al., 2003), however composite milk SCC is a less time consuming and less costly measure and is therefore commonly used on farms. In a recent study by Vissio et al. (2014) it was noted that previous estimates of the ideal threshold for diagnosing subclinical mastitis did not take into account the consideration that bacteriological culturing, used to estimate the accuracy of SCC on diagnosing IMI, is itself not a completely accurate test for diagnosing IMI. These authors further identified the most sensitive and specific threshold for composite milk SCC as a measure of IMI to be 150,000 cells/ml based on Latent-class models using Bayesian methods, using 175 composite milk samples from cows in two herds.

Variation between cows in their milk SCC due to factors such as lactation stage and parity have also been researched, due in part to a suspicion that factors other than an IMI

may make setting an equally precise threshold for all cows challenging. Laevens et al. (1997) did not find an effect of parity or stage of lactation on the milk SCC of bacteriologically negative milk, defined as a quarter milk sample with no isolates obtained from bacteriological culture, from uninfected cows. However, when including cows with an IMI (caused by CNS, *C. bovis,* or esculin-positive cocci other than *Streptococcus uberis*), they found a higher SCC in the first month of lactation (0 to 30 DIM; 55,900 cells/ml), compared to the SCC in the next month (31 to 60 DIM; 40,800 cells/ml), and again an increased SCC during later stages of lactation (> 60 DIM; 90,000 cells/ml). The authors also found a greater elevation in SCC during the first month of lactation for primiparous cows compared to cows in second and third lactation, but this elevated SCC decreased in the following month (31 to 60 DIM) for primiparous cows. Meanwhile, cows in second and third lactation had a greater elevation in SCC towards the end of lactation (> 240 DIM) compared to primiparous cows towards the end of lactation (> 240 DIM). The effects of parity and stage of lactation were generally attributed to factors present only when including infected cows. These factors likely include both an increased risk of acquiring an IMI in early and late lactation, and differences in the severity of the immune response to an IMI depending on parity and lactation stage. For example, Pyörälä and Pyörälä (1998) found that IMIs caused by coliforms and coagulase negative staphylococci (CNS) induced a larger inflammatory response in the mammary gland during early lactation compared to later stages of lactation. It is clear from this that parity and stage of lactation should be taken into account in research looking at the propensity for particular microbial groups to colonize the mammary gland and influence mammary inflammatory status.

It should be noted, that due to the relatively short duration of most clinical mastitis cases, in conjunction with its frequent occurrence, SCC is generally not an accurate indicator of the incidence rate for clinical mastitis (Erskine et al., 1987, Hogan and Smith, 2003). Along with this, SCC cannot distinguish clinical mastitis from subclinical mastitis and it is best to use visual observation of milk upon taking milk samples as a cost-effective way to diagnose and identify the severity of clinical mastitis.

Apart from visual observation, indicators of acute phase responses in dairy cows have been assessed for use in the diagnosis of clinical mastitis. The acute phase response indicator, haptoglobin (Hp), can increase to over 2 g/L in blood within two days after an infection, while a concentration below 20 mg/L in blood is considered healthy (Eckersall and Bell, 2010). Infections this may indicate include clinical mastitis, however it can also indicate enteritis, peritonitis, pneumonia, endocarditis, endometritis (Murata et al., 2004). Interestingly, Hp and a mammary isoform of SAA (M-SAA3) can be secreted in milk from cows with mastitis (Grönlund et al., 2003, Eckersall et al., 2006), and therefore within milk, there is greater potential for these proteins to be used as more specific biomarkers for mastitis in comparison to other indicators of an acute phase response (Eckersall and Bell, 2010).

1.2.2 Known bacterial causative agents of mastitis

The following section will summarize the major bacterial agents of mastitis; bacterial groups that are known to cause IMIs.

The immune/inflammatory response to an IMI tends to differ depending on the infecting bacterial agent. Coliforms are specific rod-shaped gram-negative bacteria and mastitis pathogens in this group include *Escherichia coli, and species of Klebsiella,* and

Enterobacter, which commonly originate from feces. Gram-negative bacteria that have been isolated from mastitic milk that are not coliforms, but originate from the environment, include species of *Pseudomonas,* and *Serratia* (Hogan and Smith, 2003). Infections caused by non-coliform gram-negatives are often characterized as being chronic infections extending even into the next lactation (Hogan et al., 1989a). Mastitis caused by gram-positive pathogens such as *Staphylococcus aureus* and *S. uberis* are more often characterized by milder clinical symptoms, or subclinical mastitis, however they can also present as chronic intramammary infections (Smith and Hogan, 1993). Meanwhile, mastitis caused by coliform pathogens is typically characterized by clinical mastitis (e.g. abnormal milk and swollen glands) and tends to have a short-lived infection period (Hogan and Smith, 2003), for example, Todhunter et al. (1991) found that the duration of naturally occurring *E. coli* intramammary infections was less than ten days. This has previously been attributed to coliforms such as *E. coli* often remaining in milk and not adhering to epithelial tissue (Frost et al., 1977), with the notion that adherence properties can allow a bacterial group to remain in the mammary gland, attached to mammary tissue, without being removed in milk during milking. However, more recently, Lammers et al. (2001) found specifically for *E. coli* associated with clinical mastitis*,* that adhesion to mammary gland cells is possible but efficiency of adhesion is strongly dependent on strain, and of 11 strains tested *in-vitro*, four had low adhesion to mammary cells (1 to 10 bacteria adhered per cell), three had intermediate adhesion (10 to 100 bacteria per cell) and three had strong adherence capabilities (100 to 1000 bacteria per cell) like the adherence found for 6 of the 7 *S. aureus* strains tests, with one *E. coli* strain resulting in severe damage to the cell after incubation and no specific adhesion

level recorded. Interestingly, a laboratory strain of *E. coli* (DH5α) did not adhere to mammary cells in this research, and indicated that mammary cell adhesion of the 11 strains may be specific to strains of *E. coli* isolated from mastitic quarters.

Frequently, coliforms such as *E. coli* are considered opportunistic pathogens, and previously, the duration of infection has been attributed more-so to host-characteristics rather than differences in pathogen virulence factors that were previously unable to explain differences between transient and persistent *E. coli* intramammary infections (Burvenich et al., 2003, Zadoks et al., 2011). However, recent research has shown that virulence factors and other pathogen-characteristics may in fact be important to the duration of *E. coli* infections. Specifically, *E. coli* isolates from persistent IMIs were more likely to present specific genes important to iron-acquisition (*iroN,* and *sitA*) compared to those isolated from transient IMIs, and also showed more resistance to antimicrobials with 38.9% of isolates resistant to at least one antimicrobial, compared to those isolated from transient IMIs that had 17.8% resistant to at least one antimicrobial (Fairbrother et al., 2015). While resistance to antimicrobials itself is not a virulence factor, this does indicate that the persistence of *E. coli* isolates may be dependent in part on phenotypic differences between particular *E. coli* isolates, seen as differences in resistance to antimicrobials that likely stems from genetic differences. Bacterial groups, such as possibly the *E. coli* strains specifically causing persistent infections previously discussed, along with *S. aureus* strains that are generally capable of causing chronic infections due to virulence factors such as adherence to the mammary epithelium and in some cases, invasion into cells (Dego et al., 2002), are sometimes referred to as host-adapted pathogens. Such pathogens are more frequently transferred from host-to-host, for

example through contamination of milking equipment, therefore they are termed contagious mastitis pathogens (Dego et al., 2002). Environmental mastitis pathogens on the other hand are those that transferred to the cow from the cows' environment rather than from other cows, these primarily include the coliforms and other gram-negative pathogens previously mentioned along with environmental Streptococci (i.e. those other than *Streptococcus agalactiae*) (Smith and Hogan, 2008). It is noteworthy that this classification system based on route of transmission is not clear-cut, for instance *S. uberis* is commonly found in environmental sources on dairy farms and spreads from the environment to the udder, but it is now thought that particular strains of *S. uberis* have potential to spread from cow to cow contagiously and are more adapted to the bovine mammary gland environment, as suggested by the frequent chronic infections that specific strains of *S. uberis* cause (Zadoks et al., 2003). Furthermore, at least one study has suggested certain *E. coli* strains may also be host-adapted and persist in the mammary gland, due to findings that recurrent *E. coli* mastitis was the same genotype as the first case for 85.7% of recurrent cases (Bradley and Green, 2001). Clearly, classification of microorganisms based on their level of host-adaptation and route of transmission is not straightforward. The identification of a pathogens usual mode of transmission and what pattern of mammary inflammation they typically cause (e.g. acute or chronic) are likely important considerations when identifying what avenue to focus on in the development of mastitis prevention strategies specific to different pathogens. Another important consideration may be whether either differences in pathogenicity between strains of a pathogen, or variation in host-factors like host immunity are the primary explanation for variation in the immune response elicited between IMIs by the same pathogen species.

1.3 Links between the mammary gland microbiome and mammary health

The identification of microbial groups associated with mammary inflammatory status is an important component of research into the causes and treatment of mastitis. Along with this, the environment within the mammary gland may be viewed as a unique ecological niche, with the presence of numerous microorganisms that do not necessarily cause IMIs, but may nonetheless influence mammary inflammatory status. This gives rise to a notion of mammary gland homeostasis, or the balance between factors within the mammary gland to maintain mammary health. The large number of bacterial groups within the bovine mammary gland that has been identified in healthy quarters (Bhatt et al., 2012, Oikonomou et al., 2012, Kuehn et al., 2013, Oikonomou et al., 2014) is a strong indication of commensal mammary microbial communities, defined as microbial communities that are naturally found in the mammary gland, but do not cause the host harm. These commensal mammary microbial communities may play a critical role in maintaining mammary gland homeostasis. Sections 1.2.1 and 1.2.2 will summarize important findings of studies regarding bacterial diversity of healthy and mastitic milk, and the bacterial groups associated with mammary gland health status. Along with this, a summary of the methods and the main results of studies exploring the mammary microbiome through high-throughput sequencing are included in Table 1.1.

Table 1.1 A summary of research using next-generation sequencing to investigate the milk microbiome and udder health

Study	Type of milk samples/number of samples	Sequencing technology	Most abundant bacterial groups (Phyla/Family/Genera/Species depending on focus of the study)	Associations with health/disease
Bhatt et al. (2012)	**Subclinical milk** diagnosed on a quarter basis using SCC (cut-off not specified) (3 pooled samples, each a composite of ten quarter milk samples pooled in equimolar concentrations)	Shotgun metagenomics using 454 pyrosequencing	**Phyla**: Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes **Genera/species**: *Escherichia coli, Pseudomonas* spp., *Shigella* spp., *Bacillus cereus, Salmonella* spp., *Streptococcus* spp., *Klebsiella* spp., *Enterobacter* spp., *Acinetobacter* spp.	**No control group – No associations identified**
Kuehn et al. (2013)	**Clinically mastitic milk** [at least abnormal milk (flacks, clots, serous)] with culture negative results (10 quarter milk samples) **Clinically healthy milk** from the same selected cows with a clinically mastitic quarter (10 quarter milk samples) Cows with no clinical mastitis and a low LSCC in all quarters (2 quarter milk samples)	454 pyrosequencing of the V1-V2 16S rRNA gene	**Genera**: *Ralstonia, Pseudomonas, Sphingomonas, Stenotrophomonas, Psychrobacter, Bradyrhizobium, Corynebacterium, Pelomonas,* and *Staphylococcus*	**Healthy quarters:** *Pseudomonas, Psychrobacter,* and *Ralstonia* **Culture negative clinical mastitis:** *Brevundimonas, Burkholderia, Sphingomonas,* and *Stenotrophomonas*
Oikonomou et al. (2012)	**Clinically or subclinically mastitic milk** [at least abnormal milk (flacks, clots, serous)] with conclusive culture results or culture negative results (136 pooled milk samples) **Clinically healthy milk** from cows with no history of clinical mastitis and with a SCC below 10,000 cells/ml (20 quarter milk samples)	454 pyrosequencing of the V1-V2 16S rRNA gene	**Genera***: Lactobacillus*, unclassified *Lachnospiraceae, Propionibacterium, Streptococcus, Staphylococcus, Comamonas, Bacteroides, Arcanobacterium, Geobacillus,* unclassified *Ruminococcaceae, Faecalibacterium,* and *Enterococcus*	**Clinical or subclinical mastitis:** *Escherichia coli*, *Klebsiella* spp. and *Streptococcus uberis*, *Corynebacterium pyogenes*, *Streptococcus dysgalactiae*, *Staphylococcus aureus*, *Fusobacterium necrophoxrum*
Oikonomou et al. (2014)	**Clinically healthy milk** in various SCC categories from SCC < 20,000 cells/ml to SCC > 50,000 cells/ml (110 quarter milk samples) **Subclinical milk** with culture positive results and a SCC > 400,000 (34 quarter milk samples) **Clinically mastitic milk** [at least abnormal milk (flacks, clots, serous)] with culture negative results (33 quarter milk samples)	454 pyrosequencing of the V1-V2 16S rRNA gene	**Genera**: *Fecalibacterium,* unclassified *Lachnospiraceae, Propionibacterium, Aeribacillus, Staphylococcus, Lactobacillus, Comamonas, Fusobacterium,* and *Enterococcus*	**Comparing only culture negative clinically healthy quarters (Regression against Log10SCC) Low SCC:** *Nocardiodes* and *Paenibacillus* **High SCC:** *Sphingobacterium* and *Streptococcus*
Ganda et al. (2016)	**Clinically mastitic milk** [at least abnormal milk (flacks, clots, serous)] (80 quarter milk samples (40 *E. coli,* 2 *Pseudomonas,* and also *Klebsiella,* and cluture negative results)) **Clinically healthy milk** – An ipsilateral quarter from the same selected cows with clinical mastitis (80 quarter milk samples)	Illumina MiSeq sequencing of the V4 16S rRNA gene	**Phyla**: Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes	***E. coli* and *Pseudomonas* spp. mastitis vs. healthy:** Proteobacteria. **Healthy vs. *E. coli*:** Firmicutes, Actinobacteria, Bacteroidetes, Tenericutes, Chlorobi. **Healthy vs. *Pseudomonas:*** Actinobacteria, Bacteroidets

1.3.1 Mammary gland bacterial diversity and mammary gland health status

In terms of the microbial diversity between milk samples, known as beta-diversity, distinct clustering patterns have previously been reported for milk microbiota of healthy quarters compared to culture negative clinically mastitic quarters using non-metric multidimensional scaling (nMDS) of Bray-Curtis dissimilarities ($P_{PERMANOVA}$=0.001) (Kuehn et al., 2013). Distinct clustering patterns have also been reported between milk microbial communities from quarters with *E. coli* mastitis compared to healthy quarters (P=0.001), using analysis of similarities on weighted UniFrac distances (Ganda et al., 2016).

In terms of bacterial diversity within milk samples, known as alpha-diversity, Oikonomou et al. (2014) found that clinically mastitic quarters with a culture-negative etiology had a reduced bacterial diversity (Shannon index) compared to clinically healthy quarters (P<0.05). Similarly, Ganda et al. (2016) observed a trend towards reduced diversity (Shannon index) of milk microbial communities from clinically mastitic quarters with a gram-negative bacteria or culture-negative etiology, compared to healthy quarters (P<0.1). Meanwhile Oikonomou et al. (2012) identified a variety of different bacterial diversity (Shannon index) and richness (Chao1 index) estimates for different etiologies of both clinical and subclinical mastitis. Results mainly indicated that milk from cows with clinical or subclinical mastitis as a result of *Trueperella pyogenes* and *Streptococcus* spp. had the lowest Chao1 richness and Shannon diversity estimates in comparison to mastitis caused by other pathogens such as *E. coli* (Chao1 index: 1207.39, Shannon index: 4.51) and *Staphylococcus* spp. (Chao1 index: 1015.2, Shannon index: 4.55), as well as compared to milk samples from healthy quarters (Chao index: 864.27,

Shannon index: 4.98 respectively), with a Chao1 index of 82.55 and Shannon index of 2.97 for *T. pyogenes,* and a Chao1 index of 104 and Shannon index of 3.11 for *Streptococcus* species. Though statistical comparisons were not performed for diversity and richness estimates, these results suggest that the diversity of milk from mastitic quarters may largely depend on the etiology, or causal agent, of mastitis. Nonetheless, the finding that healthy quarters had a higher bacterial diversity compared to mastitic quarters by Oikonomou et al. (2014) and Ganda et al. (2016) is in accordance with the ecological concept that has been applied to microbial communities, that high biodiversity may be an indication of community stability, with a high bacterial diversity implying that there is more likely to be a redundancy of function in the community, such that a loss of community members due to environmental perturbations will be less likely to result in community dysfunction (Girvan et al., 2005).

The overall bacterial diversity may be an important indication of community health and mammary homeostasis; however, in this scenario some specific bacterial groups are still going to be more critical to maintaining mammary gland health and homeostasis than others. Therefore, identifying associations between specific bacterial groups and mammary health status is another important component of previous research.

1.3.2 Milk bacterial composition and associations with mammary health status

At the phylum level, there has been consistency in the four main phyla identified in milk samples, regardless of the mammary glands health status. Milk from healthy and mastitic quarters alike, is found to be composed of microbial groups primarily from four major phyla, Proteobacteria, Actinobacteria, Firmicuties and Bacteroidetes (Bhatt et al., 2012, Ganda et al., 2016). Bhatt et al. (2012) identified Proteobacteria and Firmicutes as the

predominant phyla present in milk samples from subclinically mastitic cows, followed by Actinobacteria and Bacteroidetes, with no healthy quarter milk samples taken for comparison. Meanwhile, in research focusing on clinically mastitic quarters associated with *E. coli, Pseudomonas* spp., and *Klebsiella* spp., Ganda et al. (2016) identified statistical differences in the abundances of the major phyla between healthy and clinically mastitic milk associated with *E. coli* and *Pseudomonas* spp*.,* which had a higher abundance of Proteobacteria along with a lower abundance of Actinobacteria and Bacteroidetes compared to healthy quarters. *E. coli* associated clinical mastitis additionally had a significantly lower relative abundance of Firmicutes, Tenericutes, and Chlorobi compared to healthy quarters ($P<0.05$). An increased abundance of Enterobacteriaceae ($P<0.001$) as well as Pseudomonadaceae ($P=0.03$), both in the phylum Proteobacteria, in clinical quarters associated with *E. coli* and *Pseudomonas* spp., was also observed. These results concur with the fact that these families contain the primary mastitis pathogens cultured form the mastitic milk samples, *E. coli* and *Pseudomonas,* that are presumably the direct causes of the clinical mastitis.

Using 454 pyrosequencing, Bhatt et al. (2012) subjected three pooled milk samples representative of subclinically mastitic quarters to shotgun sequencing (Table 1.2). Their main results indicated that predominant bacterial groups present in milk from subclinically mastitic cows were primarily those known to be associated with mastitis, namely *E. coli, S. aureus, Pseudomonas* spp*., Shigella* spp*., Bacillus cereus, Salmonella* spp., *Streptococcus* spp., *Klebsiella* spp., *Enterobacter* spp., and in addition to this, *Acinetobacter*, a genus with no known influence on mammary health to-date. It should be

noted that this research was unable to identify associations between bacterial groups and healthy or diseased states of the mammary gland for lack of a healthy control group.

Oikonomou et al. (2014) found that the most predominant bacterial genera from 144 clinically healthy quarter milk samples, characterized as culture negative, were *Faecalibacterium,* unclassified *Lachnospiraceae, Propionibacterium* and *Aeribacillus.* In addition, the prevalence's of two genera were associated with reduced $\log_{10}$SCC (*Nocardiodes* and *Paenibacillus*), suggesting they may be contributing to a reduced mammary gland inflammation despite not being among the most prevalent genera in healthy milk samples (Oikonomou et al., 2014). Meanwhile, two genera were associated with an elevated $\log_{10}$SCC (*Sphingobacterium* and *Streptococcus*), and some bacterial species within the genera *Streptococcus* are known to cause mastitis. This suggests that these genera or specific species within these genera play an important role in mammary inflammation. Additional bacterial groups that may play a role in causing mammary inflammation may be anaerobic bacterial groups, mainly *Fusobacterium* spp. and *Porphyromonas* spp.*,* both of which Oikonomou et al. (2012) found to be elevated in mastitic milk but not in milk from healthy quarters. Species within these groups, *F. necrophorum,* and *P. levii,* have previously been found to act with *Trueperella pyogenes* as part of the summer mastitis etiology (Pyörälä et al., 1992). Additional research identified four genera to be in higher abundance in clinically mastitic milk samples, specifically *Brevundimonas, Burkholderia, Sphingomonas,* and *Stenotrophomonas,* and identified *Pseudomonas, Psychrobacter,* and *Ralstonia* to be in higher in abundance in clinically healthy milk samples ($P<0.05$) (Kuehn et al., 2013).

The above-mentioned studies do not show consistencies with each other in terms of the bacterial groups they found associated with mammary health status. However, differences in methods, such as differences in the criteria used to distinguish healthy quarters, clinically and subclinically mastitic quarters, whether only mastitis of specific etiologies were focused on, and how milk bacterial communities were identified (e.g. use of 16S rRNA gene sequencing or shotgun metagenomics), likely influence results (See Table 1.1 for specifics of each study). Along with this it is important to consider that there are likely many bacterial groups capable of influencing mammary health and inflammation in various ways.

Another complication is added when considering the large-scale changes in mammary microbial communities that may predispose a cow to mastitis, referred to as mammary dysbiosis. It is noteworthy that the presence of a mastitis pathogen in the mammary gland may not necessarily be a sign of disease, and that in at least some instances, some form of mammary dysbiosis may have to occur in order for a pathogen to cause disease. For example, major mastitis pathogens *S. uberis* and *S. aureus* have previously been identified, at low prevalence's, in milk from healthy quarters, and it was suggested that at least in low prevalence, these bacterial species may be natural inhabitants of the mammary gland (Oikonomou et al., 2014). These species of *Staphylococcus* may increase in prevalence during times of mammary microbial dysbiosis, or immunosuppression, albeit, this has yet to be confirmed and the authors cautioned that the presence of well-known pathogens in healthy milk samples could also be the result of contamination from the teat-apices.

1.4 Sources of, and barriers to microbiota colonizing the mammary gland

1.4.1 Teat-apex and Teat Canal

A wide diversity of bacteria, including pathogens, opportunistic pathogens, and commensals, that may either prefer natural skin as an ecological niche or environmental sites, such as feces, that commonly contact teat-apices, have commonly been identified on bovine teat-apices using culture and molecular techniques (Woodward et al., 1987, Braem et al., 2012, Verdier-Metz et al., 2012, Braem et al., 2013). Table 1.2 summarizes specific methods and results of selected studies referred to in this section.

Of the commonly identified bacterial groups on the teat-apex, those known to influence mammary health include coagulase negative *Staphylococci* (CNS), which were identified as the most commonly present microbiota on teat-apices of clinically healthy cows, along with *Corynebacterium* and *Enterococcus* (Braem et al., 2012, Braem et al., 2013). CNS species have arguably drawn the most attention, due in part to members of CNS being common causes of subclinical mastitis that on occasion have also been associated with clinical mastitis in heifers (Waage et al., 1999), despite it being considered a minor pathogen. On the other hand, CNS species have also drawn attention because members of this group may be beneficial to mammary health. For example, *Staphylococcus chromogenes* colonization of the teat-apex pre-partum (within two weeks before expected calving date) has been associated with a low SCC (< 200,000 cells/ml) in the 5 days after parturition (OR=0.27, P=0.048) (De Vliegher et al., 2003), however *S. chromogenes* IMIs tend to increase SCC above 200,000 cells/ml (Laevens et al., 1997), and therefore they are still a concern. Members of *Corynebacterium,* which is another common colonizer of the teat-apex (Braem et al., 2013), as well as the teat-canal (Falentin et al.,

2016), have also been implicated to protect against IMIs with major mastitis pathogens, despite being minor mastitis pathogens themselves (Brooks and Barnum, 1984). Specifically, *Corynebacterium bovis* IMIs were found to protected against infection by major mastitis pathogens, and a mechanism suggested was that the IMI induces a mild immune response sufficient to protect against infection by major mastitis pathogens such as *S. aureus* (Brooks and Barnum, 1984). This would likely only be a plausible mechanism for the findings of De Vliegher et al. (2003) discussed here, if the teat-apex colonization reflected an increased risk of IMI by *S. chromogenes*, to allow for a mild challenge to the immune system. However, De Vliegher et al. (2003) found that of 30 heifers sampled, only one had an *S. chromogenes* IMI at calving, and this was in two quarters from a single cow that was not found to have teat-apex colonization of *S. chromogenes* pre-calving, suggesting that its colonization of the teat-apex pre-calving was not necessarily a risk factor for IMI by *S. chromogenes* at calving. Another possibility is that some teat-apex microbial groups such as *S. chromogenes* are capable of inhibiting the colonization of mastitis pathogen on the teat-apex (Woodward et al., 1987, De Vliegher et al., 2004). De Vliegher et al. (2004) found evidence of this, in identifying two of ten *S. chromogenes* isolates from teat-apices of heifers that inhibited the growth of all strains of *S. aureus, S. dysgalactia,* and *S. uberis* tested *in-vitro,* however no *E. coli* strains were inhibited. The mechanism of this pathogen inhibition has recently been investigated by (Braem et al., 2014), who further identified CNS strains to have bacteriocin-like properties, such as the production of compounds that inhibit the growth of mastitis pathogens. In this study, *S. chromogenes* L217 was active against all tested mastitis pathogens (*S. uberis, Streptococcus dysgalactae,* and *S. aureus*) *in-vitro*, along

with most CNS species. *S. chromogenes* L217 was also found to produce a novel antibacterial peptide that was named nukacin L217. This research strongly suggests that specific strains of CNS play an important role as commensal teat-apex microbiota that limit mastitis-pathogen growth.

Woodward et al. (1987) assessed inhibitory effects of a broader range of microbiota that were considered natural teat skin microbiota using *in-vitro* culture-based methods. A general finding was that 25% of bacterial isolates, from teat-apex swabs (n=40) taken from non-lactating heifers, inhibited the growth of major mastitis pathogens. Greater inhibition was observed against gram-positive pathogens (*S. aureus, S. epidermidis, C. pyogenes* and *Streptococcal* spp.) compared to gram-negative pathogens (*E. coli* and *Klebsiella* spp.). The isolates with inhibitory effects against gram-positive pathogens were from the genera *Corynebacterium* (11/42 inhibitory isolates), *Bacillus* (11/21 inhibitory isolates), *Staphylococcus* (3/35 inhibitory isolates), and *Aerococcus* (2/2 inhibitory isolates), and most inhibitory *Corynebacterium*, *Bacilus,* and *Aerococcus* isolates also had inhibitory effects against gram-negative pathogens. It was suggested by the authors that this inhibitory effect was a characteristic of specific individuals within species and not a characteristic of the species containing the inhibitory isolates identified. Inhibition of pathogen growth appears to vary depending on strains within species, as shown by the previously mentioned study regarding CNS strains with bacteriocin-like properties (Braem et al., 2014). In addition, a bacterial group's inhibitory effects appear to be specific to only certain pathogens.

As discussed earlier, *C. bovis* IMIs may either elicit a mild and protective immune response or have inhibitory effects against mastitis pathogens such as *S. aureus*.

Interestingly, *Corynebacterium* as a genus has been found to co-occur in the human mammary gland with *Staphylococcus,* and was suggested to contribute to microbial dysbiosis in humans (Sam Ma et al., 2015); there may be species and/or strain level differences not captured in this particular study. Specifically for *C. bovis*, evidence exists that an IMI by this species can either reduce or increase susceptibility to infection by a mastitis pathogen depending on the mastitis pathogen. Pankey et al. (1985) challenged quarters infected with *C. bovis* to *S. aureus* and *S. agalactiae* broth culture immersion and found that while *C. bovis* infected quarters were more susceptible to *S. agalactiae* infections (8.5-fold), they were more resistant to *S. aureus* infections (0.5 fold) compared to healthy controls. Furthermore, trends towards increased rates of environmental streptococcal IMI during periods of high prevalence for *C. bovis* IMIs have also been observed (Hogan et al., 1988).

Additional genera commonly found on the teat-apex include *Acinetobacter, Aerococcus, Bifidobacterium, Facklamia* and *Jeotgalicoccus* (Braem et al., 2012). These have also been found to commonly colonize the teat-canal (Falentin et al., 2016), however they have little known influence on mammary health and likely do not cause IMIs. Bacterial groups are well known to influence mammary health through their colonization of the mammary gland. Bacterial groups may also influence mammary health through their presence on the teat-apex or in the teat-canal and influence which bacterial groups colonize the teat-apex and/or teat-canal. The idea that high bacterial diversity is an indication of microbial community stability may extend to teat-apex and teat-canal bacterial communities, along with bacterial communities in the mammary gland as discussed previously. Braem et al. (2012) found a numerically higher number of genera

on teat-apices of non-infected quarters (richness of 16) compared to teat-apices of subclinical and clinical quarters (both a richness of 12), with *Streptomyces, Propionibacterium, Myroides,* and *Weissella* only being identified from teats of healthy quarters. Along with this, teat-apices of healthy quarters had a higher bacterial diversity (Shannon index=2.47) compared to subclinical quarters (Shannon=2.27, P=0.080) and clinical quarters (Shannon=2.25, P=0.067). Similar results have been observed for the bacterial diversity of the teat-canal, which was found to be significantly higher for healthy quarters (Shannon index=7.87) in comparison to mastitic quarters (Shannon index=6.54, P<0.05) in research using amplicon Pyrosequencing of the V3-V4 region of 16S rRNA genes (Falentin et al., 2016).

Table 1.2 A summary of selected culture-dependent and culture-independent studies focusing specifically on bovine teat-apex microbiota

Study	**Number/type of samples**	**Methods**	**Most abundant bacterial groups**	**Main Findings**
Woodward et al. (1987)	40 teat-apex swabs cross-sectionally sampled from 10 heifers	Routine aerobic culturing of teat-swabs, and cross-streaking with mastitis pathogens to identify zones of pathogen growth inhibition	*Corynebacteria, Staphylococcus, Bacillus* and *Acinetobacter*	Some *Corynebacterium, Bacillus, Aerococcus*, and *Staphylococcus* isolates inhibited pathogen growth (*Corynebacterium pyogenes, S. aureus, S. epidermidis,* selected *Streptococcus* spp., *E. coli* and *Klebsiella*)
De Vliegher et al. (2003)	144 teat-apex swabs cross-sectionally sampled from 36 heifers 7-14 days before calving 144 quarter milk samples from the same heifers 3-5 days postpartum	Bacterial culture of swabs – focus on *S. chromogenes* Milk SCC measurement and bacterial culture of diagnosis of mastitis	**Not applicable** – *S. chromogenes* focused study	Pre-partum teat-apex colonization with *S. chromogenes* protected quarters against an elevated SCC
Braem et al. (2012)	48 teat-apex swabs cross-sectionally sampled from 12 lactating cows, each with a clinical mastitis infection in only one quarter.	V3-16S rRNA-PCR-denaturing gradient gel electrophoresis	*Corynebacterium, Aerococcus, Acinetobacter, Psychrobacter, Staphylococcus, Jeotgalicoccus, Streptococcus, Streptomyces, Kocuria, Bifidobacterium*	Increased bacterial richness and bacterial diversity (shannon-index) of uninfected quarters compared to infected quarters
Braem et al. (2013)	72 teat- apex swabs cross-sectionally sampled from 18 clinically healthy lactating cows from 2 herds	Culturing and $(GTG)_5$-PCR fingerprinting and culture-independent PCR-DGGE	**Culture-dependent analysis:** *Staphylococcus, Kocuria, Micrococcus, Bacillus, Corynebacterium, Weissella, Paenibacillus,* and *Enterococcus* **Culture-independent analysis:** *Aerococcus viridans, Jeotgalicoccus coquina, Lactobacillus fermentum, Staphylococcus* spp., *Corynebacterium coyleae, Propionibacterium* sp. , *Burkholderia* sp., and *Psychrobacter immobilis*	CNS were isolated most commonly (CNS isolates comprised 59% of all isolates, and were on 97.2% teat-apices) There was a large diversity of CNS species isolated form teat-apices (15 species identified in total with an average of 2 but up to 6 on a single teat-apex)

1.5 Environmental factors influencing mammary microbiota and mammary health

Maintaining mammary health is a complex challenge, due in part to the many factors of the microbe, host and environment that can influence mammary microbial communities and mammary health. The following sections will address the following environmental factors, including management practices: a) bedding management factors, b) facility design, and c) seasonal effects. This is by no means a complete list of environmental factors and other important aspects not reviewed here include a cow's exposure to dry-cow and lactational antibiotic therapy. The influence of environmental factors on the exposure of the teat-apex and mammary gland to bacterial groups will be the focus of these sections, however, it should be noted that environmental factors might also influence the risk of a cow acquiring an IMI through influencing host immunity (e.g. heat stress is thought to negatively impact host immunity).

1.5.1 Bedding material

Managing teat exposure to pathogens on bedding material is a critical factor impacting mammary gland microbial communities (Hogan and Smith, 2012). Many bedding management factors, including bedding choice, storage, moisture content, and how frequently bedding material is replaced, influence the microbial communities present in bedding and thus, those exposed to the udder. Commonly used dairy bedding materials including straw, sand, recycled manure solids (RMS), and wood shavings. Recycled manure solids are made by mechanically separating manure from pens and stalls where cows are housed. When this is used as-is, it is known as separated RMS, another type of RMS is digested RMS which goes through the additional process of anaerobic digestion,

and another yet is composted RMS, which goes through a horizontal rotary to aerate material during composting, which promotes aerobic fermentation which produces heat that limits bacterial growth (Husfeldt et al., 2012).

Different bedding materials may have distinctly different physical and chemical characteristics that influence the microorganisms capable of proliferating on them. In one study focusing on properties of RMS on 38 dairy farms in the US, comparisons of unused RMS, or RMS before being placed in stall as bedding material, revealed that composted RMS (n=4 farms) had significantly lower moisture content compared to separated (n=9) and digested RMS (n=25) (60.3% compared to 72.6% and 72.9% respectively). Further, separated RMS was found to have higher bacterial counts (identified through culturing-techniques) compared to digested RMS and composted RMS (15.70±0.75 compared to 12.00±0.46 for digested and 11.96±1.13 for composted) before use as a bedding material. However, after being used as bedding materials, only digested RMS had a lower bacterial count compared to the other two materials (15.55±0.33 compared to 17.01±0.49 for composted and 16.50±0.33 for separated) (Husfeldt et al., 2012). Disregarding likely differences in management practices between sand and RMS bedded farm, Husfeldt et al. (2012) found that the average bulk tank somatic cell count (BTSCC) of RMS bedded farms enrolled in the study of 274,000±98,000 was comparable to the average identified for sand-bedded facilities in Minnesota and South Dakota by Lobeck et al. (2011) of 305,000 cells/ml.

Godden et al. (2008) compared chemical characteristics between bedding materials, namely pH, total carbon, and total nitrogen of different clean bedding materials from 49 dairy farms, and identified their relationship with bacterial load, using culturing

techniques after inoculation with *Klebsiella pneumonia,* and *Enterococcus faecium.* While within each bedding type [clean sand (n=20), recycled sand (n=21), digested RMS (n=15), and shavings (n=15)] no chemical characteristics influenced the growth of the two bacterial species, the variability of these biochemical parameters within each bedding type was low in comparison to its variability between bedding types. When all bedding materials were combined in the model, *K. pneumonia* growth was positively associated with pH and total carbon ($P<0.05$), while *Enterococcus faecium* growth was associated with increased pH ($P<0.05$). Though these chemical characteristics may have been confounded with other bedding type differences, carbon content and pH of bedding are likely important parameters for bacterial growth. Organic beddings, digested RMS (46.22%, SD=1.22) and wood shavings (53%, SD=1.21) had a higher carbon content than sand bedding materials (clean sand mean=0.28%, SD=0.14; recycled sand mean=1.13%, SD=1.79), which provides a nutrient source to microbiota. Of note is that carbon availability is likely another important characteristic of bedding to microbial growth, though little research has been done on this. The advantage of wood shavings in limiting the growth of particular microbial groups inoculated may be partly related to its much lower pH (Mean=4.27, SD=0.14) than all other bedding types tested (pH between 8.15 and 8.90). After a 24-hour, 48-hour, and 72-hour incubation period, *K. pneumonia* counts were greatest in digested RMS, followed by recycled sand, wood shavings, and lowest in clean sand. Similarly, a slight growth of *E. faecium* was observed for both digested RMS and recycled sand, but reductions in its growth were observed for clean sand and wood shavings. While the effects that different bedding materials and physical and chemical parameters have on the ability for bacteria to proliferate were demonstrated, this research

does not mimic the on-farm scenario where microorganisms and organic material (e.g. fecal contamination) are continually added to bedding during the time the bedding is being used, such that even used sand bedding could support the growth of microorganisms. Research placing cows in the same farm-system on three bedding materials [5 cows per group, each group placed on each bedding for 3 weeks in a Latin square design], suggested particle size to be another important property of bedding, with bacterial counts on teat-ends of cows tending to be higher with the use of sawdust bedding which has a considerably lower particle size compared to the other bedding materials, wood shavings and straw (Rendos et al., 1975). This observation may be due to increased surface area for bacteria to attach to bedding as a substrate, and/or fine bedding more readily sticks to teat-apices.

Some controversy exists regarding whether RMS in particular, is a bedding material that negatively affects udder health. Wenz et al. (2007) identified herd management factors associated with bulk tank SCC (BTSCC) on 1,013 dairy farms in the United States. Note that BTSCC is an important milk quality parameter for dairy herds used as a rough estimate of a herd's overall udder health status. They found that the use of composted manure bedding increased the odds of having an increased BTSCC, with only 12% of farms using composted manure solids having a BTSCC below 200,000 cells/ml compared to 26.3% and 35.5% of straw and sand bedded farms, respectively. While increased SCC is generally associated with IMI caused by contagious pathogens, the exact cause for the association between the use of composted manure bedding and increased BTSCC was not identified. More recently, Rowbotham and Ruegg (2016b) conducted a trial over one year on a single farm to compare the effects of using different manure solid and sand based

bedding materials on bacterial counts of used bedding and teat skin (32 teats, one per cow) about three days after the last bedding replacement for each bedding material. Total gram-negative coliform, and *Klebsiella* spp. counts were significantly higher for deep-bedded manure solids compared to both shallow-bedded manure solids and recycled sand, and were significantly lower for new sand bedding; this corresponded with the number of gram-negative bacteria recovered from teat-apices of cows. However, significantly fewer streptococci and streptococci-like organisms were recovered from teat swabs from cows bedded on deep-bedded manure solids compared to new sand, recycled sand and shallow bedded manure solids. Despite these differences found, the overall incidence rate of clinical mastitis (IRCM) and incidence rate of subclinical mastitis was not significantly associated with bedding type (Rowbotham and Ruegg, 2016a). Meanwhile, Leach et al. (2015) reviewed the literature on the use of RMS as a bedding material and concluded that no consistent impact of using recycled manure solids as a bedding material on SCC or clinical mastitis could be identified and evidence to compare RMS with other bedding material was limited. Though the use of RMS that was stored before use, as a bedding material, has been linked to increases in the incidence of clinical mastitis caused by *E. coli* and *Klebsiella* spp. (Locatelli et al., 2008, Ostrum et al., 2008), whether a consistent link between the use of RMS and increased incidence of clinical mastitis or increased SCC is identified, may largely depend on the management of the RMS bedding. Harrison et al. (2008) did not identify a consistent impact of the use of RMS (combination of separated, composted and digested) on SCC. When looking at individual cow SCC records on six farms that converted to RMS, only two experienced an increase in SCC after converting to RMS and it was also noted that this change was

not different from spikes that had been observed before implementing RMS as a bedding. When focusing more closely on one farm where separated RMS, composted RMS and sand bedding materials were compared, this research also found increased levels of *Streptococcus, Klebsiella* and other bacteria on teat-ends of cows bedded on RMS (both separated and composted) compared to sand bedding, however there was no difference in the prevalence of increased SCC (>200,000 cells/ml for cows or >100,000 cells/ml for heifers) depending on bedding material. For this farm, RMS bedding was only retained in storage for three days (piled if separated, or retained in the drum composter if composted), which is a relatively short storage period, and it was placed in stalls twice a week, while the sand bedding was only placed in stalls once per week. Maintaining a low SCC may be possible when using RMS materials as bedding, however to achieve this, extra caution is likely needed in terms of its management, for example, bedding may need to be replaced more frequently in comparison to other materials such as sand, and the length of storage before use is also likely an important consideration. If RMS bedding is managed carefully to avoid the increased SCC that can be observed, benefits of its use as a bedding material are based mainly in economics. When straw or other bedding materials such as sand are not readily available, producing bedding from a continually available resource, manure, can be a favorable option (Leach et al., 2015).

1.5.2 Facility design, region, and seasonal effects

The two major types of facility designs used in Canada are tie-stall and free-stall facilities. In a Canadian study comparing the two major facility designs, cows in tie-stalls were found to have a higher incidence of *S. aureus, S. uberis,* and CNS, while cows in free-stalls had a higher incidence of *Klebsiella* spp. ($P<0.05$, 44 free-stall, 50 tie-stall

facilities), and additionally, a higher IRCM was observed for tie-stall barns compared to free-stalls (IRCM per 100 cow-years: 26.6 vs. 19.1) (Olde Riekerink et al., 2008). However, since eastern provinces (Quebec and Ontario) had a higher IRCM compared to western provinces (British Columbia, Alberta and Manitoba), and also had a higher proportion of tie-stall facilities, these results may have been confounded in part, by differences in the typical management practices used depending on region other than facility design.

The impact of seasonal housing (housed outdoors during summer months compare to indoors during the winter) for a single dairy farm on both the microbial diversity and composition of milk (bulk tank and three individual cows), teat-ends, bedding, feces, along with grass, silage, and soil was assessed by Doyle et al. (2017) using amplicon sequencing of the V3-V4 region of the 16S rRNA gene. Their main conclusions included that seasonal housing had a greater impact on the composition of milk and teat bacterial communities than the use of teat preparation prior to milking, both based on Bray-Curtis dissimilarities (between sample diversity) and within sample bacterial diversity, which was greater for milk samples from cows housed outside compared to indoors (Shannon index, P=0.026). Additionally, for milk samples from cows that underwent teat preparation prior to milking, indoor samples were generally higher in gut-associated genera such as *Bifidobacterium, Eremococcus, Corynebacterium, Ruminococcus* and *Prevotella,* and had a lower proportion of environmental bacteria such as an uncultured *Verrucomicrobia, Flavobacterium, Massilia* and *Sphingomonas* compared to outdoor samples, with a similar trend being observed for indoor teat swab samples compared to outdoor teat swab samples. In addition, despite large differences in the composition of

milk depending on housing, based on SourcTracker, a Baysian algorithm, the teat was consistently found to be the largest contributor to the milk microbiota for both indoor and outdoor milk samples, followed by feces. Feces had a greater contribution to indoor milk than outdoor milk microbiota and as expected, grass and soil were only contributors to outdoor milk samples while bedding and silage appeared to only contribute to microbiota of indoor milk samples. One might assume that coliform mastitis would be higher for this group of cows during the winter, when cows are housed indoors and exposed to more gut-associated microbiota, though neither clinical nor subclinical mastitis were monitored. However, it is a more common practice for cows to be housed primarily indoors all year round in Canada, and research focusing on this more commonly implemented system have found the opposite to be true. Gram-negative bacterial counts in bedding tend to peak during warmer months of the year (Hogan et al., 1989b), which is consistent with findings that coliform mastitis in herds always confined indoors, is more common during warm summer months (Smith et al., 1985).

1.6 Summary

A large body of research has identified a large variety of environmental factors that influence mammary health and not all of these factors could be covered here. However, important environmental factors covered here, that influence microbial communities in bedding and other sources of mammary microbiota on dairy farms (such as teat-apicies and feces), include seasonal factors and facility design along with other management differences between farm-systems, such as the use of different bedding replacement regimes and bedding types. In relation to bedding type, physical and chemical properties of bedding, is another important environmental factor that influences the proliferation of

environmental and gut associated microbiota in bedding material and on the cows' teat-apices, and likely along with this, the bacterial composition and diversity of microbial communities. This research aims to fill gaps in knowledge regarding the full diversity of mammary microbiota originating from specific bacterial sources within different farm-systems and seasons. Specifically, we will use high-throughput sequencing to identify used bedding, feces, and teat-apices as bacterial sources of commensal mammary microbiota, and specific microbial groups potentially important to mammary inflammation and health within different farm-systems and assess the overall variation among farm-systems and between season in the bacterial diversity and composition of mammary, teat-apex and environmental (i.e. fecal and bedding) bacterial communities.

HYPOTHESES AND OBJECTIVES

Hypotheses

1) The bacterial diversity and composition of bedding, and host-sites (feces, teat-apices and milk) differs among farm-systems [farms within the same regions, that differ in their bedding type used (straw, sand, and recycled manure solids) and other management factors].
2) The estimated contribution of environmental source/s (used bedding materials, feces, and/or teat-apices) to milk microbial communities, differs among farm-systems.
3) The abundances of specific bacterial groups on the teat-apex and in milk are associated with reduced mammary inflammatory status, and these bacterial groups differ in abundance within milk, teat-apices and potential environmental sources, bedding and feces, among different farm-systems.
4) For a straw bedded farm specifically, an additional hypothesis was that there was an increased mammary inflammatory status of cows, and reduced bacterial diversity and richness in milk and the teat-apices of cows, in the summer compared to the winter sampling time-point.

Objectives

1) To identify differences in the bacterial diversity and composition of host-sites (feces, teat-apices and milk) and bedding among different farm-systems.
2) To estimate the contribution of environmental sources (used bedding, feces, and/or teat-apices) to milk microbial communities and identify whether these estimated levels of contribution differs among farms-systems.
3) Identify specific bacterial groups on the teat-apex and in milk that were associated with reduced mammary inflammation, and to identify whether their abundances within milk, or teat-apices, and other potential environmental sources, bedding and feces, differ between farm-systems, to explore the possibility these bacterial groups are more important the mammary inflammatory status of cows on specific farms.
4) For a straw bedded farm specifically, an additional objective was to determine whether there were was increased mammary inflammation of cows, and a reduced bacterial diversity and richness of milk, and the teat-apex of cows, during the summer in comparison to the winter time-point.

2 INFLUENCE OF ENVIRONMENTAL BACTERIAL COMMUNITIES ON BOVINE MAMMARY BACTERIAL COMMUNITIES AND MAMMARY INFLAMMATION

Fehr KB, Derakhshani H, Sepehri S, Plaizier JC, and Khafipour E.

2.1 Abstract

Mastitis, defined as mammary inflammation, is most commonly caused by an infection. It is a major constraint to milk production and animal welfare, making its prevention and treatment critical, however most commonly addressed using antimicrobials. In light of diverse bacterial communities found in healthy bovine milk, to identify potential alternatives for mastitis prevention, aims of this research were: a) identify sources contributing to milk bacterial diversity and sources of specific microbial groups influencing mammary inflammation, and b) identify differences in milk bacterial diversity and composition, and bacterial diversity of milk bacterial sources, among different farms and seasons. Here, a cross-section survey was carried out on three dairy farms in Manitoba, Canada, each using a different bedding material [sand (SA), straw (ST), and recovered bedding material (RBM)] during the winter, with an additional sampling time-point during the summer for farm ST. To characterize the bacterial-profile of milk and potential sources contributing to its composition, composite milk samples, as well as teat-apex swab, fecal samples from selected cows, and used-bedding from each farm were collected and subjected to 16S rRNA gene sequencing. Using UniFrac distances, for all sample-types, differences in beta-diversity were identified between all pairs of farms, and between both time-points for farm ST ($P_{(PERMANOVA)} \leq 0.0002$). Additionally, using a method of multivariate linear regression, four OTUs on the teat-apex (within *Coprococcus, Aerococcus, Pseudomonas* and *Facklamia*), and two in milk (within *Lautropia* and *Rhodocyclaceae*), were associated with reduced mammary inflammation ($P_{(FDR)}<0.05$), two of which were identified as non-random OTUs (≥25% of samples) within bedding material. There were differences between farm-systems, in the

abundances of these potentially important OTUs, and in the bacterial composition and diversity of milk, and teat-apices of cows from each farm. Further, on two farms, an increased teat-apex bacterial diversity tended to be associated with reduced mammary inflammation (Spearman rank-order, $P \leq 0.058$). Our results demonstrate that the large variation in bacterial diversity and composition of milk and milk bacterial sources among farm-systems may be an important consideration for future development of mastitis prevention strategies in light of potential for teat-apex bacterial diversity, and specific bacterial groups to promote mammary health.

2.2 Introduction

Bovine mastitis is costly to dairy producers (Halasa et al., 2007, Rollin et al., 2015), limiting milk quality (Ogola et al., 2007), milk production (Wilson et al., 1997, Gröhn et al., 2004) and animal welfare (Medrano-Galarza et al., 2012). For instance, recent analyses accounting for direct and indirect costs have estimated the average case of clinical mastitis within the first 30 days of lactation to be $444 USD (Rollin et al., 2015). Meanwhile, a case of subclinical mastitis, a silent form of the disease that may not be detected, within 5 to 45 days of lactation, was estimated to result in more milk lost across the lactation compared to the average case of clinical mastitis within the first 60 days of lactation (Kirkpatrick and Olson, 2015).

Mastitis is defined as mammary gland inflammation, and is commonly triggered as a defense mechanism against intramammary infections (IMIs) (Pyörälä et al., 1992), with a common measure of increased mammary inflammation being somatic cell count (SCC).

Recently, research has implemented high-throughput sequencing techniques to identify not only the presence of microbiota in the healthy mammary gland (Oikonomou et al.,

2012, Oikonomou et al., 2014, Ganda et al., 2016), but also new associations between bacterial groups in milk and mammary gland health status (Kuehn et al., 2013, Oikonomou et al., 2014). As with pathogens causing mastitis, the primary route of transmission for commensal mammary microbiota is also thought to be through the external environment with bacterial groups first contacting the teat-apex, the area on and surrounding the teat-orifice, before entry into the mammary gland (Addis et al., 2016). Additionally, bacterial groups that influence mammary inflammation could also do so through either protective or harmful effects on the teat-apex, possibly through influencing the colonization of mastitis pathogens on the teat-apex (Woodward et al., 1987, De Vliegher et al., 2003). For example, pre-calving colonization of the teat-apex by a specific microbial group, *Staphylococcus chromogene*, a member of coagulase negative staphylococci (CNS), was associated with lower mammary inflammation within the five days post-calving compared to cows without colonization of *S. chromogene* pre-calving in previous research (De Vliegher et al., 2003). Many possible sources of microbiota capable of influencing mammary inflammation exist within the diary environment. For instance, a number of pathogens causing mastitis such as coliforms like *Escherchia coli, Klebsiella* spp., and *Enterobacter* spp. inhabit the cows digestive tract, and can also inhabit soil and water along with mastitis pathogens commonly found in the environment such as *Pseudomonas* spp. and *Proteus* species (Hogan and Smith, 2003). While a major environmental source of microbiota on the teat-apices of cows, particularly coliforms, is fecal material, an additional major environmental source of microbiota on the teat-apex that influences mammary inflammation is used bedding material. Lactating dairy cows have been reported to spend 10 to 12 hours resting in stalls daily (Haley et al., 2001), and

during this time, teat-apices contact bedding material that quickly becomes contaminated. Further, gram-negative bacterial counts in bedding peak during summer months (Hogan et al., 1989b), and coliform mastitis is more common during warm months (Smith et al., 1985), also indicating the importance of bedding material, along with seasonal influences, on the risk of acquiring mastitis infections.

The key players helping prevent mastitis in dairy herds thus far have been improvements in environmental sanitation and milking hygiene to limit transfer of bacteria to teat-apices (Bushnell, 1984, Smith and Hogan, 2008). However, despite the implementation of sanitation improvements, mastitis is still a major problem for many dairy farms, with recent estimates in the US indicating that mastitis affects nearly 25% of dairy cows yearly (USDA, 2016). Many factors influence both a cow's risk of acquiring mastitis and the severity of mastitis. These factors include those related to: a) the infectious agent, such as virulence factors, b) the host, such as immune system function, and c) the environment, such as bedding replacement regimes and other environmental sanitation practices. Challenges in controlling all of these factors is likely a primary reason that mastitis is still a major problem. Controlling the exposure of the udder to mastitis pathogens through environmental sanitation is a particularly difficult challenge, considering that microbial communities, including mastitis pathogens, will continue to exist in the dairy environment, despite sanitation improvements.

This research was set out in light of the fact that not all microbial communities a dairy cow is exposed to can be eradicated, and in light of the notion that some of these microbial communities are beneficial to mammary health. From cows that varied in their mammary inflammatory status, we characterized the bacterial-profile of milk and sources

of milk bacterial communities, teat-apices and feces, and also characterized the bacterial-profile of used bedding material as an additional source of milk bacterial communities. This was done within three distinct farm-systems that used different bedding materials [One used Straw (ST), one sand (SA) and one recycled bedding material (RBM)], during the winter. In addition, for one farm (Farm ST), we also characterized the bacterial-profile of these same sites (milk, teat-apices, feces and bedding), during an additional time-point in the summer. Aims of this research were to help address how bacterial communities within environmental sites (bedding and feces) and host-sites (feces, teat-apices and milk), differed between farms during the same season and within the same region, and also differed between seasons within the same farm, in ways that may be of importance to mammary inflammation. Further, for each farm, we aimed to identify the estimated proportion of teat-apex and milk microbiota transferred from various environmental sources, and identify possible relationships between the level of contribution that environmental sources have to a cow's milk and teat-apex microbial communities, and bacterial diversity of milk and teat-apices. Along with addressing these general aims, we aimed to identify specific bacterial groups whose abundance, either on the teat-apex or in milk, was associated with mammary inflammation, and to identify variation in abundances of these potentially important bacterial groups among farms, within milk, and on teat-apices.

In exploring the variation in bacterial diversity and composition of milk, teat-apices, and additional sources of milk microbiota, feces and bedding, among farms that varied in farm management strategies, defined here as different farm-systems, we can: a) define a clearer picture of what the bacterial-profile of milk and teat-apices of cows with low

mammary inflammation can look like within different farm-systems, and b) identify whether bedding or feces as bacterial sources, may contribute to the variation observed between farm-systems. Further, we add support to the notion that specific bacterial groups with potential to benefit mammary inflammatory status deserve attention, for use in the development of mastitis prevention strategies, and in identifying ideal conditions to support their growth within environmental sources on farms, such as bedding material, or on teat-apices of cows.

2.3 Methods

2.3.1 Herd selection and collection of herd and animal information

The Animal Care Committee of the University of Manitoba approved the sampling protocol for this project (protocol number: F14-028). Bedding samples from stalls of lactating cows, as well as fecal, composite milk, and composite teat-apex swab samples from a pre-selected set of cows, were taken on three dairy farms located in Manitoba, Canada. These farms were selected based on their use of different bedding materials: clean sand (SA), un-chopped wheat straw, (ST) and recycled bedding material (RBM). To clarify, farm RBM used drum-composted manure solids as bedding, which are processed manure recycled from cow pens on the farm. The recycling process on this particular farm was as follows: Manure solids were mechanically separated from manure liquids using a screw-press, and the solid fraction was then placed into a rotating drum that allowed the material to aerate, promoting aerobic fermentation that heated the material in the drum to around 70° C, with this heat from fermentation reducing the bacterial load of the material.

Farm RBM was sampled during a single day in October 2015, farm ST was sampled on a single day in February 2016, and farm SA was sampled over the span of two days, once on December 18th, 2015, and once January 4th, 2016, with a different set of cows being sampled on each of these days and treated as a single sampling time-point. These three sampling time-points, one for farm RBM, one for farm ST, and one for farm SA, were considered winter sampling time-points, and for farm ST, an additional summer sampling time-point occurred during a single day in August 2015. It should be noted that farm SA was sampled during two days for the winter time-point due to space constraints in the parlor that did not allow for multiple people to be in the parlor for sampling of milk, and therefore, two sampling days were necessary to sample from the desired number of cows without slowing down the milking process.

To be part of the study, these farms had to use the Canadian Dairy Herd Improvement Program, which was accessed for days in milk (DIM) and parity information used for animal selection criteria. In addition, to further compare differences in management of the lactating herd between farms, farm managers were asked to provide answers to questionnaires about housing and management practices (Table 2.1), and nutrient compositions of the total mixed rations (TMRs) fed to each pen of sampled lactating cows was determined during the day of sampling (Table 2.2). Total mixed ration samples were taken from feed bunks immediately after being dispensed during the day of sampling. After drying the TMRs, TMRs were analyzed by Central Testing Laboratory Ltd. (Winnipeg, MB, Canada) using wet chemistry, to obtain nutrient compositions of the TMRs on a dry matter basis (Table 2.2).

Table 2.1 Housing and management information gathered from farm visits and farm manager questionnaires.

	Farm SA	**Farm ST**	**Farm RBM**
Housing design	Free-stall	Tie-stall	Free-stall
Number of milking's per day	2	2	3
Breed of cows	Holstein	Holstein	Herd was 95% Holstein, 5% Jersey (All sampled cows were Holstein)
Type of bedding in lactating herd stalls	Sand, deep bedding	Straw, shallow bedding with mats	Recycled manure solids (drum composted), deep bedding
Bedding replacement regime	Removal of soiled bedding small portions at a time (randomly), about every three days.	Bedding fully replaced daily (around 10 a.m).	Full bedding replacement of each pen every other day (For pens with sampled cows).
Additives to bedding	None	Hydrated lime daily	None
Pre and post milking teat-dips	Both, iodine based	Both, iodine based	Both, iodine based
Use of blanket dry-cow therapy	Novodry antibiotic and an internal teat-sealer (Orbaseal)	Cefa-Dri antibiotic and an internal teat-sealer (Orbaseal)	Novodry antibiotic and an internal teat-sealer (Orbaseal)
Mastitis pathogen vaccination	No	Yes, For *E. coli*	Yes, For *E. coli*
General treatment protocol (for non-acute form, all farms treated acute immediately)	If no visible signs and SCC >400,000, culture milk and treat depending on results, OR if visual signs, treat immediately	Abnormal milk, and visible udder inflammation, and culture milk sample, treat depending on results	Abnormal milk, and visible udder inflammation, and culture milk sample, treat depending on results
How the decision that a cow is cured of mastitis is made	No visible signs and SCC < 400,000 cells/ml	No visible signs and SCC drops	No visible signs and SCC drops
Separate pen for cows with mastitis	No	Each cow had its own individual stall	Yes

Table 2.2 Nutrient compositions of total mixed rations (TMRs) fed to lactating herds on each farm during the day of sampling, with % on a dry matter basis.

Farm[3]	SA	SA	ST	RBM, Pen 1	RBM, Pen 2	RBM, Pen 3	RBM, Pen 10
Survey Date	Dec, 18/15	Jan, 4/16	Feb, 3/16	Oct, 8/15	Oct, 8/15	Oct, 8/15	Oct, 8/15
Acid Detergent Fiber (%)	26.23	23.10	25.21	20.74	23.19	20.51	18.81
Neutral Detergent Fiber (%)	38.40	33.64	29.32	27.40	30.69	30.07	26.24
Starch (Enzymatic) (%)	15.75	26.46	22.32	21.39	19.91	22.14	25.01
Non Fiber Carbohydrates (%)	35.27	39.99	40.98	43.30	40.29	40.50	44.48
Crude Protein (%)	15.53	15.57	18.9	18.51	18.23	18.63	18.47
Fat (%)	4.43	2.84	3.95	4.97	5.09	4.76	4.33
Ash (%)	9.38	6.44	8.06	8.60	8.53	8.52	8.62
Calcium (%)	0.56	0.40	1.34	1.18	1.12	1.31	1.66
Phosphorus (%)	0.46	0.40	0.47	0.51	0.50	0.49	0.49
Magnesium (%)	0.35	0.30	0.38	0.55	0.54	0.51	0.47
Potassium (%)	1.33	1.33	2.39	1.88	1.90	1.87	1.68
Sodium (%)	0.35	0.11	0.28	0.44	0.41	0.42	0.35
Total Digestible Nutrients (%)	70.62	73.96	71.70	76.48	73.87	76.73	78.55
Net Energy for Lactation (Mcal/kg)	1.61	1.70	1.64	1.76	1.69	1.76	1.81

One Sample was taken of the TMR provided to each lactating pen sampled cows were held in (free-stall), and one TMR sample taken for the tie-stalls facility (Farm ST, representative of what was fed to the lactating herd). All TMR samples were taken from feed bunkers immediately after dispensed in feed bunks on the day of sampling. [3]Farm RBM had different TMR ration for Pen 10 compared to the other three pens, Pen 10 had early lactation, high milk producing cows.

2.3.2 Animal selection criteria, and cows sampled

On farm ST (a 46 lactating cow herd), during the winter time-point, all 46 lactating cows were sampled from, and during the summer time-point all 49 lactating cows were sampled from. Farm SA and farm RBM were larger (200 and 500 lactating cow herds, respectively), and on each of these farms, we aimed to sample from a maximum of 30 primiparous and 30 multiparous cows. In each of these parity groups, 10 cows in early lactation (1–44 DIM), 10 cows in mid-lactation (45–99 DIM) and 10 cows in late lactation (≥100 DIM) were selected for sampling. This was to adequately account for the cow-factors known to influence mammary inflammatory status, parity and lactation stage. Table 2.3 shows the final number of cows in each parity and lactation stage on each farm with milk, teat-apex swab and fecal samples used in statistical analyses. Of note, is that cows were sampled randomly with respect to SCC.

Table 2.3 Number of cows per group for milk, teat-apex swab and fecal samples used for statistical analyses.

Milk samples	**SA farm**	**ST farm**	**RBM farm**	**ST farm (Summer)**
Primiparous	**26**	**19**	**22**	**20**
1-44 DIM (in Primiparous)	6	4	8	4
45-99 DIM (in Primiparous)	7	3	5	4
> 100 DIM (in Primiparous)	13	12	9	12
Multiparous	**22**	**27**	**21**	**28**
1-44 DIM (in Multiparous)	6	7	7	5
45-99 DIM (in Multiparous)	9	6	5	1
> 100 DIM (in Multiparous)	7	14	9	22
Total per farm	**48**	**46**	**43**	**48**
Teat-apex swab samples	**SA farm**	**ST farm**	**RBM farm**	**ST farm (Summer)**
Primiparous	**25**	**19**	**22**	**23**
1-44 DIM (in Primiparous)	7	4	7	5
45-99 DIM (in Primiparous)	8	3	6	4
> 100 DIM (in Primiparous)	10	12	9	14
Multiparous	**23**	**27**	**17**	**26**
1-44 DIM (in Multiparous)	8	7	8	5
45-99 DIM (in Multiparous)	10	6	6	1
> 100 DIM (in Multiparous)	5	14	3	20
Total per farm	**48**	**46**	**39**	**49**
Fecal samples	**SA Farm**	**ST Farm**	**RBM Farm**	**ST farm (Summer)**
Primiparous	**26**	**17**	**23**	**22**
1-44 DIM (in Primiparous)	7	4	8	5
45-99 DIM (in Primiparous)	8	3	6	4
> 100 DIM (in Primiparous)	11	10	9	13
Multiparous	**26**	**27**	**23**	**26**
1-44 DIM (in Multiparous)	8	7	8	5
45-99 DIM (in Multiparous)	11	6	6	1
> 100 DIM (in Multiparous)	7	14	9	20
Total per farm	**52**	**44**	**46**	**48**

2.3.3 Sample collection

Nitrile gloves (Thermo Fisher Scientific, Ottawa, ON, Canada) were worn during all sampling procedures to reduce the potential for contamination.

2.3.3.1 Bedding samples

For sampling of bedding material on each farm, stalls were split into sections based on location, and the number of sections depended on the size of the pen. Within each of these sections, about 100 g of bedding was sampled from the back one-third of 50% of stalls for the ST bedded farm, which, as mentioned previously, was smaller than the other two farms, and the back one-third of 25% of stalls for the larger two farms (RBM and SA bedded farms). These bedding samples were pooled into a single large garbage bag (Glad, Oakland, CA, USA). For straw on farm ST, a long arm grabber tool (ProMax Commerce, Melrose, MA, USA), wiped with 70% alcohol, was used to easily grab bedding from stalls, while on the other two farms, a plastic shovel wiped with 70% alcohol, was used to pick up the RBM and sand bedding from stalls. Each bag of pooled bedding, representing one section, was mixed and three subsamples of about 100 g each were placed into zip-lock freezer bags, this process was repeated for each section. Bedding samples were transferred to the laboratory for storage at -20°C.

The ST bedded farm was a small tie-stall facility that we split into four subsections for bedding sampling, therefore a total of 12 bedding samples were taken on this farm. All 200 lactating cows on farm SA were in a single large free-stall pen that was split into six different sections for bedding sampling purposes. A total of 18 used bedding samples were taken from this farm during each of the two sampling dates. The 500 lactating cows from the RBM bedded farm were separated into 7 smaller pens based on their milk

production, and these pens were considered sections. Since the sampled cows came from only four pens, this resulted in a total of 12 bedding samples being taken from farm RBM.

An important consideration for sample collection of bedding was the time of sampling relative to the time of bedding replacement. For the straw bedded farm, bedding was sampled between 6 and 7 pm, approximately 8 to 9 hours after the addition of fresh bedding that was reported by the farm manager to occur daily at approximately 10 am (Table 2.1). For farm RBM, since bedding was replaced from different pens during different days of the week, bedding was replaced approximately 48 hours prior to sampling bedding from pens 1 and 2, and about 24 hours prior to the sampling of bedding for pens 10 and 3. On this farm, bedding replacement of different pens occurred every other day, such that bedding was replaced at least every 48 hours for each pen. For farm SA, the last bedding replacement occurred three days before bedding was sampled and this was a partial bedding replacement. Bedding on this farm was never replaced all at once, but gradually replaced over the span of one week (Table 2.1).

2.3.3.2 Fecal samples

For collection of fecal samples, cows were restrained by performing a tail-jack and in the two free-stall facilities, also by a head-locking system. A 11-pound sampling bag over a gloved hand was inserted into the rectum to collect about 200 g of feces. These fecal samples were then mixed and 20 g of sample was transferred into a sterile 2 oz sample collection bag (Nasco Whirl-Pak, Ocala, FL, USA) and placed on ice, in a cooler, for transfer to the laboratory and stored at -20°C.

2.3.3.3 Teat-apex swab samples

Teat-apex swab samples were taken during the evening milking times, just prior to cleaning teat apices for taking milk samples, sterile swabs (polyester tipped applicators, Puritan, Guilford, ME, USA) were moistened with sterile physiological saline solution (Vétoquinol, Canada) and one swab was rubbed against each teat-apex. For each cow, four swabs (one per teat-apex) were combined into a single sterile 15 ml centrifuge tube (Thermo Fisher Scientific, Ottawa, ON, Canada), such that a single tube constituted a single composite teat-apex sample. The portion of each swab touched by the sampler was removed by snapping the swab handles over the side of the tube. Tubes were placed on ice in a cooler and taken to the laboratory for storage at -20°C.

2.3.3.4 Milk samples

Composite milk samples for microbiological analysis were collected following the recommendations by the National Mastitis Council for collection of milk samples for microbiological analysis: prior to sampling, three streams of foremilk were discarded to minimize chances of sample contamination from bacteria colonizing the teat canal, pre-milking teat disinfection was performed using 0.5% iodine pre-dip solution which was a part of all three farms usual milking procedure, teats were then thoroughly dried using individual paper towels and then scrubbed for 15 sec. using cotton pads moistened in 70% alcohol. One milk sample (~10 ml per quarter) per cow was then collected into sterile 50 ml centrifuge tubes (VWR, Radnor, PA, USA) and placed on ice until transfer to the laboratory. An additional 30 ml composite milk sample was taken from each sampled cow for SCC analysis performed by Horizon Lab Ltd. (Winnipeg, MB, Canada). In the laboratory, the 40 ml milk samples were gently inverted a few times to mix, and

placed into aliquots of 1.5 ml in a sterile 2 ml cryogenic tube (VWR, Radnor, PA, USA) for DNA extraction and a back-up 3 ml in a sterile 4 ml cryogenic tube (VWR, Radnor, PA, USA); these aliquots were placed in -80°C for storage. A third aliquot of 12 ml in a 15 ml centrifuge tube (Thermo Fisher Scientific, Ottawa, ON, Canada) was placed in -20°C for storage as a back up for SCC analysis.

2.3.4 DNA extraction

Genomic DNA was extracted from fecal, bedding and milk samples using ZR Fecal DNA MiniPrep Kits (Zymo Research, Irvine, CA, USA).

Genomic DNA extraction from fecal samples followed the manufacturer protocol, and with initial steps summarized as follows: 150 mg of fecal sample was placed in a ZR BashingBead Lysis tube and 750 µl of Lysis Solution (both provided by the manufacturer). The mixture in 2 ml tubes was then placed in a 2010 GenoGrinder (SPEX SamplePrep, Metuchen, NJ, USA) at 1750 strokes per min for 4 min, and continued to follow the manufacturers protocol exactly thereafter. Modifications from this protocol were performed for processing of bedding, milk and teat-swab samples. A modification common to bedding, milk and teat-swab sample processing was the final filtering step specified in the manufacturers protocol that was not used for these sample types. For fecal samples, this step entailed placing eluted DNA into a Zymo-Spin IV-HRC Spin Filter (Zymo Research, Irvine, CA, USA) on a 1.5 ml microcentrifuge tube and centrifuging for 30 sec at 10,000 × *g*.

For genomic DNA extraction from milk samples, pre-processing steps were performed as follows: 1.5 ml milk samples were centrifuged at 13,000 × *g* for 25 min at -4°C and supernatant was carefully removed. Then 200 µl of TE buffer and 300 µl of 0.5M EDTA

(pH = 8) were added to the remaining pellets and left to incubate at room temperature for 20 min. This mixture was then centrifuged at 12,000 × *g* for 20 min at -4°C. Supernatant was removed and the pellet was then resuspended in 150 µl of PBS by vortexing for 5 sec. The lysis beads provided in the ZR BashingBead lysis tubes and 500 µl of Lysis Solution, both provided by the kit manufacturer, were added to the PBS and pellet mixture along with 20 µl of 20 mg/ml Proteinase K (Zymo Research, Irvine, CA, USA). Mechanical cell lysis was then performed using a 2010 GenoGrinder (SPEX SamplePrep, Metuchen, NJ, USA) at 1750 strokes per min for 4 min and all tubes were incubated in a heat shaker heated shaker at 45°C for 45 min. Genomic DNA extraction continued following manufacturer protocol with the exception of the final filtering step described for fecal DNA extractions.

Pre-processing of bedding samples for genomic DNA extraction was performed as follows: A volume of approximately 10 ml of each bedding material was added to autoclaved 15 ml short polycarbonate vials (SPEX SamplePrep, Metuchen, NJ, USA). Note that in doing this, straw was chopped, using a scissor and tweezers, which were rubbed with 70% alcohol pads between each use, and other bedding types were dispensed with a spoon rubbed with 70% alcohol pads between each use. For each 15 ml tube of bedding, 5 ml of 10× PBS (pH = 7.4) was added and incubated at room temperature for one hour in a shaker at 200 RPMs, supernatant was removed and placed into a second 2 ml autoclaved tube and centrifuged for 2 min at 16,000 × g. The supernatant in the 2 ml tube was discarded and additional liquid was removed from the 15 ml vial and added to the 2 ml tube, repeating the same process until no supernatant was left in the 15 ml vial. The pellet in the 2 ml tube was then re-suspended in 1 ml of homogenization buffer (100

mM Tris, 100 mM EDTA, 1.5 M NaCl, 100 mM phosphate, final pH = 8.0) by vortexing for 1 min, scraping any remainder with a narrow pipette tip, and vortexing again until the pellet was removed. The mixture in the 2 ml tube was then returned to the 15 ml vial and an additional 4 ml homogenization buffer was added to all 15 ml tubes with two autoclaved 10-mm stainless steel grinding balls (SPEX SamplePrep, Metuchen, NJ, USA). Contents of 15 ml tubes were then homogenized using a 2010 GenoGrinder (SPEX SamplePrep, Metuchen, NJ, USA) at 1500 strokes per min for 4 min, this step was not used for the sand bedding material but rather sand was vortexed for 1 min (Breakdown of particles was not necessary). Approximately 2 ml of homogenized sample was then placed into a new autoclaved 2 ml microcentrifuge tube and centrifuged at 16,000 × g for 2 min. Supernatant was discarded and the tubes were weighed. Pellets weighed over the target 150 mg to be used for extraction and were brought down to 150 mg by removing excess liquid from the pellet using a sterile swab (polyester tipped applicators, Puritan, Guilford, ME, USA). Genomic DNA extraction from bedding material was continued using the manufacturers protocol, with the exception of the final filtering step described previously.

For teat-apex swab samples, the ZR Bacterial/Fungal DNA MiniPrep Kits (Zymo Research, Irvine, CA, USA) were used. Modifications of the kit manufacturers protocol for teat-apex swab samples were as follows: a scissor that was rubbed with cotton pads soaked in 70% alcohol was used to remove the bottom three quarters of the cotton tip into a ZR BashingBead Lysis tube (Zymo Research, Irvine, CA, USA). All four swab ends from the same cow were placed into the same lysis tube along with 750 µl of Lysis Solution (Zymo Research, Irvine, CA, USA). The mixture in 2 ml tubes was then placed

in a 2010 GenoGrinder (SPEX SamplePrep, Metuchen, NJ, USA) at 1750 strokes per min for 4 min. 400 µl of liquid was then transferred from this tube to a Zymo-Spin IV Spin Filter in a collection tube (Zymo Research, Irvine, CA, USA). Genomic DNA extraction continued using the manufacturers protocol for the kit, which was the same as the protocol for the kit used for other sample types, with the exception of the final filtering step described previously which was not in the protocol.

At least one negative extraction control (using 1 ml of sterile water) for every kit used (one kit for every 50 samples) was included in swab (negative control also included sterile swabs), bedding and fecal extraction protocols, and at least two negative extraction controls (using 1 ml of sterile water) for every kit used was included in the milk extraction protocol.

2.3.5 PCR amplification and sequencing library construction

For each sample, PCR amplification of the V1-V2 region of the 16S rRNA gene was performed with modified F27/R357 primers (See Additional file 1 for the list of primers used in PCR amplification and sequencing reactions). The forward primer (F27) was indexed with 12-base Golay barcodes that allowed for samples to be multiplexed. PCR amplification for each sample was performed in duplicate, and PCR reaction mixtures and reaction conditions differed depending on sample type. For milk samples, PCR reaction mixtures consisted of 5.0 µl of extracted genomic DNA, 1.0 µl each, of forward and reverse primer (5 µM), 0.5 µl of 20 mg/ml BSA (Thermo Fisher Scientific, Ottawa, ON, Canada), 12.5 µl of AmpliTaq Gold 360 Master Mix (Applied Biosystems, USA) and 5 µl nuclease-free water (Thermo Fisher Scientific, Ottawa, ON, Canada). For teat-apex swab samples, bedding samples, and fecal samples, PCR reaction mixtures

consisted of 2 µl of extracted genomic DNA, 1.0 µl each, of forward and reverse primer (5 µM), 12.5 µl of AmpliTaq Gold 360 Master Mix and 8.5 µl nuclease-free water (Thermo Fisher Scientific, Ottawa, ON, Canada).

PCR conditions for milk samples consisted of an initial denaturing step at 94°C for 3 min followed by 33 amplification cycles at 95°C for 40 sec, 57°C for 50 sec, an initial extension step of 72°C for 45 sec, with a final extension step at 72°C for 5 min. PCR conditions for teat-apex swab samples differed from those set for milk samples only during the initial extension step, which was 72°C for 30 sec for swab samples. PCR conditions for fecal samples and bedding samples differed by having 30 rather than 33 amplification cycles, and during the annealing step and initial extension step: for fecal samples these were 61.5°C for 40 sec and 72°C for 25 sec, respectively, and for bedding samples these were 62.5°C for 40 sec and 72°C for 18 sec, respectively. PCR amplification of all sample types occurred in an Eppendorf Mastercycler pro (Eppendorf, Hamburg, Germany).

The amplicon libraries were generated as described by (Derakhshani et al., 2016) and can be summarized very briefly as 600-cycle paired-end sequencing runs (one sequencing run for each sample type) all using the MiSeq Reagent Kit V3 (600-cycle; Illumina, San Diego, CA, USA) at the Gut Microbiome and Large Animal Biosecurity Laboratories, Department of Animal Science, University of Manitoba, Winnipeg, MB, Canada. For the metadata used to perform statistical analysis, refer to Additional file 2 [these included Sample IDs, barcode's sequences, cows' IDs, parity (primiparous vs. multiparous), SCC, DIM, and stage of lactation].

2.3.6 Bioinformatics and statistical analyses

Overlapping paired-end Illumina fastq files were first merged using the default settings of the FLASH assembler (version 1.2.11) (Magoč and Salzberg, 2011). Quality filtering and bioinformatics were then performed using QIIME (Caporaso et al., 2010b). Reads were assigned to samples using the split_libraries_fastq.py script. In addition, this step included quality filtering, where any read not meeting the following criteria was removed: A minimum read quality score of 20, no ambiguous bases, and the barcode matched a barcode provided in the mapping file. De novo (abundance based) and reference-based chimera checking were then performed against the GOLD database using the USEARCH61 algorithm (Edgar, 2010, Edgar et al., 2011) implemented in QIIME. Reads were then clustered into operational taxonomic units (OTUs) based on a threshold of 97% similarity using the pick_open_reference_otus.py script in QIIME (Caporaso et al., 2010b). To summarize, during this step reads were clustered and taxonomy was assigned using UCLUST (Edgar, 2010). Reads were first aligned to the Greengenes database (released May 2013) using the PyNAST algorithm (Caporaso et al., 2010a). Reads that failed to align to the GreenGenes database were clustered de novo, again using UCLUST, and lastly, the sequences representative of each OTU were used to build a phylogenetic tree using FastTree 2.1.3 (Price et al., 2010), and any sequence that failed to align was omitted from the OTU table.

The resultant OTU table was filtered to remove all samples below a sequencing depth of 6000 reads per sample, leaving a total of 137 milk samples, 133 teat-apex samples, 142 fecal samples, and 54 bedding samples for winter time-point downstream analyses of comparisons between farms, and for farm ST comparisons between seasons, a total of 94

milk, 95 teat-apex, 92 fecal, and 12 bedding samples for downstream analyses. The OTU-table was split based on these two comparisons at this point. Alpha-diversity and beta-diversity analysis, along with a sparse multivariate method for phylum-level compositional analyses, were performed on both of these OTU-tables using the same methods. However, 'Season' was the variable used only for farm ST comparisons between the summer time-point and winter time-point, and 'Farm' was the variable only used for comparing farm ST, farm SA and farm RBM during the winter time-point.

The alpha-diversity indicators, bacterial richness (Chao1 index) and diversity (Shannon index), were calculating after subsampling to an even depth of 6000 reads per sample using QIIME (Caporaso et al., 2010b). These alpha-diversity indicators were each used as response variables in analysis of variance (ANOVA) tests using the MIXED procedure in SAS 9.4. All pairwise comparisons of the response variable between groups were tested using the Tukey studentized range adjustment and the normality of residuals was tested using the UNIVARIATE procedure in SAS 9.4. For ANOVA models with non-normally distributed residuals, the response variable was transformed (log transformed or Box-Cox transformed) to normalize the residuals.

Using this method of ANOVA, we compared the aforementioned alpha-diversity measurements of milk between farms, SCC categories (low SCC < 150,000 cells/ml and high SCC > 150,000 cells/ml), stages of lactation (1-44 DIM, 45-99 DIM, and ≥100 DIM) and parities (primiparous and multiparous), and repeated these analyses for teat-apex samples. Additionally, these analyses were repeated only using samples from cows with a low milk SCC, and eliminating SCC as a variable, for host-site individually (milk, teat-apex and fecal samples). This was done since splitting SCC into only two discrete

groups was not expected to capture the full effect of SCC on bacterial richness diversity and therefore, including all samples may increase unexplained variation in the model.

Of note, is that the cut-off value of 150,000 cells/ml was used to differentiate high and low levels of mammary inflammation (high and low SCC) because recent research that accounted for error in standard culturing methods used for comparison to identify the sensitivity and specificity of composite milk SCC, has identified the threshold of 150,000 cells/ml to have optimal sensitivity and specificity for composite milk SCC (Vissio et al., 2014), and composite milk samples were the type of sample taken in our current study. Further, in research concerned with subclinical mastitis caused by IMIs from minor mastitis pathogens along with major pathogens, using a threshold of 150,000 cells/ml is likely advantageous, since IMIs caused my minor mastitis pathogens often result in only a small increase in milk SCC, not above 200,000 cells/ml (Sargeant et al., 2001, Schukken et al., 2009), and an SCC threshold as low as 100,000 cells/ml specifically for quarter milk samples, has been reported to have the highest sensitivity and specificity for cows on the fifth day post-calving in previous research considering IMIs by any bacterial species (Sargeant et al., 2001).

The aforementioned analyses were also performed for samples on farm ST, except using season as the main variable rather than farm, and including an additional random variable, Cow ID to the models, since 21 cows were sampled from during both the summer and the winter time-point. Within each farm during the winter time-point, further ANOVA models were used to compare the diversity of host-sites (milk, teat-apex, and feces) of cows with a low milk SCC, also using parity and lactation stage as explanatory

variables and Cow ID as a random variable, since multiple sites were sampled from the same set of cows.

For bedding samples, farm (or season) was the only variable used to explain alpha-diversity indicators in ANOVA models comparing bedding bacterial diversity between farms (and for farm ST, also between seasons). For each farm within the winter time-point, additional ANOVA models compared bedding bacterial diversity with that of feces, teat-apices, and milk, though only farm could be used as a variable in this case as well.

Of note, is that aside from microbiological analyses, comparisons of milk SCC between sampled cows were also performed using milk SCC as a response variable in ANOVA models that had the same predictor variables described previously. For the between farm analysis, these variables were farm, lactation stage and parity, and for the between season analysis these variables were season, lactation stage and parity, along with cow as a random variable.

To briefly explore the possibility that alpha-diversity of milk or teat-apices may be associated with the continuous measure of SCC, rather than associated with the discrete SCC groups we chose, on a per-farm basis, Spearman rank-order correlation was performed between milk sample SCC and alpha-diversity measurements of milk and of the corresponding teat-apex samples Chao1 and Shannon alpha-diversity indices.

Beta-diversity matrices (weighted UniFrac distances) were calculated using QIIME after the OTU-table was normalized using a cumulative sum scaling (CSS) transformation (Paulson et al., 2013). Comparisons of microbial community differences between samples, or comparisons of beta-diversity, were performed by permutational multivariate

analysis of variance (PERMANOVA) with 9999 permutations of residuals under a reduced model on beta-diversity matrices, and visualized using principal coordinate analysis (PCoA). All PERMANOVA and PCoA tests were performed with PRIMER-e v7 software (Clarke and Gorley, 2015). For these analyses, comparisons were made on a per-site basis, using samples from only low milk SCC cows, again to reduce variation that could not be completely accounted for by using a categorical variable for SCC. These PERMANOVA tests used the same explanatory variables described for alpha-diversity [Farm, lactation-stage and parity for samples within the winter time-point, and season, lactation-stage and parity for samples within farm ST in both seasons]. Bedding samples were an exception and only included farm (or season) as an explanatory variable and as such, the permutation method was switched to unrestricted permutations of raw data rather than of residuals under a reduced model. Additionally, these same PERMANOVA tests were also performed on milk from all cows, and included SCC as an additional categorical variable. Other than beta-diversity analyses that used a CSS normalized OTU table, all downstream analyses were done after rarefication of the OTU table to an even depth.

For the dataset used to compare samples between farms within the winter time-point, the relative abundances of OTUs, as well as phyla, were tested for associations with variables separately for each site using multivariate analysis with linear modeling (MaAsLin) (Morgan et al., 2012), along with the relative abundances of genera within milk [variables tested for associations within all analyses were Farm, Log_{10}SCC (continuous), parity (primiparous vs. multiparous), and DIM (continuous)]. Meanwhile, for the dataset used to compare samples between time-points in different seasons within farm ST, the final

analysis performed was to compare the bacterial composition of each site between seasons, and this was only done at the phylum-level, also using the multivariate method MaAsLin to also account for additional variables that may be important [Log_{10}SCC (continuous), parity (primiparous vs. multiparous), DIM (continuous), and again CowID as a random variable]. Briefly, these analyses were performed as follows: The OTU-tables (between-farms and between-seasons datasets) were first rarified to 6000 reads per sample, following this, OTU-tables were summarized at different taxonomic levels (using default parameters of the summarize_taxa_through_plots.py script in QIIME), to obtain phylum-level and genus-level OTU-tables. Phylum-level and OTU-level OTU-tables were separated based on site (one OTU-table for milk, one for teat-apex, one for bedding and one for fecal samples at each level), and an additional genus-level OTU-table was filtered to include milk samples only. OTU-tables created for each site were filtered to remove OTUs (or taxa) that were present in less than 25% of samples to eliminate transient members that may not be part of a sites microbial community and also to reduce the sparsity of datasets. MaAsLin was then performed on each of the above-mentioned filtered OTU-tables and worked on a per-OTU (or per-taxa) basis, boosting all metadata to select variables that showed potential to be associated with an OTUs relative abundance, and then included the selected metadata as predictor variables and arcsin-square root transformed abundances of an OTU as the response variable in a general linear model. In these models, farm (or season for the between-season dataset) was set as a forced predictor and as such, was never eliminated from the linear model by boosting. Multiple hypothesis testing was corrected for using Benjamini and Hockberg false

discovery rate (FDR). Significant associations were considered below a q-value of 0.05 and trends were also considered between a q-value of 0.05 and 0.1.

Additionally, for milk only, the relative abundances of genera with an average abundance over 0.05% were correlated with SCC using Spearman rank-order correlation. Spearman rank correlation coefficients (ρ) were visualized using the ggplot2 package (Wickham, 2009) implemented in R, and also used to visualize differences in abundance of genera between farms. For all correlation analyses, correlations below a *P*-value of 0.05 were considered significant.

SourceTracker 1.0 (Knights et al., 2011), a Bayesian inference algorithm, was used to identify the proportional contribution of environmental sources to milk microbiota on a per-farm basis, and also, environmental sources of teat-apex microbiota on a per-farm basis. In summary, within SourceTracker metadata files, milk samples were set as sinks for microbiota and teat-apex, bedding and fecal samples as sources, with an additional analysis setting teat-apex samples as sinks and milk samples removed. To create the input OTU-tables, a non-rarified OTU-table containing samples from all sites above 6000 reads was filtered to separate samples into three OTU-tables based on farm. These OTU-tables were then filtered to remove OTUs present in fewer than two samples. The SourceTracker algorithm was then used as implemented in QIIME, with script parameters specified to perform the analysis at an depth of 2000 reads per sample, with 100 burn-ins and 5 random re-starts. The output of the SourceTracker analysis showed the proportional contributions of each source to each milk sample (or teat-apex sample), and from this, we correlated the proportional contribution of each source to a milk sample, with the milk samples corresponding alpha-diversity indices (Chao1, Shannon and Simpson indices), as

well as its SCC using Spearman rank-order correlation. Similarly, the proportional contribution of each source to a teat-apex sample was correlated against the teat-apex samples alpha-diversity indices and the milk SCC of the corresponding milk sample.

2.4 Results

2.4.1 Milk somatic cell count among farms

Results of the ANOVA analysis comparing composite milk SCC of sampled cows, shown in Table 2.4, revealed that sampled cows on farm RBM had a higher SCC (524,986 cells/ml) compared to farm ST (140,181 cells/ml, $P < 0.0001$) and farm SA (119,594 cells/ml, $P=0.018$), with no significant difference in SCC between farm ST and SA ($P=0.16$). Further, across all farms, late lactation cows tended to have a higher SCC compared to cows in mid-lactation ($P=0.053$) but not cows in early lactation ($P=0.89$). Canadian dairy herd improvement program records obtained for each farm indicated that the average-herd SCC was 98,000 cells/ml for farm ST 17 days after sampling, 493,000 cells/ml for farm RBM 11 days before sampling, and 239,000 cells/ml for farm SA the day before the sampling date and 18 days before the second sampling date. Farm RBM and farm SA had an average herd-SCC well above two commonly used thresholds to indicate subclinical mastitis (150,000 - 200,000 cells/ml). Of note, is that despite efforts to sample randomly with respect to SCC, farm SA's herd-average SCC (239,000 cells/ml) appeared well above the average for sampled cows on this farm (119,594 cells/ml). Signs of clinical mastitis (clots, blood or other abnormalities) were not observed by the sampler for milk samples included in analyses and therefore, cows with

high somatic cell count in our research were assumed to most likely have subclinical mastitis.

Table 2.4 ANOVA results for comparisons of mean SCC of composite milk samples from all sampled cows between farms, parities and lactation stages.

	Farm				**Parity**		
	SA n=48	**ST** n=46	**RBM** n=43	**SED**	**1** n=67	**≥ 2** n=70	**SED**
SCC	119594[a]	140181[a]	524986[b]	0.040[†]	148055[a]	375119[a]	0.032[†]
	Lactation Stage				**P-values**		
	0–44 n=38	**45–99** n=35	**≥100** n=64	**SED**	**Farm**	**Parity**	**DIM**
SCC	229193[a]	194968[a]	360599[a]	0.040[†]	0.0001[†]	0.1905[†]	0.0621[†]

[†]Used Box-cox transformation of SCC to normalize model residuals. A difference in lettering between two least squares means for factors of a variable indicates a significant difference with $P<0.05$. SED=Standard error of the difference in least squares means. ANOVA models developed with the Mixed procedure in SAS 9.4.

2.4.2 Sequencing output and OTUs identified

For the samples included in statistical analyses, 137 milk samples comprised a total of 4,157,863 sequences that passed quality control (Mean 30,349, StdDev 11,079), 133 teat-apex swab samples comprised a total of 6,278,427 sequences that passed quality control (Mean 47,206, StdDev 10,780), 142 fecal samples comprised a total of 4,863,057 sequences that passed quality control (Mean 34,247, StdDev 24,643), and lastly, 54 bedding samples comprised a total of 2,222,038 sequences that passed quality control (Mean 41,149, StdDev 13,761).

At a similarity threshold of 97% for OTU clustering, and rarefaction to an even depth of 6000 reads per sample, a large number of OTUs were identified in each site, on each farm. For milk, 659 OTUs were identified in at least 25% of all samples overall, and 950, 773 and 578 OTUs were identified in at least 25% of milk samples from farm RBM, farm SA and farm ST, respectively. 349 OTUs (present in ≥25% of milk samples on each farm) were shared among milk from all farms, and 448, 275 and 139 OTUs were specific

to only the set OTUs for milk on farm RBM, farm SA and farm ST, respectively (Figure 2.1). Sites exposed to the external environment, the teat-apex and bedding, had a larger numbers of OTUs distinct to a single farm's set of OTUs, rather than shared between farms (Figure 2.1b and c). For instance, for the teat-apex, 638, 422 and 1031 OTUs were distinct to farm RBM, SA and ST, respectively, with only 202 OTUs being common to all farms (for OTUs present in 25% of teat-apex samples on each farm, Figure 2.1b). Meanwhile, the fecal dataset clearly demonstrating a larger number of OTUs shared between all three farms (997 OTUs) compared to the number of OTUs distinct to a single farm, ranging from 433 to 224 OTUs (for OTUs present in 25% of fecal samples on each farm, Figure 2.1b, c, and d).

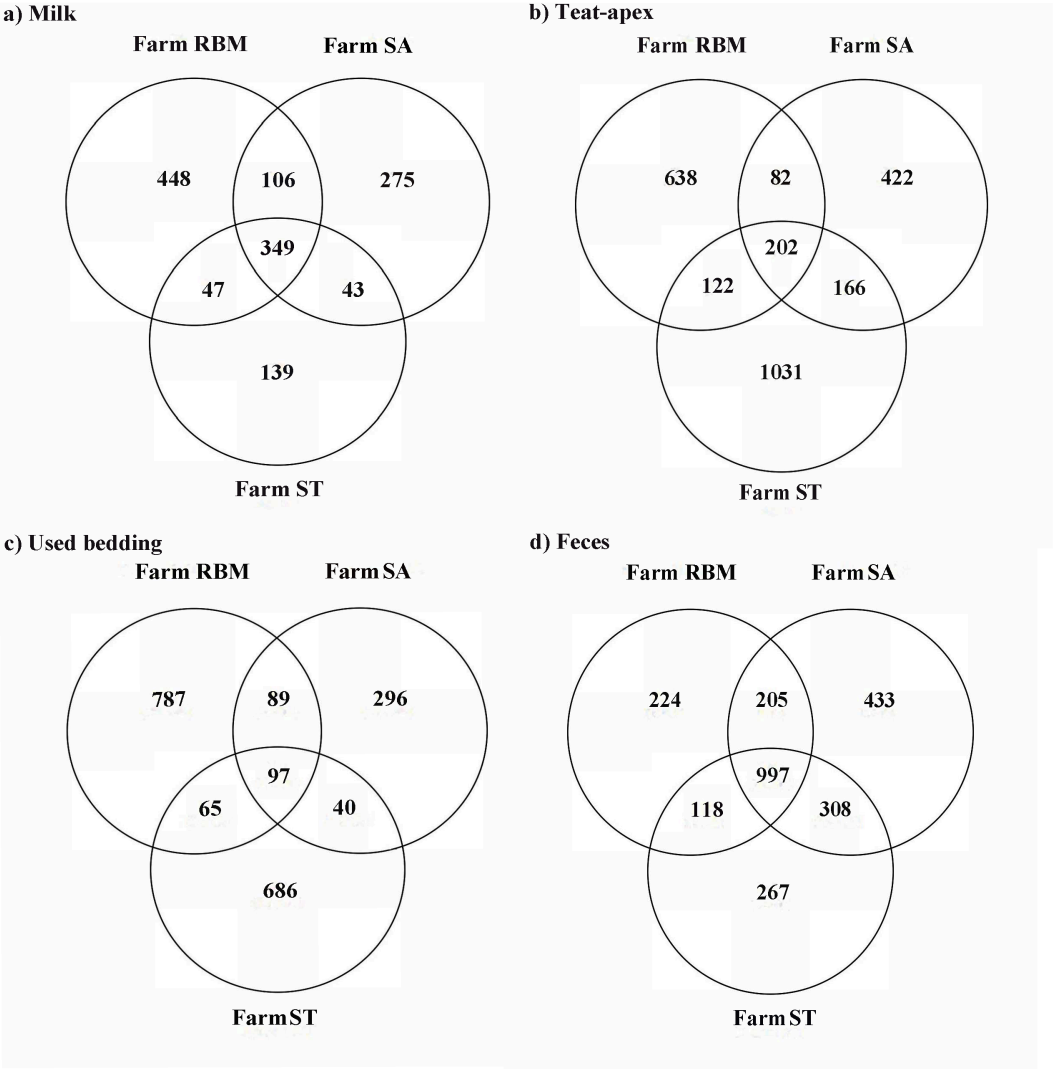

Figure 2.1 OTUs of each site shared among farms. Venn diagrams showing the number of OTUs present in at least 25% of samples on each farm, for each site. Sites: a) milk, b) teat-apex, c) used bedding, and d) feces.

2.4.3 Diversity of milk, teat-apex, fecal and bedding bacterial communities

Using principal coordinate analysis (PCoA) and permutational multivariate analysis of variance (PERMANOVA) on weighted UniFrac distance matrices between samples for each site (milk, teat-apex, fecal and bedding), diversity between samples within each site was explored (Refer to Additional file 3 for full PERMANOVA main test and pairwise test results). Comparisons between milk samples on three farms during the winter,

revealed that high SCC samples (defined as an SCC >150,000) did not cluster separately from low SCC samples (defined as an SCC ≤ 150,000) ($P_{(PERMANOVA)}$=0.3988) (Figure 2.2). Further, farm was the most significant variable in the PERMANOVA model ($P_{(PERMANOVA)}$=0.0001, Figure 2.2), with PERMANOVA t-tests also revealing all pairwise comparisons between farms to have the lowest level of significance possible ($P_{(PERMANOVA)}$=0.0001). To a lesser degree, a cow's parity (primiparous vs. multiparous) was also a significant variable differentiating milk bacterial communities ($P_{(PERMANOVA)}$= 0.0211), while lactation stage was not a significant variable ($P_{(PERMANOVA)}$=0.2723). Pairwise tests were also performed to identify whether SCC had at least a near-significant association with between-sample bacterial diversity within any group of milk samples. These analyses did not show low SCC and high SCC groups to be potentially distinct within any farm, lactation stage, or parity with all PERMANOVA *P*-values being above 0.1. Additional PERMANOVA and PCoA analyses were performed using only samples from cows with low mammary inflammation (SCC < 150,000) to limit biases that may exist due to differing SCC between samples that may not be accounted for through categorizing SCC into two discrete groups. These tests were performed on separate matrices of milk, teat-apex and fecal samples which all included farm, parity and lactation stage as main factors, and in all cases, farm was the only significant main factor identified ($P_{(PERMANOVA)}$=0.0001, Figure 2.3). Also in all cases, pairwise PERMANOVA tests revealed all farms to be distinct from one another with the lowest *P*-value possible for a PERMANOVA analysis with 9999 permutations ($P_{(PERMANOVA)}$=0.0001, Figure 2.3). For milk samples, there was a significant interaction between parity and lactation stage ($P_{(PERMANOVA)}$=0.0483), with primiparous cows having a distinct bacterial

community composition compared to multiparous cows only during late lactation (>100 DIM, Pairwise $P_{(PERMANOVA)}$=0.006), and early lactation cows (0-44 DIM) having a distinct bacterial community composition compared to late lactation cows (>100 DIM) only for primiparous cows (Pairwise $P_{(PERMANOVA)}$=0.0445, Additional file 3). Additionally, for teat-apex samples, significant interactions between farm and parity as well as farm and lactation stage were identified (P=0.0372 and P=0.0436, respectively). These interactions were attributed to parity being a significant variable only within farm ST (Pairwise $P_{(PERMANOVA)}$=0.0213) and specifically within farm SA, early lactation cows having a distinct teat-apex bacterial diversity compared to mid-lactation cows (Pairwise $P_{(PERMANOVA)}$=0.0183, Additional file 3). Lastly, for fecal samples, parity and farm had a significant interaction effect ($P_{(PERMANOVA)}$=0.0162), with primiparous and multiparous cows having distinct fecal bacterial communities only on farm RBM (Pairwise $P_{(PERMANOVA)}$=0.0462, Additional file 3).

When all milk samples were included in analyses of variance, both comparing bacterial richness (Chao1 index) and diversity (Shannon index) between groups, no significant difference was identified in the bacterial richness or diversity of milk samples with a low SCC compared to a high SCC (P=0.7622 and P=0.1111 respectively, Table 2.5). Additionally, the straw bedded farm had a significantly lower bacterial richness and diversity compared to farm RBM (Both $P < 0.0001$) and farm SA (P=0.0009 and 0.0004, respectively), and primiparous cows had a lower bacterial richness and diversity compared to multiparous cows (P=0.0282 and P=0.0357 respectively), with lactation stage having no significant effect on bacterial richness (P=0.6325) and diversity (P=0.7662) (Table 2.5). As with milk, when all teat-apex samples were included in an

analysis of variance, again no significant difference was identified in the bacterial richness and diversity of teat-apex samples with a low SCC compared to those with a high SCC (P=0.1122, and P=0.2948, respectively, Table 2.6). To elucidate the possibility that associations existed between bacterial diversity and mammary inflammation as a continuous variable, rather than through splitting SCC into two groups based on a predetermined threshold, Spearman rank-order correlation was performed between bacterial diversity indicators and SCC. This was on a per-farm basis since farm would likely be a confounding factor otherwise, because farm was a significant variable associated with teat-apex bacterial richness and diversity and farms differed in milk SCC. On farm RBM and farm SA, increased SCC of a milk sample tended to be associated with reduced bacterial diversity (Shannon index) of the corresponding teat-apex sample ($\rho = -0.31$, P=0.051, and $\rho = -0.28$, P=0.058, respectively). However, no association between teat-apex bacterial diversity and milk SCC was found for farm ST ($\rho = -0.092$, P=0.54). Further, teat-apex bacterial richness was not associated with the corresponding milk samples SCC for any farm (P=0.11 and higher). Of note is that no association between milk bacterial diversity or richness and SCC was observed on any farm through this method (P=0.13 and higher, data not shown), however additional variation not accounted for by this analysis includes variation between primiparous and multiparous cows, particularly since parity was a variable specifically associated with milk bacterial diversity as mentioned previously.

For cows with a SCC $\leq$ 150,000 cells/ml, comparisons of diversity (Shannon index) and richness (Chao1 index) between samples for each host-site were also performed, with detailed results of these analyses shown in Table 2.7. For milk samples, using only

samples below a SCC of 150,000 revealed similar results with respect to the effects of farm, lactation stage and parity on bacterial diversity and richness in comparison to when all milk samples were included in the analysis, however, the effect of parity on bacterial richness was no longer significant (P=0.0852). Meanwhile, teat-apex bacterial richness and diversity of cows from farm ST were significantly higher than cows from farm SA (Both $P < 0.0001$) and from farm RBM (Both $P < 0.0001$), with farm SA having the lowest teat-apex bacterial diversity (Figure 2.4a and b), and this was consistent with the analyses including teat-apex samples of all SCC categories. Cow factors parity and lactation stage had no significant association with teat-apex bacterial diversity or richness. No significant differences in bacterial richness of feces was identified between farms (P=0.0834, Figure 2.4a), and between stages of lactation (P=0.2990), however primiparous cows had higher fecal bacterial richness compared to multiparous cows (P=0.0071), along with a higher fecal bacterial diversity compared to multiparous cows (P=0.0197). In addition, farm SA had a higher fecal bacterial diversity compared to farm ST and farm RBM (P=0.0005 and $P < 0.0001$, respectively), with farm RBM having the lowest fecal bacterial diversity (Figure 2.4a). Further, the bacterial diversity of used bedding between farms was explored, with sand bedding on farm SA having a lower bacterial richness and diversity compared to bedding on farm ST and farm RBM, and with farm ST bedding also having a significantly lower bacterial diversity in comparison to farm RBM (Figure 2.4a, b and Table 2.8).

Comparisons of sample bacterial diversity (Shannon index) and richness (Chao1 index) indicators were also performed between cow-sites within each farm, again using only samples from cows with a low SCC, with detailed results of these analyses shown in

Table 2.9. Briefly, the ANOVA models for farm RBM and farm SA showed that bacterial richness of feces was significantly higher than both teat-apices (P=0.0234, and $P <$ 0.0001, respectively), while on farm ST, bacterial richness of teat-apex samples was higher than that of feces ($P < 0.0001$, Figure 2.5a). As expected, bacterial richness was significantly lower in milk compared to both teat-apices and feces for all farms (Figure 2.5a), however bacterial diversity of milk was no different from that of teat-apices for farm SA and farm RBM (P=0.4898 and P=0.0909, respectively, Figure 2.5b). Meanwhile, on farm ST, bacterial diversity of milk was significantly lower compared to bacterial diversity of teat-apices ($P < 0.0001$), with the bacterial diversity of teat-apices being similar to that of feces (P=0.4685, Figure 2.5b). The ANOVA models comparing bedding to the bacterial diversity of host-sites, with detailed results shown in Table 2.10, were only used to compare bedding with host-sites (and not compare host-sites among each other), since additional host-variables (parity and lactation stage) were not included. Interestingly, these results indicated that for farm RBM, no difference in bacterial diversity was identified between bedding and the other three sites (feces, teat-apices and milk), whereas for farm SA, bedding had a lower bacterial diversity in comparison to all other sites ($P < 0.0001$, Figure 2.5b). Meanwhile, bedding on farm SA had a lower bacterial diversity only compared to feces and teat-apices ($P < 0.0001$), but was no different from milk (Figure 2.5b).

Further results include that for farm SA, bacterial communities from sites of early lactation cows were significantly more diverse compared to mid-lactation cows (P=0.0184) and richer compared to both mid-lactation (P=0.0164) and late-lactation cows (P=0.0182) (Table 2.9). For farm ST, bacterial richness from sites of early-lactation

cows was significantly lower compared to late-lactation cows (P=0.0326), and bacterial communities from sites of primiparous cows were significantly less diverse compared to those of multiparous cows (P=0.0364) (Table 2.9).

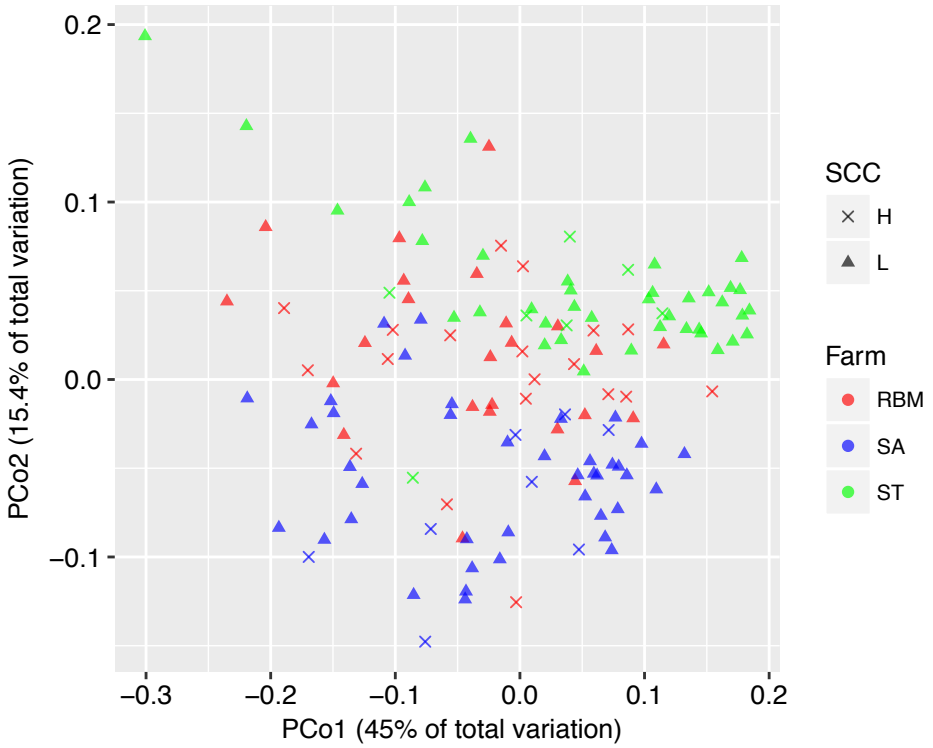

Figure 2.2 Principal coordinate analysis of weighted UniFrac distance matrices comparing bacterial diversity of milk between farms and SCC levels. Showing clustering patterns for based on farm and SCC categories (low SCC < 150,000 cells/ml and high SCC ≥ 150,000 cells/ml). Milk bacterial diversity between each pair of farms was distinctly different with all PERMANOVA P-values=0.0001. For comparisons between SCC categories, $P_{(PERMANOVA)}$= 0.3988 [PERMANOVA model also included parity (primiparous vs. multiparous) and lactation stage (early: 1-44 DIM, mid:45-99 DIM and late: ≥ 100 DIM) as additional explanatory variables].

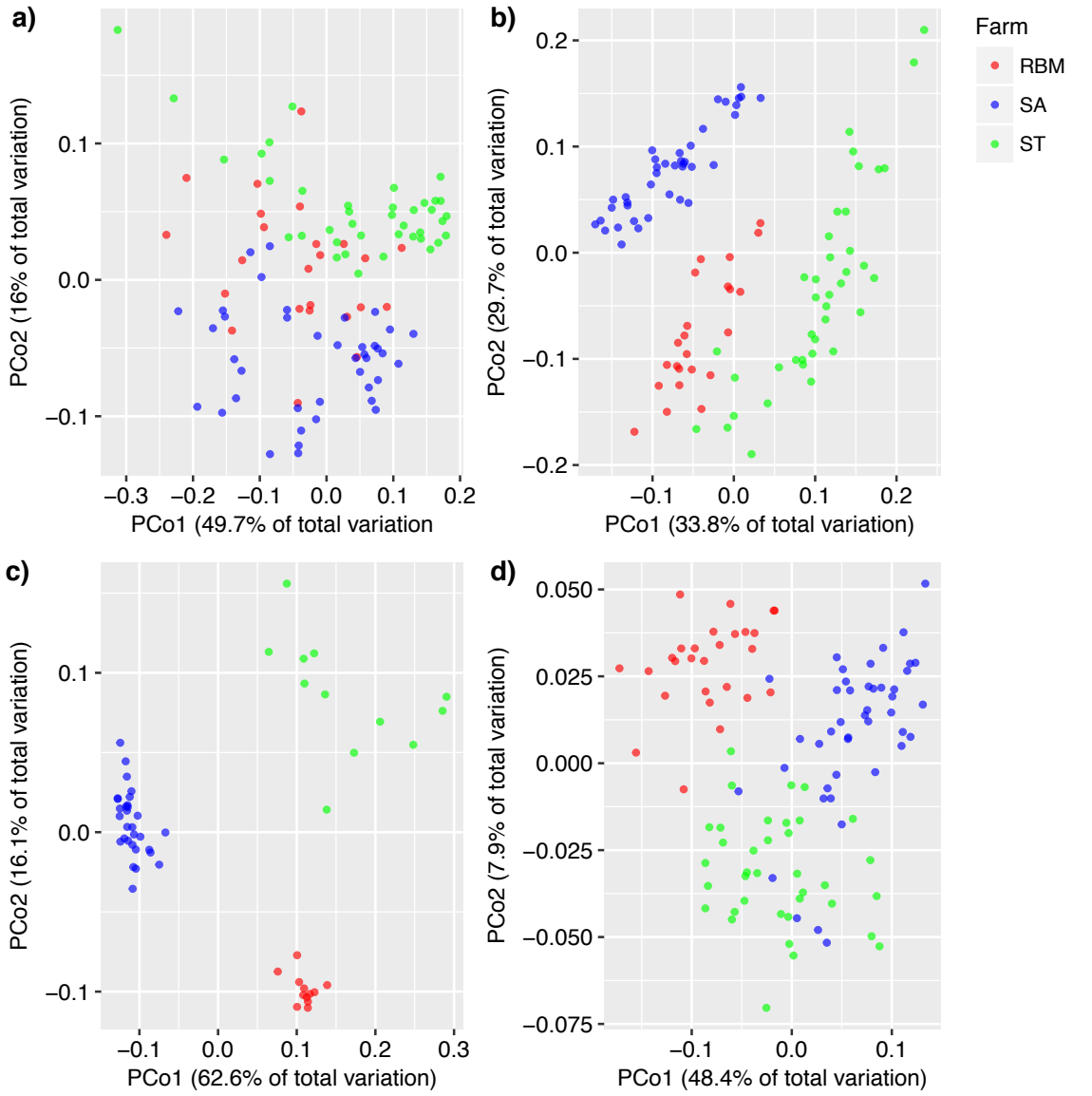

Figure 2.3 Principal coordinate analysis of weighted UniFrac distance matrices comparing bacterial diversity between farms for each site. **(a)** Milk samples **(b)** Teat-apex swab samples **(c)** Fecal samples **(d)** Bedding samples. For host-sites, only cows with a milk SCC below 150,000 cells/ml were used. For all sites, bacterial diversity between each pair of farms was distinctly different with all PERMANOVA *P*-values=0.0001 [PERMANOVA models for host-sites also included parity (primiparous vs. multiparous) and lactation stage (early: 1-44 DIM, mid: 45-99 DIM and late: ≥ 100 DIM) as additional explanatory variables].

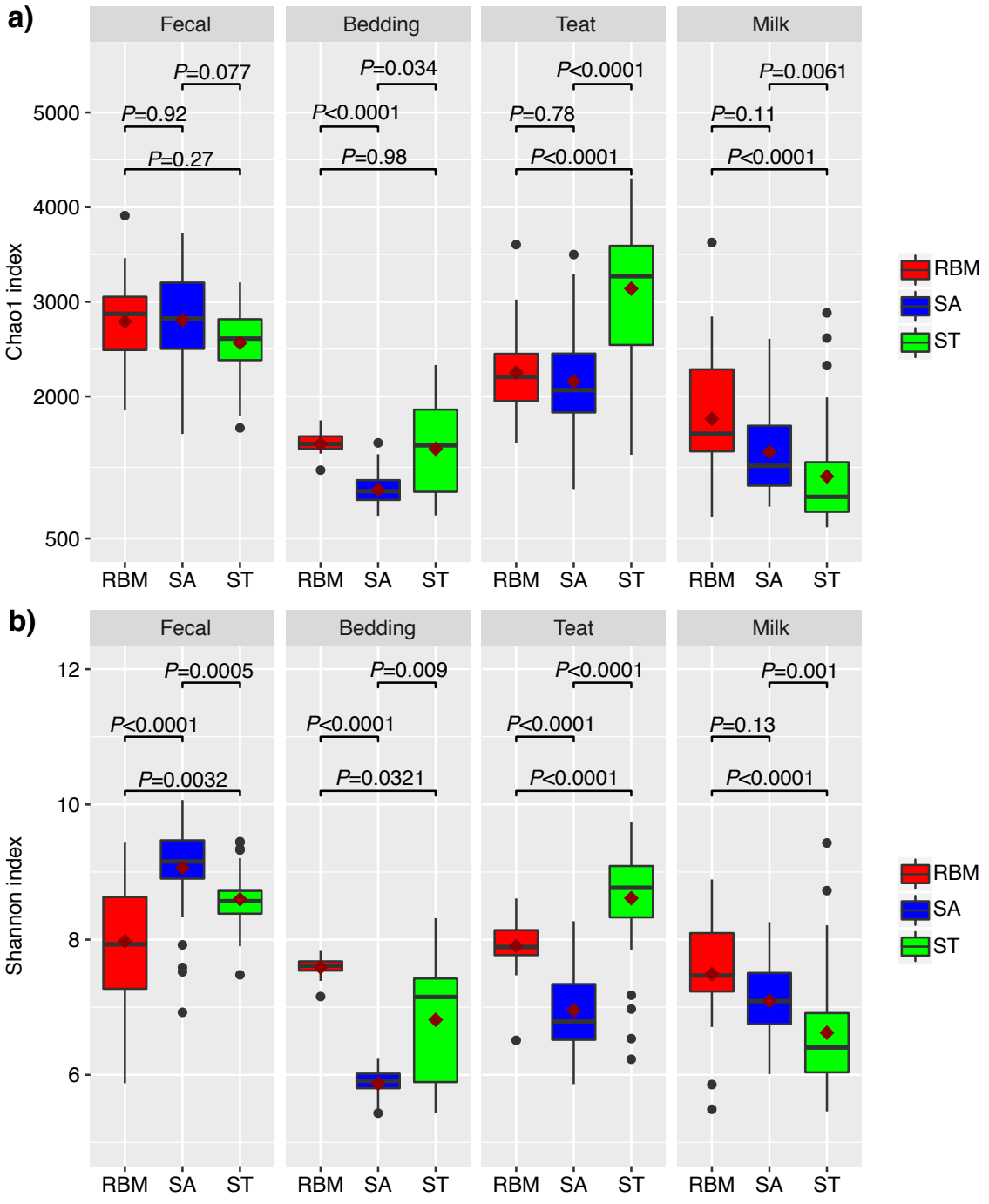

Figure 2.4 Comparisons of bacterial richness and diversity between farms for milk, teat-apices and feces and bedding. **(a)** Shannon index (bacterial diversity) **(b)** Chao1 index (bacterial richness). Showing boxplots with line as the median, and red diamond as the mean. *P*-values were obtained from ANOVA models (Proc Mixed) summarized in Table 2.6(host-sites) and Table 2.7(bedding). For fecal, teat-apex and milk analyses, only cows with a SCC ≤ 150,000 cells/ml were included, and additional variables included were parity (primiparous vs. multiparous) and lactation stage (early: 1-44 DIM, mid: 45-99 DIM and late: ≥100 DIM). For bedding, farm was the only variable used.

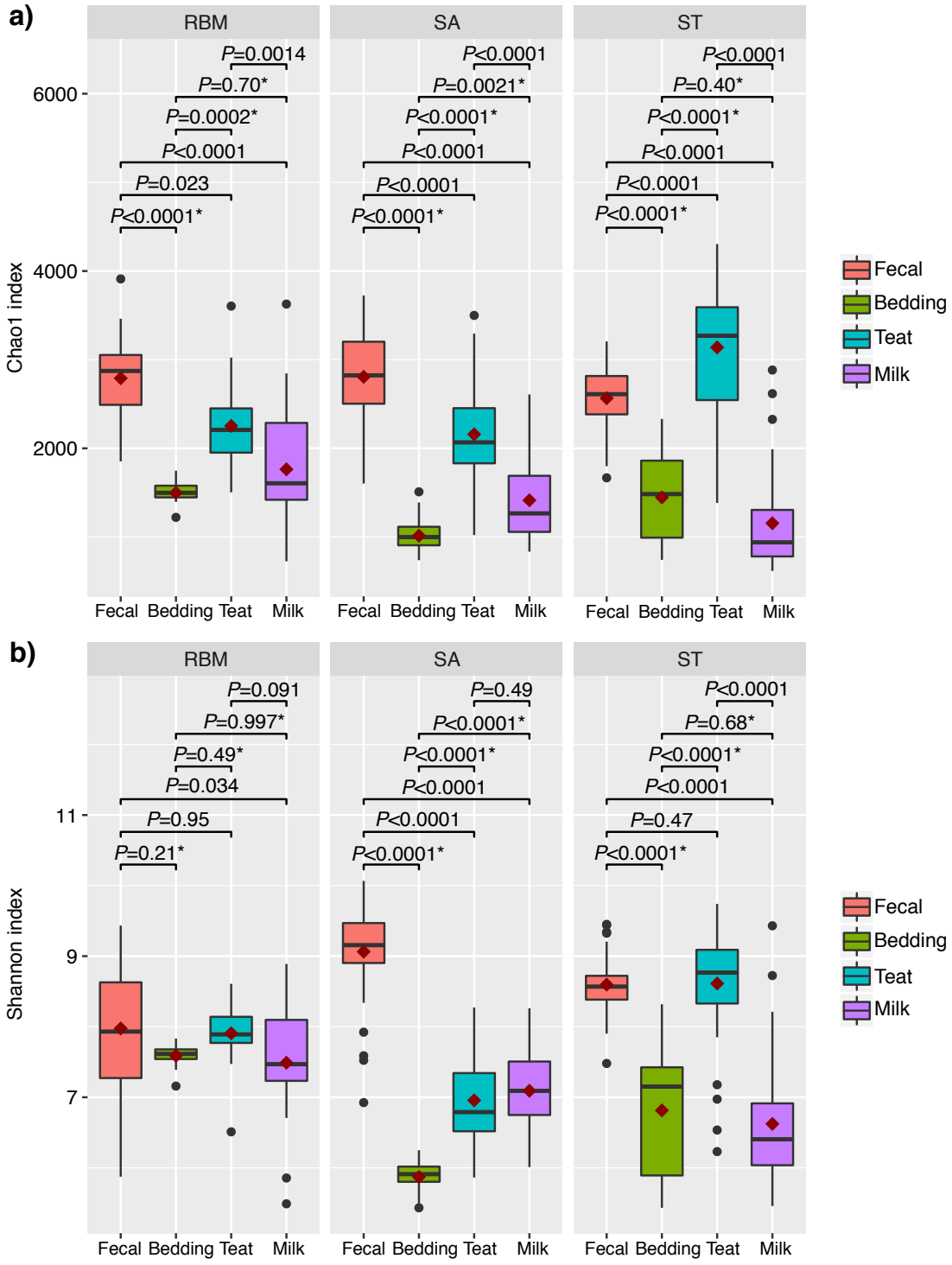

Figure 2.5 Comparison of bacterial richness and diversity between host-sites within each farm. **(a)** Shannon index (bacterial diversity) **(b)** Chao1 index (bacterial richness). Showing boxplots with line as the median, and red diamond as the mean. Only samples from cows with a SCC ≤ 150,000 cells/ml were included. *P*-values were obtained from ANOVA models (Proc Mixed) summarized in Table 2.8(host-sites) and Table 2.9(bedding). Comparisons between host-sites were from ANOVA models including additional factors: parity (primiparous vs. multiparous), lactation stage (early: 1-44 DIM, mid: 45-99 DIM and late: ≥100 DIM) and cow as a random factor. *For comparisons with bedding material, site was the only factor

Table 2.5 ANOVA results comparing milk bacterial richness and diversity indicators (Chao1 and Shannon indices) among farms.

	Farm				**Parity**			**P-values**	
	SA n=48	**ST** n=46	**RBM** n=43	**SED**	**1** n=67	**≥ 2** n=70	**SED**	**Farm**	**Parity**
Chao1	1453[a]	1137[b]	1761[a]	0.034*	1349[a]	1552[b]	0.027*	<0.0001*	0.0282*
Shannon[1]	7.084[a]	6.234[b]	7.452[a]	0.201	6.782[a]	7.064[b]	0.133	0.0001	0.0357
	Stage of lactation				**SCC**			**P-values**	
	0–44 n=38	**45–99** n=35	**≥100** n=64	**SED**	**Low**[2] n=103	**High**[3] n=34	**SED**	**Stage of lactation**	**SCC**
Chao1	1425[a]	1423[a]	1504[a]	0.035*	1439[a]	1461[a]	0.033*	0.6325*	0.7622*
Shannon[1]	6.996[a]	6.875[a]	6.900[a]	0.166	7.055[a]	6.791[a]	0.165	0.7662	0.1111

*Log transformation used. [1]removed two outliers (Sample ID's: 55.2, and 195.2), and kept an insignificant interaction in the model to maintain residual normality (Farm*SCC; P=0.32). [2]Low SCC: SCC below 150,000 cells/ml, [3]high SCC: SCC above 150,000 cells/ml. A difference in lettering between two least squares means for factors of a variable indicates a significant difference with P<0.05. SED=Standard error of the difference in least squares means. ANOVA models developed with the Mixed procedure in SAS 9.4.

Table 2.6 ANOVA results comparing teat-apex bacterial richness and diversity indicators (Chao1 and Shannon indices) among farms.

	Farm				**Parity**			**P-values**	
	SA n=48	**ST** n=46	**RBM** n=39	**SED**	**1** n=66	**≥ 2** n=67	**SED**	**Farm**	**Parity**
Chao1	2067[a]	2970[b]	2214[a]	131	2436[a]	2398[a]	105	<0.0001	0.7187
Shannon	6.88[a]	8.42[b]	7.86[a]	0.156[†]	7.80[a]	7.64[a]	0.123[†]	<0.0001[†]	0.3520[†]
	Stage of lactation				**SCC**			**P-values**	
	0–44 n=41	**45–99** n=39	**≥100** n=53	**SED**	**Low[2]** n=102	**High[3]** n=31	**SED**	**Stage of lactation**	**SCC**
Chao1	2443[ac]	2245[a]	2564[bc]	130	2520[a]	2315[a]	128	0.0536	0.1122
Shannon	7.66[a]	7.67[a]	7.83[a]	0.155[†]	7.81[a]	7.63[a]	0.152[†]	0.1634[†]	0.2844[†]

	[1]Comparing stages of lactation within farming systems									
Farm	**SA**			**ST**			**RBM**			**P-value**
DIM	**0–44**	**45–99**	**≥100**	**0–44**	**45–99**	**≥100**	**0–44**	**45–99**	**≥100**	**Farm x Stage of Lactation**
Shannon	7.09[a]	6.65[a]	6.89[a]	8.07[a]	8.67[a]	8.53[a]	7.83[a]	7.70[a]	8.05[a]	0.0423
	[1]Comparing farming systems within stages of lactation									
DIM	**0-44**			**45-99**			**≥100**			
Farm	**SA**	**ST**	**RBM**	**SA**	**ST**	**RBM**	**SA**	**ST**	**RBM**	
Shannon	7.09[a]	8.07[a]	7.83[a]	6.65[a]	8.67[b]	7.70[a]	6.89[a]	8.53[b]	8.05[a]	

A difference in lettering between two least squares means for factors of a variable indicates a significant difference with $P<0.05$. SED=Standard error of the difference in least squares means. ANOVA models developed with the Mixed procedure in SAS 9.4. [1]Shannon index had a significant interaction (Farm x stage of lactation), therefore, added "comparing stages of lactation within farming systems" and "comparing stages of lactation within farming systems"

Table 2.7 ANOVA results for each cow-site, comparing bacterial richness (Chao1 index) and diversity (Shannon index) among farms. Only cows with a SCC ≤ 150,000 cells/ml included.

	Farm				Parity			Stage of lactation				P-value		
Milk	**SA**	**ST**	**RBM**		**1**	**≥ 2**		**0–44**	**45–99**	**≥100**				
n=103	n=40	n=39	n=24	**SED**	n=54	n=49	**SED**	n=29	n=29	n=45	**SED**	**Farm**	**Parity**	**DIM**
Chao1	1420[a]	1131[b]	1777[a]	0.040*	1352[a]	1534[a]	0.032*	1456[a]	1379[a]	1494[a]	0.042*	<0.0001*	0.0852*	0.5312*
Shannon[1]	7.111[a]	6.550[b]	7.503[a]	0.011*	6.915[a]	7.195[b]	0.009*	7.135[a]	6.992[a]	7.036[a]	0.010*	<0.0001*	0.0484*	0.7221*
Teat-apex	**SA**	**ST**	**RBM**		**1**	**≥ 2**		**0-44**	**45-99**	**≥100**				
n=102	n=40	n=39	n=23	**SED**	n=53	n=49	**SED**	n=32	n=33	n=37	**SED**	**Farm**	**Parity**	**DIM**
Chao1	2179[a]	3097[b]	2275[a]	156.0	2548[a]	2480[a]	123.7	2500[a]	2396[a]	2646[a]	153.0	<0.0001	0.5847	0.2734
Shannon[2]	6.972[a]	8.679[b]	7.943[c]	0.144	7.886[a]	7.844[a]	0.115	7.811[a]	7.739[a]	8.043[a]	0.165	<0.0001	0.7157	0.0954
Fecal	**SA**	**ST**	**RBM**		**1**	**≥ 2**		**0–44**	**45–99**	**≥100**				
n=108	n=44	n=37	n=27	**SED**	n=53	n=55	**SED**	n=33	n=34	n=41	**SED**	**Farm**	**Parity**	**DIM**
Chao1	2809[a]	2594[a]	2768[a]	105	2821[a]	2595[b]	83.8	2818[a]	2665[a]	2688[a]	103	0.0834	0.0071	0.2990
Shannon	9.064[a]	8.610[b]	7.960[c]	63.8[†]	8.706[a]	8.384[b]	50.8[†]	8.545[a]	8.517[a]	8.572[a]	63.0[†]	<0.0001[†]	0.0197[†]	0.7833[†]

*Log transformation used. [†]Box-cox transformation used. [1]Removed one outlier (sample ID: 195.2). [2]Removed two outliers (sample ID's: 35.3 and 31.3). A difference in lettering between two least squares means for factors of a variable indicates a significant difference with $P<0.05$. SED=Standard error of the difference in least squares means. ANOVA models developed with the Mixed procedure in SAS 9.4.

Table 2.8 ANOVA results comparing bacterial richness (Chao1 index) and diversity (Shannon index) of used bedding among farms.

	Farm				P-value
Bedding	**SA**	**ST**	**RBM**	**SED**	**Farm**
n=54	n=30	n=12	n=12		
Chao1	1015[a]	1446[b]	1499[b]	0.036*	<0.0001*
Shannon[1]	5.87[a]	6.81[b]	7.59[c]	0.27 for ST vs. SA and RBM (0.066 for RBM vs. SA)[1]	<0.0001

*Log transformation used. [1]Satterthwaite approximation for degrees of freedom used to account for unequal variance among farms and normalize studentized residuals (Farm ST had a larger variance). A difference in lettering between two least squares means for factors of a variable indicates a significant difference with $P<0.05$. SED=Standard error of the difference in least squares means. ANOVA models developed with the Mixed procedure in SAS 9.4.

Table 2.9 ANOVA results comparing bacterial richness (Chao1 index) and diversity (Shannon index) among cow-sites, for cows with a SCC ≤ 150,000 cells/ml, on a per-farm basis.

	Site				Parity			Stage of lactation				P-Value		
Farm RBM	**Fecal**	**Milk**	**Teat–end**		**1**	**≥ 2**		**0–44**	**45–99**	**≥100**				
n=74	n=27	n=24	n=23	**SED**	n=43	n=31	**SED**	n=31	n=25	n=18x	**SED**	**Site**	**Parity**	**DIM**
Chao1	2791[a]	1757[b]	2259[c]	0.345*	2299[a]	2239[a]	0.029*	2281[a]	2199[a]	2327[a]	0.037*	<0.0001*	0.9401*	0.5146*
Shannon	7.976[a]	7.481[b]	7.914[ab]	0.195	7.884[a]	7.697[a]	0.189	7.719[a]	7.790[a]	7.862[a]	0.234	0.0295	0.3284	0.8308
Farm SA	**Fecal**	**Milk**	**Teat–end**		**1**	**≥ 2**		**0–44**	**45–99**	**≥100**				
n=124	n=44	n=40	n=40	**SED**	n=65	n=59	**SED**	n=39	n=44	n=41	**SED**	**Site**	**Parity**	**DIM**
Chao1	2818[a]	1426[b]	2147[c]	99.0	2211[a]	2050[a]	96.3	2347[a]	2024[b]	2020[b]	115.7	<0.0001	0.0970	0.0072
Shannon	9.071[a]	7.095[b]	6.949[b]	0.12	7.816[a]	7.594[a]	0.11	7.918[a]	7.547[b]	7.650[ab]	0.13	<0.0001	0.0511	0.0205
Farm ST	**Fecal**	**Milk**	**Teat–end**		**1**	**≥ 2**		**0–44**	**45–99**	**≥100**				
n=115	n=37	n=39	n=39	**SED**	n=52	n=63	**SED**	n=24	n=27	n=64	**SED**	**Site**	**Parity**	**DIM**
Chao1	2499[a]	1085[b]	3069[c]	116	2143[a]	2121[a]	119	2052[a]	2176[ac]	2168[bc]	157	<0.0001	0.4490	0.0397
Shannon[1]	8.521[a]	6.485[b]	8.679[a]	0.132	7.756[a]	8.034[b]	0.190	7.690[a]	7.947[a]	8.048[a]	0.171	<0.0001	0.0364	0.0991

Cow-ID was used a random factor. [1] Removed three outliers (Sample ID's: 31.3, 35.3, and 195.2). *Log10 transformation used. A difference in lettering between two least squares means for factors of a variable indicates a significant difference with $P<0.05$. SED=Standard error of the difference in least squares means. ANOVA models developed with the Mixed procedure in SAS 9.4.

Table 2.10 ANOVA results for each farm, comparing bacterial richness (Chao1 index) and diversity (Shannon index) among bedding and host-sites of cows with a SCC ≤ 150,000 cells/ml.

	Site					P-value
Farm RBM	**Bedding**	**Milk**	**Teat**	**Fecal**	**SED**	**Site**
Chao1[1]	1499[a]	1682[a]	2253[b]	2791[c]	152	<0.0001
Shannon	7.59[ac]	7.49[bc]	7.91[ac]	7.97[a]	3.48[†]	0.032[†]
Farm SA	**Bedding**	**Milk**	**Teat**	**Fecal**	**SED**	**Site**
Chao1	1015[a]	1414[b]	2159[c]	2806[d]	104	<0.0001
Shannon	5.87[a]	7.09[b]	6.96[b]	9.06[c]	0.0073*	<0.0001*
Farm ST	**Bedding**	**Milk**	**Teat**	**Fecal**	**SED**	**Site**
Chao1	1446[a]	1155[a]	3139[b]	2565[c]	156.7	<0.0001
Shannon[2]	6.81[a]	6.55[a]	8.61[b]	8.60[b]	0.20	<0.0001

Note: These results are referred to in text for comparisons between bedding and host-sites only, not among host-sites. *Log transformation used. [†]Box-cox transformation used. [1]Removed one outlier (milk, sample ID 94.2). [2]removed one outlier (milk, sample ID 195.2). A difference in lettering between two least squares means for factors of a variable indicates a significant difference with $P<0.05$. SED=Standard error of the difference in least squares means. ANOVA models developed with the Mixed procedure in SAS 9.4.

2.4.4 Phylum-level bacterial profile among farms for each site

Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes were predominant phyla in all sites, on all farms, with the exception of feces, which was composed of less than 2% Proteobacteria and less than 2% Actinobacteria on all farms. The phylum-level composition of each site on each farm is shown in Figure 2.6, with details in Additional file 4. A sparse multivariate method (MaAsLin) described in Morgan et al. (2012) was used to associate abundances of all phyla present in at least 25% of samples with metadata for each site (Additional file 5). Main findings were that the predominant phylum in milk, Proteobacteria, comprised a larger proportion of the milk microbial community on farm ST, with an average of 72% (StdDev 19%) compared to 50% for farm SA (StdDev 20.5%, $P_{(FDR)}<0.0001$) and 51% for farm RBM (StdDev 22%, $P_{(FDR)} < 0.0001$). Meanwhile, on the teat-apex, the average relative abundance of Proteobacteria was higher on farm ST (22.8%, StdDev 11.3%) compared to farm SA (10.7%, StdDev 3.5%, $P_{(FDR)} < 0.0001$), but not compared to farm RBM. Further, bedding on farm SA had an average proportion of Proteobacteria that was only 4% (StdDev 1.1%), which was substantially lower than its average proportion on farm RBM (22.2%, StdDev 3.5%, $P_{(FDR)} < 0.0001$) and farm ST (24.3%, StdDev 8.4%, $P_{(FDR)} < 0.0001$). The lower proportion of Proteobacteria within bedding on farm SA was accompanied by a higher average proportion of Actinobacteria in bedding on this farm (73.6% StdDev 5.4%) compared to 49.7% for farm RBM (StdDev 7.3%, $P_{(FDR)} < 0.0001$) and 45.1% for farm ST (StdDev 9%, $P_{(FDR)} < 0.0001$). In accordance with this, there was a higher proportion of Actinobacteria on both teat-apices and in milk of cows from farm SA (52.7%, StdDev 8.0%, and 24.3%, StdDev 11.5%, respectively), compared to farm ST (23.8%, StdDev

9.3% and 6.3%, StdDev 4.5%) and farm RBM (31.6%, StdDev 9.2% and 14.3%, StdDev 10.1%, respectively), with a $P_{(FDR)} < 0.0001$ for all comparisons. The average relative abundance of Firmicutes was higher in bedding, as well as on teat-apices of cows from Farm ST (26.4%, StdDev 14.5% and 44.9%, StdDev 14.0%, respectively) in comparison to bedding, and teat-apices of cows from farm RBM (14.8%, StdDev 4.0%, $P_{(FDR)}=0.0104$ and 35.7%, StdDev 15.9%, $P_{(FDR)}=0.0005$, respectively), and only on the teat-apices, Firmicutes was in a higher average proportion on farm ST in comparison to farm SA (27.5%, StdDev 6.5%, $P_{(FDR)} < 0.0001$). Interestingly, farm ST had a lower average proportion of Firmicutes in milk (17.6%, StdDev 15.1%), in comparison to milk on farm RBM (28.1% StdDev 18%, $P_{(FDR)}=0.0419$), and was not significantly different than its average proportion in milk on farm SA (20.3%, StdDev 10.8%).

Interestingly, the multivariate analysis for teat-apex samples revealed that Cyanobacteria was associated with reduced Log_{10}SCC (LogSCC) ($P_{(FDR)}=0.0390$). Cyanobacteria were in a significantly higher average proportion on teat-apices of cows from farm ST (0.10%, StdDev 0.08%) in comparison to farm SA (0.025%, StdDev 0.027%, $P_{(FDR)} < 0.0001$) and farm RBM (0.038%, StdDev 0.048%, $P_{(FDR)}=0.0030$) (Figure 2.7). Consistent with this, in bedding material, Cyanobacteria was also in a significantly higher average proportion on farm ST (0.53%, StdDev 0.57%) in comparison to farm SA (0.0011%, StdDev 0.0042, $P_{(FDR)} < 0.0001$) and farm RBM (0.0014%, StdDev 0.0048%, $P_{(FDR)} < 0.0001$) (Figure 2.7). However in fecal material, Cyanobacteria were in a higher average proportion on farm SA (0.21% StdDev 0.17%) in comparison to farm ST (0.097%, StdDev 0.081%, $P_{(FDR)} =0.0018$) and farm RBM (0.11%, StdDev 0.10%, $P_{(FDR)}=0.0158$) (Figure 2.7). Further, Spirochaetes tended to also be associated with reduced LogSCC ($P_{(FDR)=}0.0674$).

Spirochaetes were in a higher average proportion on teat-apices of cows from farm ST in comparison to farm RBM ($P_{(FDR)} < 0.0001$), and also on farm SA in comparison to farm RBM ($P_{(FDR)}=0.0002$) (Figure 2.7). Additionally, while Spirochaetes were not found in at least 25% of bedding samples (Additional file 4), they were identified in feces, with a higher abundance on farm ST in comparison to farm SA ($P_{(FDR)}=0.0004$) and farm RBM ($P_{(FDR)} < 0.0001$), and again also on farm SA in comparison to farm RBM ($P_{(FDR)} < 0.0001$) (Figure 2.7).

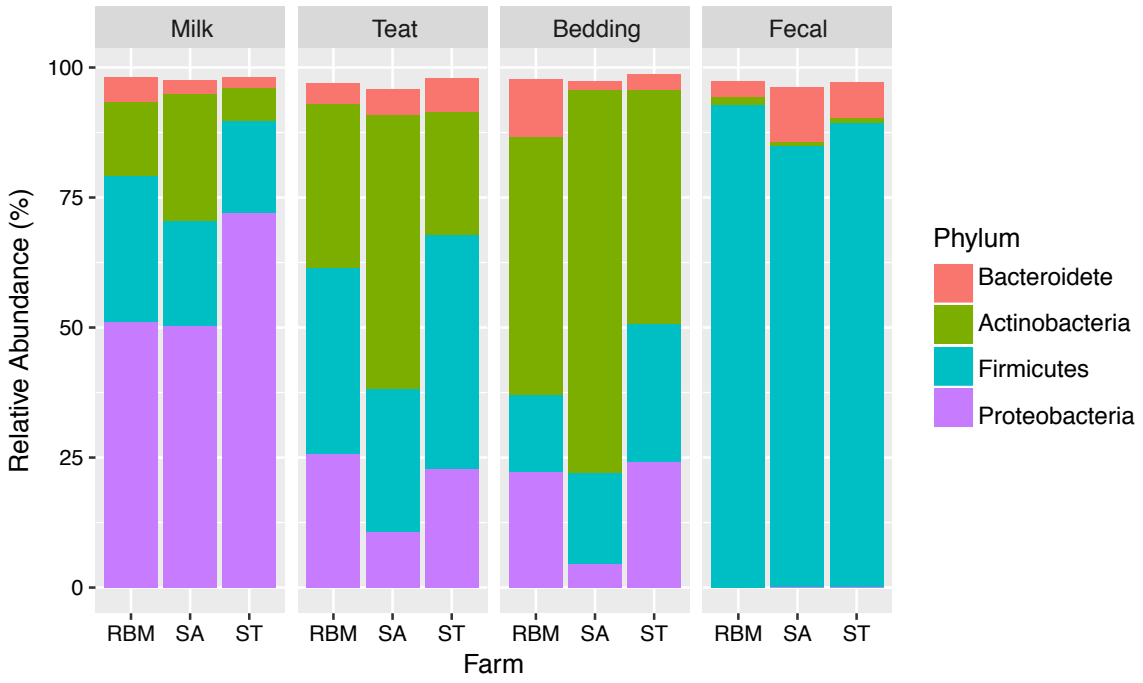

Figure 2.6 The average relative abundances of the four main phyla within each site on each farm. Comparisons are between farms, within all milk, teat-apex, bedding and fecal samples.

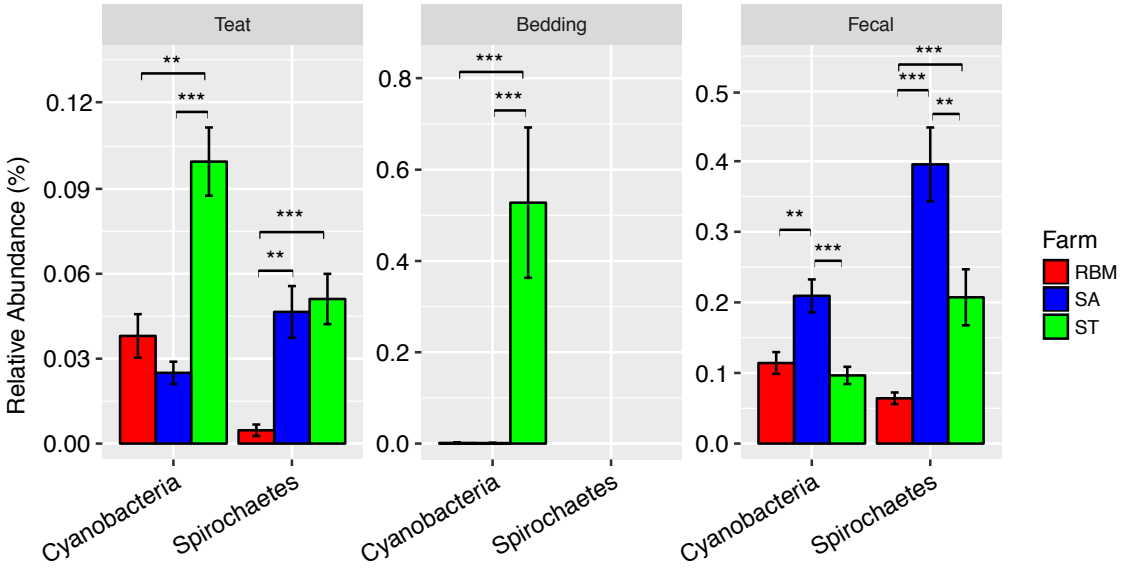

Figure 2.7 Relative abundances on teat-apices, bedding and feces, for phyla that, on the teat-apex, were associated with LogSCC. The two phyla that at least tended to be associated with LogSCC based on the multivariate analysis (MaAsLin) for teat-apex samples were Cyanobacteria ($P_{(FDR)}$=0.0390) and Spirochaetes ($P_{(FDR)}$=0.0674). *P*-values for comparisons in relative abundance between pairs of farms: * = $P_{(FDR)}$<0.05, ** = $P_{(FDR)}$<0.01, *** = $P_{(FDR)}$<0.0001. *P*-values were obtained from the MaAsLin analyses used to associate metadata with phyla abundances within teat-apex, bedding and fecal samples. Error bars represent standard error of the mean.

2.4.5 OTUs within milk or on teat-apices associated with mammary inflammation and other host-factors

Using a sparse multivariate method by implementing MaAsLin software (Morgan et al., 2012), we identified metadata features [farm, LogSCC, days in milk (DIM), and parity (multiparous vs. primiparous)] associated with the abundance of each non-random OTU within each site (present in at least 25% of samples of a site) (Additional file 6). The multivariate analysis of milk samples allowed us to identify specific OTUs whose relative abundance in milk was associated with LogSCC. Of the 659 OTUs identified in at least 25% of milk samples across all farms, the abundance of only two OTUs were significantly associated with LogSCC ($P_{(FDR)} < 0.05$), with an additional 5 having a tendency to be associated with LogSCC ($P_{(FDR)} < 0.1$). OTUs that at least tended to be associated with reduced LogSCC, defined as an FDR-corrected *P*-value below 0.1, were

primarily within the phylum Proteobacteria, these included OTU2221284 (*Lautropia*; $P_{(FDR)}$=0.018), OTU631674 (*Rhodocyclaceae*; $P_{(FDR)}$=0.027), OTU860929 (*Sphingomonadaceae*; $P_{(FDR)}$=0.06), OTU325711 (*Erythrobacteraceae*; $P_{(FDR)}$=0.073), and OTU1108062 (*Sphingomonas*; $P_{(FDR)}$=0.09), while a member of Actinobacteria, specifically new reference (N.R.)OTU1936 (*Nocardioidaceae*), was also associated with reduced LogSCC ($P_{(FDR)}$=0.08). N.R.OTU2358 (*AKIW874*), also a member of Actinobacteria, was the only OTU that tended to be associated with increased LogSCC ($P_{(FDR)}$=0.0727).

Using the same sparse multivariate method, out of the 1,156 OTUs identified in at least 25% of teat-apex samples, the abundances of six OTUs on the teat-apex were significantly associated with LogSCC ($P_{(FDR)} < 0.05$). The OTUs associated with reduced LogSCC included three members of Firmicutes, New Cleanup Reference (NCR.)OTU339570 (*Coprococcus*; $P_{(FDR)}$=0.0038), and N.R.OTU2061 (*Aerococcus*; $P_{(FDR)}$=0.0165), and N.R.OTU805 (*Facklamia*; $P_{(FDR)}$=0.034) and one member of Proteobacteria, OTU295031 (*Pseudomonas*; $P_{(FDR)}$=0.031). Two OTUs were associated with increased LogSCC, OTU1636835 (*Corynebacterium*; $P_{(FDR)}$=0.041), and OTU614083 (*Staphylococcus;* $P_{(FDR)}$=0.046). Additional host-factors included in the multivariate analyses, DIM and parity, were not associated with changes in relative abundances of the aforementioned OTUs whose relative abundances in milk or on the teat-apex were associated with LogSCC.

2.4.6 Comparisons between farms for OTUs potentially important to mammary inflammation

The multivariate analysis used to identify OTUs in milk associated with LogSCC also revealed differences between farms in the abundance of these OTUs (Additional file 6).

Specifically, of the OTUs in milk negatively associated with LogSCC, OTU325711 (*Erythrobacteraceae*) was in higher abundance in milk on farm ST in comparison to farm RBM ($P_{(FDR)}$=0.0023) and farm SA ($P_{(FDR)}$=0.0353), N.R.OTU1936 (*Nocardioidaceae*) was in higher abundance on farm ST in comparison to farm SA ($P_{(FDR)}$=0.0182), but not compared to farm RBM, and OTU2221284 (*Lautropia*) was in higher abundance on farm RBM in comparison to farm SA ($P_{(FDR)}$=0.0090) (Figure 2.8a). OTU1108062 (*Sphingomonas*), OTU631674 (*Rhodocyclaceae*), and OTU860929 (*Sphingomonadaceae*) were not significantly different in abundance in milk between farms (Figure 2.8a). Meanwhile, the OTU that tended to be associated with increased LogSCC (N.R.OTU2358, *AKIW874*) was significantly lower in relative abundance within milk from farm ST where it was not found to be present, in comparison to both farm SA ($P_{(FDR)}$=0.0085) and farm RBM ($P_{(FDR)}$<0.0001) (Figure 2.8b). This was the only OTU associated with mammary inflammation in milk that was also identified in at least 25% of samples (a non-random OTU) from any other site sampled. It was identified in bedding and on teat-apices of cows from farm RBM and farm SA, but it was not present in feces on any farm, or any site on farm ST, and as such, was in significantly higher relative abundances on farm RBM and SA in comparison to farm ST on teat-apices and in bedding (P<0.0001). The analysis (MaAsLin) for OTUs in bedding material (Additional file 6) revealed that N.R.OTU2358 (*AKIW874*) was also in higher relative abundance in bedding on farm RBM in comparison to farm SA ($P_{(FDR)}$<0.0001; Figure 2.8b). Of note, is that while all OTUs included in any multivariate analysis for any particular site were considered non-random OTUs for that site (present in at least 25% of samples), some OTUs were still in an average relative abundance below 0.05% on all farms. For milk

OTUs associated with mammary inflammation, these consistently low-abundant OTUs were OTU2221284 (*Lautropia*), OTU1108062 (*Sphingomonas*), and N.R.OTU2358 (*AKIW874*).

Focusing on the teat-apex OTUs identified as potentially important to mammary health, the multivariate analysis (MaAsLin) used to identify OTUs on the teat-apex associated with mammary inflammation also revealed differences in relative abundances on the teat-apex between farms for some of these OTUs ($P_{(FDR)} < 0.05$; Additional file 6). In summary, two out of the four teat-apex OTUs associated with reduced mammary inflammation, namely N.R.OTU2061 (*Aerococcus*) and NCR OTU339570 (*Coprococcus*), were in substantially higher average relative abundances on farm ST in comparison to farm SA (Both $P_{(FDR)} < 0.0001$) and farm RBM ($P_{(FDR)}=0.0039$, and $P_{(FDR)}=0.0003$, respectively, Figure 2.9a). Of the additional two OTUs associated with reduced mammary inflammation, OTU29501 (*Pseudomonas*) was also in a significantly higher relative abundance on farm ST in comparison to farm SA ($P_{(FDR)}=0.0015$, Figure 2.9a), and only numerically in higher relative abundance compared to farm RBM (Figure 2.9a), while N.R.OTU805 (*Facklamia*) was present in significantly higher proportions on farm RBM in comparison to both farm SA ($P_{(FDR)}=0.0149$) and farm ST ($P_{(FDR)}=0.0013$, Figure 2.9a). As for the OTUs found positively associated with mammary inflammation, OTU614083 (*Staphylococcus*), was present in higher proportions on farm RBM in comparison to farm SA ($P_{(FDR)} < 0.0001$) and farm ST ($P_{(FDR)}=0.0002$, Figure 2.9a), meanwhile, OTU1636835 (*Corynebacterium*) was in a lower average proportion on farm SA in comparison to farm ST ($P_{(FDR)}=0.0179$), and farm RBM ($P_{(FDR)}=0.0003$, Figure 2.9a). Further, two OTUs whose relative abundance on the teat-apex was associated with

reduced LogSCC, N.R.OTU2061 (*Aerococcus*) and N.R.OTU805 (*Facklamia*), were identified as non-random OTUs in bedding material, though they did not differ significantly in abundance between farms within bedding material (Figure 2.9b). No other OTUs associated with mammary inflammation on the teat-apex were identified as non-random OTUs in bedding or feces, though NCR OTU339570 (*Coprococcus*) was a low abundant OTU within feces, being present in five fecal samples (Additional file 7). Additional file 7 can be referred to for details pertaining to the OTUs associated with mammary inflammation, including details on each association identified by MaAsLin analyses for these specific OTUs, and for each site on each farm, abundance information and the fraction of samples each OTU was identified in.

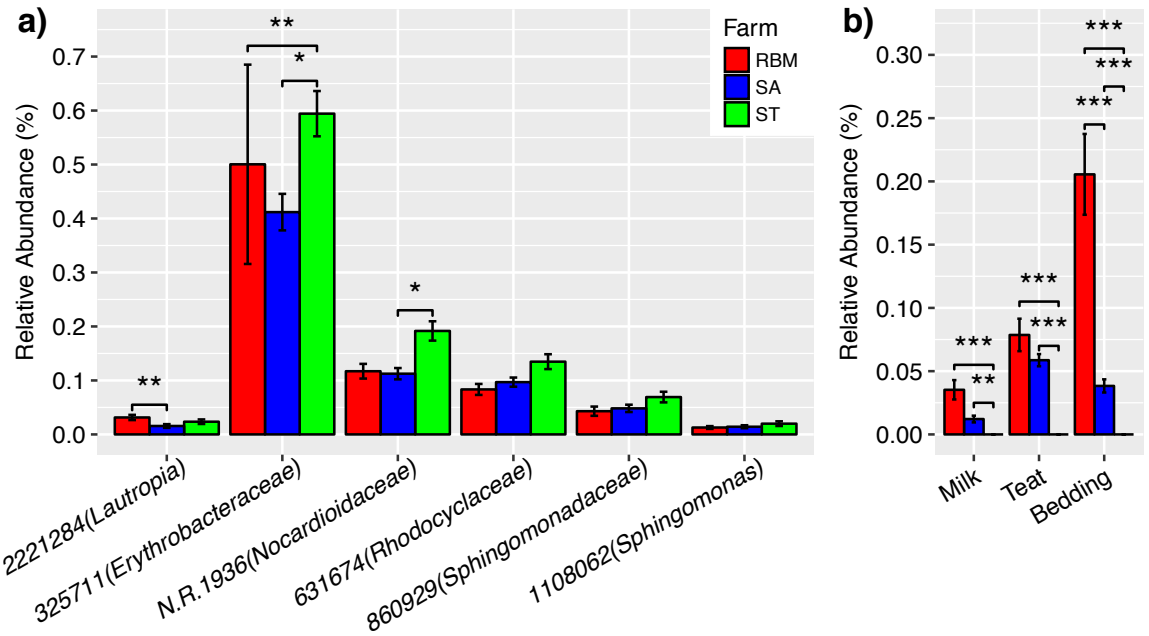

Figure 2.8 For OTUs that tended to be associated with LogSCC within milk, proportional comparisons between farms, within sites they were considered non-random OTUs. Showing non-random OTUs in milk (≥ 25% of samples) that at least tended to be associated with LogSCC ($P_{(FDR)}$ <0.1) based on the multivariate analysis (MaAsLin). **(a)** Average relative abundances within milk for OTUs associated with reduced LogSCC ($P_{(FDR)}$ <0.1). X-axis label: numbers represent each OTU's ID, and in parentheses, the lowest taxonomy assigned. **(b)** Average relative abundances in milk, teat-apex and bedding samples for the only OTU associated with increased LogSCC (N.R.OTU2358 (*AKIW874*), $P_{(FDR)}$ <0.1). *P*-values for comparisons in relative abundance between pairs of farms: * = $P_{(FDR)}$<0.05, ** = $P_{(FDR)}$<0.01, *** = $P_{(FDR)}$<0.0001. *P*-values were obtained from the MaAsLin analyses used to associate metadata with OTU abundances within milk, teat-apex and bedding samples. Error bars represent standard error of the mean.

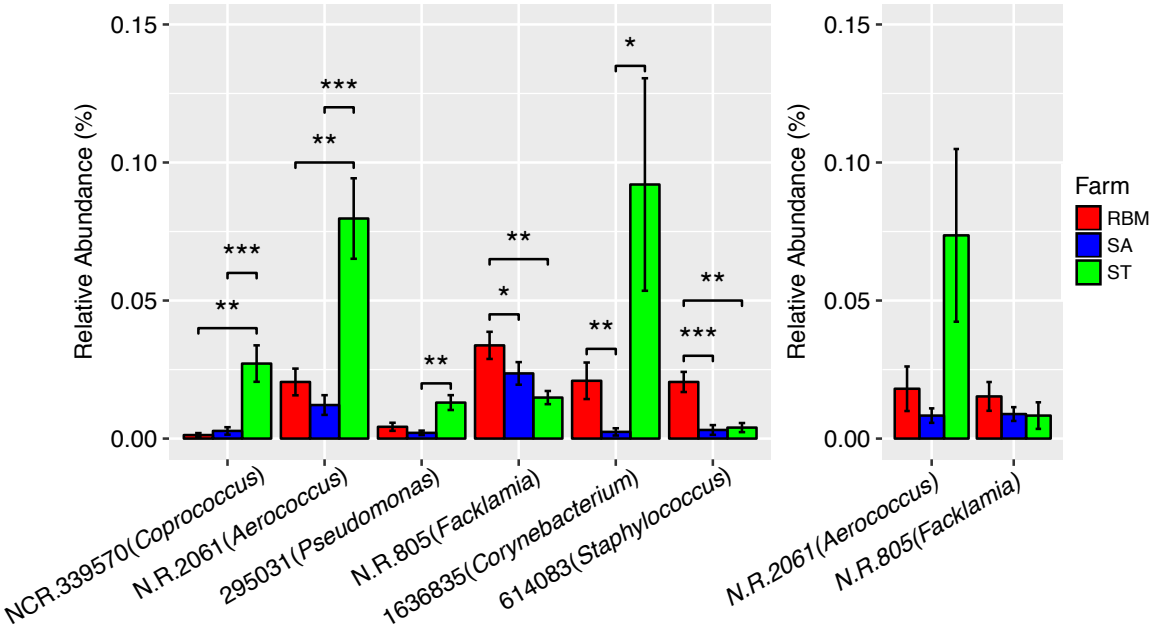

Figure 2.9 For OTUs on the teat-apex associated with milk LogSCC, proportional comparisons between farms, within sites they were considered non-random OTUs. Showing non-random OTUs on teat-apices (≥ 25% of samples) that were significantly associated with LogSCC ($P_{(FDR)}$ <0.05) based on the multivariate analysis (MaAsLin). X-axis label: numbers represent each OTU's ID, and in parentheses, the lowest taxonomy assigned. **(a)** Average relative abundances on the teat-apex. **(b)** Average relative abundances in bedding, for OTUs considered non-random (≥ 25% of samples) within bedding. *P*-values for comparisons in relative abundance between pairs of farms: * = $P_{(FDR)}$<0.05, ** = $P_{(FDR)}$<0.01, *** = $P_{(FDR)}$<0.0001. *P*-values were obtained from the MaAsLin analyses used to associate metadata with OTU abundances within milk, teat-apex and bedding samples. Error bars represent standard error of the mean.

2.4.7 Genus level associations with mammary inflammation and between-farm relative abundance comparisons

Previous research looking at the milk microbiome has focused on the genus level, therefore, to assess whether our data is comparable with previous findings, we briefly assessed the genus-level associations with available metadata. Using Spearman rank-order correlation, we found that of the 42 genera over an average relative abundance of 0.5% within milk, the proportions of 4 genera were associated with increased mammary inflammation, and 12 genera, associated with reduced mammary inflammation ($P < 0.05$) (Figure 2.10). The genus *Staphylococcus* had the strongest correlation with mammary

inflammation (ρ =0.31, *P*=0.0002), followed by an unclassified *Planococcaceae* (ρ =0.26, *P*=0.0021), and unclassified Betaproteobacteria (ρ = –0.25, *P*=0.0037), which had the strongest correlation for those associated with reduced mammary inflammation. All 12 genera associated with reduced mammary inflammation were within the phylum Proteobacteria*,* and three genera associated with increased mammary inflammation (*Staphylococcus*, unclassified *Planococcaceae*, and *Enterococcus*) were in the phylum Firmicutes*,* while one (*Arthrobacter*) was in Proteobacteria*.*

To further investigate genus-level associations, we used the multivariate method of association, MaAsLin, to identify associations between genera present in at least 25% of milk samples, and available metadata (Additional file 8). A main finding was that all genera associated negatively with mammary inflammation based on spearman rank-order correlation, were in higher relative abundance on farm ST in comparison to the other two farms, and two genera associated positively with mammary inflammation were in lower relative abundance on farm ST in comparison to the other two farms ($P_{(FDR)} < 0.05$, Figure 2.10). Meanwhile *Staphylococcus* did not differ in abundance between the three farms (Figure 2.10). Additionally, using this multivariate analysis (Additional file 8), *Staphylococcus* was the only genera with an average abundance above 0.5% that was associated with mammary inflammation ($P_{(FDR)}$=0.0021), again being a positive association. An Unclassified *Rhodospirillaceae* (f_Rhodospirillaceae_Other), associated with reduced SCC using Spearman rank-order correlation (ρ = –0.23, *P*=0.0074), also least tended to be associated with reduced LogSCC using the multivariate method ($P_{(FDR)}$=0.086) Other than *Staphylococcus,* one additional genera significantly associated with LogSCC using this multivariate method, *Lautropia* ($P_{(FDR)}$=0.026), had a negative

association with LogSCC. An additional finding regarding *Lautropia* was its lower abundances on farm RBM in comparison to farm ST ($P_{(FDR)}$=0.0388) and farm SA ($P_{(FDR)}$=0.0003) (Additional file 8). Additional file 9 can be referred to for relative abundance details for genera in average abundances of over 0.5% within milk.

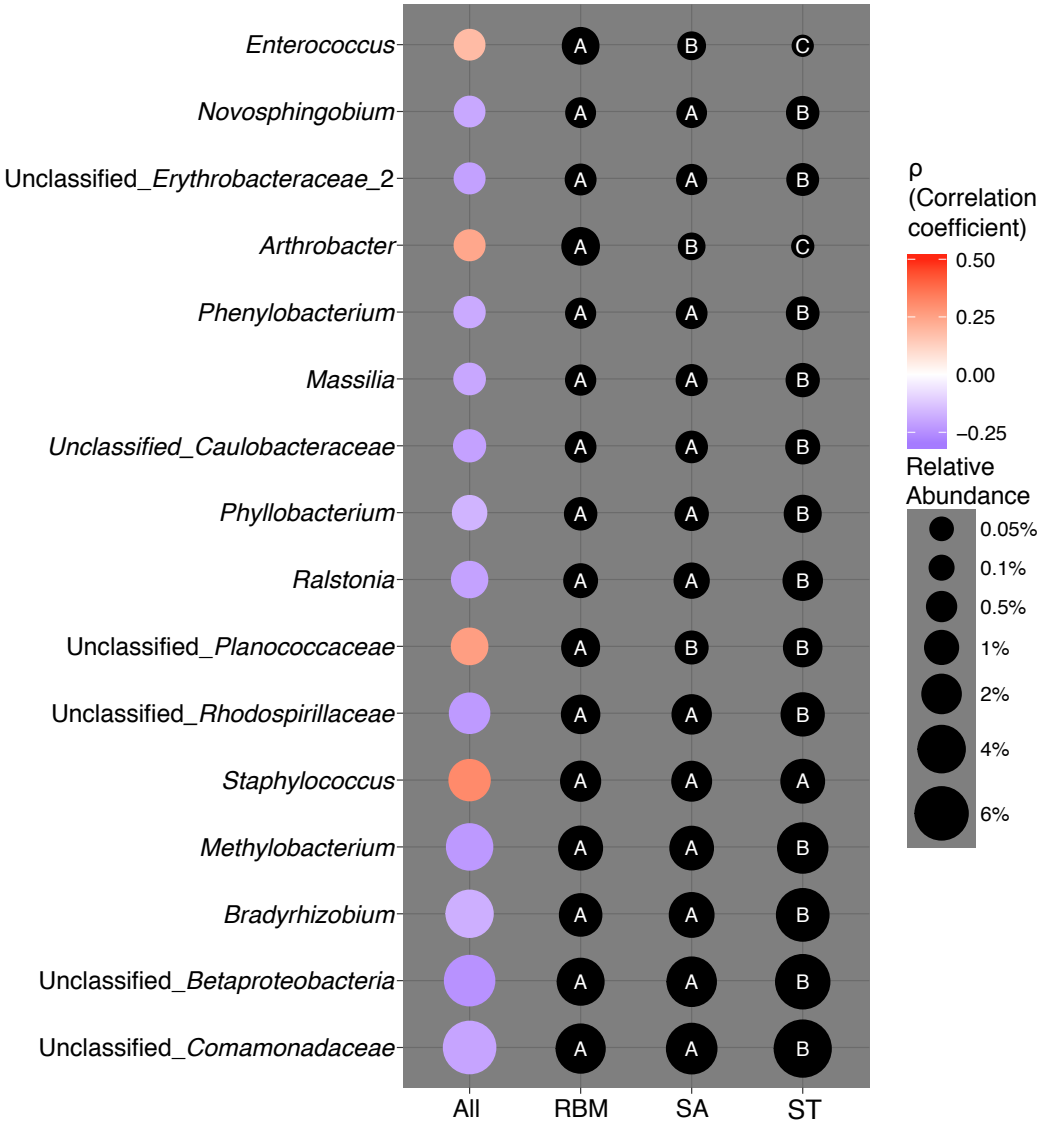

Figure 2.10 Correlations between genus relative abundances and milk SCC, with comparisons of their relative abundance between farms. Of the 42 genera above an average abundance of 0.05%, the 16 genera shown here are those significantly correlated with milk SCC (P<0.05) using Spearman rank-order correlation for all milk samples combined (All). A difference in lettering represents a significant difference in relative abundance between farms ($P_{(FDR)}$<0.05) based on the multivariate analysis (MaAsLin) for milk samples. Farm IDs are shown on the x-axis as RBM, SA and ST.

2.4.8 SourceTracker analyses: Bacterial sources of milk and teat-apex microbiota

Results of the Bayesian analysis, SourceTracker, performed to identify sources of milk microbiota, indicated that on all farms, of the sites that were sampled (teat-apex, bedding, and feces), the bovine teat-apex contributed the most to the milk microbiome on average [farm ST (16.4%), farm SA (35.8%), farm RBM (34.3%)], this was followed by bedding [farm ST (6.3%), farm SA (5%), farm RBM (7.1%)] and feces [farm ST (2.5%), farm SA (2.7%), farm RBM, (5.9%), Figure 2.11a]**.** Since the SourceTracker algorithm has been reported to not recognize bidirectional relationships, in that a source of microbiota cannot also be a sink for microbiota (Knights et al., 2011), and since bedding and feces are sources of teat-apex microbiota as well as milk, we cannot confirm that the algorithm is correctly classifying most microbiota, with a known source, to colonize the mammary gland from natural bovine teat-apex microbiota. Therefore a second analysis was performed that used the teat-apex as a sink for microbiota from bedding and feces, which were considered the only sources. The results of the SourceTracker analysis performed to identify sources of teat-apex microbiota indicated that the majority of teat-apex microbiota came from bedding [farm ST (62.1%), farm SA (88.7%) and farm RBM (75.2%), Figure 2.12a], followed by unknown source/s [farm ST (20.6%), farm SA (7.4%), farm RBM (9.1%), Figure 2.12a], and fecal material [farm ST, (17.3%), farm SA (3.9%), RBM farm (15.7%), Figure 2.12a].

Spearman rank-order correlation analysis was performed to associate the proportion of a teat-apex sample's microbiota contributed by a particular source (bedding or feces) with the teat-apex sample's bacterial richness (Chao1 index) and diversity (Shannon and Simpson indices) and the cow's corresponding milk SCC for each farm (Figure 2.12b, c,

and d). In summary, only for farm SA and farm ST was the proportion of teat-apex microbiota originating from feces associated with all teat-apex bacterial richness and diversity indicators (ρ ranging from 0.84 to 0.49, $P < 0.001$). Meanwhile, the proportion of teat-apex microbiota originating from bedding had a negative association with teat-apex bacterial richness and diversity for farm SA and farm ST. These associations were strongest for farm SA [Chao1 index ($\rho = -0.92$), Shannon index ($\rho = -0.91$) and Simpson index ($\rho = -0.78$); $P<0.0001$]. Only on farm SA was there an association between the proportion of teat-apex microbiota originating from unknown sources and both teat-apex bacterial richness and diversity indices (ρ ranging from 0.67 to 0.84; $P < 0.0001$), with bacterial richness also having a positive association with the proportion of microbiota originating from unknown sources for farm RBM ($\rho =0.42$, $P=0.008$). In terms of associations between a cow's corresponding milk SCC and the proportion of a teat-apex sample's microbiota contributed by a particular source, for farm SA, there was a tendency for an increased proportion of teat-apex microbiota contributed from bedding material to be associated with reduced milk SCC of the corresponding milk sample ($\rho = 0.28$, $P=0.051$), while this same analysis for farm RBM and ST was not near significance ($\rho = 0.15$, $P=0.37$ and $\rho = 0.08$, $P=0.62$). Meanwhile, only for farm SA, the proportion of microbiota originating from unknown sources tended to be associated negatively with SCC ($\rho = -0.25$, $P=0.08$).

An additional Spearman rank-order correlation analysis was performed to associate the proportion of a milk sample's microbiome contributed by a particular source (teat-apex, bedding or feces), with the milk sample's bacterial diversity and SCC. The general findings of this were that, consistently across farms, the proportion of milk microbiota

originating from fecal and teat-apex samples was association with an increased bacterial richness and diversity (Figure 2.11b, c, d). Meanwhile, only on farm RBM was the proportion of milk microbiota originating from bedding association with an increased bacterial diversity (Shannon index ρ =0.35, *P*=0.022; Simpson index ρ =0.37, *P*=0.015, Figure 2.11b).

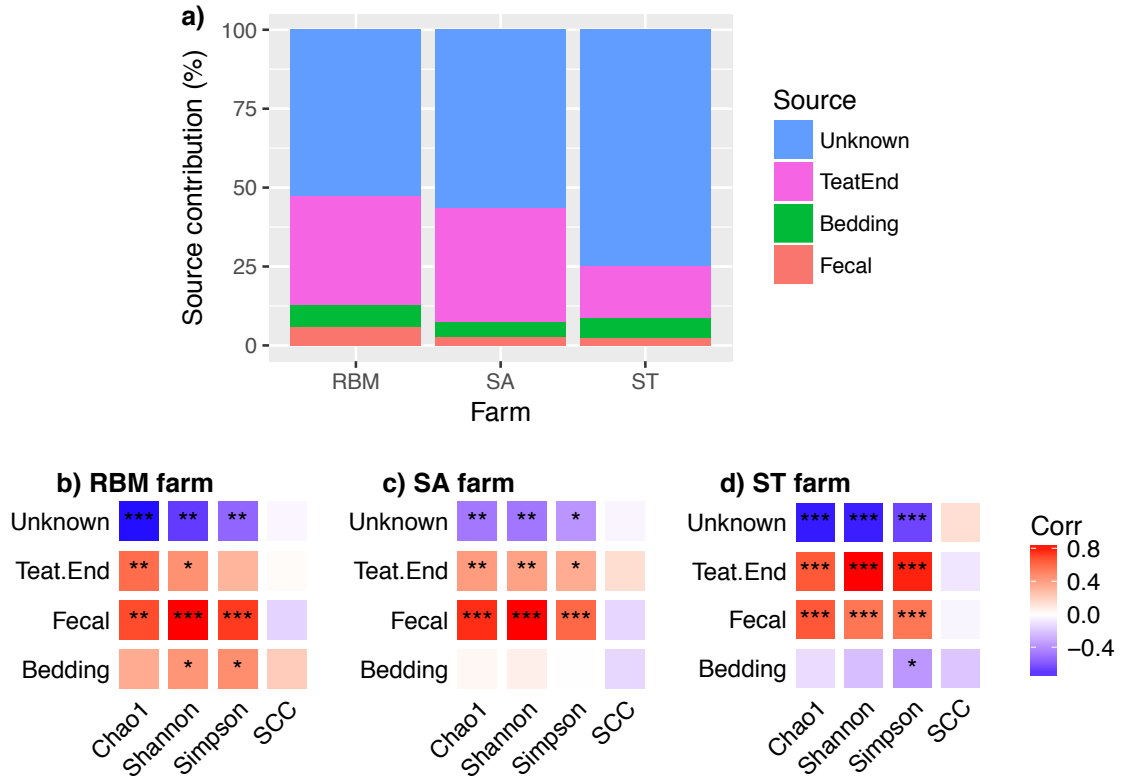

Figure 2.11 The estimated contribution of bacterial sources to milk bacterial communities and its association with milk bacterial diversity and milk SCC. **(a)** The percentage of milk bacterial communities contributed from various sources, as identified by the SourceTracker algorithm for each farm-system. **(b)** The correlation between the proportion of milk bacterial communities from different sources and bacterial diversity indicators and SCC on farm RBM **(b)**, farm SA **(c)**, and farm ST **(d)**. Heatmap colors represent Spearman rank-order correlation coefficients (ρ), with * = *P*<0.05, ** = *P*<0.01 and *** = *P*<0.0001.

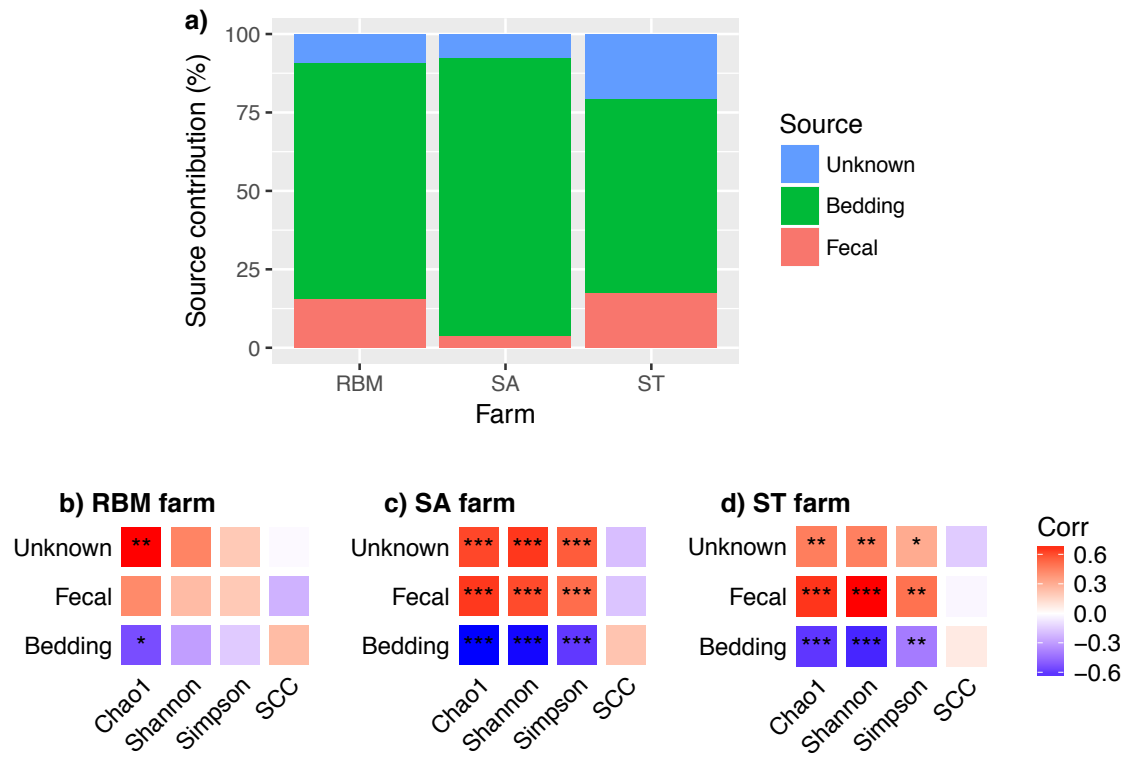

Figure 2.12 The estimated contribution of bacterial sources to teat-apex bacterial communities and its association with teat-apex bacterial diversity and the corresponding milk samples SCC. **(a)** The percentage of teat-apex bacterial communities contributed from various sources, as identified by the SourceTracker algorithm for each farm-system. **(b)** The correlation between the proportion of teat-apex bacterial communities from different sources and bacterial diversity indicators and SCC on farm RBM **(b)**, farm SA **(c)**, and farm ST **(d)**. Heatmap colors represent Spearman rank-order correlation coefficients (ρ), with * = P<0.05, ** = P<0.01 and *** = P<0.0001.

2.4.9 Mammary inflammation during summer and winter within the straw bedded farm

An ANOVA model used to compare SCC between all milk samples on farm ST did not find any significant differences between parities, lactation stages, or between the summer and winter time-points (Table 2.11). Further, winter and summer time-points both had a least squares means below the most commonly used threshold for subclinical mastitis of 200,000 cells/ml, suggesting that subclinical mastitis was likely not a greater problem during one time-point in comparison to the other.

Table 2.11 ANOVA results for comparisons of mean composite milk SCC between seasons, parities and lactation stages of sampled cows on farm ST (n=94).

	Season				**Parity**		
	Summer n=48	**Winter** n=46		**SED**	**1** n=39	**≥ 2** n=55	**SED**
SCC	118914[a]	183298[a]		0.034[†]	61989[a]	240224[a]	0.043[†]
	Lactation Stage				**P-Value**		
	0–44 n=20	**45–99** n=14	**≥100** n=60	**SED**	**Season**	**Parity**	**DIM**
SCC	191468[a]	106330[a]	155521[a]	0.0538[†]	0.4820[†]	0.7949[†]	0.0695[†]

[†]Used Box-cox transformation of SCC to normalize model residuals. Cow ID was included as a random factor. A difference in lettering between two least squares means for factors of a variable indicates a significant difference with $P<0.05$. SED=Standard error of the difference in least squares means. ANOVA models developed with the Mixed procedure in SAS 9.4.

2.4.10 Bacterial diversity during summer and winter within the straw bedded farm

When comparing the bacterial diversity (Shannon index) and richness (Chao1 index) indicators among all milk samples for farm ST, the sampling time-point in the summer had a significantly lower bacterial richness compared to the winter ($P<0.0001$), however bacterial diversity of milk was not associated with season ($P=0.4834$, Table 2.12). Similar to when comparisons of milk bacterial diversity and richness between cows from different farms was performed, SCC category and lactation stage did not have a significant association with bacterial diversity or richness indicators used (Table 2.12). However, again similar to when comparisons between farms were performed, cows within the low SCC group had at least a numerically higher bacterial diversity in comparison to cows within the high SCC group (Table 2.12). Bacterial diversity of milk from primiparous cows was also found to be significantly lower in comparison to milk from multiparous cows ($P=0.0129$, Table 2.12), and this was also consistent with our findings regarding parity when comparing milk diversity between farms.

When only samples from cows below a SCC of 150,000 were included in ANOVA models for each host-site, consistently for milk, teat-apices and feces, cows sampled during the summer time-point had a significantly lower average bacterial richness and diversity in comparison to those sampled during the winter time-point ($P<0.05$, Figure 2.13). However, for bedding material, there was no difference in bacterial richness or diversity between seasons identified ($P=0.34$ and $P=0.68$, respectively, Figure 2.13). Detailed results for these analyses, including the additional explanatory variables parity and stage of lactation, can be referred to in Table 2.13, and Table 2.14.

Using PCoA and PERMANOVA on weighted UniFrac distance matrices between samples, we found that for each site (milk, teat-apex, fecal and bedding), samples collected during the summer time-point clustered distinctly from those taken during the winter time-point ($P_{(PERMANOVA)}=0.0002$ or lower, Figure 2.14). Additionally, for milk and feces, primiparous and multiparous cows also clustered separately from each other ($P_{(PERMANOVA)}=0.0331$ and $P_{(PERMANOVA)}=0.0058$, respectively), with teat-apex samples also tending to cluster separately based on parity ($P_{(PERMANOVA)}=0.0547$). Lactation stage was not a significant explanatory variable of any PERMANOVA model. Of note is that these analyses on host-sites were performed only using samples from cows with a SCC below 150,000 cells/ml (refer to Additional file 3 for detailed results of PERMANOVA tests).

Consistent with previous between-farm analyses, all milk samples were also combined in order to additionally compare the weighted UniFrac distance matrices between low and high SCC groups. Again, no significant difference in weighted UniFrac distances was identified between cows with a low and high SCC, in comparison to their within-group

weighted UniFrac distances ($P_{(PERMANOVA)}$=0.1263, Figure 2.15). The significance of the remaining explanatory variables remained fairly consistent with what was observed when only milk from cows below a SCC of 150,000 cells/ml were included for comparisons between seasons on farm ST (Additional file 3).

Table 2.12 ANOVA results comparing least squares means of bacterial diversity and richness indicators among all milk samples on farm ST, during summer and winter (n=154).

	Season				**Parity**			**P-values**	
	Summer n=48		**Winter** n=46	**SED**	**1** n=39	**≥ 2** n=55	**SED**	**Season**	**Parity**
Chao1	775[a]		1155[b]	0.0004[†]	868[a]	1062[a]	0.00041[†]	0.0001[†]	0.0628[†]
Shannon[1]	5.96[a]		6.12[a]	0.213	5.75[a]	6.33[b]	0.209	0.4834	0.0129
	Stage of lactation				**SCC**			**P-values**	
	0–44 n=20	**45–99** n=14	**≥100** n=60	**SED**	**Low**[2] n=77	**High**[2] n=17	**SED**	**Stage of lactation**	**SCC**
Chao1	916[a]	914[a]	1065[a]	0.0006[†]	923[a]	1007[a]	0.00054[†]	0.1619[†]	0.6615[†]
Shannon[1]	6.21[a]	5.70[a]	6.21[a]	0.246	6.24[a]	5.84[a]	0.242	0.1489	0.1134

[†]Box-cox transformation used. [1]Season*SCC interaction kept in model to maintain normality (*P*-value=0.0973). CowID was included as an additional random factor. A difference in lettering between two least squares means for factors of a variable indicates a significant difference with $P<0.05$. SED=Standard error of the difference in least squares means. ANOVA models developed with the Mixed procedure in SAS 9.4.

Table 2.13 ANOVA results for each cow-site on farm ST, comparing bacterial richness (Chao1 index) and diversity (Shannon index) between seasons. Only cows with a SCC ≤ 150,000 cells/ml included.

	Season			Parity			Stage of lactation				P-value		
Milk	**Summer**	**Winter**		**1**	**≥ 2**		**0–44**	**45–99**	**≥100**				
n=77	n=38	N=39	**SED**	n=36	n=41	**SED**	n=16	n=14	n=47	**SED**	**Season**	**Parity**	**DIM**
Chao1	725[a]	1115[b]	0.00022[†]	843[a]	997[a]	0.0002[†]	858[a]	871[a]	1032[a]	0.0003[†]	0.0002[†]	0.1921[†]	0.1961[†]
Shannon	6.00[a]	6.49[b]	0.144	6.02[a]	6.47[b]	0.210	6.45[a]	5.87[a]	6.42[a]	0.229	0.0041	0.0480	0.0646
Teat-apex	**Summer**	**Winter**		**1**	**≥ 2**		**0-44**	**45-99**	**≥100**				
n=76	n=37	n=39	**SED**	n=37	n=39	**SED**	n=16	n=14	n=46	**SED**	**Season**	**Parity**	**DIM**
Chao1	1399[a]	3125[b]	133	2195[a]	2328[a]	146	2066[a]	2375[a]	2344[a]	193	<0.0001	0.3795	0.2672
Shannon[1]	6.20[a]	8.61[b]	0.164	7.33[a]	7.48[a]	0.166	7.14[a]	7.51[a]	7.57[a]	0.228	<0.0001	0.3679	0.1489
Fecal	**Summer**	**Winter**		**1**	**≥ 2**		**0–44**	**45–99**	**≥100**				
n=73	n=36	n=37	**SED**	n=34	n=39	**SED**	n=16	n=14	n=43	**SED**	**Season**	**Parity**	**DIM**
Chao1	2280[a]	2553[b]	65.1	2573[a]	2260[b]	82.3	2343[a]	2422[a]	2484[a]	70.9	0.0012	0.0025	0.2849
Shannon	8.12[a]	8.58[b]	69.1[†]	8.33[a]	8.37[a]	73.5[†]	8.22[a]	8.44[a]	8.39[a]	97.2[†]	0.0042[†]	0.7403[†]	0.4089[†]

[†]Box-cox transformation used. [1]Removed one outlier (sample ID 35.3). Cow ID was included as a random factor. A difference in lettering between two least squares means for factors of a variable indicates a significant difference with $P<0.05$. SED=Standard error of the difference in least squares means. ANOVA models developed with the Mixed procedure in SAS 9.4.

Table 2.14 ANOVA results comparing bacterial richness and diversity indicators of straw bedding between seasons. Comparisons made for a single straw bedded farm between two time-points in different seasons.

	Summer n=12	**Winter** n=12	**SED**	**P-value**
Chao1	1587	1387	0.066*	0.3379
Shannon	6.97	6.81	0.37	0.6810

*Log transformation used. SED=Standard error of the difference in least squares means. ANOVA models developed with the Mixed procedure in SAS 9.4.

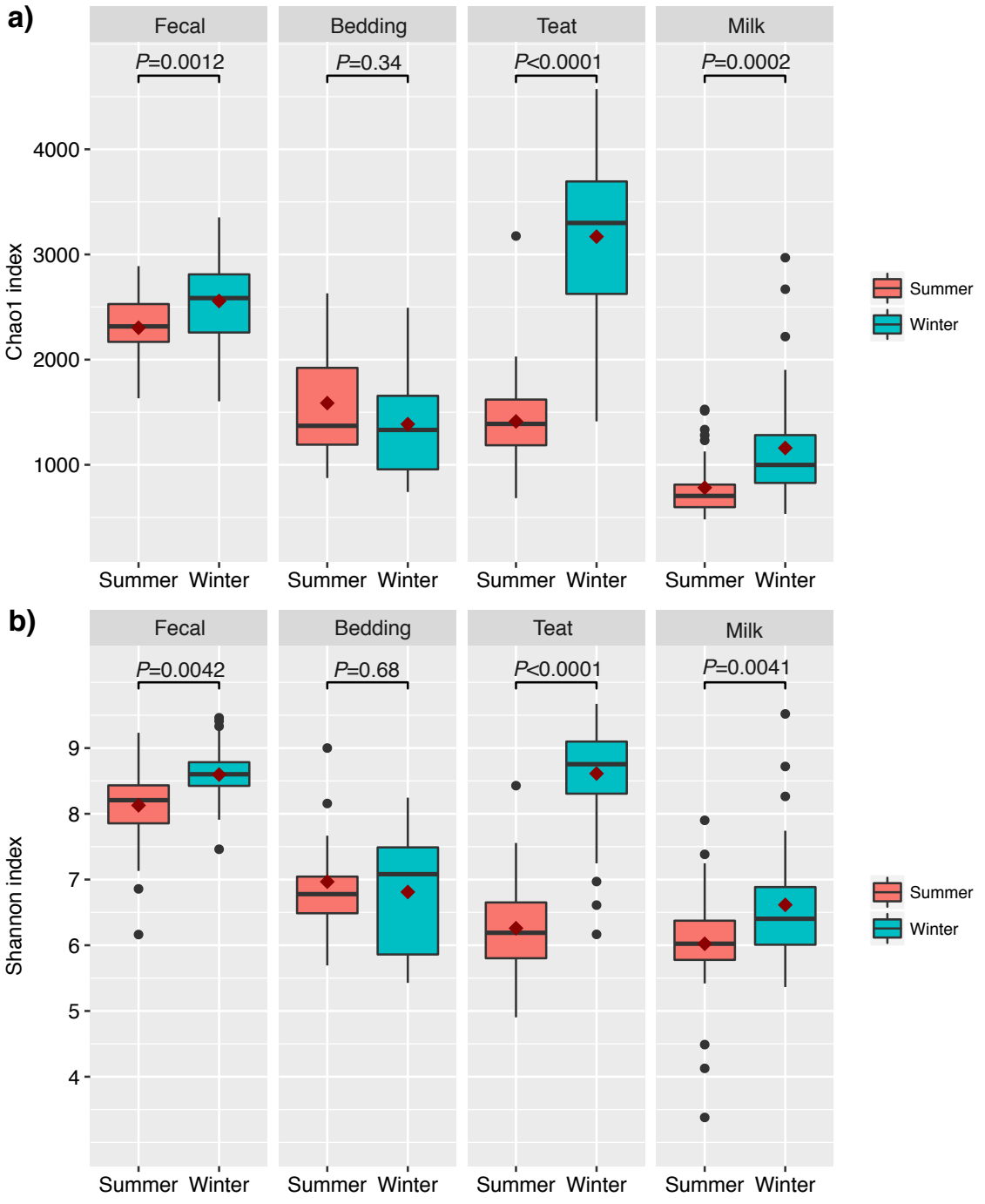

Figure 2.13 Comparisons of bacterial richness and diversity between seasons for all sites on farm ST. **(a)** Chao1 index (Bacterial richness) **(b)** Shannon index (Bacterial diversity). Boxplots with line showing the median, and red diamond showing the mean. *P*-values were obtained from ANOVA models (Proc Mixed) summarized in Table 2.10(host-sites) and Table 2.11(bedding). For fecal, teat-apex and milk analyses, only cows with a SCC ≤ 150,000 cells/ml were included, and additional variables included were parity (primiparous vs. multiparous) and lactation stage (early: 1-44 DIM, mid: 45-99 DIM and late: ≥100 DIM). For bedding, season was the only variable used.

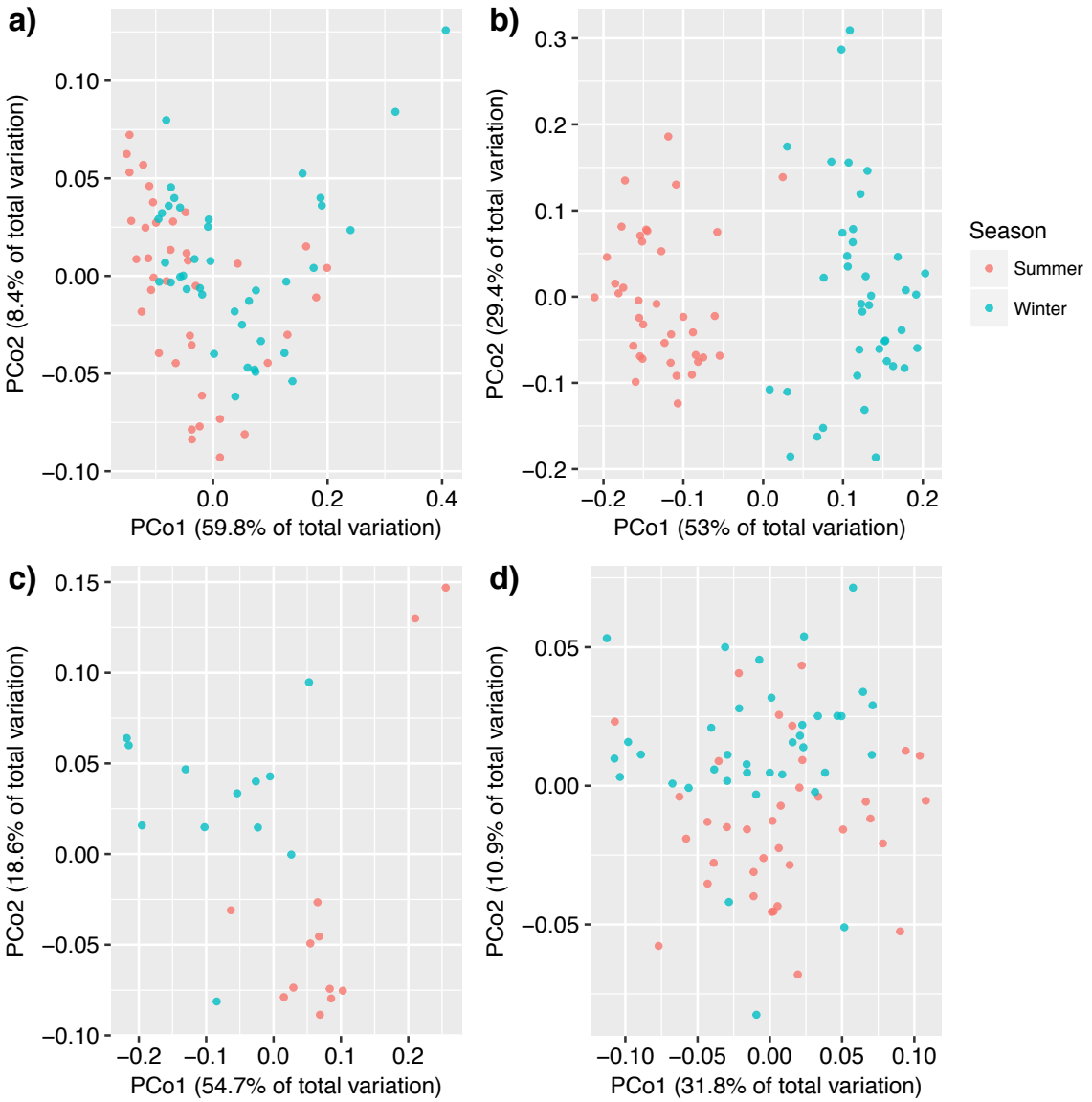

Figure 2.14 Principal coordinate analysis (PCoA) of weighted UniFrac distance matrices comparing bacterial diversity between seasons for each site on farm ST. **(a)** Milk samples **(b)** Teat-apex swab samples **(c)** Fecal samples **(d)** Bedding samples. For host-sites, only cows with a milk SCC below 150,000 cells/ml were used. For all sites, bacterial diversity was distinct between seasons with $P_{(PERMANOVA)}$=0.0002 or lower. [PERMANOVA models for host-sites also included parity (primiparous vs. multiparous) and lactation stage (early: 1-44 DIM, mid: 45-99 DIM, and late: ≥ 100 DIM) as additional explanatory variables]

2.4.11 The bacterial composition of samples during summer and winter within the straw bedded farm

We assessed associations between bacterial phyla present in at least 25% of samples and available metadata for winter and summer time-points on the straw bedded farm, once

more using the multivariate method MaAsLin (Additional file 10). Through this method, the relative abundance of Firmicutes in milk was associated positively with LogSCC ($P_{(FDR)}$=0.0277), while the relative abundance of Proteobacteria in milk was associated negatively with LogSCC ($P_{(FDR)}$=0.03). However, neither of these phyla were in differing average relative abundances between seasons (Figure 2.15).

Of the four main phyla in milk, only Bacteroidetes differed significantly in relative abundance between seasons ($P_{(FDR)}$<0.05), being in higher relative abundance during the winter sampling time-point compared to the summer time-point (Figure 2.15). In accordance with this, there was a higher average relative abundance of Bacteroidetes on teat-apices of cows sampled during the winter compared to the summer time-point ($P_{(FDR)}$ < 0.0001, Figure 2.15). Along with this, Proteobacteria was in a higher average relative abundance on teat-apices of cows sampled during the winter compared to the summer time-point ($P_{(FDR)}$ < 0.0001, Figure 2.15). Further, Proteobacteria also tended to be in a higher average relative abundance on bedding sampled during the winter compared to the summer time-point ($P_{(FDR)}$=0.0788, Figure 2.15). Additionally, Actinobacteria and Firmicutes both had lower average relative abundances on teat-apices of cows sampled in the winter compared to the summer time-point ($P_{(FDR)}$=0.0005 and $P_{(FDR)}$=0.0037, respectively, Figure 2.15). No other significant differences were observed in the relative abundances of the four main phyla, between the two sampling dates, within any site.

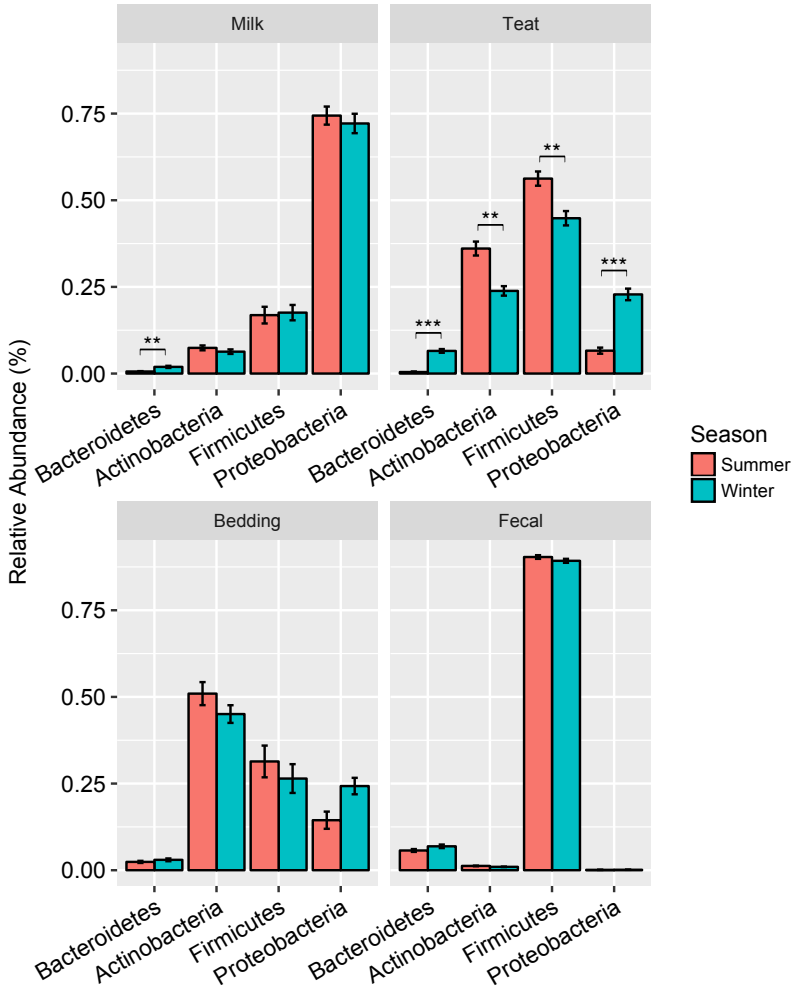

Figure 2.15. Average relative abundances of four main phyla within milk, teat-apices, bedding and feces between seasons on farm ST. *P*-values for comparisons in phylum relative abundance between seasons: * = $P_{(FDR)}$<0.05, ** = $P_{(FDR)}$<0.01, *** = $P_{(FDR)}$<0.0001. *P*-values were obtained from the MaAsLin analyses used to associate metadata with phyla abundances within teat-apex, bedding and fecal samples for the between-seasons dataset. Error bars represent standard error of the mean.

2.5 Discussion

This current research provides important insights for future research looking at commensal teat-apex and mammary microbial communities to consider. Specifically, identifying natural variation in the composition and diversity of commensal udder microbial communities is an important consideration to identify specific differences between commensal microbial communities compared to those observed in diseased states, rather than differences due to natural variation in the healthy commensal mammary microbiome. Further, the identification of teat-apex and milk microbiota associated with reduced mammary inflammation by our current research, is a first step in identifying groups of microbiota that may be used as alternatives to antibiotic therapies for the prevention and/or treatment of mastitis.

Using 16S rRNA gene sequencing, we identified large differences in the bacterial composition and diversity of milk between the three farms, as well as differences between seasons, even for Holstein cows with similarly low levels of mammary inflammation. Along with this, came differences in the bacterial profile of teat-apices and feces of these cows. Of note, is that differences observed, both in the bacterial diversity and composition of sites between farms, and in the mammary inflammatory status of cows between farms, is not necessarily due to differences in bedding material used, and is in fact more likely the result of a combination of different management practices used on each of these farms. Some of these management practices that can be of importance to the overall mammary health of a herd were recorded for each farm and are reported in Table 2.1.

We found that cows with low mammary inflammation on farm ST had substantially higher teat-apex bacterial richness and diversity in comparison to farm SA and farm RBM (Figure 2.4). Further, cows with low mammary inflammation on farm ST had a substantially lower milk bacterial diversity in comparison to milk on farm RBM and farm SA (Figure 2.4). These two findings are consistent with those identified when teat-apices as well as milk from cows of all milk SCC categories were included in similar analyses of variance (Table 2.5 and Table 2.6). It appears from these results, that a higher teat-apex bacterial richness and diversity may not in fact correspond to a higher milk bacterial richness and diversity. One possible explanation for this, is that numerous teat-apex bacterial groups found on the teat-apices of cows on farm ST are not adapted to the mammary gland environment. Additionally, cows sampled from farm ST had a significantly lower average SCC in comparison to farm RBM (Table 2.4), which was the only farm with sampled cows that had a SCC well above the common thresholds used for identifying subclinical mastitis (150,000 to 250,000 cells/ml). Farm ST having both a lower mammary inflammation and lower milk bacterial diversity compared to farm RBM appears to oppose some findings in previous research. Specifically, Oikonomou et al. (2014) and Ganda et al. (2016) found milk from healthy quarters to have a higher bacterial diversity (Shannon index), in comparison to those with clinical mastitis. It may be that exceptionally high teat-apex bacterial diversity on farm ST had an important role in protecting the mammary gland against increases in mammary inflammation for cows on this farm, despite their overall low milk bacterial diversity. This is in light of additional previous research finding that bovine teat-apices from healthy quarters had a higher teat-apex bacterial diversity (Shannon index=2.47) compared to subclinical

quarters (Shannon index=2.27; *P*=0.08) and clinical quarters (Shannon index=2.25; *P*=0.067) (Braem et al., 2012). In addition to these previous findings, a well-known ecological concept, is that communities with high diversity among members are more likely to also exhibit redundancy of functionality in the community, such that a loss in community members due to environmental perturbations, is less likely to result in community dysfunction. This results in a community that is more stable and resistant to change in composition, such as the colonization of invasive species within the community (Girvan et al., 2005). Taken in the context of commensal teat-apex and milk microbiota, which do not cause the host harm, this would include resistance to colonization by mastitis pathogens. Based on the ANOVA model used in our research, teat-apex bacterial diversity of the low milk SCC group was only numerically higher (Shannon index=7.83) compared to the high SCC group (Shannon index=7.56, *P*=0.29, Table 2.6). Similarly, high SCC milk samples had a Shannon index only numerically lower than low SCC milk samples (6.8 compared to 7.1, *P*=0.11, Table 2.5). The same analysis on milk samples between summer and winter time-points yielded similar results, with milk bacterial diversity and richness of low SCC samples again, only being numerically higher in comparison to high SCC samples (6.2 compared to 5.8, *P*=0.11, Table 2.12). Inconsistencies in our research compared to previous research could be the result of multiple factors. Firstly, this trend of reduced teat-apex and milk bacterial diversity for mastitis quarters may be more distinct when comparing healthy milk to clinically mastitic milk or quarters associated with mastitis of a specific etiology, rather than focusing on healthy compared to subclinically mastitic milk. This is particularly apparent for milk bacterial diversity research, with research finding associations between

mammary health and bacterial diversity finding these trends specifically for culture-negative and *E. coli* associated clinical mastitis (Oikonomou et al., 2014, Ganda et al., 2016). Secondly, not all cows with a SCC above the threshold of 150,000 cells/ml actually have subclinical mastitis due to variation among cows, and as such, other thresholds are also used, such as 200,000 cell/ml (Dohoo and Leslie, 1991). Since using a continuous measure of mammary inflammation sidesteps the issue of picking a discrete cutoff value for subclinical mastitis, Spearman rank-order correlation between milk SCC and the bacterial diversity (Shannon index) of teat-apices was performed. This was done on a per-farm basis because farm was found to be the only significant factor influencing teat-apex bacterial diversity, while also being associated with milk SCC, and therefore would be a confounding variable. Through this method, only on farm RBM and farm SA did an increased milk SCC tend to be associated with reduced bacterial diversity of the corresponding teat-apex sample. In general, farm ST had an overall low mammary inflammation (low SCC) and overall high teat-apex bacterial diversity, and on this farm, reduced teat-apex bacterial diversity may in fact not have been a reason for, or indication of, increased mammary inflammation. Overall, these results indicate that while a cow's teat-apex bacterial diversity may be an important indication of mammary inflammation on certain farms, teat-apex bacterial diversity appears to generally, not distinctly differ between cows with a SCC below 150,000 cells/ml and those with a SCC above 150,000 cells/ml.

Similar to alpha-diversity analyses, PERMANOVA and PCoA of weighted UniFrac distances between milk samples did not show that high and low SCC milk samples clustered separately (Figure 2.2). However, comparisons between samples from low SCC

cows showed each farm to have compositionally distinct milk, teat-apex and fecal microbial communities, along with distinct bedding microbial communities (Figure 2.3). Similarly, for farm ST, fecal, teat-apex, milk and bedding microbial communities during the summer time-point were compositionally distinct from those during the winter time-point (Figure 2.14). These analyses indicate that the bacterial communities of milk, along with other host sites, can distinctly differ in bacterial composition, based on seasonal differences, and between farm-systems, independently from milk SCC.

Interestingly on farm ST, within-sample milk bacterial diversity and richness (Shannon and Chao1 indices) of cows below a SCC of 150,000 cells/ml was lower during the summer time-point compared to those in the winter (Figure 2.13). In a consistent manner, bacterial richness and diversity of teat-apex and fecal samples was lower during the summer time-point in comparison to winter (Figure 2.13). It cannot be confirmed that the overall bacterial richness and diversity of host-sites is influenced by season in this consistent manner, since only two time-points are available, nonetheless, this observation suggests a possible link between the bacterial diversity of host-sites. It is possible that the bacterial richness and diversity of milk is dependent, or at least related to, the diversity and richness of teat-apices and feces.

OTU2061 (*Aerococcus*) and OTU805 (*Facklamia*) were two OTUs whose abundance on the teat-apex was associated with reduced mammary inflammation. *Aerococcus* and *Facklamia* are among the most frequently identified bovine teat-apex and teat-canal microbiota (Braem et al., 2012, Braem et al., 2013, Falentin et al., 2016), and to our knowledge, no species of *Aerococcus* or *Facklamia* have previously been associated with mammary inflammation or mammary health. However, research on teat-apex microbiota

found *Aerococcus* to be one of three genera isolated from the bovine teat-apex that were capable of inhibiting the growth of *E. coli* and *Klebsiella* spp. *in-vitro*, and also inhibiting gram-positive mastitis pathogens, however only two colonies of *Aerococcus* were able to be isolated from the teat-apex (Woodward et al., 1987). This research suggests that some members of bovine teat-apex bacterial communities may limit the growth of mastitis pathogens on the teat-apex and thereby reduced the likelihood of an intramammary infection. Possible mechanisms for pathogen growth inhibition include the production of antimicrobial peptides, which has been identified for a bacterial strain from the bovine teat-apex (*Staphylococcus chromogenes* L217) previously (Braem et al., 2014).

In our research, OTU2061 (*Aerococcus*) and OTU805 (*Facklamia*) were also found to be non-random OTUs within bedding material (Figure 2.9b). Though the origin of these OTUs may not be bedding material, they may have the potential for proliferation in bedding and transfer from bedding onto teat-apices of cows**.** Of note, is that the sample size of bedding material on each farm may have been too low, and in conjunction with the large variation in microbial proportions that exist in these environmental materials, resulted in low statistical power. OTU2061 (*Aerococcus*) was numerically in a higher average proportion on bedding from farm ST (0.074% StdDev. 0.11%) compared to farm SA (0.0089% StdDev. 0.014%) and farm RBM (0.018% StdDev 0.028%), though with very high variation between samples (Figure 2.9b). Nonetheless, this difference was consistent with the finding that OTU2061 (*Aerococcus*) was in significantly higher proportions on teat-apices of cows from farm ST in comparison to the other two farms (Figure 2.9a).

OTU29503, within *Pseudomonas*, another OTU whose abundance on the teat-apex was associated with reduced mammary inflammation, was not present in feces or in bedding (Additional file 7), and therefore this OTU was may have been transmitted to the teat-apex from an alternative source. A primary source of *Pseudomonas* spp. in general, is known to be water (Mena and Gerba, 2009). Further, *Pseudomonas aeruginosa*, a species that causes mastitis, was previously associated with milking parlor water system contamination and was therefore thought to be transmitted during preparation for milking (Kirk and Bartlett, 1984). From this, Kuehn et al. (2013) suggested that other species of *Pseudomonas* may also originate from on-farm water sources. Further, Kuehn et al. (2013) found *Pseudomonas* to be associated with healthy quarters compared to clinically mastitic quarters, and along with our findings, is evidence for the existence of species within *Pseudomonas* that are beneficial to mammary health that may originate from on-farm water sources.

Considering that *Coprococcus* is known to be gut associated (Ezaki, 2015), it was surprising that the OTU within *Coprococcus* whose abundance on the teat-apex was associated with reduced mammary inflammation was not identified in at least 25% of fecal samples. OTU339570 (*Coprococcus*) was present in only a very small percentage of fecal samples on farm SA (7.7% of samples, mean 0.0019%, StdDev 0.0071%) and farm ST (2.3% of sample, mean 0.0004%, StdDev 0.0025%) (Additional file 7), suggesting the possibility that this particular OTU represents a rare gut microbe. What is particularly perplexing, is that *Coprococcus* is an obligate anaerobe (Ezaki, 2015), and therefore, the ability of this OTU to proliferate on the teat-apex is questionable. Nonetheless, *Coprococcus* has previously been identified in higher abundances in foremilk and teat-

canal samples from healthy quarters, in comparison to clinically mastitic quarters (Falentin et al., 2016). This indicates the possibility that the OTU we identified on the teat-apex within *Coprococcus,* may be a teat-canal or mammary gland inhabitant, rather than an actual teat-apex inhabitant. Nonetheless, OTU339570 (*Coprococcus*) is a potentially beneficial OTU to mammary health.

The finding that the abundance of OTU1636835 within the genera *Corynebacterium,* and OTU614083 within *Staphylococcus* on the teat-apex were associated with increased mammary inflammation is in agreement with common knowledge that *Corynebacterium* and *Staphylococcus* contain mastitis pathogens such as *C. bovis* and Coagulase negative staphylococci (CNS). OTU1636835 (*Corynebacterium*) and OTU614083 (*Staphylococcus*) were not found in any fecal samples, and only OTU1636835 (*Corynebacterium*) was found very sparsely abundant within bedding microbial communities (within two samples, Additional file 7). Bacterial groups within the aforementioned genera, specifically *C. bovis, Staphylococcus aureus,* and some species of CNS, are classified as contagious mastitis pathogens. Contagious pathogens, meaning they tend to be transmitted from cow-to-cow during the milking procedure (Brooks and Barnum, 1984, Watts, 1988), and this may also be the case for OTUs within the *Staphylococcus* and *Corynebacterium* genera identified here.

The finding that abundances of OTUs on the teat-apex were associated with reduced mammary inflammation suggests an interesting idea that specific bacterial groups could limit the proliferation of pathogens on the teat-apex, and therefore limit their entry into the teat-canal and mammary gland. Three out of four of these potentially beneficial teat-apex OTUs identified here were in higher abundance on farm ST compared to the other

farms, two of which were significantly higher on farm ST compared to both farms (*Coprococcus* and *Aerococcus* OTUs, Figure 2.9a). These particular OTUs may therefore be of more importance in contributing to the low mammary inflammation of cows sampled from farm ST. Similarly, the most highly abundant OTU in milk that was associated with reduced mammary inflammation (OTU325711, *Erythrobacteraceae*) was in significantly higher abundance on farm ST in comparison to farm SA and farm RBM (Figure 2.8a), and may be one of many contributing factors to the low mammary inflammation on this farm as well.

As for the OTU within milk associated with increased mammary inflammation (N.R.OTU2358, *AKIW874*), though it was in low relative abundance in milk, it was consistently in a higher abundance on farm RBM in comparison to both farm SA and ST within milk, on teat-apices as well as bedding, and was not present in fecal material (Figure 2.8b). This particular OTU may represent an environmental bacterial group whose proliferation within bedding on farm RBM contributes to its increased relative abundance within milk, and subsequently, may at least partially contribute to the higher milk SCC of cows sampled on farm RBM in comparison to farm SA and farm ST.

Four OTUs whose abundances within milk were associated with mammary inflammation were assigned to taxa that, to our knowledge, have not previously been associated with bovine mammary health in any way. Specifically, to our knowledge, no previous research has identified the family *AKIW874*, or bacterial groups within this family, to be associated with mastitis or increased mammary inflammation, nor to our knowledge is there research implicating *Lautropia, Rhodocyclaceae,* or *Erythrobacteraceae* to be associated with healthy quarters or reduced mammary inflammation. One possible

explanation for this is that, since the majority of previous milk microbiome research has focused on the genus-level or higher, variation in effects on mammary health existing at the OTU-level was missed. As for taxa that have been associated with mammary inflammation within milk, the genera *Nocardioides* has been associated with reduced LogSCC (Oikonomou et al., 2014), and this genus is within the family *Nocardioidaceae*, which was assigned to an OTU within milk that was associated with reduced mammary inflammation within our current study (OTU1936).

Contrary to our finding that OTU1108062, within *Sphingomonas*, tended to be associated with reduced mammary inflammation, Kuehn et al. (2013) found the genera *Sphingomonas* to be associated with clinical mastitis. The OTU found here may be a non-pathogenic species of *Sphingomonas,* or alternatively, its abundance in milk was generally not great enough to result in a negative effect on mammary inflammation, which may be the case as this OTU was in a low relative abundance, being below 0.05% across all farms (Figure 2.8a). While being in a low relative abundance does not mean a bacterial group won't have the expected effect on mammary inflammation per-say, a known major mastitis pathogen, *S. aureus*, was found in low proportions within the bacterial community of healthy quarters (Oikonomou et al., 2014). From this, the authors hypothesized that in small quantities, *S. aureus* may be part of the normal bacterial community of milk, and a similar case could be made for the OTU found here, within *Sphingomonas*.

As mentioned previously, when we looked at the 42 genera above an average relative abundance of 0.5%, *Staphylococcus* had the strongest correlation with SCC (Figure 2.10a), and *Staphylococcus* was also associated with LogSCC using the multivariate

method. This genus contains major mastitis pathogens *S. aureus* and *S. aglactiae,* along with minor mastitis pathogens, namely members of CNS. It may be that a number of OTUs assigned to *Staphylococcus* in the milk dataset are either minor or major mastitis pathogens, but that any one OTU individually was not present in sufficient quantities, or sufficient number of milk samples, to adequately identify an association with milk SCC. One other genera, of the four genera associated with increased mammary inflammation, *Enterococcus* (Figure 2.10a), has been found to contain a genetically diverse group of enterococci associated with bovine mastitis, including *E. faecium* and *E. faecalis* (Petersson-Wolfe et al., 2008). Additionally, the genus *Ralstonia,* associated with reduced SCC in milk in thi s research (Figure 2.10a), was also found in a higher relative abundance within healthy quarters, in comparison to clinical mastitis with culture negative results in previous research (Kuehn et al., 2013).

Of the 12 genera associated with reduced mammary inflammation, the majority had relatively weak correlation coefficients not exceeding a strength of –0.25 (Figure 2.10a). However, all 12 of these genera were within the phylum Proteobacteria, and all in a higher relative abundance on farm ST in comparison to farm RBM and SA (Figure 2.10a). In consensus with this, farm ST cows had a higher proportion of Proteobacteria within milk, in comparison to farm SA and RBM (Figure 2.6). In addition, associations that were specific to farm ST when comparing summer and winter sampling time-points existed, where Proteobacteria was associated with reduced LogSCC, meanwhile, Firmicutes was associated with increased LogSCC. Contrary to this, previous research has identified clinically mastitic quarters associated with *E. coli* to have a higher proportion of Proteobacteria in comparison to healthy quarters, which had a higher

proportion of Firmicutes along with a higher proportion of a number of other remaining phyla, and these differences were primarily thought to be the result of an increased proportion of *Enterobacteriaciaea*, the family containing the causative agent of mastitis, *E. coli* (Ganda et al., 2016). It is clear that not all members of Proteobacteria are known to cause mastitis, and those that are known to cause mastitis are more-so known as environmental opportunists, largely coliforms, which are transmitted from the environment more commonly in farms with low environmental hygiene (Bushnell, 1984, Smith and Hogan, 2008). Meanwhile, those known to cause mastitis within Firmicutes are more often coined as being contagious mastitis pathogens, such as *S. aureus*, which are more commonly transmitted from cow-to-cow through contaminated milking equipment (Bushnell, 1984). Our finding of the opposing associations with LogSCC for Proteobacteria and Firmicutes, identified for cows on farm ST, may be indicative of a particular etiology of increased mammary inflammation that is specific to farm ST. This may simply indicate, in other words, that increases in SCC for farm ST are more-so due to mastitis pathogens that tend to transmit contagiously and are within the phylum Firmicutes (e.g. *Staphylococcus*), and not as frequently transmit from environmental sources such as used bedding. Meanwhile, increases in mammary inflammation of farm SA and farm RBM may have been more so the result of a larger combination of pathogens, from environmental mastitis pathogens in Proteobacteria (e.g. *E. coli*), to additional pathogens within Actinobacteria (e.g. *C. bovis*), and Firmicutes.

Despite differences in the relative abundances of the four major phyla on teat-apices between the two time-points for farm ST, the milk bacterial profile at the phylum level remained fairly constant, with only Bacteroidetes differing significantly, with a higher

relative abundance during the winter time-point (Figure 2.15). Along with this, no phylum in a relative-abundance over 25% differed in relative abundance within feces between time-points (Additional file 10). This is in consensus with the idea that internal host-associated sites (milk and feces) have a more consistent bacterial profile, since the conditions they are subjected to are continually regulated, for instance, body temperature should be maintained at a constant temperature. On the other hand, in the external environment, more extreme differences in temperature and other conditions occur, therefore bacterial communities exposed to environmental conditions, such as those on the teat-apex, differ largely in composition based on these environmental conditions. Within the dataset comparing farm ST, SA and RBM during the winter, the abundance of the phylum Cyanobacteria on teat-apices was associated with reduced LogSCC. Cyanobacteria have been found among the eight most abundant phyla within milk in previous research (Bonsaglia et al., 2017), and in other research within the teat-canal, Cyanobacteria was found to be one of six phyla with a higher relative abundance in healthy quarters in comparison to clinically mastitic quarters (Falentin et al., 2016). This particular phylum was also in a higher abundance on teat-apices of cows on the straw bedded farm compared to the other farms, and consistent with this, was in a higher abundance within bedding materials on farm ST, suggested that bedding may be an important source of Cyanobacteria on farm ST. Though certain strains within Cyanobacteria are known to be present on the root-system of plants such as wheat (Karthikeyan et al., 2007, Nain et al., 2010), to our knowledge, wheat stalks, the compound that straw on Farm ST was made of, is not a known habitat of bacterial groups

within Cyanobacteria. Cyanobacteria may have contaminated bedding material on farm ST from an alternate source.

In terms of the overall teat-apex microbial community, based on the SourceTracker analysis, colonization of microbiota on teat-apices and in milk from bedding was lowest for farm ST (Figure 2.12, and Figure 2.11). Sand and manure solid bedding materials, such as those used on farm SA and farm RBM have much smaller particle sizes compared to straw. Bedding materials with a small particle size such as these are more likely to stick to the teat apex, while also increasing surface area for bacterial growth, increasing bacterial counts on the teat skin (Smith and Hogan, 2000). Additionally, the colonization of microbiota on both teat-apices and in milk from feces was highest for farm RBM (Figure 2.12 and Figure 2.11). Bedding material on farm RBM was, in addition to being fine, also developed from manure and readily supports the growth of microbiota from feces that contaminates used bedding, while sand is an inorganic material thought to support the growth of fewer microorganisms (Smith and Hogan, 2000).

Based on results from the SourceTracker analysis, the majority of milk microbiota were unable to be classified as coming from either teat-apices, bedding or fecal sources (figure 2.11). Similarly, no OTU associated with reduced mammary inflammation within milk was identified as a non-random OTU within teat-apex, bedding or fecal microbial communities (Additional file 7). One possible explanation for this is that a number of OTUs within milk are relatively rare within the environment, and as such, not detected in large proportions. These OTUs within may account for larger proportions of the mammary bacterial communities compared to environmental bacterial communities

simply because the mammary gland is a niche they are more adapted too, compared to other bacterial groups and therefore, are able to be identified in milk at the rarefaction depth used. For instance, two non-random OTUs associated with reduced LogSCC within milk were present in low relative abundances on the teat-apex; these were *Lautropia* and *Erythrobacteraceae* (Present in 18 and 7 of 137 samples respectively, Additional file 7). An additional explanation is that OTUs within milk colonized the mammary gland from teat-apices, bedding and/or feces at a previous point in time, when the bacterial composition of these sources was different than their composition during the time of sampling. For example, teat-apex swab samples were taken just prior to milking, or 8 to 12 hours prior to the last milking, and therefore, the teat-apex swab samples we took were more representative of a cow's exposure to microbiota in pens or stalls in comparison to what the bacterial composition of teat-apices would be just prior to the milking process. There may be a transient group of bacteria that are present on the teat-apex due to a change in teat-apex exposure from environmental microbiota within pens and on bedding, to bacterial groups on milking equipment, that do not remain present in sufficient proportions to be identified on the teat-apex after exposure of the teat-apex to environmental microbiota within pens and on bedding. In consensus with this, bedding was found to be the primary source of teat-apex microbiota on all farms (Figure 2.12a), and this would be expected for teat-apices prior to milking, while bacteria from bedding would not be expected to, at least to the same degree, dominate the teat-apex microbial communities just after milking, since milking requires teats to be cleaned and exposes them to additional sources of microbiota, such as milking equipment.

The finding that, on all farms, the proportion of milk microbiota originating from teat-apex and fecal microbial communities was associated with increased milk bacterial diversity and richness (Figure 2.11), suggests that the more milk bacterial groups originating from feces and teat-apices, the more diverse milk microbial communities are. This is in consensus with our previous finding that in general, teat-apex and fecal bacterial sources were of higher bacterial diversity compared to milk bacterial communities (Figure 2.5). Generally, from these analyses, it appears that the bacterial diversity and richness of the main bacterial sources contributing to a milk or teat-apex samples bacterial community tends to shape the richness and diversity of that milk or teat-apex samples bacterial community (Figure 2.11 and 2.12).

Further supporting this, an increased proportion of teat-apex microbiota originating from bedding material, for farm SA, was strongly associated with reduced teat-apex bacterial diversity, whereas for farm ST these associations were more moderate, and for farm RBM, bedding was not associated with reduced bacterial diversity (Figure 2.12). Additionally, sand bedding had a lower bacterial diversity than the other known contributing source on farm SA (feces), and lower compared to the sink (teat-apices) on farm SA as well, meanwhile this was not the case for farm RBM (Figure 2.5b). The bacterial diversity and richness of bedding on farm SA was also lower in comparison to both farm RBM and farm ST (Figure 2.4) and is likely attributed in part, by sand being an inorganic material that supports the growth of fewer microorganisms. Interestingly, along with a reduced teat-apex bacterial richness and diversity being associated with an increased proportion of microbiota on the teat-apex originating from bedding for farm SA, there was an increased SCC that tended to be associated with teat-apex samples

composed of a larger proportion of microbiota from bedding (*P*=0.051). This came with a reduced SCC associated with a higher proportion of OTUs originating from unknown sources (*P*=0.081, Figure 2.12c). These unknown sources may include those previously discussed (water, milking equipment), or natural bovine teat-apex microbiota such as *Aerococcus* and *Facklamia* that were previously discussed as being associated with reduced mammary inflammation.

2.6 Conclusions

We demonstrated here, an account of the dramatic differences between different farms, in the bacterial composition and diversity of milk, and teat-apices of cows with low levels of mammary inflammation, and also of cows from all levels of mammary inflammation combined. Differences were also observed in the proportional contribution of environmental sources to milk and teat-apex microbiota between farms. For instance, there was a higher contribution to milk bacterial communities by feces and bedding for farm RBM compared to the other farms, and this may reflect differences between the farm-systems management strategies. However, despite differences in management strategies between farms, consistency among farms in the major contributing sources of teat-apex and milk bacterial communities was demonstrated, and suggests a consistency in where milk and teat-apex microbiota originates from across farm-systems. For instance, the major known source of milk microbiota was teat-apices, followed by bedding material, and lastly, a small portion of the milk microbiota on each farm originated from feces.

While no difference in teat-apex or milk bacterial diversity between cows with a SCC below 150,000 cells/ml and those with a SCC above 150,000 cells/ml was apparent, on

the sand and RBM bedded farms, an increased teat-apex bacterial diversity tended to be associated with reduced SCC. Additionally, on farm SA, there was an increased SCC along with a reduced teat-apex bacterial diversity associated with teat-apex samples composed of a larger proportion of microbiota from bedding. Along with demonstrating the substantial influence that environmental sources may have in shaping teat-apex bacterial diversity, at least for particular farm-systems, these results also demonstrate the potential importance of high teat-apex bacterial diversity in limiting mammary inflammation.

Lastly, this research as it regards comparisons between farms, also provided evidence for the existence of specific bacterial groups that may be beneficial to mammary health within milk and on teat-apices. Two of these potentially important OTUs within *Aerococcus* and *Facklamia*, along with one phylum, *Cyanobacteria*, all having abundances on the teat-apex associated with reduced mammary inflammation, may also contain bacterial groups that have potential to inhabit bedding material, possibly as a source for transmission to the teat-apices of cows. For some bacterial groups associated with reduced mammary inflammation on teat-apices and in milk, differences in abundances between farms were also observed, though more research is needed to elucidate whether this has implications for herd-level mammary inflammatory status.

Additional results specifically for farm ST indicated that both the phylum-level milk bacterial profile and SCC of cows between summer and winter sampling time-points remained fairly consistent, however despite this, bacterial diversity of all sites were distinct between sampling days. It may be that management strategies on farm ST, such as frequent replacement of bedding material, are limiting the increases in SCC that are

often expected during summer compared to winter, despite reduced bacterial diversity of mammary and teat-apex microbial communities observed for the summer time-point.

A surprising finding specifically for farm ST, was the reduced LogSCC associated with increased proportions of Proteobacteria, and reduced proportions of Firmicutes within milk. Along with previous research identifying the opposite associations for a specific form of mastitis (Ganda et al., 2016), these findings suggest a possible paradigm of how the bacterial-profile of healthy quarters compared to mastitic quarters is influenced by the prevailing etiology of mastitis on a farm.

Future research focusing on the milk microbiome should consider the etiology of mastitis infections to be an important driver in the bacterial composition of milk. Along with this, consideration should be placed on environmental differences, such as seasonal differences, and differences in bedding management (e.g. bedding material used and bedding replacement regime), as substantial drivers in the bacterial diversity and composition of milk and teat-apices of cows, possibly even more-so than differences in mammary inflammation as measured using SCC. Consideration should also be placed on the possibility of utilizing bacterial groups with potential to promote mammary health, such as those found here, for applications in alternative mastitis prevention strategies. Before this can occur however, these bacterial groups must be cultured and their effects on mammary health confirmed, which may include identifying specific mechanisms for their promotion of mammary health, such as the production of antibacterial compounds that inhibit the proliferation of mastitis pathogens. Alternative mastitis prevention strategies could include creating an optimal environment for the growth of beneficial bacterial groups within environmental sources of milk and/or teat-apex microbiota, or

applying these microbial groups directly to the teat-apex as a prophylactic therapy for mastitis.

REFERENCES

Addis, M. F., A. Tanca, S. Uzzau, G. Oikonomou, R. C. Bicalho, and P. Moroni. 2016. The bovine milk microbiota: insights and perspectives from -omics studies. Mol. Biosyst. 12(8):2359-2372.

Bey, R. F., J. K. Reneau, and R. Farnsworth. 2002. Manage bedding to control bacteria and reduce udder infections. Pages 103-113 in Minnesota Dairy Health Conference. St. Paul, MN.

Bhatt, V. D., V. B. Ahir, P. G. Koringa, S. J. Jakhesara, D. N. Rank, D. S. Nauriyal, A. P. Kunjadia, and C. G. Joshi. 2012. Milk microbiome signatures of subclinical mastitis-affected cattle analysed by shotgun sequencing. J. Appl. Microbiol. 112(4):639-650.

Bonsaglia, E. C., M. S. Gomes, I. F. Canisso, Z. Zhou, S. F. Lima, V. L. Rall, G. Oikonomou, R. C. Bicalho, and F. S. Lima. 2017. Milk microbiome and bacterial load following dry cow therapy without antibiotics in dairy cows with healthy mammary gland. Sci. Rep. 7(1):8067.

Bradley, A. J. and M. J. Green. 2001. Adaptation of *Escherichia coli* to the bovine mammary gland. J. Clin. Microbiol. 39(5):1845-1849.

Braem, G., S. De Vliegher, B. Verbist, M. Heyndrickx, F. Leroy, and L. De Vuyst. 2012. Culture-independent exploration of the teat apex microbiota of dairy cows reveals a wide bacterial species diversity. Vet. Microbiol. 157(3-4):383-390.

Braem, G., S. De Vliegher, B. Verbist, V. Piessens, E. Van Coillie, L. De Vuyst, and F. Leroy. 2013. Unraveling the microbiota of teat apices of clinically healthy lactating dairy cows, with special emphasis on coagulase-negative staphylococci. J. Dairy Sci. 96(3):1499-1510.

Braem, G., B. Stijlemans, W. Haken, S. Vliegher, L. Vuyst, and F. Leroy. 2014. Antibacterial activities of coagulase‐negative staphylococci from bovine teat apex skin and their inhibitory effect on mastitis-related pathogens. J. Appl. Microbiol. 116(5):1084-1093.

Brooks, B. and D. Barnum. 1984. The susceptibility of bovine udder quarters colonized with *Corynebacterium bovis* to experimental infection with *Staphylococcus aureus* or *Streptococcus agalactiae*. Can. J. Comparat. Med. 48(2):146.

Bryan, C. 1947. Penicillin in the treatment of infectious bovine mastitis. Am. J. Public Health N. 37(9):1147-1150.

Burvenich, C., V. Van Merris, J. Mehrzad, A. Diez-Fraile, and L. Duchateau. 2003. Severity of E. coli mastitis is mainly determined by cow factors. Vet. Res. 34(5):521-564.

Bushnell, R. B. 1984. The importance of hygienic procedures in controlling mastitis. The Veterinary clinics of North America. Large Anim. Pract. 6(2):361-370.

Canadian Dairy Information Centre (CDIC). 2017. Culling and replacement rates in dairy herds in Canada. Accessed Oct. 19, 2017. http://www.dairyinfo.gc.ca/index_e.php?s1=dff-fcil&s2=mrr-pcle&s3=cr-tr.

Caporaso, J. G., K. Bittinger, F. D. Bushman, T. Z. DeSantis, G. L. Andersen, and R. Knight. 2010a. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26(2):266-267.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Peña, J. K. Goodrich, J. I. Gordon, and G. A. Huttley. 2010b. Qiime allows analysis of high-throughput community sequencing data. Nat. Methods 7:335-336.

Clarke, K. R. and R. N. Gorley, inventors. 2015. Primer v7: User Manual/Tutorial. New Zealand Pat. No.

De Vliegher, S., H. Laevens, L. Devriese, G. Opsomer, J. Leroy, H. Barkema, and A. de Kruif. 2003. Prepartum teat apex colonization with *Staphylococcus chromogenes* in dairy heifers is associated with low somatic cell count in early lactation. Vet. Microbiol. 92(3):245-252.

De Vliegher, S., G. Opsomer, A. Vanrolleghem, L. A. Devriese, O. C. Sampimon, J. Sol, H. W. Barkema, F. Haesebrouck, and A. de Kruif. 2004. In vitro growth inhibition of major mastitis pathogens by *Staphylococcus chromogenes* originating from teat apices of dairy heifers. Vet. Microbiol. 101(3):215-221.

Dego, O. K., J. Van Dijk, and H. Nederbragt. 2002. Factors involved in the early pathogenesis of bovine *Staphylococcus aureus* mastitis with emphasis on bacterial adhesion and invasion. A review. Vet. Quart. 24(4):181-198.

Derakhshani, H., H. M. Tun, and E. Khafipour. 2016. An extended single-index multiplexed 16S rRNA sequencing for microbial community analysis on MiSeq illumina platforms. J. Basic Microbiol. 56(3):321-326.

Dohoo, I. R. and K. Leslie. 1991. Evaluation of changes in somatic cell counts as indicators of new intramammary infections. Prev. Vet. Med. 10(3):225-237.

Doyle, C. J., D. Gleeson, P. W. O'Toole, and P. D. Cotter. 2017. Impacts of seasonal housing and teat preparation on raw milk microbiota: a high-throughput sequencing study. Appl. Environ. Microbiol. 83(2):e02694-02616.

Eckersall, P. and R. Bell. 2010. Acute phase proteins: Biomarkers of infection and inflammation in veterinary medicine. Vet. J. 185(1):23-27.

Eckersall, P., F. Young, A. Nolan, C. H. Knight, C. McComb, M. Waterston, C. Hogarth, E. Scott, and J. Fitzpatrick. 2006. Acute phase proteins in bovine milk in an experimental model of *Staphylococcus aureus* subclinical mastitis. J. Dairy Sci. 89(5):1488-1501.

Edgar, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26(19):2460-2461.

Edgar, R. C., B. J. Haas, J. C. Clemente, C. Quince, and R. Knight. 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27(16):2194-2200.

Erskine, R., R. Eberhart, L. Hutchinson, and S. Spencer. 1987. Herd management and prevalence of mastitis in dairy herds with high and low somatic cell counts. J. Am. Vet. Med. Assoc. 190(11):1411-1416.

Ezaki, T. 2015. *Coprococcus*. in Bergey's Manual of Systematics of Archaea and Bacteria. W. B. Whitman, ed. John Wiley & Sons, Inc.

Fairbrother, J.-H., S. Dufour, J. M. Fairbrother, D. Francoz, É. Nadeau, and S. Messier. 2015. Characterization of persistent and transient Escherichia coli isolates recovered from clinical mastitis episodes in dairy cows. Vet. Microbiol. 176(1):126-133.

Falentin, H., L. Rault, A. Nicolas, D. S. Bouchard, J. Lassalas, P. Lamberton, J.-M. Aubry, P.-G. Marnet, Y. Le Loir, and S. Even. 2016. Bovine teat microbiome analysis revealed reduced alpha diversity and significant changes in taxonomic profiles in quarters with a history of mastitis. Front. Microbiol. 7:480.

Frost, A., D. Wanasinghe, and J. Woolcock. 1977. Some factors affecting selective adherence of microorganisms in the bovine mammary gland. Infect. Immun. 15(1):245-253.

Ganda, E. K., R. S. Bisinotto, S. F. Lima, K. Kronauer, D. H. Decter, G. Oikonomou, Y. H. Schukken, and R. C. Bicalho. 2016. Longitudinal metagenomic profiling of bovine milk to assess the impact of intramammary treatment using a third-generation cephalosporin. Sci. Rep. 6:37565.

Girvan, M., C. Campbell, K. Killham, J. Prosser, and L. Glover. 2005. Bacterial diversity promotes community stability and functional resilience after perturbation. Environ. Microbiol. 7(3):301-313.

Godden, S., R. Bey, K. Lorch, R. Farnsworth, and P. Rapnicki. 2008. Ability of organic and inorganic bedding materials to promote growth of environmental bacteria. J. Dairy Sci. 91(1):151-159.

Gröhn, Y., D. J. Wilson, R. González, J. Hertl, H. Schulte, G. Bennett, and Y. Schukken. 2004. Effect of pathogen-specific clinical mastitis on milk yield in dairy cows. J. Dairy Sci. 87(10):3358-3374.

Grönlund, U., C. Hultén, P. D. Eckersall, C. Hogarth, and K. P. Waller. 2003. Haptoglobin and serum amyloid A in milk and serum during acute and chronic experimentally induced Staphylococcus aureus mastitis. J. Dairy Res. 70(4):379-386.

Halasa, T., K. Huijps, O. Østerås, and H. Hogeveen. 2007. Economic effects of bovine mastitis and mastitis management: A review. Vet. Quart. 29(1):18-31.

Haley, D., A. De Passillé, and J. Rushen. 2001. Assessing cow comfort: Effects of two floor types and two tie stall designs on the behaviour of lactating dairy cows. Appl. Anim. Behav. Sci. 71(2):105-117.

Harmon, R. J. 1994. Physiology of mastitis and factors affecting somatic cell counts. J. Dairy Sci. 77(7):2103-2112.

Harrison, E., J. Bonhotal, and M. Schwarz. 2008. Using manure solids as bedding - Final report. Ithaca, NY.

Hogan, J., K. Smith, K. Hoblet, P. Schoenberger, D. Todhunter, W. Hueston, D. Pritchard, G. Bowman, L. E. Heider, and B. Brockett. 1989a. Field survey of clinical mastitis in low somatic cell count herds. J. Dairy Sci. 72(6):1547-1556.

Hogan, J., K. Smith, K. Hoblet, D. Todhunter, P. Schoenberger, W. Hueston, D. Pritchard, G. Bowman, L. E. Heider, and B. Brockett. 1989b. Bacterial counts in bedding materials used on nine commercial dairies. J. Dairy Sci. 72(1):250-258.

Hogan, J., K. Smith, D. Todhunter, and P. Schoenberger. 1988. Rate of environmental mastitis in quarters infected with *Corynebacterium bovis* and *Staphylococcus* species. J. Dairy Sci. 71(9):2520-2525.

Hogan, J. and K. L. Smith. 2003. Coliform mastitis. Vet. Res. 34(5):507-519.

Hogan, J. and K. L. Smith. 2012. Managing environmental mastitis. Vet. Clin. North. Am. Food Anim. Pract. 28(2):217-224.

Hogan, J. S. and K. L. Smith. 1997. Bacteria counts in sawdust bedding. J. Dairy Sci. 80(8):1600-1605.

Holdaway, R., C. Holmes, and I. Steffert. 1996. A comparison of indirect methods for diagnosis of subclinical intramammary infection in lactating dairy cows. Aust. J. Dairy Technol. 51(2):64-71.

Hugenholtz, P., B. M. Goebel, and N. R. Pace. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. 180(18):4765-4774.

Husfeldt, A., M. Endres, J. Salfer, and K. Janni. 2012. Management and characteristics of recycled manure solids used for bedding in Midwest freestall dairy herds. J. Dairy Sci. 95(4):2195-2203.

Karthikeyan, N., R. Prasanna, L. Nain, and B. D. Kaushik. 2007. Evaluating the potential of plant growth promoting cyanobacteria as inoculants for wheat. Eur. J. Soil Biol. 43(1):23-30.

Kirk, J. and P. Bartlett. 1984. Nonclinical *Pseudomonas aeruginosa* mastitis in a dairy herd. J. Am. Vet. Med. Assoc. 184(6):671-673.

Kirkpatrick, M. A. and J. D. Olson. 2015. Somatic cell counts at first test: More than a number. Pages 53-56 in Proc. Natl. Mastitis Counc. Annu. Mtg., Memphis, TN.

Knights, D., J. Kuczynski, E. S. Charlson, J. Zaneveld, M. C. Mozer, R. G. Collman, F. D. Bushman, R. Knight, and S. T. Kelley. 2011. Bayesian community-wide culture-independent microbial source tracking. Nat. Methods 8(9):761-763.

Kuehn, J. S., P. J. Gorden, D. Munro, R. Rong, Q. Dong, P. J. Plummer, C. Wang, and G. J. Phillips. 2013. Bacterial community profiling of milk samples as a means to understand culture-negative bovine clinical mastitis. PLoS One 8(4):e61959.

Laevens, H., H. Deluyker, Y. H. Schukken, L. de Meulemeester, R. Vandermeersch, L. de Muelenaere, and A. de Kruif. 1997. Influence of parity and stage of lactation on the somatic cell count in bacteriologically negative dairy cows. J. Dairy Sci. 80:3219-3226.

Lammers, A., C. J. van Vorstenbosch, J. H. Erkens, and H. E. Smith. 2001. The major bovine mastitis pathogens have different cell tropisms in cultures of bovine mammary gland cells. Vet. Microbiol. 80(3):255-265.

Leach, K. A., S. C. Archer, J. E. Breen, M. J. Green, I. C. Ohnstad, S. Tuer, and A. J. Bradley. 2015. Recycling manure as cow bedding: Potential benefits and risks for UK dairy farms. Vet. J. 206(2):123-130.

Lobeck, K., M. Endres, E. Shane, S. Godden, and J. Fetrow. 2011. Animal welfare in cross-ventilated, compost-bedded pack, and naturally ventilated dairy barns in the upper Midwest. J. Dairy Sci. 94(11):5469-5479.

Locatelli, C., L. Scaccabarozzi, A. Casula, F. Gorrieri, A. Harouna, and P. Moroni. 2008. Manure solids bedding as a source of clinical environmental mastitis. Pages 224-225 in Proc. Natl. Mastitis Counc. Annu. Mtg., New Orleans, LA.

Lok, C. 2015. Mining the microbial dark matter. Nature 522(7556):270.

Magoč, T. and S. L. Salzberg. 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27(21):2957-2963.

Medrano-Galarza, C., J. Gibbons, S. Wagner, A. De Passillé, and J. Rushen. 2012. Behavioral changes in dairy cows with mastitis. J. Dairy Sci. 95(12):6994-7002.

Mena, K. D. and C. P. Gerba. 2009. Risk assessment of *Pseudomonas aeruginosa* in water. Pages 71-115 in Reviews of Environmental Contamination and Toxicology. Vol. 201. Springer.

Morgan, X. C., T. L. Tickle, H. Sokol, D. Gevers, K. L. Devaney, D. V. Ward, J. A. Reyes, S. A. Shah, N. LeLeiko, S. B. Snapper, A. Bousvaros, J. Korzenik, B. E. Sands, R. J. Xavier, and

C. Huttenhower. 2012. Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. Genome Biol. 13(9):R79.
Murata, H., N. Shimada, and M. Yoshioka. 2004. Current research on acute phase proteins in veterinary diagnosis: an overview. Vet. J. 168(1):28-40.
Nain, L., A. Rana, M. Joshi, S. D. Jadhav, D. Kumar, Y. Shivay, S. Paul, and R. Prasanna. 2010. Evaluation of synergistic effects of bacterial and cyanobacterial strains as biofertilizers for wheat. Plant Soil 331(1-2):217-230.
National Mastitis Council (NMC). 2011. Recommended mastitis control program. North American version. Accessed Oct. 19, 2017. http://www.nmconline.org/wp-content/uploads/2016/08/RECOMMENDED-MASTITIS-CONTROL-PROGRAM.pdf, New Prague, MN, USA.
Ogola, H., A. Shitandi, and J. Nanua. 2007. Effect of mastitis on raw milk compositional quality. J. Vet. Sci. 8(3):237-242.
Oikonomou, G., M. L. Bicalho, E. Meira, R. E. Rossi, C. Foditsch, V. S. Machado, A. G. Teixeira, C. Santisteban, Y. H. Schukken, and R. C. Bicalho. 2014. Microbiota of cow's milk; distinguishing healthy, sub-clinically and clinically diseased quarters. PLoS One 9(1):e85904.
Oikonomou, G., V. S. Machado, C. Santisteban, Y. H. Schukken, and R. C. Bicalho. 2012. Microbial diversity of bovine mastitic milk as described by pyrosequencing of metagenomic 16s rDNA. PLoS One 7(10):e47671.
Olde Riekerink, R. G., H. W. Barkema, D. F. Kelton, and D. T. Scholl. 2008. Incidence rate of clinical mastitis on Canadian dairy farms. J. Dairy Sci. 91(4):1366-1377.
Ostrum, P. G., M. J. Thomas, and R. N. Zadoks. 2008. Dried manure solids for freestall bedding: Experiences from a Northeast dairy. Pages 149-156 in Proc. Natl. Mastitis Counc. Annu. Mtg., New Orleans, LA.
Pankey, J., S. Nickerson, R. Boddie, and J. S. Hogan. 1985. Effects of *Corynebacterium bovis* infection on susceptibility to major mastitis pathogens. J. Dairy Sci. 68(10):2684-2693.
Paulson, J. N., O. C. Stine, H. C. Bravo, and M. Pop. 2013. Differential abundance analysis for microbial marker-gene surveys. Nat. Methods 10(12):1200-1202.
Petersson-Wolfe, C., S. Adams, S. Wolf, and J. Hogan. 2008. Genomic typing of enterococci isolated from bovine mammary glands and environmental sources. J. Dairy Sci. 91(2):615-619.
Pillai, S., E. Kunze, L. Sordillo, and B. Jayarao. 2001. Application of differential inflammatory cell count as a tool to monitor udder health. J. Dairy Sci. 84(6):1413-1420.
Price, M. N., P. S. Dehal, and A. P. Arkin. 2010. FastTree 2–approximately maximum-likelihood trees for large alignments. PloS one 5(3):e9490.
Pyörälä, S., H. Jousimies-Somer, and M. Mero. 1992. Clinical, bacteriological and therapeutic aspects of bovine mastitis caused by aerobic and anaerobic pathogens. Brit. Vet. J. 148(1):54-62.
Pyörälä, S. and E. O. Pyörälä. 1998. Efficacy of parenteral administration of three antimicrobial agents in treatment of clinical mastitis in lactating cows: 487 cases (1989-1995). J. Am. Vet. Med. Assoc. 212(3):407-412.
Rendos, J. J., R. J. Eberhart, and E. M. Kesler. 1975. Microbial populations of teat ends of dairy cows, and bedding materials. J. Dairy Sci. 58(10):1492-1500.

Rodríguez, J. M. 2014. The origin of human milk bacteria: is there a bacterial entero-mammary pathway during late pregnancy and lactation? Adv. Nutr. 5(6):779-784.
Rollin, E., K. Dhuyvetter, and M. Overton. 2015. The cost of clinical mastitis in the first 30 days of lactation: An economic modeling tool. Prev. Vet. Med. 122(3):257-264.
Rowbotham, R. F. and P. L. Ruegg. 2016a. Associations of selected bedding types with incidence rates of subclinical and clinical mastitis in primiparous Holstein dairy cows. J. Dairy Sci. 99(6):4707-4717.
Rowbotham, R. F. and P. L. Ruegg. 2016b. Bacterial counts on teat skin and in new sand, recycled sand, and recycled manure solids used as bedding in freestalls. J. Dairy Sci. 99(8):6594-6608.
Sam Ma, Z., Q. Guan, C. Ye, C. Zhang, J. A. Foster, and L. J. Forney. 2015. Network analysis suggests a potentially 'evil' alliance of opportunistic pathogens inhibited by a cooperative network in human milk bacterial communities. Sci. Rep. 5:8275.
Sargeant, J., K. Leslie, J. Shirley, B. Pulkrabek, and G. Lim. 2001. Sensitivity and specificity of somatic cell count and california mastitis test for identifying intramammary infection in early lactation. J. Dairy Sci. 84(9):2018-2024.
Schroeder, J. W. 2009. Mastitis control programs: Bovine mastitis and milking management.
Schukken, Y. H., R. N. González, L. L. Tikofsky, H. F. Schulte, C. G. Santisteban, F. L. Welcome, G. J. Bennett, M. J. Zurakowski, and R. N. Zadoks. 2009. CNS mastitis: Nothing to worry about? Vet. Microbiol. 134(1):9-14.
Schukken, Y. H., D. J. Wilson, F. Welcome, L. Garrison-Tikofsky, and R. N. Gonzalez. 2003. Monitoring udder health and milk quality using somatic cell counts. Vet. Res. 34(5):579-596.
Smith, K. L. and J. Hogan. 2000. Bedding's contribution to mastitis in dairy cows. Pages 265-270 in Proc. Dairy Housing and Equipment Systems: Managing and Planning for Profitability, CampHill, PA. Natural Resource, Agriculture, and Engineering Service, Cooperative Extension.
Smith, K. L. and J. S. Hogan. 1993. Environmental mastitis. Vet. Clin. North Am. Food Anim. Pract. 9(3):489-498.
Smith, K. L. and J. S. Hogan. 2008. Environmental mastitis: Know your opponent. Pages 1-7 in Proc. Natl. Mastitis Counc. Reg. Mtg., Green Bay, WI.
Smith, K. L., D. Todhunter, and P. Schoenberger. 1985. Environmental mastitis: cause, prevalence, prevention. J. Dairy Sci. 68(6):1531-1553.
Sordillo, L., K. Shafer-Weaver, and D. DeRosa. 1997. Immunobiology of the mammary gland. J. Dairy Sci. 80(8):1851-1865.
Todhunter, D. A., K. L. Smith, J. S. Hogan, and P. S. Schoenberger. 1991. Gram-negative bacterial infections of the mammary gland in cows. Am. J. Vet. Res. 52(2):184-188.
Tolle, A. 1980. The microflora of the udder. p 4. In factors influencing the bacteriological quality of raw milk. Int. Dairy Federation Bulletin 120.
USDA. 2016. Dairy 2014, Milk quality, milking procedures, and masitis on U.S Dairies. USDA-APHIS-VS-CEAH-NAHMS, Fort Collins, CO.
Verdier-Metz, I., G. Gagne, S. Bornes, F. Monsallier, P. Veisseire, C. Delbes-Paus, and M. C. Montel. 2012. Cow teat skin, a potential source of diverse microbial populations for cheese production. Appl. Environ. Microbiol. 78(2):326-333.

Vissio, C., S. A. Dieser, H. L. Agnelli, L. M. Odierno, and A. J. Larriestra. 2014. Accuracy of the composite somatic cell count to detect intra-mammary infection in dairy cows using latent class analysis. Prev. Vet. Med. 113(4):547-555.

Waage, S., T. Mørk, A. Røros, D. Aasland, A. Hunshamar, and S. Ødegaard. 1999. Bacteria associated with clinical mastitis in dairy heifers. J. Dairy Sci. 82(4):712-719.

Watts, J. L. 1988. Etiological agents of bovine mastitis. Vet. Microbiol. 16(1):41-66.

Wenz, J. R., S. M. Jensen, J. E. Lombard, B. A. Wagner, and R. P. Dinsmore. 2007. Herd management practices and their association with bulk tank somatic cell count on United States dairy operations. J. Dairy Sci. 90(8):3652-3659.

Wickham, H. 2009. ggplot2: Elegant graphics for data analysis. I. Springer-Verlag New York, ed.

Wilson, D. J., R. N. Gonzalez, and H. H. Das. 1997. Bovine mastitis pathogens in New York and Pennsylvania: prevalence and effects on somatic cell count and milk production. J. Dairy Sci. 80(10):2592-2598.

Woodward, W. D., T. E. Besser, A. C. Ward, and L. B. Corbeil. 1987. In vitro growth inhibition of mastitis pathogens by bovine teat skin normal flora. Can. J. Vet. Res. 51(1):27-31.

Zadoks, R., B. Gillespie, H. Barkema, O. Sampimon, S. Oliver, and Y. Schukken. 2003. Clinical, epidemiological and molecular characteristics of *Streptococcus uberis* infections in dairy herds. Epidemiol. infect. 130(02):335-349.

Zadoks, R. N., J. R. Middleton, S. McDougall, J. Katholm, and Y. H. Schukken. 2011. Molecular epidemiology of mastitis pathogens of dairy cattle and comparative relevance to humans. J. Mammary Gland Biol. 16(4):357-372.