Ecology Of Composted Bedded Pack And Its Impact On The Udder Microbiome With An Emphasis On Mastitis Epidemiology

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ECOLOGY OF COMPOSTED BEDDED PACK AND ITS IMPACT ON THE UDDER MICROBIOME WITH AN EMPHASIS ON MASTITIS EPIDEMIOLOGY

A Thesis Presented

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Tucker D. Andrews

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ABSTRACT

Infections of the cow udder leading to mastitis and lower milk quality are a critical challenge facing northeast organic dairy farmers. Limited mastitis treatment options are available to organic producers and bedding systems impact cow health, including mastitis risk. Composted bedded pack, a system touted for increased cow comfort and well-being, allows stratified accumulation of bedding and manure in the barn. This method is gaining popularity among organic producers, yet little is known about the microbiota of the accumulated pack and its interaction with the cow mammary gland. An in-depth single farm study was conducted that surveyed bedded pack (microbiome and microarthropod community), dipteran vectors of bacterial mastitis pathogens, and the teat skin and teat cistern milk microbiomes. Comparisons were made with four additional farms utilizing bedded packs to test generality of results.

Few fly pests were observed in the bedded pack. However, bedding on all farms was found to harbor the mesostigmatid mite genus Glypholaspis, a well-established predator of nematodes and muscid fly larvae, suggesting that predators may suppress populations of biting flies in bedded pack barns. Additionally, the fungivorous genus Rhizoglyphus was commonly abundant in all farms, suggesting that the mite community regulates microbial activity at multiple trophic levels.

High-throughput sequencing of universal marker genes for bacterial and fungal communities was used to characterize the skin and milk microbiome of cows with both a healthy and infected quarter on the case study farm, and the composted bedded pack of all five farms. The bedded pack microbiome varied with bedding material and management style; fungal taxa were primarily yeasts of the Ascomycota; all farms additionally contained anaerobic fungi associated with the bovine rumen. Common bacterial genera included Acinetobacter and Pseudomonas, both of which were also commonly observed on teat skin and in milk. The udder microbiome varied through time and between skin and milk. Both healthy and infected milk microbiomes reflected a diverse group of microbial DNA sequences. Health status of the quarter changed whether taxa were shared between the teat skin, milk, and bedding. Proportion of taxa shared between healthy milk and skin was stable while taxa shared in infected quarters varied widely. Taxa shared among all habitats included yeast genus Debaryomyces and bacteria Acinetobacter guillouiae.

Results support an ecological interpretation of both the udder and the bedded pack environment and support the notion that mastitis can be described as an imbalance of the healthy mammary gland microbiome. Future work might compare udder health between common bedding practices, investigating the impact of bedding on the microbiota of the mammary gland in the healthy and diseased state.
Dedicated to the tiny Chulengo
ACKNOWLEDGEMENTS

In the summer of 2015 I made a choice to re-route my career in agriculture from field production toward science-based inquiry. Years of field observations had left me with questions about the interaction between plants and soil. I was not satisfied with the answers I found in the popular literature and did not possess the language or concepts of biology to read deeper. I began to reach out to labs around the country, hoping that my nearly complete lack of science background would not limit my pursuit of advanced scientific work. Deb Neher was willing to take a chance on me, for which I am very grateful. She helped me structure and supported me through a whirlwind education in biochemistry, metagenomics, ecology, and statistics, providing invaluable advice in navigating graduate school and scientific writing. Tom Weicht has been a patient (re)explainer many times over of both laboratory methods and theoretical aspects of microbial ecology. I am indebted to his approach to science as I continue my own journey. John Barlow has been a wellspring of creative criticisms as I waded through sequence data and has been my guide to mastitis epidemiology. John and Deb received the funding from the UVM Dairy Center of Excellence that allowed me to conduct this research and pursue my M.S.; thank you! Sid Bosworth provided a reality check for the too-often abstracted language of microbial metagenomics. I am also grateful for the help I received from Olivia Schrantz and Russell Frisch sampling bedded pack, the Barlow Lab for processing milk and teat samples, and the Soil Ecology Bioindicators Lab for enduring my lab meeting rants as I learned my way through analyzing and visualizing massive data sets. I am grateful for the generosity and encouragement of Steve Keller
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Chapter 1. Microbiome of bovine teat end skin and teat cistern

INTRODUCTION

Mastitis remains one of the costliest health concerns of dairy cattle in the US. Organic dairy farmers in the northeastern US identify mastitis as a top animal health challenge area and mastitis control as a key research priority (Pereira et al. 2013). Mastitis associated economic losses are due to lost or reduced milk production, increased veterinary and treatment costs, increased labor, low product quality resulting in low premiums, and animal replacement costs due to culling (Bar et al. 2008). Even after recovery, the udder of a cow may not return to optimal functional capacity (Akers and Nickerson 2011), resulting in prolonged production losses. Organic farmers cannot rely on antibiotics to treat infections; prevention and best management practices are, therefore, key to limiting mastitis (Ruegg, 2009). A full characterization of the microbial ecology of the udder in the healthy and diseased state presents an opportunity to explore how management practices might be used to build a mammary microbiome that is more resilient to infection, providing a tool to prevent mastitis on organic dairy farms.

Mastitis is defined as inflammation of the mammary gland, most commonly due to bacterial infection (Watts 1988, Bradley 2002). Mastitis pathogens include at least 135 culturable bacterial, fungal and algal species (Watts 1998). Classically, the common mastitis pathogens are divided into major and minor categories. Major pathogens such as Staphylococcus aureus, Streptococcus uberis and Streptococcus dysgalactiae typically cause subclinical or clinical mastitis episodes, often defined by substantial inflammation.
of the udder for a prolonged period accompanied by increased somatic cell count. Minor pathogens such as *Corynebacterium* spp. and many non-*Staphylococcus aureus* staphylococci are often associated with subclinical mastitis and typically cause less severe inflammation.

The diversity and abundance of microbes observed in healthy mammalian tissue sites using genetic sequencing of the microbial DNA is redefining the traditional understanding of the mammalian relationship with microbes, evincing a paradigm shift away from a simple host-pathogen interaction (Keeney et al. 2014). Instead, infection is linked with a dysbiosis, or imbalance, of a complex endogenous microbial community unobservable via culture-dependent techniques (Keeney et al. 2014, Rainard 2017, van Baarlen et al. 2013). Recent microbial DNA sequencing of the mammary gland has led some researchers to suggest that mastitis can be similarly understood within the framework of dysbiosis, though this is not without controversy, especially the presence of a healthy milk or intramammary microbiome (Oikonomou et al. 2014, Rainard 2017). For decades, mastitis researchers have described the healthy intramammary environment as sterile, with any introduction of bacteria leading to some degree of local or systemic inflammation (Rainard 2017). Even within the sterility paradigm, the possibility of beneficial or commensal microbiota is not without precedent. As early as 1965, the presence of corynebacteria was associated with fewer pathogen cell counts recovered from quarters challenged with *Staphylococcus aureus* (Newbould and Neave 1965). In the 1990’s it was observed that infection with environmental pathogen *E. coli* was decreased in quarters for which a post milking teat dip had been discontinued (Lam et al. 2013).
Intramammary infections with minor pathogens *Corynebacterium bovis* or coagulase-negative staphylococci had a “strong and significant” protective effect from new intramammary infection with a major pathogen in challenge studies (Reyher et al. 2012b). *Staphylococcus chromogenes* in particular has been demonstrated to provide a protective effect from intramammary infection (Matthews et al. 1990, de Vliegher et al. 2003). *In vitro* challenge studies have found isolates obtained from teat skin swabs or aseptically collected milk to be inhibitory of indicator pathogens (Al-Qumber and Tagg 2006, de Vliegher et al. 2004). Despite evidence for a protective effect from experimental studies or in vitro antagonism assays, most observational studies have provided evidence that minor pathogen infections provide no benefit or increase the risk of later infection with a major pathogen (Reyher et al. 2012a, Reyher et al. 2012b). The complexity of these dynamics provides a backdrop for recent culture-independent research that has begun to suggest a diversity and presence of microbes in milk and teats from healthy and infected quarters far greater than previously understood (Oikonomou et al. 2012, Oikonomou et al., 2014, Falentin et al. 2016, Kuehn et al. 2013, Braem 2013, Bhatt et al., 2012, Kuang et al., 2009). The sum of these approaches has led some authors to advocate using an ecological framework to assess the relationship of the microbial community to the host habitat as well as the interactions within microbial community itself (Vanderhaegen et al. 2015, Gill et al. 2006). After Vanderhaegen et al. 2015, who suggest the use of the term *habitat* to describe the udder as a microbial environment, we have adopted the use of the term “habitat state” though-
out the paper to generally describe the four habitats under scrutiny; the healthy and infected teat skin and the healthy and infected teat cistern.

A novel aspect of this study is the concurrent sampling of both teat end skin and intramammary milk to explore how the dynamics of microbial exchange between these two habitats differ in the healthy and infected state. We used the concept of a “core microbiome” to approach this question. This concept has been variously defined but typically suggests a group of microbes that are shared between environments; it must be parameterized depending on the question being addressed (Shade and Handelsman, 2012). One factor that affects the transmission of microbes between the teat end skin and the intramammary lumen is the health status of the quarter; infected intramammary glands are considered at greater risk of invasion through an inflamed teat canal or via increased milk leakage, whereas the normal functioning teat both physically occludes bacteria from the lumen and may induce the recruitment of leukocytes in the lumen upon detection in the teat canal (Peeler et al. 2000, Rainard and Riollet, 2006, Waller 1997, Bougarn et al. 2010). Thus, the proportion of the microbiome that is shared between teat end skin and intramammary milk might be expected to vary with infection status. Similarly, the relative abundance of OTUs that are found in each habitat state may vary with infection status. The parameters used to define core for this study emphasized persistence of an OTU through time and presence in each habitat state and each animal.

The present study uses high throughput DNA sequencing to compare abundances of bacterial and fungal DNA of teat end skin and teat cistern milk from mastitic and healthy quarters of organic dairy cattle. The primary objective was to characterize
putative communities and investigate dynamics of microbial DNA exchange between two host-habitats (teat end surface and intramammary milk) in the infected and healthy state.

METHODS

Experimental Design and Cow Selection

In this study, a single organic dairy herd was sampled in June, July, and August of 2015 and 2016. In year 1, sampling occurred twice per month, the first sampling served as a screening to identify animals and quarters to be sampled more extensively later in the month. For the first screening sample, all lactating quarters of all cows were sampled in duplicate using established aseptic procedures, followed by bacteriologic culture (Middleton et al. 2017). Briefly, 10 µl of each duplicate individual quarter milk samples was spread on a quarter of a tryptic soy agar with 5% sheep blood plate and incubated aerobically for 48 hours. Quarter milk somatic cell count (SCC; cells per ml milk) was measured using a Somacount 150 instrument (Bentley Instruments, Chaska, MN, USA). Quarters with three or more distinct colony morphologies were considered contaminated and removed from analysis. Mastitis was defined as a quarter with SCC ≥ 200,000 cells/ml. Intramammary infection (IMI) was designated if the same organism was isolated from the duplicate milk cultures. Quarters that had a SCC less than 100,000 cells/ml and no IMI were designated as healthy and uninfected. Cows were selected for resampling on the second visit if mastitis and IMI was detected in at least one quarter, and one or more other quarters of the same cow were defined as healthy.
On the second monthly visit, approximately two weeks later, a series of milk and teat end skin swabs was obtained from at least one infected quarter with mastitis, and one uninfected quarter from selected cows. Nitrile exam gloves were worn to sample each udder. Debris and gross visual skin contamination was removed from the udder, teat barrel and teat end by wiping with a dry disposable paper-towel. Prior to application of any pre-milking teat disinfectant, teat ends were swabbed using a nylon flocked swab (FloQSwab 502CS01, Copan Diagnostics Inc., Murrieta, CA, USA) moistened in sterile molecular grade DNA-free water. The distal portion of the swabs were returned to 5 ml molecular grade water in a 15 ml conical tube by breaking the shaft at the break-point, and swab samples were immediately placed on ice for transport to the laboratory. The teat end was then cleaned and disinfected using a series of 10x10 cm cotton gauze pads moistened in 70% ethanol, initial fore-milk was hand stripped from the quarter and subsequently milk samples were collected in duplicate by hand stripping (conventional milk sample). Teat cistern milk samples were then collected using a modification of the teat cannulation technique previously reported (Friman et al. 2017). In this modification, after the sterile plastic 34 mm teat cannula (J-12 Teat infusion Cannula, Jorgensen Laboratories, Loveland, CO, USA) was inserted into the teat canal, a sterile closed tip semi-rigid polypropylene 14 cm catheter (Argyle Tom Cat Catheter, Medtronic Animal Health, Minneapolis, MN, USA) was passed through the teat cannula approximately 10 cm into the teat cistern, and 5 ml of cisternal milk was aspirated directly from the teat cistern using a syringe attached to the catheter.
Control teat swab samples were collected in the barn, by removing swabs from individual wrapping, moistened in sterile molecular grade water, holding in the air adjacent to a cow flank for approximately 10 seconds, and returned to the transport vial. Aliquots of the sterile molecular grade water, not exposed to the barn environment, were also processed as parallel negative control samples in the laboratory each month.

Conventional milk samples were cultured and SCC measured as previously described for the first monthly sample to confirm infection and mastitis status. For initial processing, teat end swab samples were vortexed in a multi-tube platform vortex mixer at 2,500 rpm for 5 minutes, the swab was removed and 1 ml aliquots of suspensions stored at -20°C until further processing for culture independent analysis. Cisternal milk samples were divided into 1 ml aliquots and stored at -20°C until further processing for culture independent analysis.

IMI and subclinical mastitis due to Staphylococcus species was most common on this farm, and cows were selected for the second monthly sampling to represent the most common genera of pathogens causing IMI in the herd. From the selected cows for the second monthly sampling (6 to 12 per month), at least six cows were selected randomly for DNA sequencing of microbial community from teat end swab and teat cistern milk samples. Sequencing was performed on one healthy and one mastitic quarter from each cow; if more than one healthy or mastitic quarters had been identified, preference was given to quarters that would create an even distribution of udder sampling locations (left-front, rear-hind, etc.). In year two (2016), no screening was conducted prior to quarter sampling in each month; instead, cows identified in 2015 were tracked for a second year.
As in 2015, quarter SCC was measured, and culture was performed in duplicate to identify pathogens; one healthy and infected quarter from a random subset of sampled cows was submitted to microbial DNA sequencing. If a cow that had been sequenced was removed from the herd or became unavailable for sampling, another cow was randomly selected from the screened group as a replacement. In some cases, over the course of the study, individual quarters changed status from healthy to subclinical mastitis with IMI ($n = 10$). To preserve balanced observations between healthy and infected teats within cows, only paired observations of one healthy and one infected quarter for each cow on each date were kept. A total of 28 pairs of healthy and infected teat cistern milk and 29 pairs of healthy and infected teat end skin from 12 cows were included in the dataset ($n = 114$).

**Farm Description**

The farm studied is a 150-cow certified organic pasture-based dairy that feeds an all grass and hay ration. The herd is a mix of Holstein and Jersey that yield a farmer reported average of 20 kg milk per cow per day. While pasture is inaccessible (between November and April, weather dependent), cows are housed on a shredded dry hay bedded-pack with a wood chip base in two separate 18 x 43 m fabric covered “solar” hoop barns where they are fed round bale silage in circular feeders. Cows are milked twice per day with a high-line system in a tie-stall barn, with concrete cubical flooring bedded with sawdust or wood shavings.

The farm discontinued the use of teat disinfection for one year prior to the initiation of the study and resumed the practice at the mid-point of the study, in
September 2015. During the two years when there was no application of teat disinfectants, pre-milking hygiene consisted of dry wiping cows with disposable paper towels to remove debris and gross contamination, and there was no post-milking udder hygiene or management.

After reinstituting the use of teat disinfection, pre-milking hygiene consisted of dry wiping cow teats with disposable paper towels to remove debris and gross contamination, fore-stripping quarters, application of a pre-milking teat end disinfection, allowing for 60–90 seconds contact time, followed by removing teat disinfectant from all four teats with a single use disposable paper towel. The teat disinfectant used for both pre- and post-milking application was Quadra-Plex iodine liquid (IBA Inc., Millbury, MA, USA) containing 5% Nonylphenoxypolyethoxyethanol Iodine Complex (1.0% minimum titratable iodine), 10% emollients, and 85% other inert ingredients including buffering agents and surfactants (pH 5.5 at time of manufacture). All milking personnel routinely wore disposable nitrile gloves during all milking procedures.

**Extraction, Sequencing, Taxonomic Mapping**

DNA was extracted from 1 ml aliquots of preserved milk and teat swab samples using the MoBio PowerSoil Soil DNA Isolation kit following the manufacturer’s instructions, using methods and exceptions adapted from Lauber et al. (2006) as follows: bead tubes were incubated in a 65°C water bath for ten minutes and then vortexed for two minutes before proceeding with manufacturer’s instructions.
Extracted DNA samples were frozen at -80°C until shipped to the University of Colorado Next Generation Sequencing Facility (Boulder) for PCR amplification, sequencing, initial data filtering and taxonomic reference mapping. Amplification was performed in triplicate using 515F and 806R primers for V4-V5 region of bacterial/archaea 16S rRNA genes and ITS-1F and ITS-2R primers for fungal ITS-1 region. Sequencing was performed on the Illumina MiSeq platform. Cleaned and quality filtered reads were dereplicated before clustering at 97% nucleotide identity into representational OTU sequences via UCLUST (version 7) (Edgar 2010). Sequences were referenced to the August 2013 version of the Greengenes database (bacteria) and UNITE database (fungi) to produce combined Operational Taxonomic Unit (OTU) count and taxonomic information tables. Methods are as described in Emerson et al. (2015).

Normalization

A proportional scaling approach was used to compare between samples with disparate library sizes (McMurdie and Holmes, 2014). OTU counts within each library were converted to a proportion of total library size and scaling to the mean of all library sizes. This scaled value was truncated to obtain a whole number, further reducing OTU richness by eliminating OTUs with a scaled proportion less than 1.0 (OTUs with a scaled count of 0.0 were removed from the dataset). ITS and 16S sequence counts were normalized separately. This process retained 5328 of 6021 16S OTUs, which represented a 16% loss of OTU richness in 2015, zero loss in 2016 and preserved all samples.

ITS sequences were sparser; samples with less than 100 sequences read counts were removed retaining a total $n = 70$. Paired infected and healthy quarter observations
within animal were unable to be maintained. Normalization of ITS data retained 789 of 949 OTUs, a 17% loss of richness.

**Statistical Analysis**

Bray-Curtis dissimilarity matrices were generated to compare samples and non-metric multidimensional scaling was used to visually represent similarities between sample groupings. Abundances were square-root transformed before calculating dissimilarity. Permutational multivariate analysis of variance (PERMANOVA) was conducted using the *adonis* function in vegan (Oksanen et al., 2013) for multivariate analysis of main effects. For bacteria, models testing herd-level variation in the microbiome due to sampling year, sample type (skin or milk), and infection status were permuted within cow and restricted to a one-way time series to control for the effect of the host on the microbiome and relatedness due to repeated measures. Variation associated with the individual cow and sampling date within year were also tested separately. For fungi, free permutations were used. Due to the relative sparsity of fungal observations, permutations would have been overly restricted if the nested series design was maintained, thus free permutations were used to assess significance; results should be interpreted with this in mind. The function *betadisper* in vegan was used to compare homogeneity of within group variance as measured by the distance between sample locations in Euclidean space and their geometric centroid (Anderson 2006). For bacteria, a co-inertia analysis was performed using the *cia* function in the *made4* package for R to assess global relative similarity (RV), a multivariate extension of the Pearson correlation coefficient, between healthy and infected milk and teat end skin community matrices.
Kruskal-Wallis tests were used to assess differences in taxa abundance between sample types using the package *mctoolsr* for R (https://github.com/leffj/mctoolsr/). Pielou index \( J \) was used to assess evenness of OTU abundances within a sample, it was calculated as \( J = \text{Shannon}/\log(\text{richness}) \). \( P \) values generated from multiple tests were false discovery rate adjusted using \texttt{p.adjust} in R and are referred to as \textit{fdr} in the text. Bartlett’s test was used to compare variance of diversity within sample type.

**Core Microbiome**

The core microbiome was calculated for bacterial sequences in year 2015 using the *mctoolsr* package. OTUs were first subset by presence at any abundance within each habitat state; of this group, OTUs were retained that were observed in all animals, in at least 50% of observations from that animal; this group was further refined to include only OTUs that were observed in 25% of samples on each sampling date. This threshold retained OTUs that were observed in two of four habitat states, i.e., only milk or teat end; or only infected milk and teat end. Hierarchical clustering of untransformed Bray-Curtis dissimilarity distances between taxa in each habitat state was performed using Ward’s minimum variance method via the \texttt{hclust} function in R (Murtagh and Legendre 2014) and taxa were sorted via the resulting dendrogram. All analyses were conducted in R.

**RESULTS**

This study provides an initial characterization of the healthy and mastitic, teat end skin and milk microbiome of organically managed dairy cattle. Microbial DNA observed
on teat ends and within the teat cistern was represented by 6021 bacterial and 949 fungal OTUs. OTU abundance varied by seasonal and individual animal effects as well as the infection status of the quarter. The infected and healthy state of both teat end skin and teat cistern milk shared 8.1% of bacterial and 3.2% of fungal OTUs. Taxa shared between the teat end and teat cistern varied by infection status. Key bacterial taxa observed in all habitats included genera *Staphylococcus*, *Corynebacterium*, *Acinetobacter* and order Clostridiales. Key fungal taxa observed in all habitats included genera *Penicillium*, and *Debaryomyces*.

**Bacteria: Global Trends**

Variation in the global community was primarily associated with year (Figure 1) and host habitat (milk or teat end) (Figure 2). Teat swab samples contained more homogenous communities than teat cistern milk samples ($p < 0.001$) and were most similar if they were sampled on the same date ($p = 0.005$) (Figure 3). Intramammary (teat cistern milk) microbiomes were not clearly affected by seasonal factors within each year, but 2015 and 2016 milk samples were distinct ($p = 0.005$). 2016 milk samples were more homogenous than 2015 ($p < 0.001$).

Community composition of infected and healthy teats were similar ($p = 0.394$) and no effect was observed between the interaction of infection status and date ($p = 0.285$) (Figure 4). Both milk and teat end skin communities were affected by the host animal ($p = 0.015$, $p = 0.005$), though animal effect on the microbiome is difficult to characterize due to 7 of the 12 animals having been sampled on only one date. Some
clustering of quarters by animal and date regardless of infection status is evident, especially in teat swab samples from June of 2016 (Figure 3).

More taxa were detected in 2015 than 2016 ($p = 0.002$), and milk contained fewer taxa than teat in both years ($p < 0.001$) (Figure 5). Despite reduced richness, 2016 taxa tended to be more evenly abundant ($p = 0.027$). Infected milk was more likely to have an uneven distribution of taxa abundances compared to healthy milk ($p = 0.008$), but minimal difference in richness ($p = 0.556$). Evenness and richness remained similar in both infected and healthy teat end swabs ($p = 0.879, p = 0.331$), though richness tended to decrease slightly in infected teat end swabs.

431 of 5328 (8.1%) OTUs were observed in healthy milk, healthy teat end, infected milk, and infected teat end in both years. At the family level, taxa with $\geq 2\%$ relative abundance (RA) in both teat end and milk communities in both years included Corynebacteriaceae, Moraxellaceae, and Ruminococcaceae. Staphylococcaceae was greater than 8% RA in each habitat and infection state except 2015 healthy milk, where it was 1.9% RA. Approximately a quarter of OTU’s in each state of each habitat were observed only in that habitat state. Teat end skin communities had the greatest number of unique OTUs; approximately a quarter of OTUs in each habitat state were only observed uniquely therein (Figure 6).

**Bacterial Microbiome: Teat End Skin**

Overall, the teat end skin microbiome showed greater sensitivity to factors associated with the passage of time rather than infection status. For example, 120 genera
were identified as significantly different between dates \((p \leq 0.05)\). Of these, five taxa had a median RA greater than 2\% on each date: *Staphylococcus, Corynebacterium, SMB53, Acinetobacter*, and *Clostridium*. However, the structure of the teat end skin community was associated with mastitis status. In July 2015, 5 out of 6 cows sampled had at least a two-fold increase in *Staphylococcus* spp. on the skin of infected teats. This trend was contrary to other months, in which *Staphylococcus* spp. were often greater on healthy teats. Host animal also affected teat end skin community. In 2016, infected and healthy teats from the same cow visually cluster together rather than by infection status.

**Bacterial Microbiome: Teat Cistern Milk**

The intramammary microbiome was predominantly structured by sampling year. Quarter milk communities were more similar in 2016 than in 2015 \((p < 0.001)\). Over both years, genera above 2\% RA in both infected and healthy quarters included *Acinetobacter, Corynebacterium, Escherichia, Methylobacterium, Pseudomonas*, and *Staphylococcus*. At the genus level, taxa that were two orders of magnitude greater in healthy milk included *Bacillus, Lactococcus*, and *Sediminibacterium*. Genera that were two orders of magnitude greater in infected milk included *Pseudomonas, Streptococcus*, and *Staphylococcus*. *Staphylococcaceae* was both the most common family and that with the greatest difference between healthy and infected milk samples (Figure 7). The genus *Staphylococcus* dominated infected teat cisternal milk and consisted of 2 OTUs (Figure 4). 81\% was an unidentified species (OTU_001), and 19\% was *S. sciuri*. Genera below 1\% mean RA represented 32.7\% of the healthy community and 23.4\% of the infected community.
Bacteria: Habitat Comparison

Infected milk and teat communities were somewhat more similar than healthy milk and teat end communities. In 2016, similarity of the microbiome between healthy milk and teat ends (RV = 0.8) was equivalent to the similarity between infected milk and teat end microbiomes (RV = 0.78). In 2015, infected milk and teat ends were slightly more similar (RV = 0.85) than healthy milk and teat ends (RV = 0.74). In both years combined, 53 of 245 families were significantly different in relative abundance between infected milk and teat end swab samples; 78 of 262 families were different between healthy milk and teat end swab samples. 46 of the identified families were significantly different between milk and teat end swabs in both infection states. Most abundant of the 46 families included Lachnospiraceae, Aerococcaceae, Clostridiaceae, and Peptostreptococcaceae: greater than 2% RA in teat; Enterobacteriaceae, Bacillaceae, and Methylobacteriaceae: greater than 2% RA in milk. Of the 7 families that were only different between infected milk and teat, Comamonadaceae, Sphingobacteriaceae, Pseudomonadaceae, Veillonellaceae, and Lactobacillaceae were increased in infected milk. Cytophagaceae were increased in infected teat. Of the 32 families only different between healthy milk and teat, 11 were not detected in milk. Taxa with most significant difference included Alcaligenaceae (greater in teat), Dehalobacteriaceae (absent in milk), and Brevibacteriaceae and Oxalobacteraceae (both greater in milk).

Core Bacterial Microbiome

The core bacterial microbiome was different between infected and healthy milk ($p = 0.001$), while it was not different between infected and healthy teat end skin ($p = 0.789$).
(Figure 8). Of shared taxa, genus *Staphylococcus* was most abundant in each habitat/infection state except healthy milk where genera *Micrococcus* and *Acinetobacter* were most abundant. Shared genera that were similarly abundant in healthy and infected teats but increased in healthy milk included *Micrococcus* \((fdr = 0.380)\), and *SMB53* \((fdr = 0.794)\). Genus *SMB53* was only observed in increased abundance in one healthy quarter while genus *Micrococcus* was commonly observed. Of the genus *Acinetobacter*, the two OTUs shared between all habitat states exhibited opposite (weak) trends in milk. *A. lwoffii* was increased 2-fold in infected milk while *A. guillouiea* was increased 1.7-fold in healthy milk.

**Fungi: Global Trends**

The fungal community was structured by year, month, and sample type (quarter milk or teat end swab); significance was assessed at \(p < 0.01\) for each (Figure 9). There was wide variation in evenness of OTU abundances; milk samples had significantly more variance than teat samples, \(p < 0.001\). Milk samples also contained significantly fewer OTUs than teat (median 13 versus 30 respective, \(p < 0.001\)), regardless of infection status. Infection status did appear to effect OTU richness in 2015, trending lower in infected teat and milk than healthy without any detectable change in the evenness of abundances. Teat skin had the greatest number of fungal OTUs uniquely associated with a habitat (healthy 281, infected 157). Intramammary milk had 7-fold fewer unique OTUs (healthy 34, infected 28). Overall, 25 (3.2%) OTUs were shared between healthy and infected milk and teat. Of the shared OTUs, genus *Penicillium* was most commonly abundant (Figure 11).
Fungal Microbiome: Teat

The teat fungal microbiome was primarily structured by year and month. 42% of fungal taxa in 2015 were less than 2% RA, compared to 29% in 2016. Taxa that was more abundant in 2016 included *Caecomyces (fdr < 0.001), Cryptococcus (fdr = 0.1), Preussia (fdr = 0.05), Cystofilobasidium (fdr = 0.03), Coprinopsis (fdr = 0.04), Mortierella (fdr = 0.05). Debaryomyces* was more abundant in 2015 (*fdr = 0.05*).

*Debaryomyces prosapidis* was the most commonly occurring taxon; observed in 69% of samples.

Genera that varied significantly among months included *Caecomyces, Sporobolomyces, Coprinopsis, Cystofilobasidium, and Debaryomyces.* Cryptococcus was observed on each sampling date RA ≥ 2%. *Caecomyces, Penicillium* and *Rhodotorula,* were also observed on all sampling dates mean RA ≥ 1%. Cryptococcus primarily consisted of 3 taxa, *C. paraflavus* (greater prevalence in 2015), *C. oeirensis,* and *C. chernovii,* both more prevalent in 2016. *Caecomyces* consisted primarily of *C. communis,* although another Chytrid in the family Neocallimastigaceae was identified as an OTU and was similarly more abundant in 2016. *Penicillium* was primarily *P. olsonii* and was more prevalent in 2015.

In animals that were consistently sampled through time, quarters were typically distinct fungal communities, yet the significance of a quarter effect was marginal (*p = 0.14 for cow 59, p = 0.81 for cow 66*). Dominant taxa often differed between individual teats, even on the same animal, but this effect was confounded by variation over time.
For example, the cow 59 teat fungal community was 48% *Pleosporales* sp. in June of 2015, but by August 2015, this taxon had completely disappeared.

**Fungal Microbiome: Teat Cistern Milk**

Fungal community composition of the teat cistern milk was similar between infection status, animal, or month, though some visual clustering by month and infection status can be identified. There was an increase in the mean distance to centroid in infected samples ($p = 0.1$), indicating that the fungal community may be less similar between infected quarters than healthy quarters. Known genera ≥ 2% RA in both infected and healthy samples included *Hydropisphaera, Mortierella, Penicillium*, and *Sistotrema*. Known genera < 2% RA amounted to 26.3% of healthy milk fungal communities and 14% of infected milk.

Dominant fungal genera within a local community were often specific to quarter. Genera such as *Aspergillus, Basidiodendron, Bjerkandera, Cryptococcus, Lophiostoma, Pseudocercosporella, Scleroconidioma* and *Sordaria* were detected in approximately one infected quarter in relative abundances ranging between 30 to 57%. Dominant genera only observed in approximately one healthy quarter included *Chaetomium* (RA 89%) and *Curvibasidium* (RA 25%). There were three genera exceeding 2% mean RA that were observed in approximately 50% of all 23 quarters. *Debaryomyces* (10 quarters), *Penicillium* (18 quarters), and *Mortierella* (17 quarters). The next most observed genus, *Rhodotorula*, was observed in 4 quarters (all healthy). *Debaryomyces*, consisting of a single taxon *D. prosopidis*, tended to be greater in infected quarters ($p = 0.08$), even in animals where the genus was observed in both infected and healthy quarters on the same
date. *Mortierella* and *Penicillium* tended to be greater in healthy intramammary glands. *Mortierella* consisted of seven OTU’s, most commonly *M. exigua* and two other unknown taxa. *Penicillium* consisted of nine taxa and was most commonly *P. olsonii*. *Penicillium* species were greater in healthy than infected quarters (mean counts 3216 vs 2197 respective, \( p = 0.1 \)). In 2015, there was considerable variation in the proportion of taxa shared between infected and healthy quarters at the cow level. Some cows shared zero OTUs between healthy and infected quarters while others shared up to 24%, median 11%.

**Fungi: Habitat Comparison**

In both years combined, two OTUs were shared between at least 30% of healthy milk, healthy teat, infected milk, and infected teat samples: *Debaryomyces prosopidis*, and OTU 74, an unknown fungal taxon. In 2015, the proportion of taxa that that was shared (observed at any abundance) between milk and teat varied widely by animal. Healthy quarters were more likely to share taxa between skin and milk than diseased quarters (\( p = 0.13 \)) (Figure 10). The mean proportion of milk taxa that was also observed on the teat of each animal was similar between healthy and infected quarters, but there was greater variation of among infected quarters than healthy quarters (Figure 10).

**Core Fungal Microbiome**

Though not considered a core OTU, *Conocybe apala* was commonly shared among healthy quarters at low abundance but not infected quarters, it was most enriched in healthy milk, and was greater in healthy than infected teats (\( p = 0.05 \)). Core taxa *Debaryomyces prosopidis* and *Penicillium* spp. were commonly shared between infected
teat and milk (Figure 11). Lower classification of *Penicillium* was commonly *P. olsonii*. Others included *P. chrysogenum* and *P. decumbens*. *Penicillium olsonii* was more abundant in milk than teat (*fdr = 0.03*), with the most observed in healthy milk.
Figure 1. 16S and ITS rRNA sequence counts before normalization by year and habitat state. Points represent quarter samples. Upper plot: 16S sequences, \( n = 114 \). Sequence counts varied between years, median counts 2015 = 14484, median counts 2016 = 1752, \( p < 0.001 \). Lower plot: ITS sequence counts. Note the difference in scale between the two plots.
Figure 2. Non-metric multidimensional scaling of Bray-Curtis dissimilarity matrix of bacterial community of intramammary milk and teat skin over both years. Points represent quarter samples (n = 114) colored by sampling year. Lines represent distance to centroid of sample type. Abundances were transformed as square root prior to analysis.
Figure 3. Non-metric multidimensional scaling of Bray-Curtis dissimilarity matrix of bacterial community of teat skin quarter samples. Points are shaped by infection status and colored by sampling date. Number next to point represents the animal from which the quarter was sampled.
Figure 4. Twenty most abundant categories of 16S rRNA OTUs as arranged by bacterial family in each habitat state. Abundances have been normalized between samples. “Unknown” category denotes unique OTUs that are unable to be classified at the family level, or potentially only as bacteria. “Other” category denotes those families that are not in the top twenty.
Figure 5. Bacterial OTU diversity as represented by richness and evenness of OTUs in each habitat state and year. Richness is calculated as number of OTUs; evenness is measured using Pielou's J (J = Shannon/log(richness)). Index values closer to 1 indicate increasingly even distributions of OTU abundances.
Figure 6. Venn diagram shows overlapping OTUs between habitat states. Numbers represent OTUs that are only observed within overlapped ellipses. For example, 431 OTUs were observed in all habitat states, while 81 OTUs were only observed in both healthy and infected milk. The addition of all numbers within an ellipse represents the total number of OTUs observed in that habitat state. Teat had more unique OTUs; 1066 of 3621 (29%) healthy and 731 of 3198 (22%) infected. Milk had fewer unique OTUs; 377 of 1314 (29%) healthy and 384 of 1441 (27%) infected.
**Figure 7.** Fifty bacterial families with the greatest difference in relative abundance (RA) between healthy and infected quarters. Difference is calculated as RA healthy minus RA infected. Taxa along the y axis are sorted by absolute value of the difference. Color and direction of bar indicates whether healthy or infected quarters was enriched in each taxa. x-axis represents difference in units of relative abundance.
Figure 8. Heat map depicts known core bacterial genera above 1% relative abundance in year 2015. To be considered core, OTUs must first be observed in each habitat state in the global data set; of this group, OTUs are retained that were observed in all animals, in at least 50% of observations from that animal. This threshold retains OTUs that may only be seen in two of four habitat states i.e. only milk or teat; or only infected milk and teat. Dendrogram representing the hierarchical clustering of Euclidean distances groups taxa based on similarity of relative abundances in each category. Relative abundance within each group on x-axis is noted in each cell. Color is scaled from light yellow to red to visualize abundance (red is most abundant).
Figure 9. Non-metric multidimensional scaling of Bray-Curtis dissimilarity matrix of fungal community of intramammary milk and teat skin over both years. Points represent quarter samples \((n = 70)\) colored by sampling year. Lines represent distance to centroid of sample type. Abundances were transformed as square root prior to analysis.
Figure 10. Proportion of fungal taxa shared between the teat and mammary in both infection states. Proportion of shared OTUs was calculated for each cow in both directions. Left plot depicts proportion of TEAT taxa that are also observed in milk in both the healthy and infected state; right plot depicts proportion of MILK taxa that are also observed on the teat. Note difference of scale between y-axes.
Figure 1. Heat map depicts known core fungal genera above 1% relative abundance in year 2015. To be considered core, OTUs must first be observed in each habitat state in the global data set; of this group, OTUs are retained that were observed in all animals, in at least 50% of observations from that animal. This threshold retains OTUs that may only be seen in two of four habitat states i.e. only milk or teat; or only infected milk and teat. Dendrogram representing the hierarchical clustering of Euclidean distances between taxa in each habitat state groups taxa based on similarity of relative abundances in each category. RA within each group on x-axis is noted in each cell. Color is scaled from light yellow to red to visualize abundance.
DISCUSSION

The results support the dysbiosis hypothesis. Infection status is being reconceived as an imbalance in the microbiome, or dysbiosis, of the mammary gland. The dysbiosis hypothesis imagines a network of microbial interactions in the healthy habitat that affects the mechanics of infection; an infection event is linked with an ecological disruption of the proposed endogenous community manifest as an imbalance of the microbiome relative to the healthy state. While there was not strong evidence, in this study, that the microbiome of the teat skin experienced dysbiosis during infection, the shift in the structure of the milk microbiome from infected mammary quarters was characterized by dominance of a single *Staphylococcus* OTU and downward trend in OTU richness for both fungi and bacteria. This shift is consistent with the aerobic culture results and selection of infected quarters, as the dominant intramammary pathogens on this farm were staphylococci. In contrast, and in agreement with other sequencing based assays of healthy quarters, the healthy intramammary microbiome exhibited greater OTU richness and evenness than diseased microbiomes (Oikonomou et al. 2014). The dominance of genus *Staphylococcus* was an expected result because most infected quarters were selected based on the presence of a *Staphylococcus* intramammary infection (IMI) using well-established definitions. The greater abundance of the genus *Staphylococcus* in infected intramammary milk samples does not prove that other taxa are reduced or impeded. It may be the case that genus *Staphylococcus* itself was increased and the community remained stable; fewer organisms were perhaps detected only due to the greater likelihood of replicating and sequencing *Staphylococcus* DNA.
While replication likely explains some of the reduced diversity of the mastitic mammary community, variations in relative abundances of core microbial DNA between the infected and healthy state were inconsistently scaled. Some milk taxa such as genera *Acinetobacter* and *Corynebacterium* remained a similar proportion of the community in both infected and healthy milk despite the dominance of the genus *Staphylococcus*. Likewise, a similar proportion of OTUs in both healthy and infected milk were detected only in their respective habitat state. If a dominant sequence reduced the likelihood of rare DNA from being amplified, rare OTUs might be expected to be a lesser proportion of infected milk, yet this did not occur. Though not conclusive, this result is consistent with the understanding that microbial DNA in the healthy mammary gland is modulated by the infection status of the quarter (Oikonomou et al. 2014). It supports the notion that “infection” describes a community-wide rearrangement that would be expected of a transitioning or disrupted ecosystem.

The dysbiosis concept is not without controversy, and we agree that equating the observation of microbial DNA in milk with the presence of an endogenous microbial community contradicts the immunobiology of the mammary gland (Rainard 2017). Another recent pilot study, using an alternative extreme aseptic sampling technique and cisternal fine needle puncture, obtained quantities of microbial DNA from milk samples that yielded no bacterial colony growth from 100 µL of milk (Metzger et al. 2018). Others have used a teat cannula to bypass the teat canal and teat apex (Hiitio et al. 2016, Friman et al. 2017). These studies also reported microbial DNA in the teat cistern, though diversity was reduced compared to milk collected via traditional aseptic technique. In the
current study, we adapted an alternative sampling technique of passing a catheter through a teat cannula to avoid potential microbial contamination from the teat canal and the skin of the teat apex. Further research is required to understand the significance and relevance the presence of microbial DNA and genes (i.e., the microbiome) in teat cistern milk, and what component, if any, of this microbiome represents a functional ecosystem of the teat cistern. This question does not discount the potential importance of a teat apex and teat canal microbiota in mastitis epidemiology and control, nor the potential value of collecting milk samples to characterize the milk microbiome of healthy and infected quarters to explore mastitis and mammary gland biology.

Results from sequencing studies are difficult to compare due to disparity of methods (Metzger et al. 2018), though the observation of similar trends via different extraction kits, PCR primers and sequencing platforms supports the use of molecular methods to investigate mastitis epidemiology. The genera *Staphylococcus* and *Corynebacterium* have been found in many culture independent assays of the milk microbiota from both healthy and infected teats (Metzger et al. 2018, Hiitio et al. 2016, Friman et al. 2017, Kuehn et al. 2013, Oikonomou et al. 2014, Ganda et al. 2016, Bonsaglia et al. 2017) though many of these reports used a traditional aseptic technique to collect milk samples. *Turicibacter* spp. and *Clostridium* spp. were increased in composite samples of aseptically collected foremilk and teat apex interiors from healthy teats (Falentin et al. 2016) and in aseptically collected milk (Ganda et al. 2016), which mirrored the result from the current study, though these genera were almost nil in milk collected by extreme aseptic methods (e.g., this study and Metzger et al. 2018) and more
prevalent in teat samples, suggesting possible contamination of the milk by the teat in all studies. Other studies report an association between Actinobacteria and healthy quarters of animals infected in separate quarter with *Klebsiella* spp., *Escherichia coli*, *Pseudomonas* spp. Likewise, Actinobacteria, Micrococcaceae, and Corynebacteria were associated with healthy milk. However, many more discrepancies in differential abundance exist between the current study and the previous, possibly due to the effect of the infecting organism (Ganda et al. 2016), bedding type (Meztger et al. 2017), days postpartum (Bonsaglia et al. 2017), and/or the many other factors that may influence the microbiota of the mammary gland. There is a paucity of studies that use molecular techniques to describe the microbial ecology of the teat end skin. Similar to the current study, these reports are characterized by a relative abundance of *Staphylococcus* spp., *Micrococcus* spp., *Acinetobacter* spp. observed in both infected and healthy teats. (Gill et al. 2006, Braem et al. 2012). Few differences were found between healthy and infected teats, though richness was decreased in infected teats (Braem et al. 2012), which matched results of this study.

A novel aspect of this study was the concurrent sampling of both teat ends and teat cisternal milk to characterize both communities and describe how taxa was differentially abundant between habitats of both infection states. There was relatively little overlap of OTUs between all habitat states. Infected milk and skin were slightly more similar than healthy milk and skin. Most bacterial families were primarily associated with habitat differentiation, while other families were only observed to vary between milk and teat in either the infected or the healthy state, suggesting that infection
status of the host habitat creates an environment that selects some taxa over others. The concept of “core” taxa enables a combined approach to both prevalence and incidence of OTUs. By concurrently observing both habitats, OTUs that are putatively transmitted from skin to milk or from milk to skin can be inferred and compared between infection state. *Staphylococcus* OTU_001 was common to all habitat states, though it was less dominant in healthy milk than infected. While this does not confirm directionality of transmission, it suggests that *Staphylococcus* spp. either flow differently between skin and milk in the infected state than the healthy state or accumulate differently within each habitat. For example, the flow of *Staphylococcus* spp. between teat cistern and teat end may be similar in both states but the normal functioning of the healthy teat immune response is able to lyse invaders more effectively, resulting in a reduced abundance. Both scenarios may explain the 13-fold increase observed in the infected teat cistern. While this is the most extreme example, all core taxa were found to be significantly different in at least one habitat state. For example, *Micrococcus* sp. and *Acinetobacter guillouiae* were common and similar abundance in healthy and infected skin but elevated in healthy milk, suggesting an increased flow or accumulation in the healthy teat cistern. An accumulation of shared teat taxa in the healthy intramammary lumen is further confirmation that a commensal microbiome of the teat is a critical research objective with potential to provide insight into both management practices that foster healthy teat microbiota and the immunobiology of the mammary.

A further novel aspect of this study is the culture independent characterization of the fungal microbiome of the udder. Differential abundance of fungal DNA detected
within the milk samples is an interesting example of how the intramammary-as-
ecosystem hypothesis is potentially borne out. The yeast *Debaryomyces prosopdis*,
though greater in infected mammary glands, is not a noted mastitis pathogen. Its
relatively greater abundance suggests that the infected state affords it greater competitive
advantage. It is unknown what this advantage is and what effect it has on the host animal.
Likewise, the increased presence of *Penicillium* spp. in the healthy state raises questions
about the effect of the mechanism of cross-kingdom interaction within the teat, such as
the production of antibiotics, on the resistance of the quarter to infection or colonization.
Interesting to note was the contrasting (though weak) trend in diversity of fungi between
infection states when compared to bacteria. In 2015, fungal richness decreased in
infected milk, yet no dominant organisms emerged. Bacterial richness remained stable
despite the emergence of a dominant OTU. In an ecological framework, this suggests
that infection disrupts fungal niche habitat, excluding teat taxa mostly observed in
healthy milk and creating new opportunities for the establishment of teat genera such as
*Aspergillus*, *Cryptococcus*, and *Debaryomyces*, observed mostly in infected milk. This
hypothesis is also suggested by greater similarity of the proportion of milk OTUs
observed on healthy teats through time when compared to the proportion shared between
habitats of infected quarters (Figure 10). The tight clustering around the healthy mean
suggests a more stable interaction between teat and intramammary communities in the
healthy state, while the infected teat cistern is less stable, variably sharing a much
increased or decreased proportion of OTUs with the teat. Notably, this trend did not
emerge for in the 16S data, again raising questions about kingdom level mechanisms of microbe-host interaction.

It is important to note that taxa such as *D. prosopidis* and *Staphylococcus* spp. were often members of both the healthy and infected community. This result matches that of other microbial sequencing studies that found mastitis pathogens or *Staphylococcus* spp. in both healthy and infected quarters but elevated in infected quarters (Oikonomou et al. 2014, Falentin 2016). This raises several questions. First, sequencing data obtained in this study is detailed enough to group OTUs by genus and occasionally species. Though more than 80% of the identified *Staphylococcus* genus consisted of a single OTU (OTU_001), it is likely that many strain types and possibly species are represented (Gill et al. 2006). Thus, a definitive statement cannot be made about whether OTU_001 represents an opportunistic pathogen or a non-pathogenic commensal organism. Second, the aerobic culture tests used to define infection are possibly not sensitive enough to detect shifts in the microbiome that are found in early or late stages of the transition between states. Perhaps sequencing was able to detect potential pathogens in what was only nominally the healthy state, and what was truly an undetected infection (albeit with a low quarter somatic cell count). This theory is supported by the experimental design, which only tested cows with at least one infected quarter. It is possible that infection of one quarter affects the microbiome of the remaining quarters, explaining the presence of opportunistic taxa or low abundance of potential pathogens on nominally healthy teats and mammary glands. This is supported by the current 16S rRNA data indicating that microbiomes are somewhat unique to the
individual animal, indicating more sharing of taxa between quarters on the same cow. Third, if taxa indicative of the infected state are also present in the healthy state, what factors prevent these taxa from becoming dominant/pathogenic? Fourth, from an animal health perspective, can the microbiome (for example of the teat skin) be managed to increase the resilience of the teat community to prevent ecosystem disruption and progression to the infected state?

While it was clear that some variation in the microbiome was associated with factors related to the sampling date, it might have been expected to see similar trends emerge through the same months in both years. This was not the case, as much of the variation in the global microbiome was observed between years. Some of the variation between years may be attributable to the commencement of teat-dipping between year 1 and year 2, but because year and teat-dipping factors are exactly confounded it is impossible to reach conclusions. That said, fewer sequences and OTUs detected in 2016 (during teat-dipping) align with the objective of teat-dipping and seem to confirm the intended effect, meriting further research, especially in the light of the increase of fungal sequences and opposing decrease of bacterial sequences on 2016 teat end skin.

As noted above, it is possible that the DNA detected in the milk is not from living organisms, but rather fragments from lysed cells that have migrated into the teat cistern, or organisms that were lysed during normal immune function of the intramammary gland. If that were true, it might be hypothesized that an infected mammary gland would experience a differential abundance of lysed DNA material than a healthy mammary
gland, accounting for some of the difference observed. This question is an important one and should be addressed in further studies.

Conclusion

This study concurrently sampled teat end skin and teat cistern milk of healthy and mastitic quarters of organic dairy cows during June July and August of two consecutive years. DNA sequencing revealed that habitat (skin or milk), date of sampling, and infection status effect the diversity and composition of the microbiome. The fungal microbiome of teat was characterized with molecular methods for the first time. An unexpected abundance of *Penicillium* spp. were observed in most samples, though increased in healthy milk. Overall OTU richness was dramatically greater on the teat end than in the cisternal milk samples. Teat end skin was not found to vary with infection status. Infected milk was dominated by an OTU of the genus *Staphylococcus* common to most samples, reflecting the experimental selection of mastitic quarters infected by a cultured *Staphylococcus* species. Abundances of taxa shared between milk and skin in both states varied depending on the infection state of the quarter. Though infected skin and milk bacterial microbiomes were slightly more similar than healthy milk and skin, *Micrococcus* spp. and *Acinetobacter guillouiea* were shared milk and teat taxa that were commonly elevated in healthy cisternal milk. Though no conclusions can be drawn regarding the actual presence of living microbes, the evidence accumulated here suggests that the healthy mammary gland is characterized by an even distribution of taxa, some of which are also observed on the teat skin. The presence of commensal organisms within the teat cistern remains unknown, though variance in the putative sharing of taxa between
the skin and milk depending on infection suggests that continued use of refined molecular methods should be used to understand the potential for management practices to create a beneficial teat skin microbiome.
Chapter 2. Microbiome of Bedded Pack

INTRODUCTION

Bedded pack dairy systems that use a carbon rich substrate such as straw or wood chips to create a comfortable and clean surface on which animals move freely are used worldwide (Aztori et al. 2009, Albino et al. 2017, Barberg et al. 2007, Galama et al. 2014). Traditionally, bedded pack is similar to the straw-yard style of bedding, a loose housing system that adds bedding when needed without removal, though manure may be removed to reduce bedding needs (Bewley 2017). A refined version of the straw-yard style of bedding is beginning to be reconceived of as “deep bedded pack” (Benson 2012). Though understudied, deep pack systems retain feces and urine in carefully managed strata, usually comprised of wood chips and hay or straw. Detrital decomposition, 15 cm below the surface, warms the freshly applied bedding above (Benson 2012). Mechanically aerated bedded pack, commonly referred to as “compost bedded pack”, relies primarily upon a tractor-drawn chisel or rotary tiller to bring up partially decomposed, heat-dried wood sawdust or chips to the surface to be re-used (Barberg 2007). In principle, both methods rely on composting action of aerobic, thermophilic microbes in the deeper pack layers to decompose manure and bedding, warming and drying the surface layer. Within each method, factors such as cow stocking density, time spent on pack, bedding rate, mechanical aeration rate and bedding material can alter the composition of compost feedstock and potentially alter the microbial community, changing rate of decomposition and heat production (Neher et al. 2013).
These systems have gained popularity with dairies throughout Vermont due to the perception of increased cow comfort and increased soil fertility from spreading composted pack on crop fields (Gilker et al. 2012). Yet the potential for biological interaction between the bedded pack and pathogens that cause bovine mastitis remains unclear. Despite the culture of mastitis pathogens from pack (Albino et al. 2017, Black et al. 2014), some studies report the decreased incidence of mastitis and somatic cell count on bedded pack (Astiz et al. 2014, Barberg 2007). Physical characteristics of bedding including temperature, moisture, C:N ratio, and ambient barn temperature effect the abundance of mastitis pathogens cultured from pack (Black et al. 2014, Favero et al. 2015, Eckelkamp et al. 2016). Odds of observing a case of environmental mastitis increases with bedding moisture (Favero et al. 2015), though cow cleanliness does not necessarily decrease if moisture is kept low (Eckelkamp et al. 2016). Increasing ambient barn temperature and moisture content of bedding increase mastitis pathogens cultured from pack, while increasing bedding temperature decreased staphylococci, streptococci and bacilli species in pack (Eckelkamp et al. 2016). While the carefully managed aerobic decomposition of bedded pack creates much of the value in this system, little is known about the microbial community that underpin these effects.

Inspiration for the study is drawn from the limitation of certified organic dairy management which requires that no antibiotics be used to treat mastitis (or any other issue) on animals that are used to produce goods sold as organic, and preventative methods of mastitis control are codified (Dehne and Green 2014). The possibility that the teat cistern milk microbiome is shaped by bedding type (Metzger et al. 2018), raises
questions about whether bedding can be used to help promote a healthy udder, and the mechanisms behind this effect. This study is designed to test the hypothesis that teat cistern milk microbes are associated with the microbiome of the compost bedded pack. Concurrent measures of bedding, teat end skin and teat cistern milk microbiomes were compared on a single dairy farm. The study represents a first effort to characterize the bacterial and fungal community of bedded pack using a high-throughput sequencing approach. We seek to identify relationships between microbial taxa and bedding and herd management techniques. The term “core” microbiome is used to identify taxa that are persistent through time and shared between sample types, as noted in Chapter 1 of this thesis. The core microbiome concept is necessarily plastic and has been variously defined, though typically suggests a group of microbes that are shared between environments (Shade and Handelsman 2012). While it does not have an ecological meaning per se, it is helpful to create hypotheses about taxa associations, functional groups and treatment effects.

METHODS

Experimental Design

Bedded pack, teat cistern milk and teat-end skin was sampled at a single farm (Farm C) in northwestern Vermont over the period of approximately one year. To test for the possibility of farm level and management practices effects, bedded packs were sampled at four additional organic dairies (D, L, B, S) within an 80 km radius of Burlington, Vermont.
Table 1. Bedding material, stocking density and median temperature for each farm. Wood shavings were dried compressed pine (*Pinus*), sawdust was green pine. Wood chips were unidentified type. Hay was farm-grown. Straw was purchased (Farm S) or farm-grown (B). Primary bedding represents approximately 75% of bedding while secondary represents 25%. Farm L was the only farm that mechanically aerated bedding.

<table>
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<th>Farm</th>
<th>C</th>
<th>D</th>
<th>B</th>
<th>S</th>
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<td>Hay</td>
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Farm C Profile

Farm C is a 150-cow dairy that feeds an all grass and hay ration (Chapter 1, this thesis). The herd lives on bedded pack when pasture is inaccessible (between November and April, weather dependent) and is divided into two barns approximately depending on lactation schedule. Lactating cows are kept isolated from heifers in separate barns. Beginning in November of the study year, the farmer chose to rotate groups between barns to allow consistent bedding management between barns. Lactating cows are moved into a tie-stall milking barn twice per day for several hours. Each barn contains approximately 75 cows at a stocking density of 8 m²/cow. Bedding material is a mix of purchased hardwood chips and mulch hay produced on the farm. The farmer manages the quality of the pack by visually and tactiley assessing temperature, moisture, sponginess and cow cleanliness. Hay bedding application rate is increased as cow cleanliness decreases and when moisture is in excess. Wood chips are added to increase structure and aeration. A temperature probe is occasionally used to measure heat.
generated by microbial decomposition. The mix and application rate of bedding is varied by the farmer to facilitate a pack temperature of 32°C.

Sampling and Metadata Collection

Prior to sampling, a 45-minute verbal interview was conducted with farmers to assess pack management priorities and strategies. Farmer estimates of bedding volume were also asked, though not reported here due to evidence of inaccuracy of memory.

**Table 2.** Farm C bedded pack was sampled ten times between July 2015 and June 2016. Farms D, L, B and S bedded packs were sampled on the same day as Farm C, four times between March and June 2016. Colored bars indicate months in which sampling occurred for each sample type and farm.

<table>
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<th>Au</th>
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</thead>
<tbody>
<tr>
<td>C</td>
<td>Milk &amp; Teat</td>
<td></td>
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<tr>
<td></td>
<td>Bedding</td>
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</tr>
<tr>
<td>B, D, S, L</td>
<td>Bedding</td>
<td></td>
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</tr>
</tbody>
</table>

Five 3.75 l samples were collected at both surface (0-15 cm) and depth (15-35 cm) along random transects in each bedded pack barn at each sampling date. Occasionally, when the pack was less than 15 cm deep, a composite depth/surface sample was collected. Each barn at Farm C was sampled independently. Temperature and percent oxygen were also measured at time of sampling using an OxyTemp™ (Reotemp, San Diego, CA) probe. Samples were transported to the lab in insulated containers for same-day processing. A composite 18.75 l sample was formed for each barn, resampled at approximately 1 cm³ and frozen at -80 °C until DNA was extracted.
On Farm C, cows were selected for sampling based on clinical identification of the presence of mastitis in one or more of the teat quarters. Teat and milk were sampled from cows with paired healthy and infected quarters. Milk was collected using a catheter within cannula technique that eliminated potential contact between sampled milk and the teat or teat orifice. Samples were chilled on site before being frozen at -80°C. Before DNA extraction, teat and milk samples were surveyed for bacterial species causal of mastitis using restrictive culturing techniques. Complete methods and sampling design are described in Chapter 1. Two sampling dates were concurrent for skin, milk, and pack, July and August of 2015. For comparisons between bedding and cow taxa, data was subset to include all samples on these dates.

**Extraction, Sequencing, Taxonomic Mapping**

Extraction was performed using the MoBio PowerSoil Soil DNA Isolation kit. DNA was extracted following the manufacturer’s instructions, using methods of Lauber et al. (2006). Frozen bedded pack samples were kept on ice before removing a 0.15-0.25 g subsample into the spin column of the PowerSoil kit. Subsample mass varied due to density differences between bedded pack samples; less dense samples filled the bead tube at a lower weight. Bead tubes were then incubated in a 65 °C water bath for ten minutes and then shaken for two minutes before proceeding with manufacturer’s instructions.

Extracted DNA samples were frozen at -80 °C until shipped to the University of Colorado Next Generation Sequencing Facility (Boulder) for PCR amplification, sequencing, initial data filtering, and taxonomic reference mapping. Amplification was performed in triplicate using 515F and 806R primers for V4-V5 region of
bacterial/archaeal 16S rRNA genes and ITS-1F and ITS-2R primers for fungal ITS-1 region. Sequencing was performed on the Illumina MiSeq platform. Cleaned and quality filtered reads were dereplicated before clustering at 97% nucleotide identity into representational OTU sequences via UCLUST (version 7) (Edgar 2010). Sequences were referenced to the August 2013 version of the Greengenes database (bacteria) and UNITE database (fungi) to produce combined Operational Taxonomic Unit (OTU) count and taxonomic information tables. Methods are as described in Emerson et al. (2015).

Normalization and Statistical Analysis

OTUs with a single count were removed. For comparison between farms, Farm C samples taken in other months were removed. Without the need to preserve a paired design as described in Chapter 1, a rarefaction approach was used to normalize library sizes between samples. Samples were removed that had fewer counts than the rarefaction threshold. Farm C was rarified separately for analyses that did not compare between farms. Bacterial and fungal sequences were rarified separately; thresholds were chosen that minimized sample loss while including the most counts.

For comparison between farms, bacterial counts were rarified to a depth of 4437, leaving 55 of 60 samples and 5360 of 6895 OTUs; fungal counts were rarified to 2194, leaving 51 of 54 samples and 955 of 1195 OTUs. For the separate analysis of Farm C, bacterial sequences were rarified to 4437 leaving 28 of 31 samples; fungal sequences were rarified to 2219, leaving 29 of 29 samples. For calculation of shared ITS OTUs between pack, skin, and milk, 5 pack samples, 22 milk and 26 teat samples were retained.
after removing samples below 200 sequences; for 16S rRNA OTUs, 5 pack samples and 24 of both teat and milk samples were retained.

Bray-Curtis dissimilarity matrices were generated to compare community composition of samples and non-metric multidimensional scaling was used to visually represent similarities between sample and OTU groupings. Abundances were square root transformed prior to dissimilarity calculation. Contour maps of temperatures on each sampling date were fitted to sample ordinations and overlaid on projections of OTUs to suggest associations between taxa and temperature associated with season. Permutational multivariate analysis of variance (PERMANOVA) was performed to assess pairwise differences in the microbiome between farms. PERMANOVA was used to assess contribution of sampling depth, date, and barn to variation in Farm C microbial sequence relative abundances in a 3-way additive model. Variation due to temperature was tested separately with a reduced data set with eight samples removed due to missing temperature measurements on those dates.

To compare between OTUs at a higher taxonomic resolution, OTUs were first converted to a relative abundance (RA) within sample. Mean RAs were computed among all sample dates for each farm. Hierarchical clustering of untransformed Bray dissimilarity distances between farms was performed using Ward’s minimum variance method via the `hclust` function in R (Murtagh and Legendre 2014) and taxa were sorted via the resulting dendrogram. Mean RA was additionally calculated by date in the case of Farm C.
The Kruskal-Wallis test was used to assess contribution of commonly abundant families to differences between the microbiome of farms. Family relative abundance was the dependent variable and farm was the independent variable in the model. Families included in the model were restricted to those that were greater than 5% RA in at least one farm. Families with fdr-value less than 0.05 were compared between farms using the Wilcoxon rank sum test.

Shannon diversity, a combined index of both evenness of distribution and richness of OTUs within a sample, was compared between each habitat and date using non-normalized data. Higher index value indicates a more even distribution and greater richness or number of OTUs. Loess curves were fitted to diversity index values by date to visualize and compare trends.

Core Microbiome

Core microbiome between all farms was explored in two ways, first by comparing number and proportion of shared and unique OTUs and second by limiting the core to OTUs that existed on all dates in at least 25% of samples from each farm. To compare proportion of overlapping OTUs between samples of varying sequence depth, shared and unique OTUs were calculated between farms using rarified data. To capture low-abundance OTU’s, date-based core microbiomes were calculated using un-rarified data converted to RA of each sample; samples fewer than 1000 sequences were dropped. Core microbiome of Farm C was calculated as all OTUs that existed in at least 75% (bacteria) or 25% (fungi) of samples from each sampling date. Similarity of relative abundances through time was visualized with a dendrogram and variation was compared
between taxa using the coefficient of variation (CV) calculated as CV = standard deviation/mean of taxon relative abundance among all samples.

Core microbiomes of bedding, teat and milk were calculated between samples that were concurrently collected in July and August of 2015 using non-normalized data to preserve rare species. Samples below 200 sequences were removed. OTUs were considered core if they were observed in at least 25% of samples from skin, milk, and pack.

All statistical analysis was conducted in R version 3.3.4, PERMANOVA was performed using the adonis function in vegan (Oksanen et al. 2018). Contour maps were fitted with the ordisurf function in vegan and plotted using ggplot2 (Wickham, 2016). Core microbiome calculation, sample ordinations, and Kruskal Wallis tests were conducted using the package mctoolsr for R (https://github.com/leffj/mctoolsr/). Taxa based ordinations were performed using the phyloseq package (McMurdie and Holmes, 2013).

RESULTS

Comparison Between Farms: Global Trends

Farm-level effects had a clear impact on the bedding microbiome (Figure 1,2). Farms L and S were more clearly segregated from farms B, C and D. Bacterial microbiomes differed between farms L and S (fdr = 0.003) and between L and S versus C, B and D (fdr ≤ 0.004), but no significant differences between farms B, C and D. Fungal microbiomes differed between all farms (fdr ≤ 0.027) (Figure 2).
Taxonomic characterization of the bacterial microbiome

The phylum Proteobacteria had the greatest mean RA among all farms followed by Bacteroidetes and Firmicutes in descending order. The dominant class of Proteobacteria was γ-Proteobacteria, which contained the family with the highest mean RA, Pseudomonadaceae. Other common families within γ-Proteobacteria were Moraxellaceae (predominantly *Acinetobacter lwoffi* and *A. guillouiae* on all farms) and Alteromonadaceae (Figure 3). Of the Pseudomonadaceae, *Pseudomonas* was the predominant genus in all farms. Farm S had the highest overall mean RA of Pseudomonadaceae, yet 62% of OTUs at this farm were distinct from the predominant Pseudomonadaceae OTU in all other farms. A mean of 15% of Pseudomonadaceae in both Farms D and B were *P. thermotolerans*. Orders of Bacteroidetes with the greatest mean RA through time at each farm included Sphingobacteriales, Flavobacteriales, and Bacteroidales. Orders of Firmicutes with the greatest mean RA through time at each farm included Clostridiales, Bacillales, and Lactobacillales. The greatest number of both OTUs unknown at the family level and families in less than 1% abundance were observed in Farm L. At the family level, hierarchical clustering segregated farms L and S from B, D and C (Figure 3). Variation in relative abundance of commonly abundant bacterial families that explained differences among farms were typically associated with farms L or S (Figure 4).

Taxonomic characterization of the fungal microbiome

Hierarchical clustering segregated farms L and S from B, D and C at the family level (Figure 5). Fungal communities in all farms were dominated by phylum
Ascomycota. Of the Ascomycota, yeasts were predominant; *Candida* was commonly the most relatively abundant genus (Figure 5). Of the B, D, C farm group, genus *Thermomyces* was more relatively abundant in Farm B, and was also notable in Farm L, consisting of the single species *T. lanuginosus* in both farms. *Aspergillus* was prominent in farms B and D. Farm S had the most genera in less than 1% abundance and had the highest abundance overall of genus *Parastagonospora* (17.7%). Many farms were void or close to void of species that were in found in abundances greater than 5% RA in another farm (Figure 6).

Though neither the Saccharomycetaceae nor genus *Candida* was identified as significantly different between farms, differences did exist at the species level. Farm L was dominated by *C. glaeosa* while farms D, C and B were primarily *C. rugosa*. The genus *Candida* contained 28 known species, 14 of which were in greater than 1% mean RA, and five of which had greater than 5% mean RA, and three of which (*C. rugosa*, *C. glaeosa* and *C. catenulata*) greater than 25% mean RA. These species were significantly different between farms (*fdr* = 0.0008, 0.0011, and 0.0013 respectively). Farms C, D and B had the greatest mean RAs of *C. rugosa*; Both farms L and S were elevated in *C. glaeosa*, while C and D were elevated in *C. catenulata*.

Proportion of Overlapping OTUs and the All-Farm Core Microbiome

All farms shared 8% (341 OTUs) of total bacterial OTUs and 2% (18 OTUs) of total fungal OTUs. Farm B had the greatest percentage of unique bacterial OTUs followed by Farm L. Farm L had the greatest percentage of unique fungal OTUs followed by Farm S. Bacterial OTUs shared by all farms except C were the fewest, while
the OTUs shared by all but Farm S were greatest. Fungal OTUs shared by all farms but L were the greatest; (Table 2). Most abundant bacterial core OTUs included *Pseudomonas* sp. and *Acinetobacter lwoffii*. Most abundant core fungal OTUs included *Candida rugosa* and an OTU from the family Pichiaea (Table 3).

Table 3. Describes overlap between number of unique OTUs. Samples: samples included after rarefaction. Total: total observed OTUs in farm community. Shared excepting: specific OTUs shared by all other farms, for example three OTUs were shared between all other farms that were not also observed in Farm B. Unique: percentage of the total OTUs observed in a farm that were not observed in any other farm.

<table>
<thead>
<tr>
<th></th>
<th>Farm</th>
<th>Samples</th>
<th>Total</th>
<th>Shared Excepting</th>
<th>Unique</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fungi</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>9</td>
<td>265</td>
<td>5</td>
<td>31%</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>7</td>
<td>304</td>
<td>3</td>
<td>31%</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>6</td>
<td>230</td>
<td>7</td>
<td>29%</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>5</td>
<td>211</td>
<td>22</td>
<td>41%</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>3</td>
<td>257</td>
<td>6</td>
<td>36%</td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>9</td>
<td>2035</td>
<td>6</td>
<td>19%</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>8</td>
<td>2191</td>
<td>74</td>
<td>44%</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>6</td>
<td>1375</td>
<td>24</td>
<td>11%</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>8</td>
<td>2094</td>
<td>63</td>
<td>34%</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>5</td>
<td>1092</td>
<td>205</td>
<td>19%</td>
</tr>
</tbody>
</table>
Table 4. Bacterial and fungal OTUs are represented by the last known taxonomic classifier, if family is the last know classifier, it is designated in parentheses. Mean RA: mean relative abundance of OTU within sample among all samples on all dates.

<table>
<thead>
<tr>
<th>Core Microbiome All Farms</th>
<th>Bacterial OTU</th>
<th>Mean RA</th>
<th>Fungal OTU</th>
<th>Mean RA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Luteimonas sp</em></td>
<td>0.7%</td>
<td>Pichiaceae (Family)</td>
<td>13.5%</td>
</tr>
<tr>
<td></td>
<td>Alcaligenaceae (Family)</td>
<td>0.8%</td>
<td><em>Candida rugosa</em></td>
<td>29.6%</td>
</tr>
<tr>
<td></td>
<td>Rhodobacteraceae (Family)</td>
<td>0.3%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Stenotrophomonas sp</em></td>
<td>0.7%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Acinetobacter lwoffii</em></td>
<td>2.5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas sp</em></td>
<td>2.9%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Farm C Global Trends

Month of sampling, source barn, and depth of sampling contributed to variation in the fungal microbiome ($p = 0.001, 0.017, 0.069$ respective) and the bacterial microbiome ($p = 0.001, 0.008, 0.047$) (Figure 7). Temperature of the bedding, modeled separately, also affected the fungal and bacterial microbiome ($p = 0.006, 0.023$ respective) (Figure 8).

Shannon diversity varied through time for both bacteria and fungi (Figure 9). Opposing trends were observed through December of 2015; bacterial diversity increased while fungal diversity decreased. A fungal spike occurred in two samples in February before steadily rising again in the spring. Bacterial diversity decreased in January before rising through late winter and falling slightly in the spring.

Taxonomic Characterization of the Farm C Bedding Microbiome

*Candida* was consistently the dominant fungal genus, of which *C. rugosa*, *C. catenulata*, were the most relatively abundant. *Candida* spp. appeared to be positively
associated with increased temperature (Figure 10). Other genera with a mean RA ≥ 5% through time included *Pichia* (all of which was *P. fermentans*), another Pichiaceae family of unclassified genus, and *Wickerhamomyces* (all of which was *W. anomalus*).

Bacterial orders with a mean relative abundance ≥ 5% included Bacteroidales, Pseudomonadales, Clostridiales, Flavobacteria, and Burkholderiales (Figure 11). OTUs with mean RA ≥3% at the species level included *Acinetobacter lwoffi*, Porphyromonadaceae (unknown genus and species), *Pseudomonas* sp., and Ruminococcaceae unknown genus and species.

**Taxa driving variation in the Farm C microbiome**

Bacterial families ≥1% RA Porphyromonadaceae, Tissierellaceae, Clostridiaceae, Caldicoprobacteraceae, and Spirochaetaceae were greater in samples taken below 15 cm (*fdr* = 0.036, 0.024, 0.074, 0.076, 0.067 respective). Bacterial orders were discriminated seasonally, winter months were associated with similarly occurring OTUs in the Clostridiales and Bacteroidales while Burkholderiales and Flavobacteria were primarily associated with warmer pack temperatures in the spring, though patterns of abundance between samples was less similar (Figures 8, 11).

Comparison of the fungal community between barns was characterized by similar top abundance genera, and similar trend in low abundance genera increasing in the spring with an uptick in February (Figure 12). Relative abundance of genus *Candida* was 7.5 and 4.25-fold greater in the cow barn for the months of December and January. In both barns combined, phylum Ascomycota, representing 88% of all OTUs, were similar.
between depth and surface samples (fold change 1.05) while Basidiomycota were 3-fold (mean RA 14%) higher in surface and Neocallimastigomycota were 13-fold higher (mean RA 4.5%) in depth samples.

**Farm C Bedding Core Microbiome**

Both Farm C core bacterial and fungal taxa exhibited seasonal variation. Core bacterial taxa greater than 1% RA that exhibited the most relative variation through time included *Acinetobacter lwofii*, *Comamonas* sp. *Pseudomonas* sp. was both greater than 1% RA and exhibited the lowest relative standard deviation from the mean (CV) (Table 5). Fungal taxa exhibiting similar pattern of abundance through time included *Candida rugosa* and an OTU from the Pichiaceae family (Figure 13). The OTU Pichiaceae family was the most consistent RA through time, though it still exhibited wide swings in abundance. Low abundance fungal core taxa included *Apiotrichum* sp. and *Aspergillus intermedius* (Figure 13). While both these OTUs were low abundance, their relative standard deviations were among the highest, owing to spikes in abundance in August of 2015 and June of 2016.
Table 5. Mean relative abundance of Farm C fungal and bacterial core taxa through four sampling months. OTUs are labeled by last known taxonomic classifier. Standard deviation (sd) and standard deviation relative to the mean of OTU relative abundance mean among all samples \( n = 34 \) (CV) are presented. To be considered core, OTUs existed on all dates in at least 25% of samples from each farm.

<table>
<thead>
<tr>
<th>Core bacterial taxa</th>
<th>sd</th>
<th>mean</th>
<th>cv</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acinetobacter lwoffii</em></td>
<td>0.044</td>
<td>0.052</td>
<td>0.841</td>
</tr>
<tr>
<td><em>Comamonas</em> sp.</td>
<td>0.026</td>
<td>0.033</td>
<td>0.794</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp.</td>
<td>0.011</td>
<td>0.023</td>
<td>0.508</td>
</tr>
<tr>
<td>Alcaligenaceae family</td>
<td>0.009</td>
<td>0.010</td>
<td>0.910</td>
</tr>
<tr>
<td><em>Solibacillus</em> sp.</td>
<td>0.009</td>
<td>0.006</td>
<td>1.464</td>
</tr>
<tr>
<td><em>Flavobacterium gelidilacus</em></td>
<td>0.006</td>
<td>0.010</td>
<td>0.623</td>
</tr>
<tr>
<td>Porphyromonadaceae family</td>
<td>0.006</td>
<td>0.007</td>
<td>0.903</td>
</tr>
<tr>
<td><em>Luteimonas</em> sp.</td>
<td>0.005</td>
<td>0.007</td>
<td>0.692</td>
</tr>
<tr>
<td>Porphyromonadaceae family</td>
<td>0.005</td>
<td>0.008</td>
<td>0.611</td>
</tr>
<tr>
<td>Weeksellaceae family</td>
<td>0.005</td>
<td>0.007</td>
<td>0.719</td>
</tr>
<tr>
<td>Rhodobacteraceae family</td>
<td>0.002</td>
<td>0.004</td>
<td>0.512</td>
</tr>
<tr>
<td><em>Corynebacterium</em> sp.</td>
<td>0.002</td>
<td>0.003</td>
<td>0.720</td>
</tr>
<tr>
<td>Phyllobacteriaceae family</td>
<td>0.001</td>
<td>0.002</td>
<td>0.671</td>
</tr>
<tr>
<td>Xanthomonadaceae family</td>
<td>0.001</td>
<td>0.003</td>
<td>0.660</td>
</tr>
<tr>
<td><em>Sphingobacterium</em> sp.</td>
<td>0.001</td>
<td>0.002</td>
<td>0.817</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Core fungal taxa</th>
<th>sd</th>
<th>mean</th>
<th>cv</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida rugosa</em></td>
<td>0.176</td>
<td>0.182</td>
<td>0.966</td>
</tr>
<tr>
<td>Pichiaceae family</td>
<td>0.095</td>
<td>0.134</td>
<td>0.709</td>
</tr>
<tr>
<td><em>Candida catenulata</em></td>
<td>0.090</td>
<td>0.092</td>
<td>0.979</td>
</tr>
<tr>
<td><em>Pichia fermentans</em></td>
<td>0.069</td>
<td>0.064</td>
<td>1.081</td>
</tr>
<tr>
<td><em>Candida tropicalis</em></td>
<td>0.064</td>
<td>0.049</td>
<td>1.300</td>
</tr>
<tr>
<td><em>Wickerhamomyces anomalus</em></td>
<td>0.048</td>
<td>0.060</td>
<td>0.800</td>
</tr>
<tr>
<td><em>Apiotrichum</em> sp.</td>
<td>0.039</td>
<td>0.022</td>
<td>1.805</td>
</tr>
<tr>
<td><em>Debaryomyces</em> sp.</td>
<td>0.032</td>
<td>0.039</td>
<td>0.807</td>
</tr>
<tr>
<td><em>Aspergillus intermedius</em></td>
<td>0.008</td>
<td>0.004</td>
<td>1.943</td>
</tr>
</tbody>
</table>
Comparison Between Bedding, Teat and Milk Microbiomes

Shannon diversity of both bacterial and fungal communities were greatest in bedding decreasing progressively for teat and milk (Figure 14). Of bacterial sequences on the two dates that teat skin, cistern milk, and bedding were concurrently sampled, bedded pack exclusively shared 93 OTUs with healthy teats and 152 OTUs with infected teats; 466 OTUs were shared between bedding and both healthy and infected teats. Bedding exclusively shared 80 OTUs with healthy milk samples and 163 with infected milk samples; 146 were shared among all.

Of fungal taxa, bedded pack exclusively shared 25 OTUs with healthy teats and 26 OTUs with infected teats; 28 OTUs were shared between bedding and both healthy and infected teats. Bedding exclusively shared 11 OTUs with healthy milk samples and 16 with infected milk samples; 10 were shared between all.

One OTU, the bacteria *Acinetobacter lwoffii*, was core to all farms as well as teat end skin on Farm C (Table 6). Two OTUs, the yeast *Debaryomyces prosopidis* and bacteria *Acinetobacter guillouiea*, were considered core to all three habitats on Farm C. *Pseudomonas umsongensis* was 3-fold greater in healthy milk, and an unknown fungal OTU was 6.5-fold greater in healthy milk. *D. prosopidis* was 3.7-fold greater in infected milk.
Table 6. Farm C OTUs shared between all three habitat types presented as percent relative abundance within sample type. Core microbiomes of bedding, teat and milk were calculated between samples that were concurrently collected in July and August of 2015 using non-normalized data to preserve rare species. Samples below 200 sequences were removed. OTUs were considered core if they were observed in at least 25% of samples from skin, milk, and pack. OTUs were included in the table if mean percent RA of bedding samples ≥ 0.1 and if the summed value of milk and teat samples mean percentage RA ≥ 1.0.

<table>
<thead>
<tr>
<th>Bacteria: teat skin and bedding</th>
<th>healthy</th>
<th>infected</th>
<th>pack</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acinetobacter guillouiae</em></td>
<td>2.4</td>
<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Acinetobacter lwofii</em></td>
<td>0.9</td>
<td>0.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Order Clostridiales</td>
<td>0.5</td>
<td>0.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bacteria: milk and bedding</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas umsongensis</em></td>
<td>1.5</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td><em>Ochrobactrum sp.</em></td>
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**Figure 1.** Non-metric multidimensional scaling of Bray-Curtis dissimilarity matrix of square root transformed bacterial sequence abundances. Points represent bedding samples colored by farm origin. Hulls connect outer points of farm sample group.
Figure 2. Non-metric multidimensional scaling of Bray-Curtis dissimilarity matrix of square root transformed fungal sequence abundances. Points represent bedding samples colored by farm origin. Hulls connect outer points of farm sample group.
**Figure 3.** Heat map of bacterial families in greater than 1% RA at each farm. Increasingly red: higher mean relative abundance; increasingly blue: lower mean relative abundance; increasing transparency: mean relative abundance. Unclassified: OTUs that match reference database but are not classified. Other: OTUs ≤ 1% RA. Unknown Family: OTUs with known higher classification, but unidentified at the family level. Adjacent dendrogram clusters farms based on mean similarity of OTU abundance distances between samples.
Figure 4. Bacterial families with greater than 5% relative abundance in at least one farm were compared between farms using the Kruskal-Wallis test. False discovery rate (fdr) corrected p-values were used to infer which families could be driving community differences observed in the PERMANOVA and ordination results. Families with fdr less than or equal to 0.05 are included in the figure. Each panel compares bacterial family relative abundance among farms. Mid-line represent median, hinges are at 25th and 75th quantiles. The Wilcoxon test was used to make pairwise comparisons of relative abundances of each bacterial family between farms; relationships noted below indicate fdr less than or equal to 0.05. Pseudomonadaceae (L less than all, S greater than all), Comamonadaceae (D greater than all but B, S less than all), Weeksellaceae (L and S less than all, not different from each other), Alcaligenaceae (B greater than L), Sphingobacteriaceae (L less than all), Flavobacteriaceae (S greater than B), class Clostridia order MBA08 unknown family (S less than all but D), and Alteromonadaceae (S greater than L and B).
**Figure 5.** Heat map of fungal genera ≥ 1% RA in 1 of five farms. Increasingly red: higher mean relative abundance; increasingly blue: lower mean relative abundance; increasing transparency: mean relative abundance. An unclassified genus of the Pichiaceae family is included. Unclassified: OTUs that match reference database but are not classified at the genera level. Other: OTUs ≤ 1% RA. Adjacent dendrogram clusters farms based on average similarity of OTU abundance distances between samples.
Figure 6. Fungal families with greater than 5% relative abundance in at least one farm were compared between farms using the Kruskal-Wallis test. False discovery rate (fdr) corrected p-values were used to infer which families could be driving community differences observed in the PERMANOVA and ordination results. Families with fdr less than or equal to 0.05 are included in the figure. Each panel compares bacterial family relative abundance among farms. Mid-line represent median, hinges are at 25th and 75th quantiles. The Wilcoxon test was used to make pairwise comparisons of relative abundances of each bacterial family between farms; relationships noted below indicate fdr less than or equal to 0.05. Basidiomycota (L greater than all but S), Cystofilobasidiaceae (L greater than all), Phaeosphaericeae (no difference), Pichiaceae (L and S less than C and D), Tremellales (B greater than C), Wallemiaceae (B greater than C and L).
Figure 7. Non-metric multidimensional scaling of Bray-Curtis dissimilarity matrix of square root transformed ITS OTU abundances. Points represent bedding samples colored by month. Points are shaped by barn of origin. Hulls connect outer points of each month.
Figure 8. Non-metric multidimensional scaling of Bray-Curtis dissimilarity matrix of square root transformed 16S rRNA OTU abundances. Both samples and OTUs are included in the ordination. Points represent OTUs colored by order. Orders are limited to the 5 most abundant. Shapes represent samples and are designated by month. Temperatures observed for each sampling date was fit as a contour to the ordination. Contour lines are shaded blue by temperature; darker blue is cooler, lighter blue is warmer.
Figure 9. Shannon diversity of Farm C bedded pack through time. Higher index values indicate a more even distribution and/or greater number of OTUs. Loess curves were fitted to diversity index values by date to visualize and compare trends.
Figure 10. Mean monthly relative abundance as a function of bedding temperature (right y-axis) and percent relative abundance (left y-axis) of Farm C heifer barn *Candida* spp. through time.
Figure 11. Non-metric multidimensional scaling of Bray-Curtis dissimilarity matrix of square root transformed bacterial abundances. Points represent OTUs colored by order. Orders are limited to the five most abundant. Orders are broken out by panel.
**Figure 12.** Stacked bar plot of 15 top relatively abundant fungal genera observed in Farm C heifer and cow barn through time. Other: genera ranked 16th or lower. Unclassified: OTU reference matched but without taxonomic classification. Unclassified Pichiaceae family: OTU reference matched at family level only. Note that colors do not represent the same taxa in both plots.
Figure 13. Farm C core fungal community was defined as any OTU observed in ≥25% of samples from all dates. Raw counts were used to capture rare taxa. Abundance is relative to sample. Cluster diagram calculated using Euclidean distance between mean RA of taxa on each date. Clustering of high abundance/high variation vs low abundance/low variation taxa suggest different life history strategies and changing habitat conditions, including local variation such as larval hatches, calf births or unequal distribution of bedding material.
Figure 14. Comparing Shannon OTU diversity in samples within each habitat at Farm C. Non-normalized data was used from all sample dates.
DISCUSSION

Farms C, B and D utilized similar bedding material and strategy. Farm D used Farm C as a model for pack management, increasing the chance of similar microbial communities between these two farms. Though Farm B bedded more frequently (twice daily, rather than once), all used hay as primary bedding material with woodchips added to provide structure and increased aeration. Ordination and hierarchical clustering group these farms together while segregating farms L and S, suggesting that bedding material has a role in shaping the microbiome.

Farm S bedding material was similar to farms C, B, and D; it used a wood chip base and chopped straw (rather than hay), yet the microbiome was distinct. Several factors unique to Farm S that may have contributed to this distinction. Farm S had the most barn space per cow and cows were not restricted to the barn, they could range into the snowy, frozen or muddy pasture. As a result, less manure and bedding accumulated in the barn and lower temperatures were observed (Farm S median 18 C, all others median 34.5 C), likely because decomposition was not as well insulated from freezing temperatures. Outdoor access also may have brought unique environmental microbes into the barn. Farm S also had a different lactation schedule; all other farms were milking cows while sampling was occurring. Farm S cows were dried off in early winter and calved in March-April; milk was not being produced on the first sample date and just begun to approach full production by the second sample date in April. Farm S contained different relative abundances of common bacterial families such as Alteromonadaceae, Pseudomonadaceae and Flavobacteriaceae and fungal families Phaeosphaericeae and
Tremellales incertae familiae for which it had significantly elevated abundance. These differences in the microbiome may be associated with unique management choices such as cooler pack temperatures, outdoor access, or differing lactation schedule rather than bedding material.

Pseudomonadaceae was the most relatively abundant bacterial family. It was greatest in Farm S. This family contained mostly the genus *Pseudomonas*. Of this genus, most OTUs were unknown species, except for *P. thermotolerans*. *P. thermotolerans* has a maximal growth temperature of 55°C and an optimal of 47°C (Manaia and Moore 2002). Pack temperatures were generally lower than the optimal growth point, ranging between 24°C and 49°C on farms all farms but S, but could have attained higher temperatures during the days between sampling times. Despite the greatest relative abundance of Pseudomonadaceae in Farm S, *P. tolerans* was absent in this farm, further indicating an ecological distinction between mesophilic and thermophilic pack conditions. This further suggests that despite management differences, the bedded pack environment remains similar enough between farms that higher taxonomic groupings, especially diverse groups such as Pseudomonadaceae, may not be an adequate indicator of ecological function.

Sphingobacteriaceae was the bacterial family with second greatest mean RA. The dominant genus in all farms except S was *Sphingobacterium*. This genus has been identified in bovine raw milk samples (Zhang et al. 2015, Oikonomou et al. 2014). It was significantly lower in Farm L despite observation in sawdust manure compost (Yamada et al. 2008),
Alteromonadaceae family had the third greatest mean RA across all farms. The most predominant genus therein was *Cellvibrio* on all farms except Farm L. *Cellvibrio* is a gram negative, aerobic organism with the capacity to degrade cellulose and glucose. The cellulose:lignin ratio of straw is typically 50% greater than wood (Tullus et al. 2009, Lawther et al. 1995). Its greater abundance on farms with hay and straw as primary bedding would be expected to be greater than on Farm L which used only sawdust as bedding. In addition to sewage sludge (Syazni et al. 2015), *Cellvibrio* species have been cultured from polluted waterbodies and found to be lytic of cyanobacteria (Granhall and Berg 1972), raising questions about built-in bio-control for bedded pack run-off.

Moraxellaceae family was the fourth greatest mean RA across all farms and contained the genus *Acinetobacter*, which along with *Pseudomonas*, were the most relatively abundant genera overall. The predominant known species in the genus were *A. lwoffii* and *A. guillouiae*. *Acinetobacter* spp. are gram negative, aerobic and unable to catabolize glucose (Whitman 2015); they have been isolated from sewage gas effluent (Nemec et al. 2010), soil (Singh et al. 2014), and found in cow feces (*A. lwoffii*) (Poirel et al. 2012). *Acinetobacter* spp. are known to degrade antibiotic compounds and to rapidly develop resistance (Poirel et al. 2012). Both *A. lwoffii* and *A. guillouiae* were also considered core between teat skin, milk and bedding.

Farm S was about 30% less abundant in *Acinetobacter* than other farms and about 3-fold more abundant in *Psychrobacter*, a psychrotrophic taxa that tolerates temperatures up to -10 C. Some strains of *Psychrobacter* have been isolated from fresh and spoiled milk and cheese (Gennari et al. 1992). Thermophilic farms were mostly void in this
genus. Farm S Preincubation Index (PI) counts were high for milk samples as cows began their lactation cycle in the spring [personal communication with farmer]. PI estimates bacterial colony-forming-units (CFU) of cold tolerant taxa after incubation of cultures at 55 C for 18 hours (Richardson 1985). The problem was eliminated after teat dipping protocol was adjusted. The situation is suggestive that the cold bedded pack provided habitat for cold tolerant bacteria observed in the milk.

Comamonadaceae is a large and diverse bacterial family that has been observed in diverse environments including the rumen and plants (Rosenberg et al. 2013). In a 2016 community sequencing study Wong et al. observed Comamonadaceae in fresh cowpats, declining slightly as the manure aged. It was significantly elevated in Farm D, where, as in all farms but L, the predominant genus was Comamonas. In Farm L, the predominant (63% of Comamondaceae) genus was Hydrogenophaga, a hydrogen-oxidizing bacteria (Willems et al. 1989). Hydrogenophaga was also observed in all other packs, but in reduced abundance. The elevated presence of Hydrogenophaga in the only pack to utilize wood shavings suggests a more acidic environment that may have contributed to the segregation of this community in the NMDS visualization despite sharing a similarly thermophilic profile with farms C, B and D. Future studies may consider including a chemical profile of the bedding environment to reinforce ecological observations.

Six OTU's were considered core between all farms. Because all dates and farms were included in the core test, OTUs that are uniquely associated with bedding material, lactation, pack temperature, cow stocking density and other factors unique to farms are theoretically removed while OTUs that remain are associated with factors common to all
farms. Most identified core species have been associated with manure or compost. *Luteimonas* spp. (Xanthomonadaceae family) have mostly been found in marine and fresh water environments, but one species has been isolated from food waste compost (Young et al. 2007). Alcaligenaceae have been observed to increase abundance through time to become the dominant β-Proteobacteria in cowpats that had been experimentally unshaded from the sun (Wong et al. 2016). *Acinetobacter lwofii*, noted above, is commonly isolated from the dairy environment. It has been associated with cow manure, isolated from chilled raw milk (Ribeiro et al. 2018), higher SCC in bulk tank milk (Rodrigues et al. 2017), and is suggested to affect immune function in mammals (Debarry et al. 2007). Genus *Stenotrophomonas* has been isolated from soil, compost and sewage (Rosenberg et al. 2013). Core taxa *A. lwofii, Stenotrophomonas* sp., *Pseudomonas* sp. and *Luteimonas* sp. are gram negative, strictly aerobic bacteria as well as the type genus *Alcaligenes* of the Algaligenaceae family (Whitman 2015).

Most of the fungi across all farms belonged to the phylum Ascomycota (median 83.6%), while 13.6% belonged to the Basidiomycota. Farm L was the most enriched in Basidiomycota (mean RA 33.9%). The predominant classes of Basidiomycota in Farm L were Tremellomycetes and Agaricomycetes as with all other farms. Farm L was void in class Wallemiomycetes, which contained a single genus *Wallemia*. *Wallemia* spp. were significantly more abundant in Farm S and B but also was present in C and D. *Wallemia* is a xerophilic mold that has been isolated from hay and some species are known to be associated with "Farmers Lung Disease" (Roussel et al. 2003). Farms S and B had divergent management practices, yet both used a dried grass product for bedding, which
suggests that the bedding they used was possibly drier than other hay-bedded farms. Because Farm L, which did not use dried grass bedding, was void in this genus also suggests that *Wallemia* is a bedding, rather than management related taxa. Farm L was significantly enriched in the family Cystofilobasidiaceae, suggesting tilled saw dust bedding may contribute to this success of this family. Cystofilobasidiaceae was identified as a major component of the fungal rumen microbiome in one 12-animal pyrosequencing study (Fouts et al. 2012).

Of the Ascomycota, the major classes were Saccharomycetes in all but S, where the major class was Dothideomycetes. Eurotiomycetes were predominant in all but farms C and S. Class Saccharomycetes, the true yeasts, was the most abundant class overall and included two predominant families, Saccharomycetaceae and Pichiaceae. Pichiaceae family is associated with food and grain spoilage and is a fermentation contaminant (Kurtzman 2011). Bedded pack contains analogs to many of these environments, possibly accounting for its common occurrence. Pichiaceae was lower on farms L and S. While B, C and D are mostly grass ration farms, mulch hay often is left longer in the field and contains seed heads at harvest. Additionally, these farms fed hay on the pack, which may have contained seed heads. Farms L and S did not use mulch hay, or feed at all in the barn, possibly limiting habitat for Pichiaceae by removing grain as an available substrate.

The predominant genus of family Saccharomycetes, *Candida*, was also the most abundant genus overall. Genus *Candida* was about half as prevalent in Farm S, which had an equivalent abundance of *Wickerhamomyces anomalus*, a yeast noted for high
osmotolerance, utilization of excess nitrate, and the production of antimicrobial "killer toxins" that control other fungi; it has been found in sewage, mammalian feces, and the rumen of pastured cows (Abrao et al. 2014, Kurtzman 2011, Capelli et al. 2014, Gouliamova 2012).

Candida species are common in the dairy farm environment; they have been associated with raw milk cheese (Lavoie et al. 2012, Ceugniez et al. 2017), feed ration, water trough, teat, and feces (Scaccabarozzi et al. 2011). Candida has often been associated with mastitis, though it is considered a facultative pathogen that establishes in animals with weakened immune systems (Scaccabarozzi et al., Richard et al. 1980, de Casia dos Santos and Marin 2005). For example, C. rugosa was observed in elevated abundance in mastitic milk after treatment with antibiotics for an infection from Streptococcus agalactiae (Crawshaw 2005). The present study did not find elevated levels of Candida spp. in teats or milk of concurrently sampled cows, despite observing C. rugosa, C. tropicalis, both considered opportunistic mastitis pathogens (Scaccabarozzi et al. 2011).

Of the Dothideomycetes, order Pleosporales and family Phaeosphaeriaceae therein were the predominant taxa in all farms but L, which was enriched in an unclassified family. Farm S had an elevated abundance of Phaeosphaeriaceae, which was also the second greatest median family overall farms. The predominant genus on Farm S within the Phaeosphaeriaceae family was Parastogonaspora, which was also observed at lesser levels in all other farms but L which was void. A species of Parastogonaspora is a noted pathogen of wheat (Syme et al. 2016), suggesting that the observed OTU is also
associated with grain and grass. Further suggesting this is the void of the genus in the Farm L community, which did not use hay or straw for bedding material.

Class Eurotiomycetes (enriched in farms B, D, and L) consisted mostly of family Trichocomaceae within order Eurotiales. The Trichocomaceae family is ecologically diverse and abundance was distributed more evenly over genera, however, Farm D had an elevated abundance of *Aspergillus*, a highly aerobic yeast, which was common to all farms, though lower in Farm L. *Thermomyces lanuginosus* was the only species in genus *Thermomyces*, a thermophilic fungus that has been extensively mined for its biochemical properties. It was greatest in farms L and B, which had thermophilic packs, but was not abundant in the equally thermophilic pack of Farm C, suggesting that some other factor beyond temperature effects its prominence in the community. *T. lanuginosus* is reported to be the dominant species in corn biomass and cow manure fed compost (Zhang et al. 2015).

Core fungal taxa was defined as all OTUs that existed on all dates in at least 25% of samples from each farm. Three species were identified, *C. rugosa*, an unclassified OTU of the Pichiaceae family, and *Wickerhamomyces anomalus*, all yeasts that have been associated with cows, as noted above. While *C. rugosa* was present on all farms, its abundance was highly variable between farms, suggesting that aspects of bedding material, pack management or herd management (diet, lactation, cow cleanliness etc.) have a role in shaping the bovine yeast community.

Diversity is greatest when dominant groups are less relatively abundant. Because the data are inherently compositional, a higher diversity score does not necessarily
indicate that there are fewer of the dominant species in the environment. It could instead indicate that there are relatively more of the rare species. Biologically, greater diversity could suggest a general die back of facultative, copiotrophic or syntrophic organisms which would allow more of the rare species to be counted, or it could suggest an event that enables establishment of less dominant species at an increased rate than that of more populous organisms. For example, rare species could benefit from biofilm formation as common organisms also thrive. Or a localized disruption such as a recent calf birth or larval hatch could create a new habitat niche for unique species, leaving the endemic population unchanged. Future sequencing queries can estimate absolute abundances to shed more light on these dynamics.

Apparent cyclic patterns of abundance and diversity are contradicted between bacteria and fungi, though trends are obfuscated by scant data. Opposing trends occurred until December, at which point there is no clear relationship between kingdom trends. Higher diversity is a result of a higher ratio of rare to abundant taxa. Thus, when diversity trends are inversely related, rare bacterial taxa decrease and more rare fungal taxa increase.

Possibly the most important yearly disruption of the microbial habitat occurs when the barn is scraped clean in mid-summer, allowed to remain relatively vacant for several months before adding bedding and cows to the barn in November. In the sampling period, bedded pack was intact in July 2015, scraped by August and began accumulating by November. August and October 2015 samples are reflective of a composite of the barn floor before cows and bedding are added full time, it contained
remnant bedding from the previous year, sandy floor material, traces of manure from occasional cow visits etc. Bacterial diversity was rising slightly through this period, while fungal diversity is dropping, dominated by yeasts *Candida*, *Coprinopsis* and *Ascobolus*, the latter two are coprophilous genera (Amandeep et al. 2014, Gloer and Truckenbrod 1988). December samples reflect the pack after about one month of cow presence. At this point, fungal diversity is at its first low point; more than 50% of the community is *Candida*. Bacterial diversity drops in January, dominated by the Pseudomonadales and Bacteroidales. By February, the RA of rare fungal taxa has increased in the surface (but not depth) of both barns, increasing diversity, though the community is still dominated by yeasts. Rare bacterial taxa become more relatively abundant into late winter before falling somewhat before both kingdoms become more diverse in June. Speculatively, the contradicted patterns could result from interaction between kingdoms, but also points to other factors that affect kingdoms differently, such as pH or moisture content.

The fluctuation of bacterial and fungal core microbiome adds detail to the picture of overall abundances and diversity scores. Both the bacterial core and the fungal core have low variation and high variation taxa. Low variation organisms possibly occupy consistent functional niches, while high variation organisms are able to respond to environmental stimulus such as habitat disruption, excess nutrient, extreme temperature or changes in moisture content. The bacterial core is most divided into three high variation taxa, and thirteen lower variation taxa. *Acinetobacter lwoffii*, *Comamonas sp.*, *Pseudomonas* sp. and an OTU from the Alcaligenaceae family increase RA after cows
return to the pack, peaking in February, which drives down diversity scores for this month. The introduction of cows may have changed the environmental conditions or availability of substrate to promote the increase in population of this group. The fungal core is comprised only of yeasts, and appears more confounded, though *Apiotrichum* sp. and *Aspergillus intermedius* both remain at relatively low variation and abundance, suggesting that their persistence is not related to changing environmental conditions. *Candida* spp. while consistently present, appear to respond to increasing pack temperatures, or other factors that increase temperature such as rate of decomposition or ambient barn temperature.

The bedded pack is a unique environment because during use, it is constantly replenished with both bedding and fresh manure; feed stocks contain unique microbiota. One possibility is that low abundance/variation taxa are those that are inoculated via feedstock but are not adequately fit for survival in the pack; the population is in a constant state of die-off even as it is freshly deposited. It is clear that the low-level core responds differently to the conditions that enable other organism (other yeasts, in the case of fungi) to thrive.

An interesting example that may contradict this hypothesis is the increased relative abundance of fungal phylum Neocallimastigomycota deeper in the bedding. This anaerobic phylum has been identified uniquely within the rumen of mammalian herbivores and lack of its presence experimentally results in decreased voluntary feed intake and dry matter degradation (Gruninger et al. 2014). Though oxygen measurements were not included in this study for scarcity of readings, depth samples were often
anaerobic, judging by the fluid accumulating beneath the surface substrate that would
clog pores in the instrument tip, suggesting that Neocallimastigomycota deposited with
fresh manure may be able to persist in the deeper pack layers. The observation of OTUs
associated with increased rumen function in bedding is worthy of continued research.
Are these OTUs associated with living organisms, and if so what is their morphology and
persistence? Are they also apparent in fresh manure and the rumen? Is there a
mechanism that transfers anaerobic fungi from the bedded pack to the calf rumen?

A single organism, *C. rugosa*, approaches 50% of the fungal community on two
dates for Farm C and was the dominant fungal organism overall. *Candida* poses a
potential management threat due to its association with mastitis in immune deficient
animals. Lower observed abundances of *Candida* in the only mesophilic pack suggests
that lowering the temperature of the pack strategically during peaks in the population
cycle could provide control. However, the low abundance of *Candida* spp. observed on
concurrently sampled teats and milk suggests that elevated abundance in bedding does
not guarantee transmission to the mammary gland. The hypothesis that pack management
can affect the mammary gland microbiome, not just taxa associated with bedding
material or manure, will be important to explore more fully in future research.
Additionally, strain type and absolute abundances must be assayed to help establish risk
of exposure to pathogens in different management styles.

OTUs were commonly shared between all three habitats. Limiting the definition
of core reflects a more conservative approach to whether an OTU was truly common in
each habitat. Concurrent sampling only occurred on two dates. On the first date, July,
cows were living on the pack sporadically; it had been a wet summer and the barn was still in use as weather made pastures unusable. On the second date, the pack had been removed from the barn and cows were no longer living on it. In a typical summer, cows would not be accessing the pack during these months. It is possible that sampling during a time of heavy pack use would yield different shared taxa and abundances within each habitat.

The meaning of finding shared taxa between bedding, teat and milk is unclear. The presence of shared OTUs across all habitats cannot be interpreted as the presence of a living, persistent, or active organism. Some core taxa, such as *Acinetobacter lwoffii* have been isolated from cow or manure associated habitats, while others like *Debaryomyces prosopidis* have been isolated from mesquite trees (Phaff et al. 1998), though other *Debaryomyces* have been isolated from soil, feces, skin lesions, cheese, and the gut of vertebrates (Phaff et al. 1998, Saluja et al. 2007). The OTU *D. Prosopidis* was increased in infected milk, while *Pseudomonas umsongensis*, *Penicillium chrysogenum*, and an unknown fungal OTU were increased in healthy milk, which may indicate that environmental bedding taxa proliferate differently between the healthy and infected mammary gland. Whether the shared taxa increased in healthy milk can be considered part of a commensal mammary gland microbiota is unknown, though the preliminary evidence presented here does not discount the possibility. It is valuable to continue efforts to characterize the microbiomes of the mammary gland and bedding habitats to compare transference of taxa between infection state.
Conclusion

This study of five different Vermont farms is a first attempt to characterize the microbial community of bedded pack using molecular techniques. Results suggest that both pack management and bedding materials affect microbial community structure and merit further investigation of how management practices affect the transmission of microbes between the bedding and mammary gland. Some bedding OTUs were associated with key ruminal organisms, raising questions about the relationship between bedding and the gut.

The core fungal community of bedded pack was dominated by yeasts including *Candida* spp., *Wickerhamomyces* spp. and family Pichiaceae, of which some species have been implicated in the literature as opportunistic mastitis pathogens. Most abundant core bedded pack bacterial OTUs were grouped into genera *Acinetobacter*, *Comamonas*, and *Pseudomonas*. Though there were only 2 overlapping sampling dates for bedding, teat cistern milk, and teat end skin, many OTUs were shared between these two habitats. One OTU, the bacteria *Acinetobacter lwoffii*, was core to all farms as well as teat end skin. Two OTUs, the yeast *Debaryomyces prosopidis* and *Acinetobacter guillouiea*, were considered core to all three habitats on Farm C.

It will be important to establish an appropriate time-scale to capture community structure dynamics. While this study was conducted monthly, communities may change much faster than this, especially during localized events such as cow births or larval hatches. A better understanding of these dynamics is necessary to understand the composition of the bedding microbiome.
Bedding temperature was related to the composition of the bedding microbiome and may affect the prevalence of taxa that are observed on the mammary gland. From a biocontrol perspective, inoculation of packs with hypothetical beneficial microbes or microarthropods may need to be correctly synchronized with microbial community structure dynamics for maximum benefit. Furthermore, pack management tools could be utilized to disrupt or control the structure of the microbial community to create a more suitable habitat for beneficial organisms.

Understanding the microbiome of bedding is critical to animal health. Important questions remain about how bedded pack management practices affect microbial transference to the mammary gland or the rumen. For example, bedded packs that utilize long fiber substrate such as hay are typically not mechanically aerated, hypothetically leaving microhabitats intact while tilled packs using smaller, denser substrate such as sawdust are tilled, homogenizing microbial communities and bringing deeper material to the surface where it is more likely to contact with the udder. More targeted sampling and sequencing is required to understand how these and other management practice differences effect shared taxa between the bedding and the animal.
Chapter 3. Arthropod Community of Bedded Pack

INTRODUCTION

Muscid flies, including horn fly (*Haematobia irritans*), stable fly (*Stomoxys calcitrans*), house and face fly (genus *Musca*) are all costly and aggravating pests on dairy farms (Taylor 2012, Byford 1992). Horn and stable flies are known vectors for bacterial pathogens that cause mastitis (Gillespie et al. 1999, Ryman et al. 2013, Anderson et al. 2012) and *Musca* spp. are considered vectors of human and cattle disease (Calvo et al. 2010, Braverman et al. 1999). Bedded pack, built from saw dust, straw, or hay potentially provides ample habitat for stable fly and *Musca* spp. larvae (Schmidtmann, 1989) while fresh manure and its microbiota (Perotti et al. 2001a, Perotti and Lysyk 2003) is the requisite material for horn fly larval survival and development. Bacteria are also critical to *Musca* spp. larval development (Watson et al. 1993, Lysyk et al. 1999) as well as to compost bedded pack functionality.

Mites (Acari) have been established as a potential biological control on cattle and other agricultural manure and bedding with the observation of predation of muscid flies by coprophilous Macrochelidae on cattle manure (Wade and Rodriguez 1961, Axtell 1963a,b). Later studies established phoresy of macrochelid mites on stable flies as means of dispersal to new habitat (Beresford and Sutcliffe 2009). Studies focused primarily on *Macrocheles muscaedomesticae*, noting seasonal predation on Muscidae eggs and first instar larvae or nematodes based on availability, and the evolution of a rapid life cycle that mirrors the short-notice and quick duration of egg and larvae meal availability.
(Rodriguez et al. 1962, Hunter and Rosario 1988, Axtell 1963b). In his 1999 review, Hartmut H. Koehler paraphrases Karg 1994 (translated from the German) to suggest that Gamasina (including among others, families Macrochelidae, Uropodidae and Parasitidae) will opportunistically predate mass population development of nematodes or microarthropods such as Collembola, Enchytraeidae or soft bodied Astigmatida mites. This assessment is consistent with the general classification of the Gamasina as r-strategists, such that the instability of their habitat and short duration of prey availability is overcome by an opportunistic feeding strategy and an accelerated reproductive cycle that goes from egg to adult in three days (Hunter and Rosario 1988).

North American observations of predatory coprophilous mites on cattle and other manures were conducted as far north as Ithaca, New York (Axtell 1963a), and included Macrochelidae *Macrocheles*, *Glypholaspis*, as well as non-Macrochelidae families Uropodidae and Parasitidae. Bedded pack shares many of the same characteristics of habitat associated with these mites, i.e., decaying surface organic matter, litter, debris and manure (Koehler, 1999). Predatory mites are considered top predators at the mesotrophic level and are a pivotal regulator in soil food webs, integrating energy flows from bacteria and fungi (Ruf and Beck 2005). They can quickly establish enough population density in disturbed ecosystems to predate other quick-establishing groups such as nematodes, shaping the direction of species diversity as the system stabilizes (Koehler 1997). Some common factors are shared between soil-based agroecosystems and the bedded pack environment. For example, disturbance via tillage is known to effect soil mesofauna
(Koehler 1997), common to compost bedded pack systems. The bedded pack environment has not been tested for presence of predatory arthropods.

Bedded pack can either be mechanically aerated or materially aerated. In principle, both methods rely on the microbial community to provide bedding functionality. Bedding microbial communities vary between farm possibly due to differences in composition of bedded pack (Chapter 2, this thesis), which is likely to alter the rate of decomposition and heat production (Neher et al. 2013). In detrital and soil systems predatory mites can affect the fungal and bacterial community by selectively preying on bacterivorous and fungivorous nematodes (Beaulieu and Weeks 2007). Further, mites are known to disseminate bacteria and fungal propagules between different levels of the soil profile (Moore et al. 1988), which may likewise help distribute microbes in bedded pack systems. The potential complexity of these relationships raises the possibility that bedded pack might be better understood in an ecological context. The objective of the study was to test the hypothesis that bedded pack provides habitat for predatory mites that could provide control for fly pests. Associations between the mite community and the fungal microbiome were measured in addition to management practices that may shape the arthropod community. This study represents a first effort to characterize the microarthropod community of bedded pack and test for relationships between microbial and microarthropod taxa, pack and herd management techniques, and bedding materials. We expected to find both fly pests and predatory mites live in bedded pack, bedded pack management strategies (temperature, bedding material, removal from barn) affect the
mite community, and that a relationship exists between the microbial and mite communities of bedded pack.

METHODS

Experimental Design

The microbiomes of bedded pack and microarthropods at single farm (Farm C) in northwestern Vermont over the period of approximately one year. To test for the possibility of farm and management level effects, bedded pack microbiome and mites were sampled at four additional organic bedded pack dairies (D, L, B, S) within an 80 km radius of Burlington, Vermont on four of the Farm C sampling dates. Farm C bedded pack was sampled 10 times between July 2015 and June 2016. Farms D, L, B and S bedded packs were sampled on the same day as Farm C, four times between March and June 2016.

To compare bedded pack between all farms, Farm C data was restricted to only dates that all farms were sampled and non-barn samples were removed, yielding a total of 38 bedded pack observations spread across 4 months. For comparisons of bedded pack at Farm C, only barn samples from Farm C were retained, $n = 18$. To explore the effect of removing the bedding from the barn at Farm C, only pack samples that had been removed were retained, $n = 5$.

On Farm C, pack was sampled once at each of three stages of maturity after it had been removed from the barn. Time-stage 1 was collected from windrow compost piles of barn C and barn H bedding within 10 days of removal from the barn. On the same
sampling day, bedded pack that had been contemporaneously removed from these barns and spread on a recently cut hay field rather than windrowed was sampled. Time-stage 2 sampling occurred one month after removal of a mixed barn bedded pack in the summer of 2015. Time-stage 3 sampled this same bedded pack compost pile approximately one year later, in June of 2016.

Sampling and Metadata Collection

Prior to sampling, a 45-minute verbal interview was conducted with farmers to assess pack management priorities and strategies. Five 3.75 l samples were collected at both surface (0-15 cm) and depth (15-35 cm) along random transects in each bedded pack barn at each sampling date. Occasionally, when the pack was less than 15 cm deep, a composite depth/surface sample was collected. Farm C used two barns; each was sampled separately. Additionally, independent samples were taken of pack after it had been scraped from the barn, composted, or spread on the field in some farms. Temperature and percent oxygen were also measured at time of sampling using an OxyTemp™ (Reotemp, San Diego, CA) probe. Samples were transported to the lab in insulated containers for same-day processing. In the lab, depth and surface sub-samples for each barn were combined separately and mixed thoroughly before resampling a volume of approximately 3 l to create a single sample representing the entire space. Representative surface and depth samples were placed in a Berlese Funnel and exposed to surface heat and light via a 40-watt incandescent lightbulb to extract arthropods into 70% ethanol, 5% glycerol solution over a period of 10 days, or until the bedded pack was completely dry. Counts of microarthropods were standardized as number per gram of
dried pack material. Sample density varied considerably depending on condition of the pack when sampling occurred; this was reflected in the variation of dry weights per liter (mean 185 g, standard deviation 214 g). High density samples consisted of mostly sand (the bottom floor of the barn, when pack was absent), while low density samples were mostly dry hay. Extracted mites and pseudoscorpions were identified to lowest possible taxonomic level (Krantz 2009) using an Olympus BX53 microscope; other taxa were identified to order with a Zeiss Discovery V12 stereo microscope. Dipteran larvae were differentiated into potential pest fly (Muscidea) and non-pest (other Diptera) by presence of head capsule (Stehr, 1991). Identified taxa were enumerated by hand using the stereo microscope.

Some collection cups became contaminated with fungi and mites were not able to be enumerated. On several dates, bedding had been removed from the barn and only the parent floor material remained. Temperature of the floor was not taken on these days. Missing temperature data was estimated using average day-time temperatures from National Weather Service data loggers nearest to the farm (NCEI 2018). This was the case for 5 out of 39 total observations of bedded pack barns.

Statistical Analysis

Bedded pack fungal DNA sequences were extracted, amplified, sequenced, cleaned, filtered and counts rarefied as described in Chapter 2 of this thesis.

To assess whether bedded pack provides habitat for Muscidea larva and predatory mites, presence or absence of identified taxa was compared by farm on dates that bedded
pack was intact in the barn. Mite abundances were compared among farms using the Kruskal-Wallis test. Shannon diversity index was used to compare diversity of mite taxa between bedding that had been removed from the barn. Mite community and fungal microbiome were converted to Bray-Curtis dissimilarity matrices for multivariate analysis using permutational multivariate analysis of variance approach (PERMANOVA) and generation of non-metric multi-dimensional scaling (NMDS) ordinations.

To test if management strategy affects the mite community, a multivariate model was built using mite counts as the dependent variable. Covariates found to be significant in separate prior tests were included in the model. Because any management practices effect was inextricable from a possible farm effect, farm was included as a factor in the model, rather than bedding style. All interactions were included in the model. Rather than treat temperature as a linear variable in the unconstrained ordination environment, a generalized additive model was used to fit a non-linear contoured surface. \( P \)-values suggest significance relative to the linear fit. Linear fit \( p \)-values are provided for comparison.

Mite abundance and temperature gradient were compared to NMDS ordination of fungal taxa using the envfit function in vegan. \( P \)-values suggest confidence of the association by reporting the proportion of times the randomly permuted \( r^2 \) value is equal to or greater than the actual \( r^2 \) of the vector fit. The fungal microbiome was limited to the top five orders to assess potential taxonomic associations. As a post-hoc analysis, associations between mite genera of interest and Diptera larvae, temperature of pack, and abundance of yeast were investigated separately with regression analysis. The
association between mite and temperature was visualized with non-linear model under an assumption of Poisson-distributed data.

All analysis was performed in R version 3.4.2 (R Core Team). The *vegan* package was used to calculate dissimilarity matrices and NMDS ordinations, perform PERMANOVA, and fit variables to ordinations (Oksanen et al. 2017). Non-linear regression was performed using the *mgcv* package (Wood, 2017).

RESULTS

Three farms (D, B, C) utilized a deep bedded pack system primarily comprised of hay with periodic layering of wood chips. Pack temperature on these farms was median 35°C. Farm L used a compost bedded pack system primarily built from green sawdust that was aerated with a chisel plow daily, temperatures were typically warm on this pack. Farm S cows were not lactating during the winter and had free choice access to frozen pastures and barnyards. Pack was constructed from wood shavings and chopped straw. It was not tilled, nor did it accumulate bedding as rapidly as the deep bedded packs; it was typically cooler (Chapter 2, this thesis).

Non-mite taxa extracted from bedded pack included Coleoptera, Collembola, Diptera and pseudoscorpions, representing less than 3% of the total organisms observed. On a given date, mites represented between 97.0% and 100% of arthropods extracted from bedded pack samples (Figure 1). It was common to observe wide variation of abundances between consecutive sampling dates, up to 100-fold in the case of Farm B acari between May and June.
The structure of the mite community varied by farm (Figure 2). Mites identified to genus include Acaridae *Rhizoglyphus*, Macrochelidae *Glypholaspis*, and Parasitidae (Parasitinae) *Eugamasus*. Mites identified to family include Dinychidae and Oppioidea (superfamily). Some mites were identified only to Parasitiformes and Acariformes divisions within Acari due to immaturity. Oppioidea, the only orbatic mite, was observed only once and accounted for less than 0.1% of the mean overall relative abundance mite community of Farm C barn c. Dinychidae was observed twice during the study period, accounting for 0.2% of the mite community in Farm D and 0.6% in Farm L. Immature (unidentified) Parasitiformes were observed in all farms ranging from less than 0.1% of the community to 15.3% in Farm C barn h. Parasitidae *Eugamasus* was observed in all farms, ranging from less than 0.1% in Farm C barn c to 47.9% in Farm S. The two most abundant taxa were Acaridae *Rhizoglyphus*, and Macrochelidae *Glypholaspis*, present in all farms. Though abundances varied greatly between sampling dates, overall *Rhizoglyphus* was most abundant in Farm B, while *Glypholaspis* was most abundant in Farm C barn c. Only Oppioidea and Dinychidae were not observed on all farms.

Discounting unidentified organisms in the Acariformes and Parasitiformes groups, the core group of mites shared between all farms included *Eugamasus*, *Glypholaspis* and *Rhizoglyphus*.

Similar to all farms, Farm C mite taxa varied dramatically between sampling dates (Figure 3). *Glypholaspis* were most commonly occurring. Parasitiformes were only observed when pack was in use in the barn, increasing through time until the pack was removed in June of 2016. Acaridae (genus *Rhizoglyphus*) also reappeared as cows
made the transition back to pasture, though this group was also present in December, while cows were on the pack full time. Wide variation between sampling dates is apparent in Macrochelidae between August and January. August and October of 2015 were the only sampling dates on which potential pest fly larvae was present in the bedded pack.

At Farm C, all samples of bedded pack after it was removed from the barn contained both *Glyptolaspis* and *Rhizoglyphus* mites; the latter genus was dominant (≥75% of community) in both piles of bedding from time-stage 1, but not in the sample spread on the field (Figure 4). Time-stage 2 had the fewest number of counts per gram dry matter and the fewest number of arthropods. Time-stage 3 (the aged compost pile) and the time-stage 1 field sample were the most diverse (Simpson diversity index value = 0.81 and 0.75 respectively), while the immature compost piles from time-stage 1 were the least diverse (mean Simpson diversity = 0.21). The recently spread bedding sample had the greatest abundance of both *Glyptolaspis* mites and non-pest dipteran larvae.

Of dipteran taxa, potential pest Muscidae larvae was never observed in bedded pack while it was actively used by cows. Potential pest fly larva was observed in the barn in August (0.3 counts per 100 g dry matter) and October (21 counts per 100 g dry matter) samples of bedded pack from Farm C, however the bedded pack had been removed and sampled substrate contained primarily sandy material with traces of bedding and manure. Non-pest dipteran larvae was observed in June 2015 (1.4 counts per 100 g dry matter) at Farm C, while cows had occasional access to bedded pack due to a wet spring. Additionally, non-pest dipteran larvae were observed in June 2016 (483.9 counts per 100 g dry matter) at Farm C.
g dry matter) at Farm D, but pack had been removed. Non-pest dipteran larvae were also observed at Farm C (498.0 counts per 100 g dry matter) in samples taken from bedded pack that had been spread on the field within two weeks of removal from barn, and in bedded pack that had been piled outside for one year (18.2 counts per 100 g dry matter). Predatory mite *Glypholaspis* was also present in all samples in which fly larvae were observed. No statistical evidence of a relationship between abundance of larvae and predatory mites was found \((r^2 = -0.25, p = 0.94)\).

Sample depth, whether the barn was scraped clean of pack, and sample date were not statistically significant factors shaping the mite community. Both farm and bedding temperature affected the mite community \((p = 0.027 \text{ and } p = 0.048 \text{ respective})\). The combined model further confirmed the importance of a farm effect \((p = 0.005)\), but no further variation was explained by the temperature \((p = 0.265)\) or interaction variable \((p = 0.068)\). NMDS ordination shows clustering of samples within farm, visually confirming that farm factors affect the mite community (Figure 5). Farm S, a pack managed at a median 14 °C, clusters together along a cooler gradient, while Farm B, a warm pack managed at a median of 38 °C is aligned along MDS2 and spread out along MDS1 in a warmer temperature gradient. Approximate significance of the non-linear fit relative to the fit of the linear model was calculated at \(p = 0.003\); approximate significance of linear fit was \(p = 0.55\). Samples of the barn floor typically fell within cooler temperature gradients. Contrasting the test result, Farms D and C barn floor sample communities are well discriminated from bedded pack samples. In Farm S, only one of the floor samples is well discriminated. Farms L and B barn floors were not sampled.
Predatory mites *Glyptolaspis* and *Eugamasus* contributed to the difference between farm mite communities (Figure 6). Farms C and S were enriched in *Glyptolaspis*, while only Farm S was enriched in *Eugamasus*. Farms using a deep bedded pack did not necessarily contain higher abundances of *Glyptolaspis*. The only farm using a cooler bedded pack experienced higher abundances of *Eugamasus*.

Bedded pack fungi is primarily comprised of yeasts, with Saccharomycetales abundance ranging from a high of 75% of the community in Farm C to a low in the coldest bedded pack, Farm S, which had 17%. Rather than Saccharomycetales, Farm S contained a plurality of Pleosporales (43%) (Chapter 2). NMDS ordination of fungal OTUs from top orders reflects this divergence (Figure 7). Vectors of mite abundance and temperature fitted to the ordination show possible associations between the mite and fungal community. Macrochelid mites were more likely to be associated with Saccharomycetales and warmer temperatures while Acaridae, Parasitidae and Dinychidae were more likely to be associated with Pleosporales and Eurotiales, and potentially cooler temperatures. *P*-values reflecting the strength of the fit relative to permuted data suggest greater confidence for Macrochelidae and Acaridae.

Macrochelids were observed to have the greatest abundance between approximately 23 °C and 37 °C (Figure 8). Yeast was positively correlated ($r^2 = 0.10, p = 0.083$) with temperature. There was a slight positive relationship between Macrochelids and abundance of yeast in the bedding (Figure 9). However, many samples, including those with high yeast abundance, contained very low or zero Macrochelids and the resulting model is a weak predictor.
Figure 1. Each panel describes total counts per gram dry matter of arthropods extracted from bedded pack on each farm while bedded pack remained in barn. Taxa are described at the level of order, excepting collembola, described at the level of subclass. Cc and Ch denote barns C and H at Farm C.
Figure 2. Horizontal stacked bar denotes mean taxa abundance in counts per gram dry matter found in farms across all sampling dates. Mite taxa is reported at the level of family excepting Acariformes and Parasitiformes, reported at the level of sub-order. Cc and Ch denote barns C and H at Farm C.
Figure 3. Stacked bar denotes mean taxa abundance in counts per gram dry matter found in Farm C at each sampling date. Mite taxa is reported at the level of family excepting Parasitiformes, reported at the level of sub-order. Dates range from July 2015 to June 2016.
Figure 4. Arthropod communities at different time-stages of bedded pack after it had been removed from the barn. Each stacked bar represents a single sample. Time-stage 1 was collected from windrow compost piles of barn C and barn H bedding within 10 days of removal from the barn (c_compost and h_compost). On the same sampling day, bedded pack that had been contemporaneously removed from these barns and spread on a recently cut hay field rather than windrowed was sampled (field). Time-stage 2 sampling occurred one month after removal of a mixed barn bedded pack in the summer of 2015 (mixed_one.month). Time-stage 3 sampled this same bedded pack compost pile approximately one year later, in June of 2016 (mixed_one.year).
Figure 5. Non-metric multidimensional scaling of Bray-Curtis dissimilarity matrix of square root transformed mite taxa abundances. Points represent samples colored by farm of origin. Points are shaped respective to whether the pack was in situ and being used by cows (round dot) or whether the pack had been removed and only floor material remained at time of sampling. Surface overlay represents the 2-dimensional fit of temperature to MDS1 and MDS2. Surface fit lines are colored by temperature value, cooler is bluer, warmer is yellow.
Figure 6. Predatory mite genera that vary between farm are presented. Both *Glypholaspis* and *Eugamasus* were significantly different (p = 0.03 and 0.04, respective) via Kruskal-Wallis rank sum test.
Figure 7. Non-metric multidimensional scaling of Bray-Curtis dissimilarity matrix of square root transformed fungal OTU abundances from the top 5 most abundant fungal orders observed in bedded pack from all farms. Points represent fungal OTUs colored by taxonomic order. Arrows represent positive relationships between mite abundance and fungal OTUs within the ordination space. Direction of arrow indicates OTUs associated and length of arrow indicates strength of the association. \( P \)-values suggest confidence in the association by reporting the proportion of times the randomly permuted \( r^2 \) value is equal to or greater than the actual \( r^2 \) of the vector fit.
A generalized additive model using the Poisson distribution is used to visualize the relationship between temperature and genus *Glyphotholaspis* mites on five farms.

**Figure 8.** A generalized additive model using the Poisson distribution is used to visualize the relationship between temperature and genus *Glyphotholaspis* mites on five farms.
**Figure 9.** The relationship between the abundance of predatory genus *Glyptolaspis* mites and relative abundance of fungal OTUs of the fungal order Saccharomycetales of bedded pack on five farms. $r^2 = 0.05$  $p = 0.193$. 
DISCUSSION

The three-part hypothesis that both fly pests and predatory mites inhabit bedded pack, bedded pack management strategies affect the mite community, and interaction exists between the mite and fungal communities was conditionally accepted, with important insights explored below for future investigations that more fully develop and test potential management techniques.

Predatory mites observed in bedded pack

Predatory *Glyptholaspis* mites were observed in bedded pack barns on all farms sampled, and in composting piles of bedding after removal and several days after spreading on the field on Farm C. Both *Eugamasus* and *Glyptholaspis*, observed in all farms, have been established in the literature as predatory on small arthropods and other invertebrates (Axtell 1963b, Hyatt 1980). *Glyptholaspis* sp. are commonly found in manure piles, stables, and compost and have been identified as phoretic on several coprophilus organisms including dung beetles and muscid flies (Niogret, 2006). Macrochelid mites have been long explored as biocontrol organisms of muscid flies and all but the first instar of *Macrocheles Muscaedomesticae* is predatory upon the eggs of *Musca domestica* (Axtell 1963b). *Glyptholaspis confusa* has been identified as predatory of horn fly eggs and first instar larvae in the laboratory and field setting (Perotti 2001b, Axtell, 1963a,b).

The presence of macrochelids in the winter months in the present study was likely due to elevated temperatures of bedding and manure via microbial composting activity
that was increased in a sheltered area rather than outdoors. Macrochelid mite populations were greatest in summer and decreased by late fall to negligible in winter months in outdoor manure and bedding piles in Kentucky (Rodriguez et al. 1970).

Macrochelids feed on nematodes when fly larvae are unavailable (Rodriguez et al. 1970). Though nematode populations were not measured, collection cups contained nematodes that had dropped through the Berlese funnel. It is possible that Glypholaspis and other predatory mites in bedded pack survive on nematodes during the winter months, when dipteran larvae are not available. Likewise, macrochelids will feed on other micro-arthropods such as Collembola and astigmatid mites such as Rhizoglyphus, both observed in bedded pack. One hypothesis is that microbial activity in the composting pack provides warmer temperatures and greater microbial food supply for bacterivorous and fungivorous nematodes, Collembola, and astigmatid mites which in turn become a food supply for predatory mites. In this sense, predatory mite populations are regulated by abundance of both microbial, nematode and soft-bodied microarthropod populations. Complicating this interaction, nematodes have shown preferential feeding between Candida species and strains (Chang et al. 2013), a major component of the bedding community, which could have a selection effect on the nematode community, thereby potentially effecting feeding habits of predatory mites. Further, the extreme variation in abundance of Rhizoglyphus by sampling date would regulate predators’ opportunity to access this food source. The abundance of Glypholaspis had a slightly positive relationship with the abundance of yeast, suggesting the presence of an unmeasured regulatory variable. These possibilities fit well with the understanding of
species or assemblages centrally positioned between bottom-up and top-down regulation of the soil food web (Neher 2010, Moore et al. 2003). While predatory mites are dependent upon nematode and arthropod populations for non-larval food supply, they can also effect decomposition rate by preying on bacterivorous and fungivorous nematodes (Beaulieu and Weeks 2007).

Pest fly larvae in bedded pack

Very few fly larvae were found in bedded pack barns, primarily because adult flies are actively feeding and reproducing during July, August and warm Septembers, when bedding is mostly likely to have been removed from the barn. Potential pest fly larvae were observed only twice in the study, in bedded pack barns after the pack had been removed, in August and October of 2015. Mite communities remained similar and Glypholaspis in particular was observed after the pack had been removed, suggesting that any bio-control effect of the bedded pack would be retained in the barn.

Organic dairy cows are typically not housed on pack during the summer months in Vermont, due to the seasonal availability of required time spent on pasture. Therefore, any control of flies offered by predatory mites would likely not occur in the bedded pack barns but rather after it has been removed from the barn. While there was some evidence to suggest that Glypholaspis was able to transition from the pack to the field, it is unclear whether any larvae control could occur. A significant reduction in adult flies emerging from calf pen areas contained endogenous predatory Parasitidae and Macrocheles mites compared to acaricide-treated control pens (Axtell 1963b). This suggests that bedded pack could serve as an incubator of predatory mite species used to “seed” areas of the
farm that might be more prone to proliferation of flies, such as return lanes or pre-milking holding areas. This hypothesis is consistent with the increase in *Glypholaspis* mites in the sample of bedded pack spread on the field, though more extensive sampling is needed to confirm this result.

In the laboratory environment, 100 *Macrocheles* and 100 *Glypholaspis* mites/L of manure with 20,000 *Musca domesticae* eggs resulted in an average 94% reduction in adult flies compared to the control (Axtell 1963b). In an indoor calf pen environment, an approximate population of 144 macrochelid and parasitid mites/L of manure was able to provide an average 64% reduction in adult flies from the addition of 20,000 eggs. In outdoor manure piles, an approximate existing population of 69 macrochelid and parasitid mites/L reduced adult fly populations by an average of 38% from the addition of 50,000 eggs.

This study suggests that while the mite community shifts during the time-period after removal of bedding from the barn, this may not limit the efficacy of potential opportunities for bio-control. Relative to Axtell’s ratio for effective biocontrol, in the newly removed outdoor piles of bedding, macrochelids averaged approximately 24 mites/L of material, and fly counts were nil. Zero fly counts may be due to heavy mite feeding, flies not laying eggs, and/or eggs not yet hatching in the material. Similar bedding that had been spread within two weeks of removal and sampled several days after spreading contained approximately 371 counts of dipteran larvae/L, 435 counts of *Glypholaspis* and 165 counts of (Parasitidae) *Eugamasus* mites/L. The approximately two-week time difference between removal from the barn may account for the emergence
of fly larvae, and the resulting increase of predatory mite genera. The number of mites per liter in the present single observation of outdoor manure piles is 14-fold greater than that observed by Axtell. While this discrepancy could be an outlying observation and must be further investigated, some of the difference is potentially attributable to the substrate composition, the type and amount of bedding mixed with the manure, or other farm specific factors. Relative to Axtell’s observations, the number of mites observed in the Farm C bedded pack spread on the field was enough to provide some extent of fly control.

Managing the arthropod community of bedded pack

While it was clear that farm related factors influenced the mite community, uncertainty remains about which factors changed the mite community and promoted predatory mites. Despite this uncertainty, factors that were measured in this study establish potential trends for further exploration. Temperature appeared to effect *Glyptholaspis* and *Parasitidae* differently. The coldest bedded pack, median temperature 14 °C, was the highest in *Parasitidae*, while *Glyptholaspis* appeared to have an average optimal temperature of 30 °C. However, temperature is not the only predictor of *Glyptholaspis*; Farms B and C, which use a similar deep static pack, had similar median temperatures and similar cow stocking density, had significantly different observed populations of this mite. A primary difference between the two farms was the higher rate of bedding application in Farm B, which resulted in a drier pack surface. The lack of moisture may have changed the diet of predatory mites to *Rhizoglyphus*, as nematodes rely on water surfaces to forage for food (Neher, 2010). If mite prey is not the preferred
diet, a less abundant predator population could result. This trend was observed at Farm B; fewer far greater *Rhizoglyphus* mites were observed along with very high relative abundance of yeast *Candida* spp. (Chapter 2, this thesis) yet low abundance of *Glyptolaspis* mites. This hypothesis runs contrary to the general association of Macrochelidae with Saccharomycetales and Acaridae (*Rhizoglyphus*) with Pleosporales observed in this study, suggesting that the dynamics of this relationship are more complex.

Though sample $n$ was not great enough to establish bedded pack beta-diversity (differences between species assemblages between samples) at a single farm, the wide variation in mite abundance between sample dates may be associated with sample location within the barn. Events such as manure and urine deposits, larval hatches, inconsistencies in bedding distribution or material are likely to create resemblance to a soil-like system in which microhabitats are formed based on localized availability of dietary nutrients, water films, and prey (Gorres et al. 1999). This dynamic may be different between the deep bedded pack and compost bedded pack, which relies on daily tilling for aeration, homogenizing physical attributes of the bedding habitat and possibly contributing to differences seen in the biotic community of Farm L. Likewise, the location of waters, whether cows are fed on or off the pack, barn features like scratching posts etc., and the location of mineral supplements are relevant to the distribution of bedding material, manure, and moisture on the pack surface. If these features are static through the accumulation of bedding, unique local habitats may form that preclude mites but encourage the establishment of fly larvae.
Conclusion

Whether the presence of predatory mites in bedded pack affects fly control potential in the field is unknown. This study suggests that while the arthropod community changes after bedding is removed from the barn, the move does not exclude predatory mites from the new bedding habitat and may create greater opportunity to feed on fly larvae. It is unclear if predatory mites observed in the outdoor bedding are moved with the bedding or gain access via phoresy. From a farm management perspective, it stands to reason that more predatory mites in the bedded pack would result in greater potential fly control once the pack is removed from the barn. Producers may benefit from further studies that test the effect of management strategies on predatory mite populations. Another possible management application is the use of bedded pack in the summer months, while flies are active, as a holding area prior to milking in a parlor. Though some evidence of a relationship between mites and the microbial community was determined, this dynamic is complex and contains mediating factors that were not measured, though theoretical precedent and findings from soil-based research suggest that mites may help shape the microbial community of bedded pack. Further research is necessary to understand how management practices may affect the food web of the bedded pack habitat.
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